Regulation of Human Eosinophil Apoptosis

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by

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Abstract

Eosinophils play a pivotal role in the pathogenesis of asthma and allergic disease. The accumulation and persistence of eosinophils at sites of inflammation are mediated at least in part by the extended survival of eosinophils in response to circulating hematopoietins IL-3, IL-5 and GM-CSF. The apoptosis and subsequent clearance of eosinophils without histotoxic mediator release is thought to be crucial in the resolution of airway inflammation in asthma. This study characterised the morphological and biochemical events of human eosinophil apoptosis *in vitro* and investigated the mechanism by which IL-5 induces eosinophil survival. Peripheral blood eosinophils have a distinct expression profile of Bcl-2 homologues, critical regulators of apoptosis, with detectable expression of pro-apoptotic family homologues Bax, Bcl-x_S, Bim, Bak, Bid, and Bad, and anti-apoptotic homologue Bcl-x_L, with little or no detectable Bcl-2 expression. Stimulation with IL-5 induced modest upregulation of Bcl-2 mRNA and protein, with no significant modulation of the other Bcl-2 homologues examined.

Caspases are the conserved executioners of the apoptosis. Eosinophils endogenously expressed 'initiator' caspase-8 and -9, and 'effector' caspase-3, -6, and -7. Spontaneous eosinophil apoptosis involved caspase-independent translocation of Bax to the mitochondria, resulting in perturbation of the mitochondrial membrane, cytochrome *c* release, and subsequent activation of caspase-3, -6, -7, -8, and -9. IL-5 inhibited constitutive eosinophil apoptosis at a site upstream of Bax translocation to the mitochondria, thereby preventing cytochrome *c* release and caspase activation. Eosinophils constitutively expressed the conformationally active form of Bax diffusely in the cytosol, but predominantly in the nucleus. Apoptosis induced by Fas receptor ligation involved detectable activation of caspase-3 and –8, and caspase-dependent Bax translocation to the mitochondria, supporting classification of eosinophils as a Type II cell in terms of apoptotic control. The data implicate Bax and mitochondria as pivotal regulators of eosinophil apoptosis in response to diverse stimuli.

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Abbreviations

AA	arachidonic acid
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
ATP	adenine triphosphate
BAL	bronchoalveolar lavage
BH	Bcl-2 homology
BHR	bronchial hyperresponsiveness
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
CARD	caspase recruitment domain
CD	cluster of differentiation
cDNA	complementary DNA
ced-	*Caenorhabditis elegans* death gene-
CLC-P	Charcot-Leyden crystal protein
dbcAMP	dibutyryl cAMP
DD	death domain
DED	death effector domain
DISC	death-inducing signalling complex
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
EPO	eosinophil peroxidase
Erk	extracellular regulated kinase
FACS	fluorescence-activated cell scan
FADD	Fas-associated death domain
FasR	Fas (CD95/ Apo-1) receptor
FasL	Fas (CD95/ Apo-1) ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
gapdh	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' balanced salt sloution
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP	immunoprecipitation
Jak	Janus kinase
kDa	kilodalton
LT	leukotriene
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MBP	major basic protein

mCCP	carbonylcyanide m-chlorophenyl hydrazone
MEK-1	MAPK/Erk kinase-1
mRNA	messenger RNA
NO	nitric oxide
pAb	polyclonal antibody
PAF	platelet activating factor
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
pHi	intracellular pH
PI	propidium iodide
PI-3-K	phosphatidylinositol-3-kinase
PK	protein kinase
PMA	phorbol 12-myristate 13-acetate
PT	permeability transition
PtdSer	phosphatidylserine
PTP	permeability transition pore
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SHPTP-2	Src homology 2 phosphatase 2 tyrosine phosphatase
SMF	specific median fluorescence
STAT	signal transducers and activators of transcription
STS	staurosporine
tBid	truncated Bid
TBS	Tris-buffered saline
TBS-T	TBS-Tween 20
TEMED	N,N,N'N'-tetramethylethylenediamine
TGF	transforming growth factor
Th	T helper cell
TM	transmembrane
TMRE	tetramethylrhodamine ethyl ester
TNF	tumour necrosis factor
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
VDAC	voltage-dependent anion channel
zVAD.fmk	benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone
$\Delta\Psi m$	mitochondrial membrane potential
βc	common β

1.1. The Eosinophil

Originally defined by Ehrlich in 1879 (Ehrlich, 1879) as a white blood cell containing cytoplasmic granules with high affinity for the dye eosin, the eosinophil is a terminally differentiated, submucosal tissue residing leukocyte that has been the subject of much investigation and debate in terms of function and its role in host defence.

Eosinophilia was first observed to be associated with helminth infections, asthma and following acute anaphylaxis, and as a result, eosinophils were believed to actually ameliorate mast cell-dependent hypersensitivity. The observation that the release of eosinophil granular proteins was toxic to the larval stages of *Schistosoma mansoni* (Butterworth *et al.*, 1975) indicated that the principal role of the cell was in host defence, generating adaptive immunity to helminth infections (Butterworth and Thorne, 1993; Wardlaw *et al.*, 1995). Subsequently, the release of mediators such as major basic protein (MBP) and eosinophil cationic protein (ECP) were found to be potentially histotoxic to the host, resulting in tissue lesion development and airway inflammation. The tissue localisation of such toxic components during allergen challenge, in conjunction with the ability of eosinophils to generate pro-inflammatory cytokines such as interleukin-5 (IL-5), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF- α), suggested a critical role for eosinophils in the mediation of allergic responses.

1.2. Morphology and maturation

1.2.1. Eosinopoiesis

Primitive hematopoietic progenitor cells are characterised by the expression of an *O*-sialylated cell-surface glycophosphoprotein, CD34 (Civin *et al.*, 1984). Granulocytes are derived from common myeloid precursors, termed GEMM-CFU (granulocyte/ erythroid/ macrophage/ megakaryocyte colony forming unit), which, under defined cytokine conditions, differentiate into GM CFU (neutrophils, macrophages) or Eo/B CFU (eosinophils, basophils). A marker of commitment of $CD34^+$ progenitors to eosinophilic differentiation is the expression of the IL-5 receptor α-subunit (IL-5Rα; Sehmi *et al.*, 1997), induced by IL-5 itself (Tavernier *et al.*, 2000). IL-5Rα has been shown to be upregulated in allergic asthmatics and in response to allergen stimulation, and is proposed to favour eosinopoiesis and the resultant blood and tissue eosinophilia associated with allergic inflammatory diseases. Other characteristics that determine commitment to the eosinophilic lineage include expression of IgE receptors

(Rottem *et al.*, 1994), β7 integrin (mediates adhesion to fibronectin; Lundahl *et al.*, 2000) and CCR3 (eotaxin receptor; Tiffany *et al.*, 1998). These phenotypic alterations during maturation are proposed to enhance the migration of eosinophil progenitors form bone marrow to blood during allergic reaction.

IL-3 and GM-CSF are pluripotent stem cell factors, also capable of supporting eosinopoiesis, although mice transgenic for IL-3 and GM-CSF expression exhibit only minor eosinophilia (Ema *et al.*, 1990). Therefore, it is thought that although IL-3 and GM-CSF induce a commitment of $CD34^+$ myeloid progenitors to the eosinophilic lineage, IL-5 is necessary and sufficient to stimulate terminal differentiation (Sanderson, 1992). IL-5, IL-3 and GM-CSF propagate their signal via a common intracellular β subunit (βc). Mice lacking βc are still capable of eosinopoiesis, although they lack a normal eosinophilic response to parasite infection (Nishinakamura *et al.*, 1996). These findings indicate that there must be an as yet undetermined, alternative mechanism for eosinophil production and maturation, independent of these hematopoietins. Whether the two pathways of eosinophil development act in concert or are compensatory also remains to be seen. Following maturation and differentiation, eosinophil emigration from bone marrow to blood is mediated by IL-5, eotaxin, the expression of α_4 and β_2 integrins, which suppress and promote migration respectively, and the shedding of L-selectin (Palframan *et al.*, 1998a; Palframan *et al.*, 1998b). Eosinophils have a half-life in circulation of 18-20 hours prior to trans-endothelial migration into tissues, where eosinophils predominantly reside, accumulating at several sites including the lung, skin, and gastrointestinal tract.

1.2.2. Eosinophil morphology

Mature healthy eosinophils are morphologically distinct from other granulocytes, possessing a characteristic bi-lobed nucleus of condensed, marginated chromatin (Fig. 1.1). However, the most marked characteristic of eosinophils is the abundance of spherical or ovoid cytoplasmic granular organelles, which contain numerous proteins, many of which have enzymatic activity and are amongst the most abundant proteins in the eosinophil (reviewed by Dvorak and Weller, 2000). The granules are separated into four distinct categories, secondary (specific), small, primary and lipid bodies, based upon structure and developmental stage occurrence.

The secondary granules predominate and define the eosinophil. Observed during the myelocyte maturation stage as large, unicompartmental granules, maturation results in condensation and crystallisation of the granule components to form bi-compartmental membrane-bound granules. These secondary granules contain many of the highly toxic cationic proteins largely responsible for the deleterious effect of eosinophils in allergic disease, and have a distinct crystalline core predominantly of MBP, although GM-CSF, IL-4, and IL–5 have also been localised to this region (Dubucquoi *et al.*, 1994; Levi-Schaffer *et al.*, 1995; Moqbel *et al.*, 1995). The non-crystalline matrix contains many proteins, including ECP, eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), and cytokines such as IL-6 and TNF- α (Giembycz and Lindsay, 1999). Raised levels of MBP have been observed in the airway and bronchoalveolar lavage (BAL) of asthmatics (Frigas *et al.*, 1981), and marked deposition of granule proteins is observed in the bronchial mucosa in fatal asthma (Filley *et al.*, 1982). As well as their potent effect on helminths, MBP, EPO and ECP damage respiratory epithelial cells, *in vitro*, by targeting cell membranes or via their highly cationic nature, resulting in denudation of surface epithelium (Frigas *et al.*, 1981), and interaction between MBP and airway epithelium are believed to induce BHR (Wardlaw *et al.*, 1988). Importantly, this damage occurs at concentrations observed in the body fluid of asthmatics. These observations strengthened the role of eosinophils in asthma and allergic disease from mere association to potential causal factor of the tissue damage characteristic of these conditions.

Primary granules are homogenous, core-less, and, in resting eosinophils, the storage compartment for Charcot-Leyden crystal protein (CLC-P), specific to eosinophils and basophils. CLC-P protein is often found free within the cytoplasm indicating intracellular secretion and observed at inflammatory sites distinguishing eosinophil infiltration (Dvorak *et al.*, 1990).

Small granules are not observed in bone marrow or circulating eosinophils, and have been shown to contain ECP and catalase. Lungarella *et al* (1992) have shown that small granules also contain elastase, the release of which may have an important role in airway remodelling during asthma.

Finally, the non-membrane bound lipid bodies provide the intracellular store of arachidonic acid (AA), and leukotriene C_4 (LTC_4) synthase. Lipid bodies are important intracellular organelles for the synthesis of eicosanoids from AA precursor, such as cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 (Bozza *et al.*, 1997). Release of eicosanoids by eosinophils plays an important role in the pathophysiology of asthma and allergic disease, as these inflammatory mediators are potent inducers of bronchoconstriction, mucous hypersecretion and endothelial membrane permeability (Leff, 2000). They may also induce selective recruitment of eosinophils into the allergic airway, and therefore are vital mediators of the eosinophilia and airway damage characteristic of asthma. Lipid bodies are relatively few in number in quiescent eosinophils, but are observed to increase during activation.

Within the cytoplasm of the eosinophils, there are also large numbers of vesiculotubullar organelles (Komiyama and Spicer, 1975). These electron-lucent structures occur in various forms, which have been shown in the rat to be involved in endocytosis, and therefore implicated in host defence. The occurrence of this class of organelle within the cytoplasm, along with the secondary granules, can be used as a diagnostic marker of eosinophilic lineage.

1.2.3. Eosinophil surface phenotype

Recent studies have suggested that the cell surface phenotype of the eosinophil in terms of receptors more closely resembles that of the monocyte and basophil than the neutrophil. Eosinophils express a wide array of surface structures including receptors for immunoglobulin subclasses, chemokines, adhesion molecules, cytokines, and apoptotic stimuli (reviewed by Tachimoto and Bochner, 2000; Figure 1.1).

1.2.3.1. Adhesion receptors

The accumulation of eosinophils at sites of allergic inflammation is governed, at least in part, by selective adhesion and transendothelial migration, which is mediated by the activation state-dependent expression of receptors and ligands on the eosinophil cell surface. Leukocyte adhesion to the post-capillary venular endothelium is a multi-step process involving rolling, tethering, adhesion and transmigration, with sequential receptor-ligand interactions regulating specific steps in the pathway. The selectins and their glycoprotein counterligands mediate the initial selective eosinophil-endothelium interaction and tethering, whilst integrins facilitate

firm attachment to the endothelium and subsequent migration through the blood vessel tight junctions.

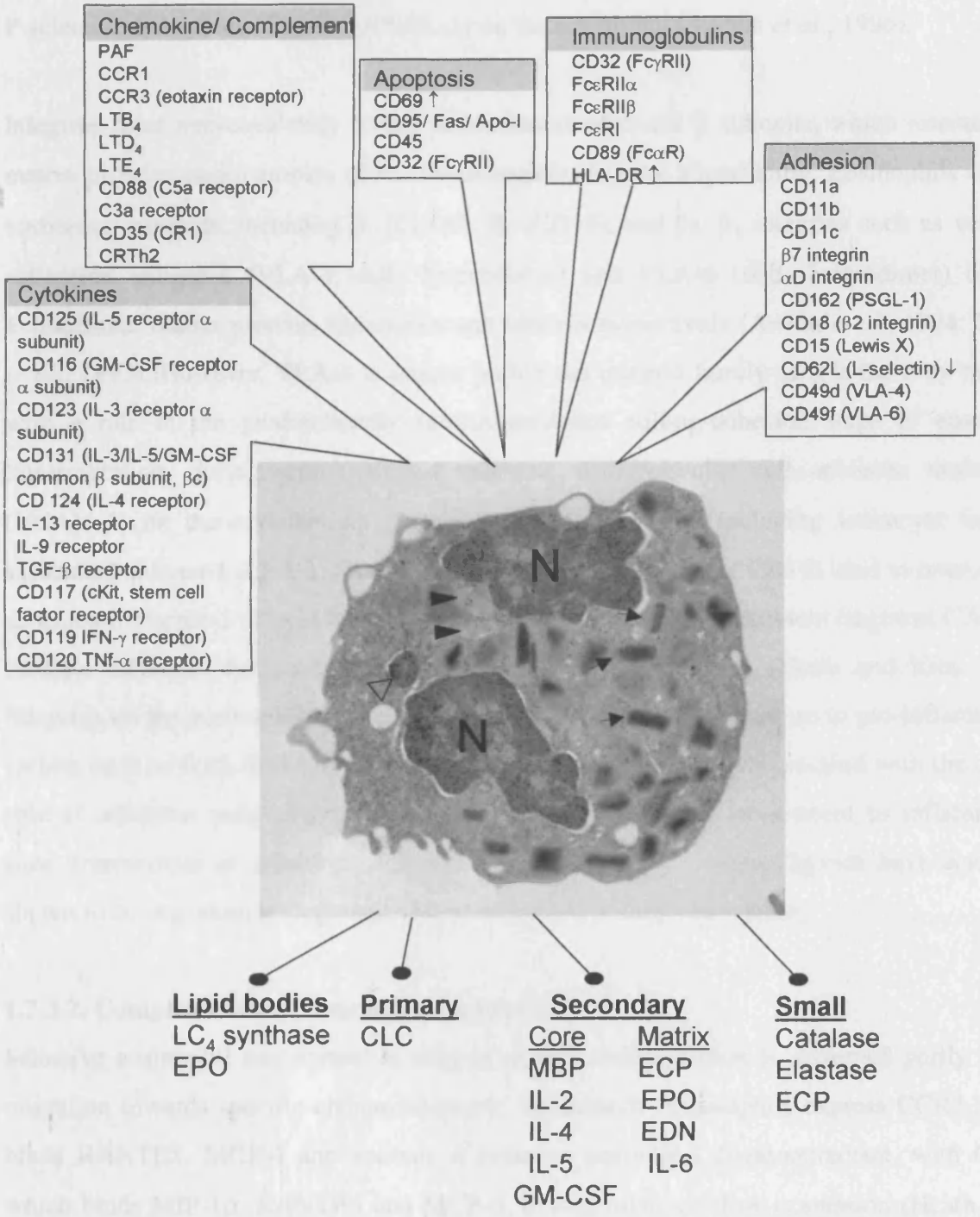

Figure 1.1. Eosinophil granule composition and surface phenotype. Eosinophils are characterised by a wide array of granule proteins (see text for details). CLC-P, Charcot-Leyden crystal protein; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EPO, eosinophil peroxidase; GM-CSF' granulocyte macrophage colony-stimulating factor; IL, interleukin; LT, leukotriene; N, lobe of bi-lobed nucleus; PAF, platelet-activating factor; TGF, transforming growth factor. Secondary granules (*arrow*), mitochondria (*solid arrowhead*) and lipid bodies (*hollow arrowhead*) are indicated.

Constitutively expressed L-selectin (CD62L) mediates the initial rolling step (Sriramarao *et al.*, 1994), interacting with endothelial ligands such as MadCAM-1 and GlyCAM-1 (Berg *et al.*, 1993), and is shed upon activation with IL-5 and platelet-activating factor (PAF; Knol *et al.*, 1994). Consequent tethering is mediated by P-selectin expressed on the endothelium and P-selectin glycoprotein ligand-1 (PSGL-1) on the eosinophil (Symon *et al.*, 1996).

Integrins form non-covalently bound heterodimers of α and β subunits, which interact with matrix proteins and members of the immunoglobulin gene superfamily. Eosinophils express numerous integrins, including β_1 (CD29), β_2 (CD18), and β_7. β_1 integrins such as very late activation antigen-4 (VLA-4, $\alpha_4\beta_1$ heterodimer) and VLA-6 ($\alpha_6\beta_1$ heterodimer) bind to extracellular matrix proteins fibronectin and laminin respectively (Anwar *et al.*, 1994; Georas *et al.*, 1993). However, VLA-4 is unique within the integrin family as it is the only member with a role in the predominantly selectin-mediated rolling-adhesion stage of eosinophil transmigration, during which VLA-4 interacts with vascular cell adhesion molecule-1 (VCAM-1) on the endothelium (Patel, 1998). β_2 integrins, including leukocyte function associated antigen-1 (LFA-1; CD11a/ CD18) and Mac-1 (CD11b/ CD18) bind to intercellular adhesion molecule-1 (ICAM-1), connective tissue matrix and complement fragment C3bi, and mediate adhesion, transendothelial migration, and degranulation (Horie and Kita, 1994). Integrins on the eosinophil surface are markedly upregulated in response to pro-inflammatory factors such as IL-5, GM-CSF, PAF and lipopolysaccharides. In conjunction with the critical role of adhesion molecules in regulating selective eosinophil recruitment to inflammatory sites, interactions of adhesion molecule receptors and their counterligands have also been shown to be important in degranulation, survival and eicosanoid release.

1.2.3.2. Complement and chemokine receptors

Selective eosinophil recruitment at sites of allergic inflammation is governed partly by the migration towards specific chemoattractants. Specifically, eosinophils express CCR3, which binds RANTES, MCP-4 and eotaxin, a selective eosinophil chemoattractant, with CCR1, which binds MIP-1α, RANTES and MCP-3, having relatively low expression (Heath *et al.*, 1997; Ponath *et al.*, 1996). Chemokinetic responses of eosinophils to lipid mediator cysteinyl leukotrienes, LTD_4 and LTE_4, and PAF indicate cell surface receptors for these mediators (Spada *et al.*, 1986; Warringa *et al.*, 1992). Eosinophils express receptors for C3a, C5a (CD88) and also Type I complement receptor (CR)1, CR3 and CR4. Activation of the

complement system can result in chemotaxis and recruitment of eosinophils. C5a is a neutrophil and eosinophil chemoattractant, whereas C3a specifically attracts eosinophils (Daffern *et al.*, 1995).

1.2.3.3. Cytokine receptors

Cytokine receptor expression is relatively low and often outside the limits of detectability by conventional methods such as flow cytometry. However, functional analyses has implicated the expression of numerous cytokine receptors on the eosinophil surface including IL-4 (heterodimer of IL-4 α chain, CD124 and a common γc chain, CD132), TNF-α (CD120) (Wallen *et al.*, 1991), IL-9 (heterodimer of IL-9 α chain, CD129 and a common γc chain, CD132), IL-13 (heterodimer of IL-13 α chain and IL-4 α chain, CD124) (Dubois *et al.*, 1994), TGF-β (Alam *et al.*, 1994), stem cell factor (c-kit receptor, CD117) (Yuan *et al.*, 1997). The specific α chains specific for IL-5 (CD125), IL-3 (CD123), and GM-CSF (CD116) share a βc chain, CD131, to form the functional receptor (Lopez *et al.*, 1991; Yamada *et al.*, 1998).

1.2.3.4. Immunoglobulin receptors

IgG receptors- Eosinophils express receptors for IgG (FcγRII/ CD32), which has a higher affinity for IgG_1 and IgG_4 (Ravetch and Kinet, 1991). FcγRII recognises multivalent IgG, in the form of immune complexes or opsonised surfaces, as opposed to the monomeric form more readily recognised by FcγRI. FcγRIII (CD16) is not expressed by resting eosinophils, but is expressed on IFN-γ-stimulated eosinophils (Hartnell *et al.*, 1992), and highly expressed by neutrophils, and therefore provides the basis for the, now standard, negative immunomagnetic selection protocol widely used for the purification and investigation of peripheral blood eosinophils (Hansel *et al.*, 1991).

IgE receptors- Resting eosinophils were reported to bind IgE via the low-affinity FcεRII (CD23), also expressed on T and B lymphocytes, and the expression of FcεRII increased upon eosinophil activation (Capron *et al.*, 1992). As with the eosinophil IgG receptor, FcεRII has higher affinity for IgE dimers and complexes. This is particularly important considering the prevalence of such immune complexes in association with parasitic infection and allergic disorders. However, more recent studies have proven contradictory, indicating expression of at least some components (α and γ chains) of the high affinity IgE receptor (FcεRI) (Gounni *et*

al., 1994). FcεRI expression has also been detected on eosinophils isolated from hypereosinophilic donors, although this remains somewhat controversial (Sihra *et al.*, 1997).

IgA receptor- Expression of FcαR (CD89) on eosinophils shows a large patient to patient variability, although a correlation has been observed between FcαR expression and allergy or parasite infection (Monteiro *et al.*, 1993).

1.2.4. Cytokine generation

Originally described as 'bystanders', the eosinophil is now known to be a potentially vital source of cytokines, chemokines and growth factors, which exhibit effector function on numerous cell types (Lacy and Moqbel, 2000; Moqbel and Lacy, 2000). The *de novo* synthesis and granule storage of eosinophil-derived mediators are proposed to play a significant role in the development of the Th2 response characteristic of allergic inflammation and helminth infection, with the generation of such pro-inflammatory cytokines as IL-4 (Velazquez *et al.*, 2000), IL-5 (Dubucquoi *et al.*, 1994), IL-3, IL-6 (Lacy *et al.*, 1998), IL-9 (Shimbara *et al.*, 2000), and GM-CSF (Gauvreau *et al.*, 1998). However, conversely, eosinophils also have the capacity to synthesise anti-inflammatory Th1-type cytokines IL-2 and IFN-γ (Woerly *et al.*, 1999a). Interestingly, whereas Th2-type cytokines are more readily upregulated in asthmatics compared with normals, and are predominantly expressed in maturing eosinophils, IL-2 and IFN-γ are constitutively and more consistently expressed (Gauvreau *et al.*, 1998; Lacy *et al.*, 1998; Velazquez *et al.*, 2000; Woerly *et al.*, 1999b). This, in conjunction with the selective release of stored Th2-type cytokines, indicates that eosinophil-derived cytokines may play an important role in the polarisation of the immune response. However, it must be recognised that eosinophils generate relatively low amounts of cytokine in comparison with other cell types, and therefore the physiological relevance of eosinophils as a cytokine source may be questioned.

1.2.5. Degranulation and mediator release

The regulated secretion of intracellular components following receptor-mediated stimulation occurs in eosinophils by two distinct mechanisms, 'compound granule extrusion' or 'piecemeal degranulation'. Analogous to the anaphylactic degranulation exhibited by mast cells and basophils, granule extrusion involves the fusion of the membrane-enclosed granules with the plasma membrane and consequent focused exocytosis of its contents, resulting in

cells with a marked hypo or agranular cytoplasmic appearance. Such compound exocytosis is observed during granule deposition following adhesion to the opsonised surfaces of parasitic helminths (McLaren *et al.*, 1977; McLaren *et al.*, 1978). Piecemeal degranulation is employed by eosinophils, but also mast cells and basophils, and involves vesicular transport of granule proteins from their intracellular stores, via secretory vesicles, to the plasma membrane, without loss of the granule structures themselves (Dvorak *et al.*, 1992; Dvorak *et al.*, 1994; Dvorak *et al.*, 1991). In contrast to granule extrusion, there is no hypogranular morphology, although the granule containers appear electron-translucent due to depletion of the electron-dense granule components (Dvorak *et al.*, 1991). The selective release of eosinophil granule proteins MBP (Torpier *et al.*, 1988) and EPO (Dvorak *et al.*, 1991) in response to specific stimuli also supports a role for piecemeal degranulation in eosinophils.

Although not strictly a secretory mechanism, necrosis and consequent cytolysis of eosinophils similarly results in the release of their toxic mediators. This may be of crucial importance as eosinophil necrosis and extrusion of its phlogistic contents may enhance allergic inflammation. This is in stark contrast to apoptosis, whereby eosinophils are cleared without histotoxic mediator release (see below).

1.3. Role of eosinophils in host defence and disease

Original definition of the role of the eosinophil was to ameliorate the pro-inflammatory functions of the mast cell. More recent evidence indicates a vital role in the regulation of allergic asthma and allergic disease (Wardlaw *et al.*, 1995), and possible limitation of parasitic infection (Meeusen and Balic, 2000). Investigation into the role of the eosinophil in both host defence and disease has centred on their capacity to release cytotoxic granule proteins, inflammatory mediators, and generate free radicals and reactive brominating oxidants (Mayeno *et al.*, 1989; Wu *et al.*, 2000), which are potentially damaging to both parasite and host tissue. Eosinophils are now known to be effector cells in numerous disorders such as, atopic dermatitis, allergic rhinitis, Crohns disease, chronic eosinophilic pneumonia and hypereosinophilic syndrome to name but a few. However, investigation over recent decades has largely concentrated on the critical role of the eosinophil in asthma and allergic disease.

1.3.1. Role of eosinophils in helminth Infection

The protective role of eosinophils during helminth infection was originally indicated by the observations of Butterworth *et al* (1975), who described eosinophil-mediated antibody-dependent damage to schistosomula. Eosinophil-derived factors such as MBP, ECP and leukotrienes have been shown to kill newborn *Trichinella spiralis* larvae, and *Nippostronglyus brasiliensis in vitro* (Coffman *et al.*, 1989; Lee, 1991). Mackenzie *et al* (1981) demonstrated that eosinophils bind to *N. brasiliensis* larvae in the presence of antibody or complement, and proteins disgorged from eosinophilic granules damaged the larval cuticle (Rotman *et al.*, 1996; Shin *et al.*, 2001). The association of IL-5 with eosinopoiesis, recruitment and activation is conserved in mammals, and mice deficient in IL-5 are incapable of mounting an eosinophilic response (Coffman *et al.*, 1989). Several reports have supported a role for IL-5-mediated eosinophilia in the host defence against parasite infection. IL-5-deficient mice and mice transgenic for IL-5 were found to be more or less susceptible respectively to infection with *Angiostrongylus cantonensis*, in terms of cranial worm burden and length (Yoshida *et al.*, 1996). Similarly a protective role for the eosinophil has been observed in infections with *Onchocerca volvulus*, *O. lienalis*, *Strongyloides ratti*, and *S. stercoralis* by ablation of IL-5 with the monoclonal antibody TRFK-5 (Folkard *et al.*, 1996; Lange *et al.*, 1994; Ovington *et al.*, 1998; Rotman *et al.*, 1996).

However, a definitive role for eosinophils in protective immunity to parasite infection is contentious as eosinophil-mediated protection is dependent upon parasite species, maturation stage and prior exposure. Depletion of IL-5 with antibody, and therefore inhibition of the eosinophil response, failed to have any effect on infection with *T. spiralis* (Herndon and Kayes, 1992) or *Schistosoma mansoni* (Sher *et al.*, 1990). Infection with *Heligosomoides polygyrus* was also unaffected by depletion of eosinophils (Urban *et al.*, 1991). Interestingly, IL-5 deficient and wild-type mice expelled a primary *T. spiralis* infection with equal efficacy, indicating that immunity to this species of nematode was both IL-5 and eosinophil independent. However, upon challenge infection, IL-5 deficient mice suffered higher gastrointestinal worm burden and impaired expulsion rate compared with the wild-type (Vallance *et al.*, 1999). This indicated that the important role of IL-5 and eosinophils in enteric host defence might not be in the initial infection, but in mediating subsequent, challenge infections. At present the role of the eosinophils in parasite killing remains unclear,

although current evidence suggests that infective larval stages, but not adults of the majority of helminthic parasites are susceptible to eosinophil-mediated killing.

1.3.2. Role of eosinophils in asthma

There have been several definitions of asthma since the Ciba Foundation Symposium in 1959, reflecting our increasing investigation into and subsequent knowledge of the complex aetiology of this disease. Although asthma is multi-factorial in origin, it is generally accepted that asthma is a chronic inflammatory disease of the airway, characterised by bronchial hyperresponsiveness (BHR) and variable airway obstruction, which is, at least in part, reversible. In 1995 the National Institute of Health (NIH) issued guidelines for the definition of asthma (National Heart and Lung Institute, 1995):

> *Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest-tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli.*

Despite advances in our understanding of the disorder, epidemiological studies have revealed a substantial pandemic increase in the incidence, morbidity, and mortality of asthma over recent decades in both adults and infants (Evans *et al.*, 1987; Gergen *et al.*, 1988). Take, for example, the dramatic increase in asthmatic prevalence in 8-12 year olds in Australia, from 12.9% in 1982 to 29.7% only ten years later (Britton *et al.*, 1986; Peat *et al.*, 1994). 'Extrinsic' asthma in response to allergens is the commonest form of asthma (Cockcroft *et al.*, 1977; Sears *et al.*, 1991), and was shown to be mediated by a novel class of immunoglobulin, IgE (Ishizaka *et al.*, 1966). A familial association with asthma is now well recognised (Holgate, 1997) and the genetic predisposition to the development of an IgE-mediated response is termed 'atopy'. Exposure to allergens causes atopics to develop specific IgE antibodies,

thereby triggering a humoral response upon subsequent exposure. Inhaled allergen challenge of allergic individuals induces an acute inflammatory response, which is sometimes bi-phasic, separated into pathophysiologically distinct early and late phases (Nadel and Busse, 1998).

The early-phase reaction occurs within minutes and is characterised by the rapid and predominant activation of airway mast cells bearing allergen-specific IgE (Liu *et al.*, 1991), causing release of pro-inflammatory mediators such as cysteinyl leukotrienes (Wenzel *et al.*, 1989), histamine (Jarjour *et al.*, 1997), and reactive oxygen species (ROS). In response to these acute mediators, mucosal oedema, vasodilatation, increased airway smooth muscle tone, and exudation of plasma into the lumen, results in airway obstruction and bronchospasm (Greiff *et al.*, 1993; Persson *et al.*, 1995). Activation and degranulation of mast cells also releases preformed chemotactic factors and pro-inflammatory cytokines including IL-4, IL-5, and GM-CSF. Local mediator release induces recruitment of effector cells to the inflammatory site and mediates the transition to the late-phase reaction of the allergic response (Austen and Orange, 1975).

The late-phase reaction, occurring 6-9 hours after initial allergen challenge, involves infiltration of the lung parenchyma and epithelium with eosinophils, $CD4^+$ T cells, macrophages, and neutrophils (Azzawi *et al.*, 1990; Calhoun *et al.*, 1993; De Monchy *et al.*, 1985; Koh *et al.*, 1993; Robinson *et al.*, 1993). Distinct functional subpopulations of $CD4^+$ T cells were defined in mice in terms of their profile of cytokine expression (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). Th1 cells predominantly elaborate IL-2, interferon-γ (IFN-γ), and TNF-α, and are vital regulators of cell-mediated immunity to infection and immune surveillance. In contrast, Th2 cells produce IL-4, IL-5, IL-13 and GM-CSF, and are instrumental in the development of the allergic response (Corrigan and Kay, 1992). IL-13 and IL-4 induce immunoglobulin isotype switching from IgM to IgE synthesis (Vercelli and Geha, 1993), and IL-5 stimulates eosinophil function (Lopez *et al.*, 1988). $CD4^+$ T cells were elevated in the bronchial biopsies and BAL of asthmatics, whereas $CD8^+$ T cell levels remained unchanged (Azzawi *et al.*, 1990; Gerblich *et al.*, 1991). Also, depletion of $CD4^+$ T cells prevented BHR and eosinophilic inflammation following ovalbumin sensitisation and antigen challenge in a murine model, whereas depletion of $CD8^+$ cells had no effect (Gavett *et al.*, 1994; Gonzalo *et al.*, 1996). The $CD4^+$ lymphocytes had increased levels of IL-5, IL-4, GM-CSF and IL-13 mRNA expression, whereas IFN-γ levels were similar between asthmatics

and normals (Robinson *et al.*, 1992; Walker *et al.*, 1992). Definitive evidence of the role of Th2 cytokines in the pathophysiology of allergy in mice was provided by the inhibition of IL-4 and IL-5 at both gene and protein level, which also prevented BHR and eosinophil infiltration (Brusselle *et al.*, 1995; Coyle *et al.*, 1995; Foster *et al.*, 1996; Kung *et al.*, 1995). Therefore allergic inflammation has been associated with the predominant activation of Th2-like $CD4^+$ T cells, and as such Th2 cells are regarded as the orchestrators of the chronic inflammatory response (Umetsu and DeKruyff, 1997). The relatively short time lag between early and late-phase is probably insufficient to allow for biosynthesis of cytokines within the activated Th2 subset. Instead, initial recruitment of inflammatory cells is more likely to be in response to the release of pre-formed cytokines from mast cells during the early-phase (Bradding *et al.*, 1994). Subsequent cytokine generation by Th2 cells is likely to maintain the responses associated with chronic inflammation (Fig. 1.2).

The reasons underlying the aberrant expansion in Th2 cell differentiation in asthmatics remain unclear. It has been postulated that the general decline in exposure to infectious disease, the 'Hygiene Hypothesis', has lead to a shift in immune response to favour a Th2-type response, and as a result increased atopy (Strachan, 1989). This hypothesis argues that systemic infection with pathogens that elicit cell-mediated immunity during infancy such as *Mycobacterium tuberculosis* (Shirakawa *et al.*, 1997), hepatitis A (Matricardi *et al.*, 1997), and measles (Shaheen *et al.*, 1996), results in a predominant Th1-type response, expression of Th1 cytokines and consequent inhibition of a Th2-type response. Decreased exposure to such pathogens during immune system development due to increased hygiene, use of antibiotics and reduction in family size (and therefore reduced sibling cross-infection) causes bias towards generation of a Th2 response (Droste *et al.*, 2000; Strachan, 2000).

Murine models have proven invaluable in studies of bronchial asthma, with major advances possible due to manipulation at the genetic level (reviewed by Kips *et al.*, 2000). The development of transgenics and knock-outs has provided insights into roles of specific genes and gene products in asthma. However, it must also be appreciated that spontaneous, chronic asthma does not develop in mice, or in any animal model, which has led some investigators to suggest studies be restricted to humans. Using such a murine model, some doubt was recently cast on the role of IgE, and therefore mast cells, in bronchial asthma, due to the observation that BHR and inflammation could still develop in mice deficient in IgE due to a null mutation

in the Cε gene (Mehlhop *et al.*, 1997). However, murine mast cells can be sensitised by IgG as well as IgE, so it is possible that the animals developed an IgG response to allergen challenge in this model (Oettgen *et al.*, 1994). Therefore, although animal models are valuable tools in the study of asthma and allergic disease, caution must be used when interpreting the results in relation to humans.

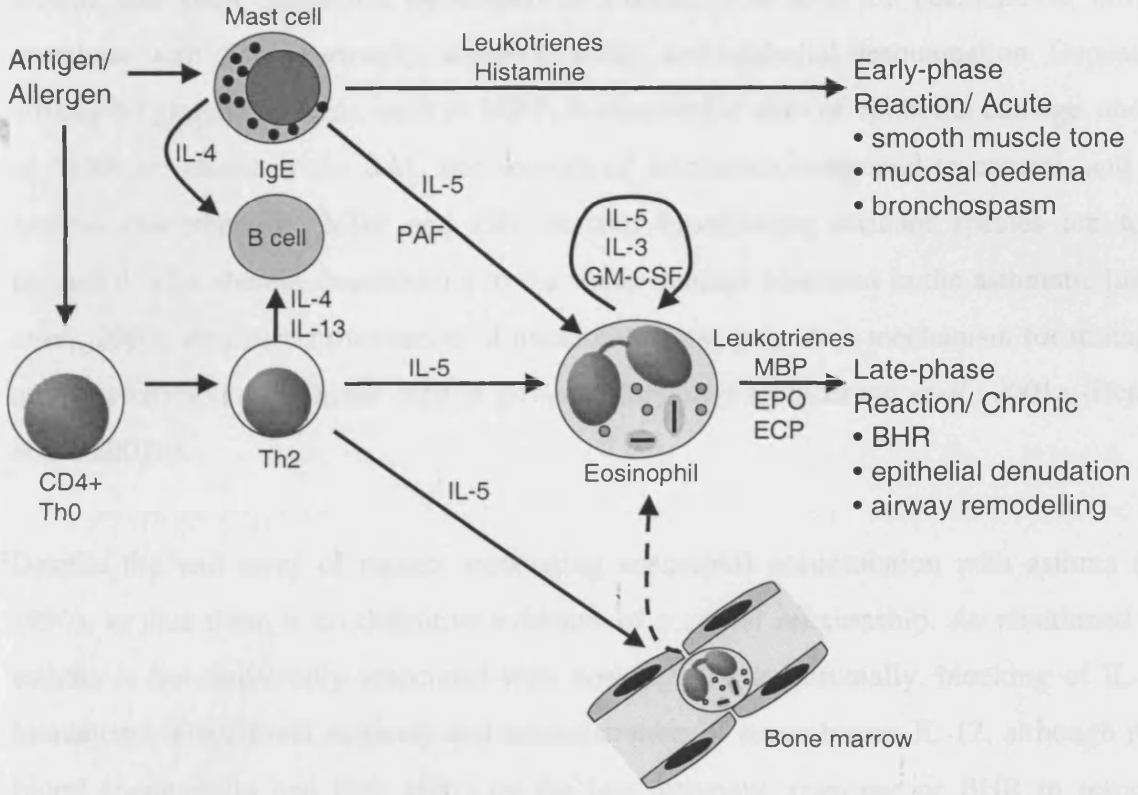

Figure 1.2. Multicellular, multifactoral basis of the early- and late-phase reactions of bronchial asthma.

'Intrinsic' (nonatopic) asthma is distinct from extrinsic (atopic) asthma as patients have total serum IgE levels within the normal range and do not respond to common aeroallergens by skin test. However, intrinsic asthma has a similar pathophysiology to extrinsic asthma, both being associated with eosinophilic infiltration of the bronchial mucosa. Also, an increase in the number of cells bearing the high-affinity IgE receptor (FcεRI) and recruitment of cells expressing Th2 cytokines was observed in both atopic and nonatopic asthma, indicating similarities in the immunopathogenesis of these clinically distinct forms of the disease (Humbert *et al.*, 1996a; Humbert *et al.*, 1996b).

The generation of a Th2 response and the consequent microenvironment of eosinophil active cytokines causes the selective accumulation and persistence of eosinophils within the asthmatic airway. Tissue, blood and BAL eosinophilia are characteristic of both allergic and non-allergic asthma, although it is important to note that in some cases the bronchi of acute asthmatics fail to exhibit marked eosinophilia. An association of eosinophils with allergic asthma has been supported by numerous studies. The level of eosinophilic infiltration correlates with disease severity, degree of BHR, and epithelial desquamation. Deposition of eosinophil granule proteins, such as MBP, is observed at sites of epithelial damage, and levels of MBP are raised in the BAL and sputum of asthmatics compared to control, and during asthma exacerbations. MBP and EPO-derived brominating oxidant species are toxic to epithelial cells, thereby contributing to the tissue damage observed in the asthmatic lung (Wu *et al.*, 2000), and such bromination of nucleotides may provide a mechanism for mutagenesis and cytotoxicity of epithelial cells at inflammatory sites (Henderson *et al.*, 2001a; Henderson *et al.*, 2001b).

Despite the vast array of reports associating eosinophil accumulation with asthma (Gleich 1990), to date there is no definitive evidence of a causal relationship. As mentioned above, asthma is not universally associated with eosinophilia and crucially, blocking of IL-5 with humanised monoclonal antibody and administration of recombinant IL-12, although reduced blood eosinophilia had little effect on the late asthmatic response or BHR in response to histamine (Bryan *et al.*, 2000; Leckie *et al.*, 2000). Therefore it remains to be seen whether eosinophils have a role in the aetiology of asthma.

1.3.3. Eosinophils and cancer

Eosinophilia has been observed in both hematologic (such as Hodgkin's disease, acute myelogenous leukaemia, and plasma cell myeloma), but also non-hematologic tumours (such as colon, breast, and lung) (reviewed by Samoszuk, 1997). Although some reports indicate that tumour-associated eosinophilia is beneficial (Bethwaite *et al.*, 1993; Dalal *et al.*, 1992; Goldsmith *et al.*, 1992), and eosinophil-derived mediators have been shown to be toxic to some tumour cells, evidence suggests that the infiltration of tumours with eosinophils has little or no effect on the prognosis of the disease (Pastrnak and Jansa, 1984). Rather, eosinophils are implicated to play a role in tissue regrowth and fibrosis following tumour invasion, attracted to the site of tumourgenesis by the chemoattractant-rich microenvironment,

including IgE and IL-5, detectable in lymphomas (Bassett, 1983; Bassett, 1962; Samoszuk and Ramzi, 1993; Samoszuk *et al.*, 1993).

1.4. Apoptosis

Apoptosis is a genetically programmed, physiologically distinct form of cell death, vital in the homeostasis and normal development of multicellular organisms (Ellis *et al.*, 1991; Steller, 1995). Defects in this process are major components of the aetiology of auto-immune disease, cancer and degenerative diseases of the immune system (Thompson, 1995). The term 'apoptosis' was first coined and defined by Kerr *et al* (1972) as a conserved, ordered pathway of cell death characterised by distinct morphological features, including chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing, and fragmentation of the cell into discrete 'apoptotic bodies' which were subsequently engulfed by phagocytes. It is now well recognised that the ability to ablate damaged misplaced or supernumerary cells is an essential aspect of animal development (Meier *et al.*, 2000). Major advances in our understanding of this complex process have come from studies performed in the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986), and the fruitfly, *Drosophila melanogaster* (Bangs *et al.*, 2000). It is evident that the basic apoptotic machinery is remarkably conserved throughout metazoan evolution, with the majority of the proteins that regulate *C. elegans* apoptosis finding homologues within the mammalian system.

A crucial aspect of apoptosis is that cell membrane integrity is maintained throughout, with the disassembly of the cell into 'apoptotic bodies' allowing clearance by resident phagocytes without the release of histotoxic and inflammatory mediators. This is in stark contrast to the alternative death process of necrosis, whereby the cell loses osmotic control, eventually undergoing cell lysis. This facet of apoptotic cell death is all too often overlooked, but is of vital importance especially in regard to inflammatory cells such as eosinophils and neutrophils and their battery of cytotoxic components. The major physiological differences between apoptotic and necrotic cell death are outlined in Table 1.1.

Apoptosis can be divided into three phases initiation, effector and execution. The initiation phase is dependent upon reception of an apoptotic stimulus such as growth factor deprivation, DNA damage and ligation of cell surface death receptors such as TNFα and CD95 (Fas/ Apo-1) (Bratton *et al.*, 2000). The effector phase, during which the apoptotic process is regulated

and the cell is committed to die, is regulated by the evolutionarily conserved Bcl-2 family of anti- and pro-apoptotic proteins (Kroemer, 1997). The effector phase also holds a critical role for the mitochondria as central to the apoptotic pathway (Green and Reed, 1998).

Table 1.1. Morphological characteristics of mechanisms of cell death.

Apoptosis	Necrosis
Internucleosomal fragmentation and compaction of chromatin	Ill-defined chromatin degradation
Cytoplasmic condensation and cell shrinkage	Osmotic cell expansion and lysis
Membrane blebbing without loss of integrity	Loss of membrane integrity
Retention of cytotoxic mediators	Cytotoxic mediator release
Redistribution of membrane phospholipids	
Phagocytotic clearance of intact 'apoptotic bodies'	
No inflammatory response	Significant inflammatory response

The execution phase involves protein degradation and segregation of the cell into distinct vesicles for phagocytosis (Savill and Fadok, 2000). Degradation of the cell is mediated by a conserved family of aspartate-specific cysteine proteases, known as the caspases, which cleave a number of cellular targets, and are responsible for most of the morphological features characteristic of apoptosis (Cohen, 1997). As such, caspases are considered the executioners of apoptotic cell death.

1.4.1. Mitochondria and apoptosis

Traditionally viewed as the 'powerhouses' of the cell due to their central role in oxidative phosphorylation and ATP generation, recent evidence has implicated mitochondria as major effector organelles in apoptosis (Green and Reed, 1998). A role for mitochondria in cell death was first indicated by the observation that the organelle was absolutely required for cytosolic extracts to induce apoptotic changes in *Xenopus* nuclei (Newmeyer *et al.*, 1994). Since, it has been shown that the release of the electron carrier, cytochrome *c*, from the intermembrane space of mitochondria is a crucial event during apoptosis and is required for the activation of

caspase-9 (Li *et al.*, 1997) and therefore subsequent downstream caspases in the cytosol (see below). Although cytochrome *c* release is an almost universal feature of apoptosis, numerous factors are released from the mitochondria during apoptosis, including apoptosis-inducing factor (AIF), which induces caspase-independent apoptotic nuclear changes (Susin *et al.*, 1999b), procaspase-2, and –9 (Susin *et al.*, 1999a), heat shock proteins (Samali *et al.*, 1999), and Smac/ DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000). The mechanism by which cytochrome *c* is released from the intermembrane space is unclear, although an integral role for the Bcl-2 family proteins is implicated and discussed below.

1.4.2. Bcl-2 homologues

The *bcl-2* gene was originally identified at the chromosomal breakpoint of t(14; 18)-bearing B-cell lymphomas (Tsujimoto and Croce, 1986; Tsujimoto *et al.*, 1985). Bcl-2 is the mammalian homologue of *C. elegans* death-suppressor Ced-9 (Hengartner and Horvitz, 1994), and belongs to a large family of proteins associated due to homology within four distinct regions of their primary structure known as Bcl-2 homology (BH) domains 1-4 (reviewed by Antonsson and Martinou, 2000). They often have a C-terminal transmembrane domain, which allows targeting to intracellular membranes, such as endoplasmic reticulum, outer mitochondrial membrane and outer leaflet of the nuclear membrane. Based on structure and function, members of the Bcl-2 family can be subdivided into three groups (Fig. 1.3):

Group I/ Anti-apoptotic- (e.g. Bcl-2, Bcl-x_L, and Bcl-w) Group I Bcl-2 proteins are characterised as having anti-apoptotic activity, bearing four BH domains 1-4, all of which seemingly required for anti-apoptotic activity (Boise *et al.*, 1993; Gibson *et al.*, 1996).

Group II/ Pro-apoptotic- (e.g. Bax, Bcl-x_S, Bak, and Bad) Group II proteins predominantly have similar structures to Group I members, but contrastingly are pro-apoptotic (Boise *et al.*, 1993; Kiefer *et al.*, 1995; Oltvai *et al.*, 1993). Structure and function analysis indicates that the divergence of function is determined by the α-helices 5 and 6, which are involved in pore-forming activity. The BH3 domain of pro-apoptotic Bcl-2 homologues is both necessary and sufficient to induce apoptosis (Wang *et al.*, 1998; Holinger *et al.*, 1999).

Group III/ BH3 only proteins- (e.g. Bik, Bim, and Bid) Group III members are potent inducers of cell death, but are structurally more divergent. With the exception of the conserved BH3

domain, their primary structures bear little resemblance to Bcl-2 family or any other known proteins (Boyd *et al.*, 1995; O'Connor *et al.*, 1998; Yang *et al.*, 1995).

1.4.2.1. BH domains and homologue dimerisation

The conserved BH domains facilitate homo and heterodimerisation of Bcl-2 proteins (Yin *et al.*, 1994), and the susceptibility of a cell to an apoptotic stimulus is governed, at least in part, by the relative abundance of pro- and anti-apoptotic proteins, and their resultant interaction (Oltvai *et al.*, 1993). BH domains 1-3 of Bcl-2 and Bcl-x_L are crucial for heterodimerisation with pro-apoptotic Bax and subsequent suppression of apoptosis (Sedlak *et al.*, 1995). However, it has also been shown that disruption of heterodimerisation between Bax and Bcl-x_L, by mutation of these domains, does not completely inhibit the protective effect of Bcl-x_L, indicating that heterodimerisation and death-repression were to a certain extent independent (Simonian *et al.*, 1996). Bcl-2 proteins bearing truncation of their C-terminal hydrophobic domain are still observed to associate, albeit to a lesser degree, with intracellular membranes, implicating that heteromeric interaction occurs at these membranes. Nuclear magnetic resonance (NMR) spectroscopic analysis has shown that the α-helices that make up BH1-3 domains form a hydrophobic cleft on the surface of Bcl-x_L. This cleft forms the docking-site for the amphipathic BH3 domain of pro-apoptotic homologues, such as Bid, Bax, and Bak (Sattler *et al.*, 1997). The BH4 domain is conserved amongst anti-apoptotic proteins, but less conserved or absent from pro-apoptotic members. Deletion of this domain from Bcl-2 has no effect on dimerisation *in vivo* (Hanada *et al.*, 1995), but rather imparts a dominant-negative phenotype, converting the protein from death-inhibitor to death-inducer (Hunter *et al.*, 1996), further supporting the separation of Bcl-2 homologue heterodimerisation from their role in repressing apoptosis. It is also important to note that the BH4 region appears critical for the association of Bcl-2 proteins with unrelated proteins, such as Raf-1 (Wang *et al.*, 1996).

1.4.2.2. Bcl-2 proteins and cytochrome *c* release

Despite recent advances in determining structure and function of the Bcl-2 homologues, the precise mechanism by which these proteins regulate apoptosis remains unclear. Their main function in controlling apoptosis appears to be regulating the permeability of the outer

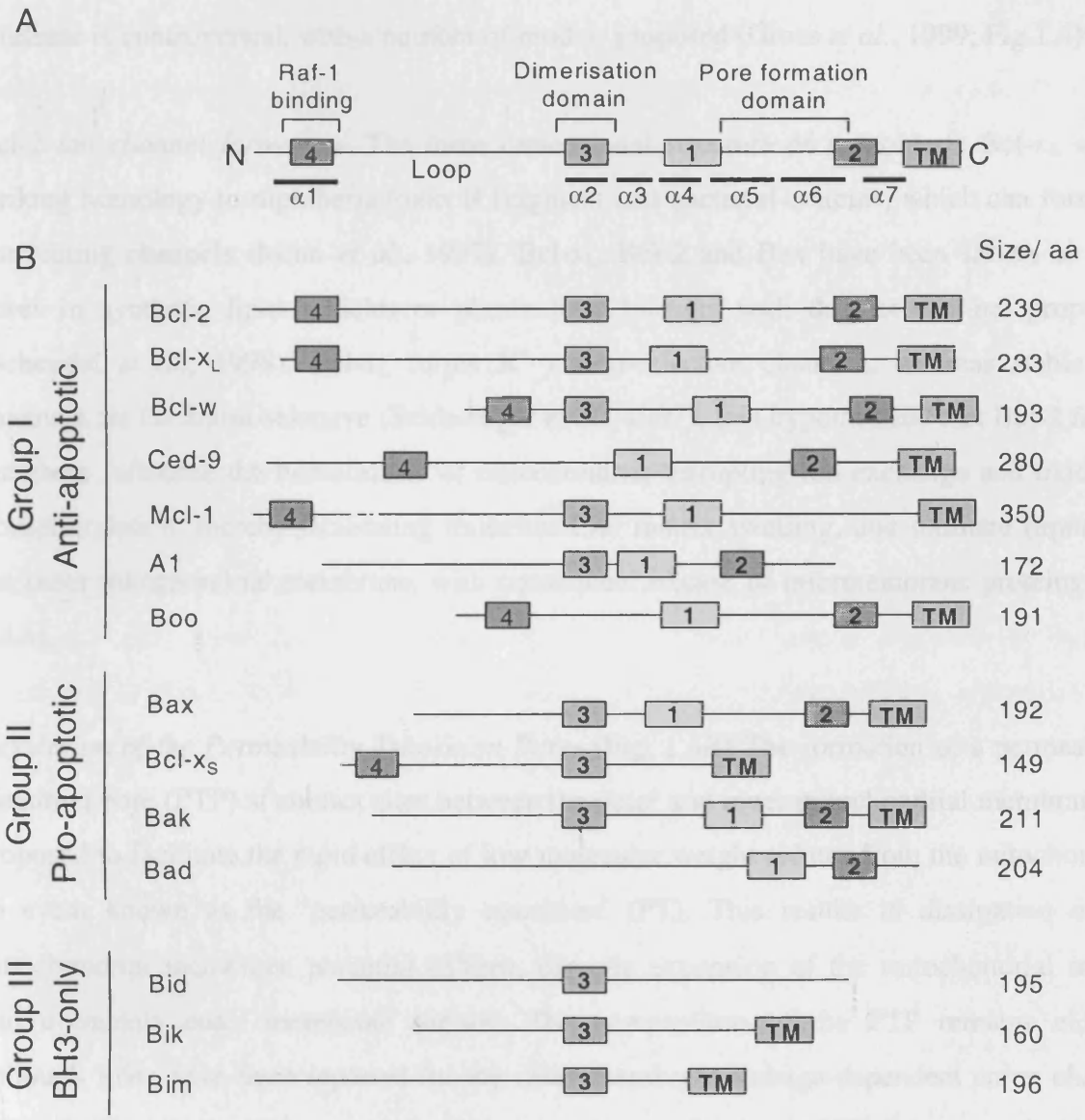

Figure 1.3. Structural comparison of the Bcl-2 family proteins. A) Topology of Bcl-2, depicting the Bcl-2 homology (BH) domains, location of α-helices, and transmembrane (TM) region. Bcl-2 functions are dependent on distinct α-helical segments. B) The domain maps of the Bcl-2 homologues are represented showing the relative positions of the BH domains 1-4 and TM region. The Bcl-2 family can be divided into three groups, I (Anti-apoptotic), II (Pro-apoptotic), III (BH3 only) based on structure and function.

mitochondrial membrane, and the resultant release of the apoptogenic factor cytochrome *c*. Overexpression of Bcl-2 and Bcl-x_L prevents cytochrome *c* release in response to numerous cell death stimuli, and requires association of the anti-apoptotic proteins with the mitochondrial membrane. Conversely, pro-apoptotic members such as Bax directly induce cytochrome *c* release from isolated mitochondria, a process blocked by Bcl-x_L (Jurgensmeier

et al., 1998; Rossé *et al.*, 1998). The mechanism by which Bcl-2 proteins mediate cytochrome *c* release is controversial, with a number of models proposed (Gross *et al.*, 1999; Fig.1.4):

Bcl-2 ion channel formation- The three dimensional structure of monomeric Bcl-x_L shows striking homology to diphtheria toxin B fragment and bacterial colicins, which can form ion conducting channels (Minn *et al.*, 1997). Bcl-x_L, Bcl-2 and Bax have been shown to form pores in synthetic lipid vesicles or planar lipid bilayers with distinct channel properties (Schendel *et al.*, 1998). Bcl-x_L forms K^+ cation-selective channels, whereas stable Bax channels are Cl^- anion-selective (Schlesinger *et al.*, 1997). It is hypothesised that Bcl-2 family members influence the homeostasis of mitochondria, disrupting ion exchange and oxidative phosphorylation, thereby facilitating mitochondrial matrix swelling, and ultimate rupture of the outer mitochondrial membrane, with consequent release of intermembrane proteins (Fig. 1.4A).

Regulation of the Permeability Transition Pore- (Fig. 1.4B) The formation of a permeability transition pore (PTP) at contact sites between the outer and inner mitochondrial membranes is proposed to facilitate the rapid efflux of low molecular weight solutes from the mitochondria, an event known as the 'permeability transition' (PT). This results in dissipation of the mitochondrial membrane potential ($\Delta\Psi m$), osmotic expansion of the mitochondrial matrix and ultimately outer membrane rupture. The composition of the PTP remains elusive, although roles have been reported for the outer membrane voltage-dependent anion channel (VDAC), the inner membrane adenosine nucleotide translocator (ANT) (Marzo *et al.*, 1998a), and the matrix protein, cyclophilin D. A role for Bcl-2 proteins in regulating the PTP has been proposed (Marzo *et al.*, 1998), supported by the aggregated distribution of Bcl-2 at the mitochondrial membrane contact points. Also, Bax directly associates with VDAC in the PTP and induces loss of $\Delta\Psi m$, mitochondrial swelling and release of cytochrome *c* (Narita *et al.*, 1998; Shimizu *et al.*, 1999). However, at present there is conflicting data regarding the role of PTP in cytochrome *c* release. There is evidence that Bax induces cytochrome *c* release in the absence of, or preceding, mitochondrial membrane depolarisation, by a mechanism that is insensitive to the PTP inhibitors cyclosporin A (CsA, ligand of cyclophilin A) and bongkrekic acid (antagonising ligand of ANT) (Eskes *et al.*, 1998). It has therefore been argued that PTP formation and the resulting dissipation of transmembrane potential is a consequence of cytochrome *c* release as opposed to a cause. Lack of Bax-induced PTP opening may have been

due to lack of Ca^{2+} in the system, a cation essential for permeability transition and subsequent $\Delta\Psi m$ loss, and that in physiological concentrations of Ca^{2+} Bax induces both PT and dissipation of membrane potential. Although the rupture of the outer mitochondrial membrane is an attractive explanation for the release of the variety of intermembrane proteins during apoptosis, current evidence suggests that PT and depolarisation of the mitochondria occur downstream of cytochrome *c* release, possibly involving caspase-dependent PTP opening, or inhibition of electron transport as a result of the cytochrome *c* efflux (Gross *et al.*, 1999).

Bcl-2 megachannel formation- The recognised membrane targeting and channel forming ability of Bcl-2 members lead to the suggestion that pro-apoptotic Bcl-2 proteins formed cytochrome *c* specific pores in the mitochondrial membrane (Fig. 1.4C). This argument was countered by the fact that Bcl-2, Bcl-x_L and Bax channels appeared to be too small to allow passage of cytochrome *c* (~14 kDa). However, recently, Antonsson *et al* (2001) reported the occurrence of Bax in the mitochondrial membrane as high molecular weight oligomers, which did not involve either VDAC or ANT, and postulated the formation of a Bax megachannel sufficiently large to facilitate cytochrome *c* efflux from the intermembrane space.

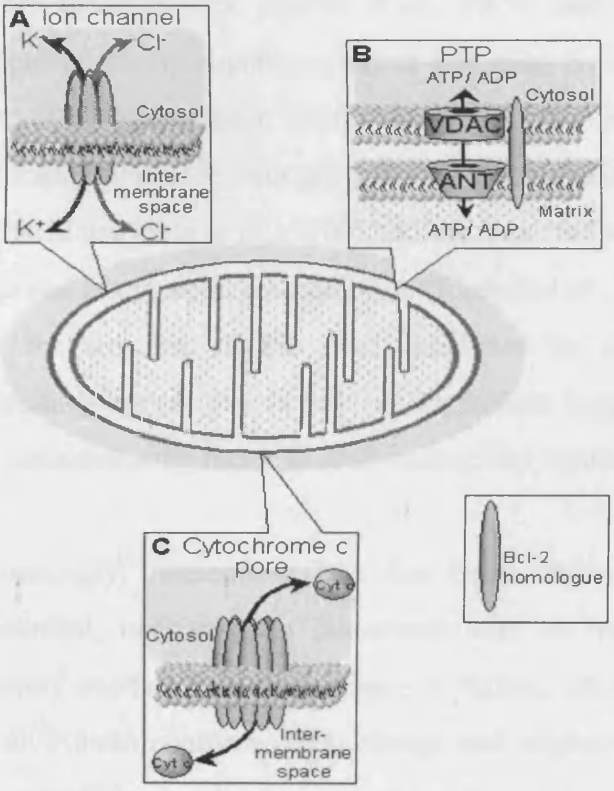

Figure 1.4. Bcl-2 homologue-mediated mechanisms for regulation of cytochrome *c* release. Various mechanisms for Bcl-2 homologue-mediated cytochrome *c* release have been proposed. The formation of an ion selective pore (A) or interaction with the permeability transition pore (PTP) (B) facilitates transmembrane ionic imbalance and ultimately outer membrane rupture, resulting in release of inter-membrane components. Alternatively, formation of a cytochrome *c* channel by pro-apoptotic Bcl-2 homologues such as Bax and Bak may allow selective release of cytochrome *c* (C).

1.4.2.3. Bax subcellular localisation and activation

Bax was first identified due to its heterodimerisation with Bcl-2, and over-expression resulted in the induction of apoptosis, leading to its description as a death agonist (Oltvai *et al.*, 1993). A 21 kDa protein with the ability to form high molecular weight homo-oligomers, Bax is widely expressed in tissues. Its activity is governed by its conformational state and intracellular localisation. In a non-apoptotic cell, Bax normally resides in the cytoplasm in soluble, monomeric form (Hsu and Youle, 1998). Following an apoptotic stimulus, Bax undergoes a conformational change that permits both dimerisation and insertion into the mitochondrial membrane (Gross *et al.*, 1998; Wolter *et al.*, 1997), where it is proposed to facilitate apoptogenic factor release by an as yet unknown mechanism (see above). Recent elucidation of the three-dimensional structure of Bax by NMR has shed new light on the structural changes required for the activation of this potent pro-apoptotic protein (Fig. 1.5). Bax consists of 9 α-helices, of which the structure of α1- α8 closely resembles the structure of Bcl-x_L (Suzuki *et al.*, 2000). The C-terminal α9 encodes the putative transmembrane domain and occupies the BH1-3 hydrophobic cleft, which mediates heterodimer formation. In this conformation, Bax has significant structural homology to Bcl-x_L bound with the BH3 domain of pro-apoptotic Bak (Sattler *et al.*, 1997), and similarly to Bcl-x_L, to exert its effect on apoptosis, the hydrophobic cleft of Bax must be unoccupied, therefore disengagement of α9 from the hydrophobic cleft facilitates both membrane insertion and oligomerisation. Alteration in intracellular pH (pHi) has been observed in a number of cell types (Khaled *et al.*, 1999; Matsuyama *et al.*, 2000) and the Bax conformational change was proposed to occur in response to cytosolic alkalinisation (Khaled *et al.*, 1999). However, Suzuki *et al* (2000) report that the structure of Bax was unaffected by pH, and although such conditions do not necessarily reflect events *in vivo*, the authors suggest that an unknown energy-driven step is the predominating factor in determining Bax conformation, and not pHi.

Interestingly, monomeric Bax has been observed to associate with the mitochondrial membrane, indicating an interaction with an unknown mitochondrial membrane protein, possibly another Bcl-2 homologue or VDAC. However, insertion into the membrane cannot occur without conformational change and oligomerisation. A similar situation is reflected in the subcellular localisation of various Bcl-2 proteins, such as Bcl-x_L, Bcl-2, and Bak, where the putative transmembrane domain is not absolutely required for membrane association.

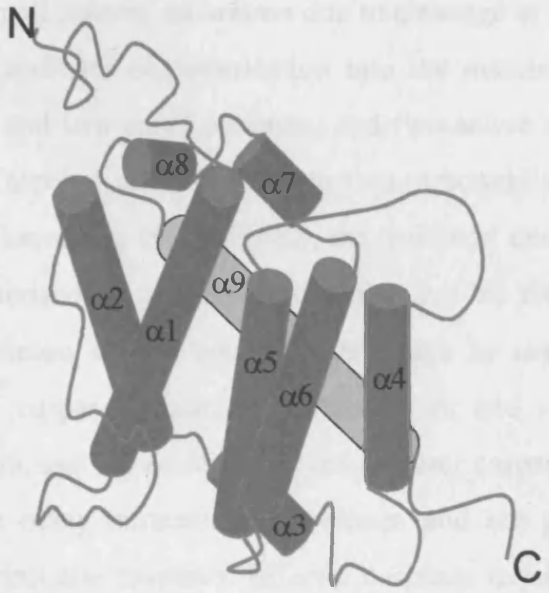

Figure 1.5. Model of Bax structure. Bax consists of 9 α-helices as deduced by NMR analysis (Suzuki *et al.*, 2000). The long C-terminal α9 occupies the hydrophobic cleft in the monomeric, soluble form of Bax.

1.4.3. Caspases

Caspases, previously known as Interleukin-1β-converting enzyme (ICE)-like proteases, are evolutionarily conserved aspartate-specific cysteine proteases. Homologues of *C. elegans* Ced-3, thirteen mammalian caspases have so far been identified, with the majority implicated in the apoptotic process (Nicholson and Thornberry, 1997). Most of the morphological and biochemical features that characterise an apoptotic cell can be directly attributed to the activity of caspases and their, near one hundred, potential substrates reported to date. For example, loss of cell shape and facilitation of cytoplasmic condensation is due to caspase cleavage of cytoskeletal components such as fodrin and gelsolin (Kothakota *et al.*, 1997), nuclear condensation is caused by caspase-mediated cleavage of nuclear lamins (Rao *et al.*, 1996), and the characteristic internucleosomal laddering is due to the caspase-3-dependent removal of the inhibitory subunit, iCAD or DNA fragmentation factor (DFF) from caspase-activated DNase (CAD) (Enari *et al.*, 1998; Liu *et al.*, 1997).

Synthesised as proenzymes, caspases consist of three main subunits, an N-terminal prodomain, and the large (p20) and small domains (p10), which contribute to the active

enzyme (Cohen, 1997; Fig. 1.6) Activation of caspases involves removal of the prodomain, followed by large and small subunit separation due to cleavage at specific aspartate residues. This ordered cleavage facilitates oligomerisation into the mature heterotetrameric caspase, consisting of two large and two small subunits, and two active sites (Walker *et al.*, 1994; Wilson *et al.*, 1994). Caspases are divided into two structurally and functionally distinct groups (Thornberry and Lazebnik, 1998). Firstly, the 'initiator' caspases, such as caspase-8, -9, and –10, are characterised by a long prodomain, and as the name suggests are held responsible for the initiation of the apoptotic pathways in response to various stimuli. Secondly, the 'effector' caspases, such as caspase-3, -6, and –7, are characterised by a relatively short prodomain, and are activated by the initiator caspases (Orth *et al.*, 1996). The effector caspases cleave many intracellular substrates and are generally more active and abundant than upstream initiator caspases. Effector caspases require processing by upstream caspases prior to becoming active, and are therefore rigidly constrained. In contrast, processing of initiator caspases is neither sufficient nor necessary, with activation determined by cofactor association. This is reflected in the 'zymogenicity' of caspases, the ratio of activity of the processed enzyme to the activity of the proenzyme (Salvesen and Dixit, 1999). Table 1.2 compares the zymogenicities of two effector caspases, two initiator caspases, and two proteases of the chymotrypsin family. tPA (tissue plasminogen activator) and trypsin are initiator and effector respectively of the fibrolytic pathway (Tachias and Madison, 1996). Like caspase-9, tPA requires an allosteric regulator, fibrin, for its activation.

1.4.3.1. Caspase activation

There are three general mechanisms by which caspases are activated in mammalian cells:

Induced proximity- In response to death stimuli, initiator caspases can be recruited to a molecular scaffold, thereby increasing the local concentration of caspases. Under such conditions of induced proximity it is postulated that the low intrinsic activity of the procaspases is sufficient to allow transcatalysis into fully active proteases. Such a model has been implicated in the processing and activation of caspase-1, -2, -8, -10, and *C. elegans,* Ced-3 (Muzio *et al.*, 1998; Salvesen and Dixit, 1999; Yang *et al.*, 1998). Caspase-8 is the major initiator caspase in death-receptor mediated or 'extrinsic' apoptosis (Sun *et al.*, 1999). Ligation of TNF-R1 and CD95 (Apo-1/ Fas) with TNF-α and Fas ligand (FasL) respectively

results in trimerisation of the receptor subunits at the cell surface, triggering the interaction via death domains (DD) with adaptor proteins such as TRADD (TNF-receptor associated death

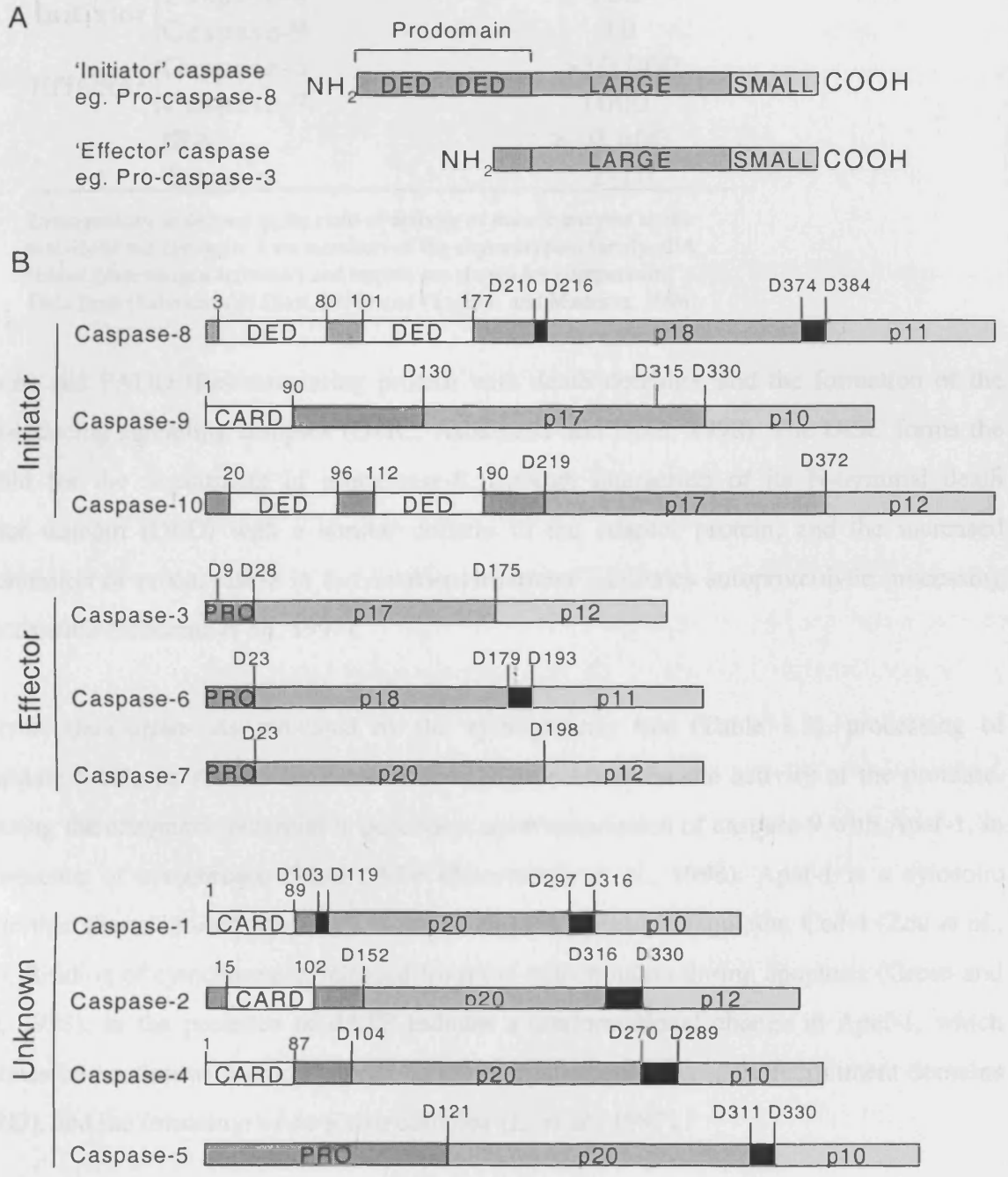

Figure 1.6. Zymogen organisation of the caspases. A) Caspases are synthesised as proenzymes consisting of a prodomain (PRO), and a large and a small subunit often separated by a linker peptide (*black box*). Caspases are subdivided into two structurally and functionally distinct groups, 'initiator' or 'effector', characterised by long and short prodomains respectively. B) Proenzyme organisation of mammalian caspases. Caspase-8 and –10 contain N-terminal death effector domains (DED) involved in interaction with adaptor molecules such as FADD. Caspase-9, -1, -2, and –4 have N-terminal caspase recruitment domains (CARD) involved in interaction with other caspases or adaptor proteins such as IAPs (see text for details).

Table 1.2. Zymogenicity of caspases compared with two serine proteases.

	Protease	Zymogenicity
Initiator	Caspase-8	100
	Caspase-9	10
Effector	Caspase-3	>10,000
	Caspase-7	1000
	tPA	>10,000
	Trypsin	2-10

Zymogenicity is defined as the ratio of activity of mature enzyme to the activity of the zymogen. Two members of the chymotrypsin family, tPA (tissue plasminogen activator) and trypsin are shown for comparison. Data from (Salvesen and Dixit, 1999) and (Tachias and Madison, 1996).

domain) and FADD (Fas-associating protein with death domain), and the formation of the death-inducing signalling complex (DISC; Ashkenazi and Dixit, 1998). The DISC forms the scaffold for the recruitment of procaspase-8, through interaction of its N-terminal death effector domain (DED) with a similar domain in the adaptor protein, and the increased concentration of procaspase-8 in the microenvironment facilitates autoproteolytic processing and activation (Medema *et al.*, 1997).

Cofactor association- As indicated by the zymogenicity rate (Table 1.2), processing of procaspase-9 into its mature form has relatively little effect on the activity of the protease. Releasing the enzymatic potential is dependent upon association of caspase-9 with Apaf-1, in the presence of cytochrome *c* and dATP (Srinivasula *et al.*, 1998). Apaf-1 is a cytosolic protein that shares limited homology to the *C. elegans* apoptosis regulator, Ced-4 (Zou *et al.*, 1997). Binding of cytochrome *c*, released from the mitochondria during apoptosis (Green and Reed, 1998), in the presence of dATP induces a conformational change in Apaf-1, which facilitates interaction with procaspase-9 via their complementary caspase recruitment domains (CARD), and the formation of an active complex (Li *et al.*, 1997).

Activation by upstream caspases- It was observed that proteolytic processing of most caspases occurs at Asp-X sites, potential caspase cleavage sites, between the large (p20) and small (p10) fragments, which indicated that some caspases are activated by autoproteolysis (Thornberry and Lazebnik, 1998). Although effector caspase-3, -6, and –7 have been shown to self activate *in vitro*, no evidence to date supports such a mechanism *in vivo*. Instead, the

major pathway for caspase activation involves proteolytic processing dependent on an upstream caspase, and the formation of a 'caspase cascade' (Slee *et al.*, 1999; Fig.1.7).

Two distinct cascades of caspase activation have been identified (Fig. 1.7) dependent upon whether apoptosis is induced by a cell surface death receptor ('extrinsic') or intracellular stress ('intrinsic') (Bratton *et al.*, 2000; Sun *et al.*, 1999).

1.4.3.2. Stress-induced apoptosis

In response to numerous 'stress' stimuli, such as growth factor withdrawal, chemical treatment (e.g. staurosporine, etoposide) and DNA damage, as yet undetermined intracellular signals induce perturbations of the mitochondrial membrane, facilitating the release of apoptogenic factors such as AIF (Susin *et al.*, 1999b) and cytochrome *c* (Liu *et al.*, 1996). As discussed above the mechanism by which these factors gain release is unclear, although the Bcl-2 proteins appear vital in regulating the phenomenon. Upon release, cytoplasmic holo-cytochrome *c* (with haem group attached) interacts with Apaf-1, dATP/ ATP, and pro-caspase-9 to form a high molecular weight (~700 kDa) complex termed the 'apoptosome' (Cain *et al.*, 1999). Apaf-1 is a 130 kDa protein and the first identified homologue of *C. elegans* apoptosis regulator, Ced-4. Its N-terminus has homology with the prodomains of several caspases including caspase-1, -2, and –9, and functions as the caspase recruitment domain (CARD). The central portion of Apaf-1 bears the structural and functional homology with Ced-4, being a nucleotide-binding domain, essential for both Apaf-1 and Ced-4 function (Zou *et al.*, 1997). The C-terminus is made up of 12-13 WD-40 repeats, which mediate protein-protein interaction. Apaf-1 requires both cytochrome *c* and ATP as cofactors to enable the formation of a multimeric complex capable of binding (in an Apaf-1: procaspase-9 ratio of 1:1) and activation of procaspase-9. Mutation of the caspase-9 active site prevents complete activation of procaspase-9, even after recruitment to the apoptosome, suggesting that Apaf-1-mediated activation of procaspase-9 occurs by autocatalysis. Active caspase-9 is then released from the complex allowing activation of downstream effector caspase-3 and –7.

1.4.3.3. Receptor-mediated apoptosis

Cell surface death-receptors are transmembrane receptors expressed by many cells, which upon binding of their specific ligand, induce rapid caspase activation and apoptosis. Death receptors are members of the tumour necrosis factor (TNF) receptor superfamily and include

CD95 (Fas/ Apo-1), TNFR1 and the TRAIL (TNF-related apoptosis-inducing ligand) receptors 1 and 2. They have a cysteine-rich extracellular region, and a conserved intracellular DD that is required for apoptotic signalling. Ligation of the receptors with cognate ligand induces receptor trimerisation and subsequent recruitment of adaptor proteins (Ashkenazi and Dixit, 1998). Such adaptor proteins have a C-terminal DD and an N-terminal DED, which allow binding of the trimerised receptor and procaspase-8 respectively. Ligation of CD95 (Fas/ Apo-1) with FasL induces binding of adaptor protein FADD, which in turn induces recruitment of procaspase-8 to the complex (DISC). TNF-α induces TNFR1 to recruit TRADD, which consequently binds FADD. The formation of this multiprotein DISC complex results in transcatalysis of procaspase-8 by the induced proximity model described previously. Caspase-8 is the major apical caspase of receptor-induced apoptosis, although caspase-10 is also recruited following ligation of TNFR1 and TRAIL, and so may also be an important caspase in some receptor-mediated caspase-cascades (Fernandes-Alnemri *et al.*, 1996). Trimerised TNFR1 also recruits serine-threonine kinase RIP (receptor-interacting protein) and TRAF2 (TNF receptor-associated factor 2), which activate NF-κB and JNK signalling pathways respectively (Reinhard *et al.*, 1997; Kasof *et al.*, 2000). Caspase-8 appears to be a pan-caspase activator, as it is capable of activating all caspases tested *in vitro*. Therefore recruitment and activation of procaspase-8 induces downstream activation of effector caspases-3 and –7. Caspase-8 also cleaves a pro-apoptotic Bcl-2 homologue, the BH3 only protein Bid. The truncated form of Bid (tBid) translocates to the mitochondria and induces the release of cytochrome *c*, thereby providing a link between the receptor-mediated and stress-induced caspase cascades, possibly forming an amplification loop (Wei *et al.*, 2000). Interaction of Bid with Bax and Bak, mitochondrial perturbation, and consequent release of cytochrome *c* have been shown to be critical in receptor-induced apoptosis *in vitro* and *in vivo* (Srinivasula *et al.*, 1996). However, CD95-induced apoptosis can occur in the absence of cytochrome *c* release (Strasser *et al.*, 1995). It has been hypothesised that death receptors induce distinct pathways of apoptosis dependent upon cell type, being either dependent or independent upon mitochondrial cytochrome *c* release for effective initiation of the apoptotic programme (Scaffidi *et al.*, 1998).

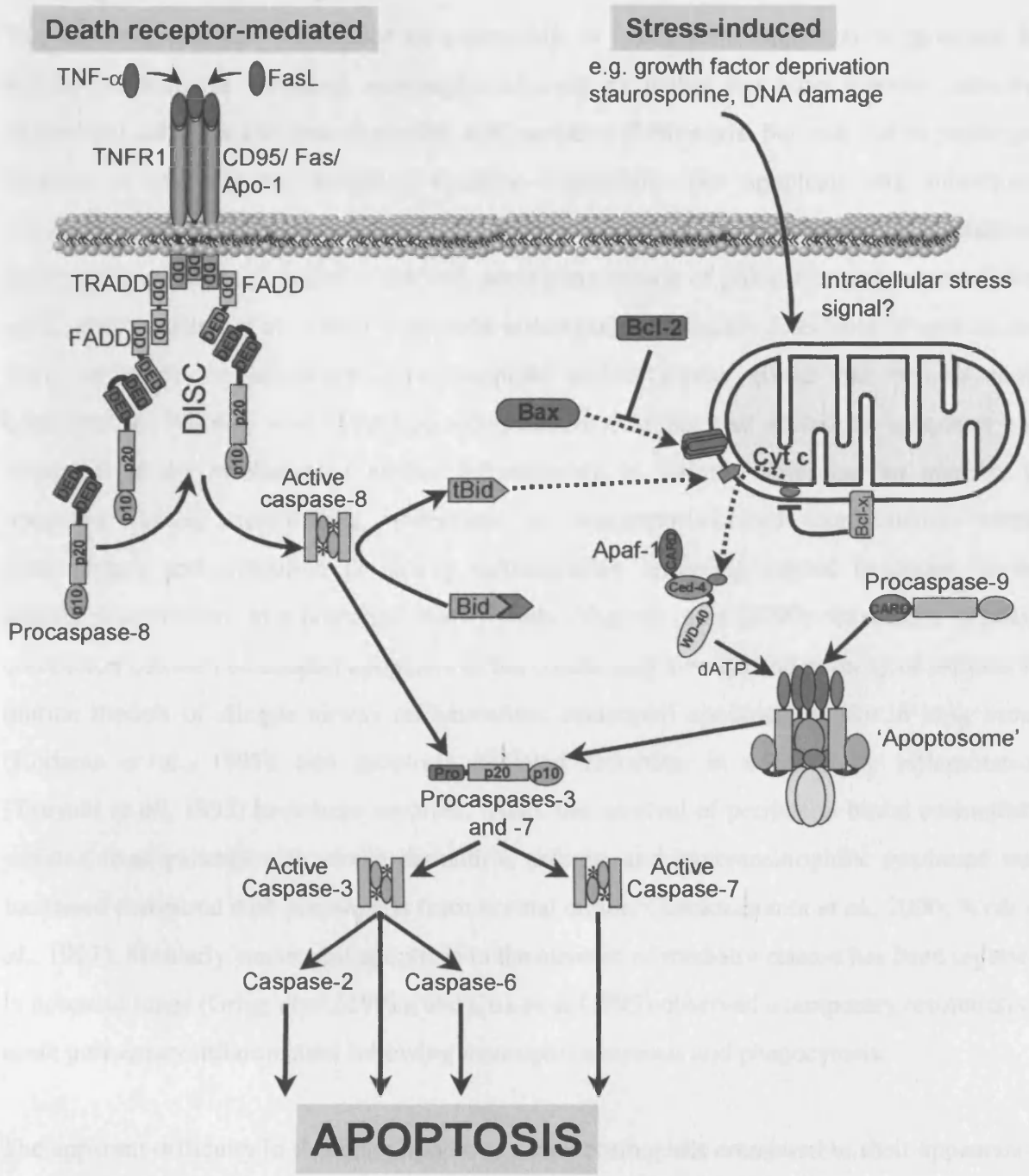

Figure 1.7. Distinct pathways of receptor-mediated and stress-induced apoptosis. Ligation of cell surface death receptors induce apical processing of caspase-8 and subsequent activation of effector caspases. Caspase-8 mediated cleavage of Bid induces release of cytochrome *c* from the mitochondria. Stress/ chemical induced apoptosis results in perturbation of the mitochondria and cytochrome *c* release, possibly involving Bax, causing apical processing of caspase-9 within the apoptosome complex (see text for details). Hatched line represents protein translocation.

1.5. Regulation of eosinophil survival

The accumulation and persistence of eosinophils at sites of inflammation is governed by several mechanisms including increased eosinopoiesis within the bone marrow, selective endothelial adhesion and transmigration, and increased chemotaxis, but also due to prolonged survival in response to prevailing cytokine conditions. The apoptosis and subsequent phagocytosis of granulocytes at inflammatory sites is proposed to be an important mechanism limiting the cytotoxic potential of the cell, preventing release of phlogistic components (Stern *et al.*, 1992, Haslett *et al.*, 1994). Apoptotic eosinophils are readily detectable in sputum and BAL, although the apoptosis of eosinophils within airway tissue has proven more controversial. Woolley *et al* (1996) provided *in vivo* evidence that eosinophil apoptosis was important in the resolution of airway inflammation in asthma, observing an increase in apoptotic airway eosinophils, detection of eosinophil-derived components within macrophages and resolution of airway inflammation following steroid treatment during asthma exacerbation. In a bronchial biopsy study, Vignola *et al* (1999) observed a negative correlation between eosinophil apoptosis in the conducting airways and severity of asthma. In murine models of allergic airway inflammation, eosinophil apoptosis *in situ* in lung tissue (Kodama *et al.*, 1998), and apoptosis-mediated reduction in eosinophilic inflammation (Tsuyuki *et al.*, 1995) have been reported. Also, the survival of peripheral blood eosinophils isolated from patients with atopic dermatitis, asthma, and hypereosinophilic syndrome was increased compared with eosinophils from normal donors (Kankaanranta *et al.*, 2000; Wedi *et al.*, 1997). Similarly, neutrophil apoptosis in the absence of mediator release has been reported in neonatal lungs (Grigg *et al.*, 1991), and Cox *et al* (1995) observed a temporary resolution of acute pulmonary inflammation following neutrophil apoptosis and phagocytosis.

The apparent difficulty in detecting apoptotic tissue eosinophils compared to their appearance in the airway lumen has lead to questioning the *in vivo* relevance of this mechanism in asthma (Erjefält *et al.*, 1999). It has been argued that the difficulty in detecting eosinophils exhibiting apoptotic morphology (condensed cytoplasm and nucleus) *in situ* is due to the efficient phagocytosis by resident macrophages, which are capable of recognising membrane alterations of an apoptotic cell that precede nuclear and cytoplasmic condensation. However, an alternative hypothesis has been proposed, whereby luminal entry (and subsequent apoptosis thereby explaining the appearance of apoptotic eosinophils in sputum and BAL) is the major

mechanism responsible for the removal of viable eosinophils from the asthmatic airway (Erjefält and Persson, 2000).

1.5.1. Cytokines

Eosinophils are terminally differentiated cells, which undergo rapid apoptosis unless exposed to cytokines IL-5, IL-3, and GM-CSF both *in vitro* and *in vivo* (Simon *et al.*, 1997; Stern *et al.*, 1992; Tai *et al.*, 1991; Yamaguchi *et al.*, 1991). IL-5 is proposed to be a specific eosinophil survival factor, with increased IL-5, and consequently eosinophilia, associated with chronic allergic disorders. Also, IL-5- and GM-CSF-dependent suppression of eosinophil apoptosis has been observed in nasal polyps explants, used as a model for chronic airway inflammation (Simon *et al.*, 1997). The predominant source of survival enhancing hematopoietins is T lymphocytes, although mast cells and endothelial cells are also capable of their release. In conjunction with exogenous growth factor, eosinophils themselves also have the capacity to generate IL-3, IL-5, and GM-CSF. Interestingly, autocrine elaboration of IL-3 and GM-CSF in response to VLA-4-mediated fibronectin binding results in enhanced eosinophil survival *in vitro* (Anwar *et al.*, 1993; Walsh *et al.*, 1996), with preferential survival on tissue as opposed to plasma fibronectin (Walsh *et al.*, 1995).

TGF-β is a pleiotropic anti-inflammatory cytokine, which antagonises the effects of IL-5, IL-3, and GM-CSF on eosinophils, preventing activation, degranulation and survival (Alam *et al.*, 1994). TGF-β mRNA expression is detectable in eosinophils of chronically inflamed upper airways, implicating a potentially crucial role for eosinophil-derived TGF-β in modulating the chronic inflammatory response. High concentrations of IL-5 can partially overcome the pro-apoptotic effects of TGF-β, at least *in vitro*, suggesting a possible homeostasis of growth factors regulating eosinophil survival *in vivo*. The mechanism by which TGF-β blocks the effects of hematopoietins is unclear, although Pazdrak *et al* (1998) have reported inhibition of phosphorylation and activation of the pivotal IL-5 signal transduction tyrosine kinases Janus kinase (Jak)2 and Lyn.

1.5.1.1. IL-5 signal transduction

IL-5, IL-3 and GM-CSF receptors have distinct, ligand-specific α subunits, but share a βc subunit. The βc lacks kinase activity, but ligation of the IL-5Rα induces recruitment and activation of intracellular, juxtamembrane tyrosine kinases, possibly involving receptor

dimerisation or α and β subunit cooperation. In eosinophils, the IL-5 signal is propagated via Jak/ signal transducers and activators of transcription (STAT), and Ras-Raf-1-mitogen activated protein kinase (MAPK) cascades (Fig. 1.9A and B). The activation of Jak2, Lyn, and Syk tyrosine kinases, and Src homology 2 phosphatase 2 tyrosine phosphatase (SHPTP-2) is necessary for the IL-5-induced suppression of eosinophil apoptosis (Yousefi *et al.*, 1996; Pazdrak *et al.*, 1997). SHPTP-2 activation and association with the adaptor protein, Grb2, is proposed to couple the IL-5 receptor to the Ras signalling pathway, supported by the necessity of Raf-1 serine/ threonine kinase activation for the IL-5-induced inhibition of eosinophil apoptosis (Pazdrak *et al.*, 1998). The regions of the IL-5R required for the binding of the various tyrosine kinases have been deduced in cell lines (Fig. 1.8A). Lyn and Jak2 are constitutively bound to residues 450-465 and a region termed box1 of βc respectively. Although the βc box 1 is required for Jak2 binding it is not sufficient, as physical association with a similar region in IL-5Rα is also necessary. IL-5 induces rapid tyrosine phosphorylation of Lyn and Jak-2, tyrosine phosphorylation of kinase binding sites on βc and subsequent recruitment of adapter proteins Shc and SHPTP-2. The kinase cascades initiated on reception of IL-5 are likely to converge at various points, e.g. Jak2 has been shown to associate with Vav and activate the Raf-1 pathway. However, some degree of functional divergence in the eosinophil is suggested by the fact that whereas Jak-2, Lyn and Raf-1 are all vital for IL-5-induced inhibition of apoptosis, only Raf-1 is required for IL-5-mediated activation and degranulation (Pazdrak *et al.*, 1998). Despite recent advances in elucidating the IL-5-induced signal transduction schemes, the mechanism responsible for the integration of the kinase cascades and the apoptotic machinery remains unclear.

1.5.2. Ligation of cell surface receptors

As well as growth factor deprivation, the apoptosis of eosinophils may also be initiated by the ligation of specific cell surface receptors.

CD95 (Fas/ Apo-1) is a 'death-receptor' of the TNF-receptor family (see above), the ligand of which, FasL, is expressed on the surface of activated T cells (Wang *et al.*, 1997). Studies have indicated that eosinophils express CD95 and that engagement of the death receptor induces the apoptosis even in the presence of survival-enhancing growth factors (Hebestreit *et al.*, 1998; Hebestreit *et al.*, 1996). Tsuyuki *et al* (1995) have also reported that intranasal administration of anti-Fas antibody completely ablates eosinophilic inflammation in the

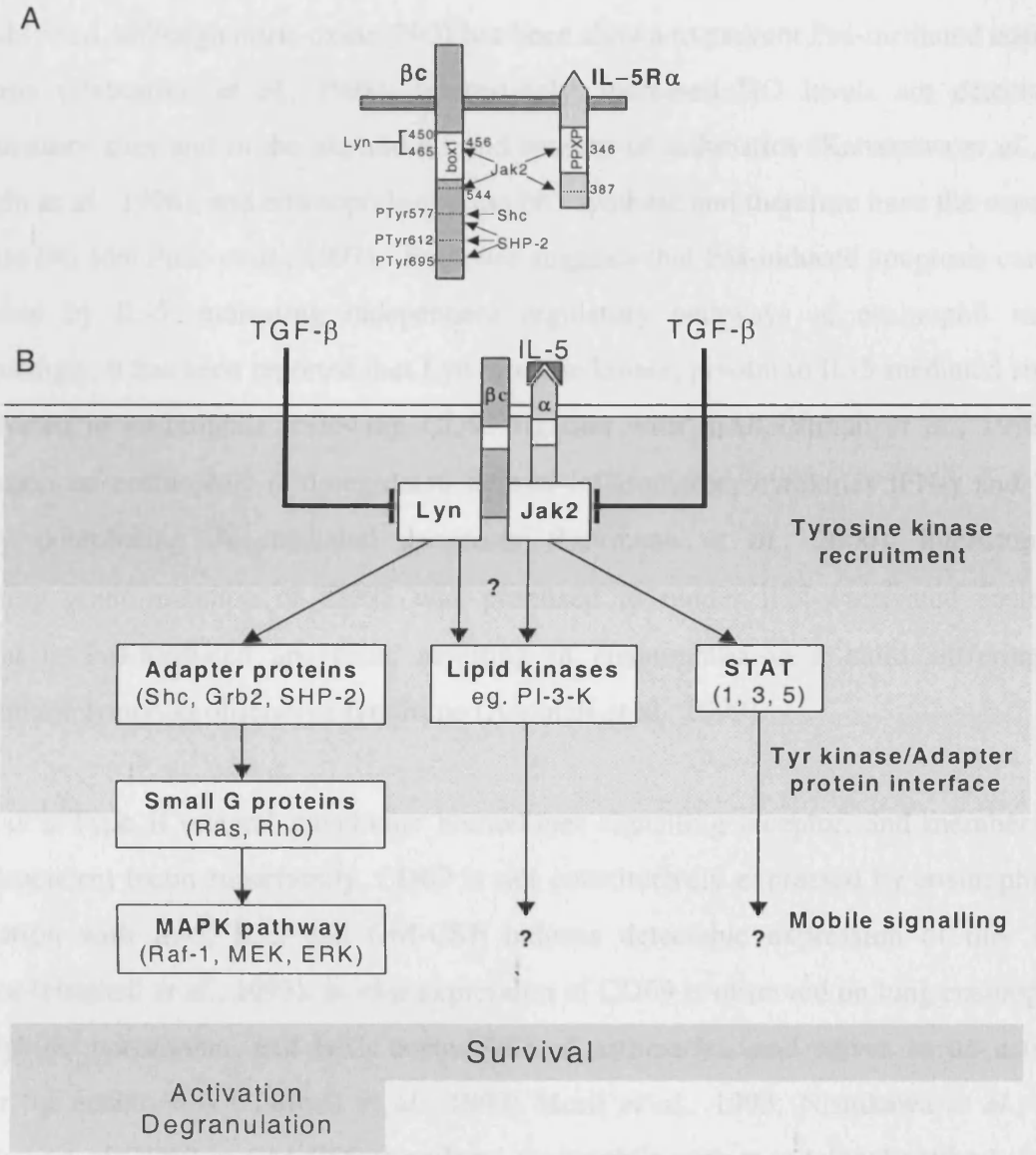

Figure 1.8. IL-5 signal transduction. A. The IL-5 receptor is composed of a unique α subunit and a shared βc subunit. B. Ligation of the IL-5R induces rapid tyrosine phosphorylation and activation of intracellular kinases, and the initiation of functionally divergent kinase cascades within the eosinophil. TGF-β antagonises the pro-survival effects of IL-5, possibly by preventing phosphorylation and activation of Jak2 and Lyn.

airway of mice. The direct induction of apoptosis may provide a mechanism for tempering eosinophil expansion within inflammatory sites, independent of the eosinophil-active hematopoietins present. A phenomenon known as 'Fas resistance' has been described, whereby ligation of the death receptor does not induce eosinophil apoptosis (Hebestreit *et al.*, 1996). This has been observed in nasal polyps, and may explain the dysregulated persistence

of eosinophils at such inflammatory sites. The mechanism underlying Fas resistance has yet to be established, although nitric oxide (NO) has been shown to prevent Fas-mediated eosinophil apoptosis (Hebestreit *et al.*, 1998). Interestingly, increased NO levels are detectable at inflammatory sites and in the expired air and sputum of asthmatics (Kanazawa *et al.*, 1997; Satouchi *et al.*, 1996), and eosinophils express NO synthase and therefore have the capacity to generate NO (del Pozo *et al.*, 1997). Evidence suggests that Fas-induced apoptosis cannot be overcome by IL-5, indicating independent regulatory pathways of eosinophil survival. Contrastingly, it has been reported that Lyn tyrosine kinase, pivotal to IL-5-mediated survival, is activated in eosinophils following CD95 ligation with mAb (Simon *et al.*, 1998). Fas expression on eosinophils is upregulated by anti-inflammatory cytokines IFN-γ and TGF-β thereby potentiating Fas-mediated apoptosis (Luttmann *et al.*, 2000). Interestingly, a disrupting point mutation of CD95 was proposed to render IFN-γ-activated eosinophils resistant to Fas-mediated apoptosis, resulting in eosinophilia in a child suffering from autoimmune lymphoproliferative syndrome (Aspinall *et al.*, 1999).

CD69 is a Type II integral membrane homodimer signalling receptor, and member of the Ca^{2+}-dependent lectin superfamily. CD69 is not constitutively expressed by eosinophils, but stimulation with IL-5, IL-3 and GM-CSF induces detectable expression of this surface receptor (Hartnell *et al.*, 1993). *In vivo* expression of CD69 is observed on lung eosinophils in eosinophilic pneumonia, and BAL eosinophils of asthmatics, and serves as an activation marker for eosinophils (Hartnell *et al.*, 1993; Morii *et al.*, 1993; Nishikawa *et al.*, 1992). Perturbation of CD69 in GM-CSF stimulated eosinophils with monoclonal antibody induces apoptosis, independent of mediator release or production of antagonising TGF-β (Walsh *et al.*, 1996). Cross-linking of CD69 has also been shown to induce apoptosis in lipopolysaccharide-preactivated monocytes by multiple, independent pathways, which, similarly to CD95-induced apoptosis, is inhibited by NO (Ramirez *et al.*, 1996). At present, the mechanism by which CD69 induces the apoptosis of activated eosinophils remains undetermined.

CD45 is a transmembrane protein tyrosine phosphatase (PTPase) and the predominant PTPase expressed on hematopoietic cells. Encoded by a single gene, the leukocyte common antigen is expressed as various differentially glycosylated splice variants including CD45RA, RB and RO, which have distinct expression profiles (Ledbetter *et al.*, 1988). CD45 is involved in the regulation of apoptosis and MHC-restricted negative selection during the maturation of

$CD4^+8^+$ thymocytes (Ong *et al.*, 1994), and soluble CD45 mAb induced the apoptosis of T and B cells *in vitro* (Klaus *et al.*, 1996). Eosinophils constitutively express CD45 isoforms in the following order of abundance RO>RB>RA. Ligation with mAb specific for CD45, CD45RB and RA isoforms induces apoptosis (Blaylock *et al.*, 1999). Interestingly, it has recently been shown that CD45 is a Jak phosphatase, and therefore potentially mediates eosinophil apoptosis by negatively regulating pro-survival cytokine receptor signalling (Irie-Sasaki *et al.*, 2001). However, the findings of Blaylock *et al* (1999) suggested abrogation of eosinophil CD45 induced apoptosis by a mechanism independent of GM-CSF.

CD32 (FcγRII), the low affinity IgG receptor, is constitutively expressed by eosinophils and thought to be crucial in the activation of eosinophils in allergic disease (Kaneko *et al.*, 1995; Kita *et al.*, 1999). Further to its ability to initiate degranulation, the ligation of FcγRII induces the apoptosis of murine granulocyte precursors and mature eosinophils, by a mechanism dependent on Fas/ FasL interaction (de Andres *et al.*, 1997). Although the physiological relevance of this death-induction has not been determined, it is proposed that binding of IgG immune complexes links the degranulation and apoptosis of eosinophils, and thereby mediates the inflammatory response.

1.5.3. Glucocorticoids and drug therapy

Glucocorticoids have long been exploited for therapeutic purposes due to their potent anti-inflammatory action. Eosinophils express high affinity receptors for steroids that exhibit an array of eosinopenic effects. Glucocorticoids can antagonise eosinophilic inflammation by inhibiting chemotaxis and adhesion of eosinophils to endothelial cells, and modulate the release of pro-inflammatory mediators such as IL-5 and PAF. However, the reduction of eosinophil numbers in response to glucocorticoid treatment appears to be governed, at least in part, due to induction of eosinophil apoptosis. *In vivo*, steroids induced the apoptosis of airway eosinophils in patients receiving treatment for asthma exacerbation (Woolley *et al.*, 1996), and in a rat model, caused apoptosis and subsequent phagocytosis of intestinal eosinophils, without histotoxic mediator release, during infection with *Nippostronglyus brasiliensis* (Nittoh *et al.*, 1998). Glucocorticoids may reduce eosinophil survival by inhibiting NF-κB-dependent production of survival enhancing cytokines (Schwiebert *et al.*, 1996), inhibiting the activity of eosinophil survival factors (Wallen *et al.*, 1991), or directly inducing eosinophil apoptosis (Meagher *et al.*, 1996). A recent study has also shown steroid-

induced apoptosis of eosinophils infiltrating nasal polyp tissue (Saunders *et al.*, 1999). Interestingly, dexamethasone exhibits contrasting effects on the survival of neutrophils, where it is a potent inhibitor of apoptosis (Meagher *et al.*, 1996). Glucocorticoids have also been shown to potentiate the engulfment of eosinophils by monocyte-derived macrophages (Liu *et al.*, 1999).

Lidocaine has also been reported to reduce eosinophil survival by inhibiting the effects of IL-5, IL-3, and GM-CSF, possibly involving inhibition of protein kinase C (Okada *et al.*, 1998). It was observed that BAL fluid from bronchial asthmatics inhibited eosinophil viability *in vitro* by a mechanism independent of glucocorticoids (Ohnishi *et al.*, 1996). This lead to the discovery that lidocaine, used as an anaesthetic during bronchoscopy, could inhibit eosinophil survival, and effectively replace oral glucocorticoid treatment of asthmatics, potentially circumventing the side effects of long term steroid therapy (Hunt *et al.*, 1996).

Theophylline has been widely used as a treatment for asthma primarily due to its broncho-dilatory properties (Pauwels, 1989; Weinberger, 1984), although an anti-inflammatory role has also been reported (Kraft *et al.*, 1996). Theophylline accelerates spontaneous apoptosis and apoptosis of eosinophils following Fas ligation, and inhibits survival induced by IL-5 and GM-CSF (Ohta *et al.*, 1996; Momose *et al.*, 1998; Yasui *et al.*, 1997; Yasui *et al.*, 2000). The pro-apoptotic effects of theophylline are proposed to be due to the inhibition of phosphodiesterase and resultant increase in intracellular levels of cAMP, as other inhibitors of phosphodiesterase can also function as anti-inflammatory agents in bronchial asthma (Banner and Page, 1996). However, this is in apparent contrast to the reported effects of other cAMP elevating agents such as rolipram and dibutyryl camp (dbcAMP), which actually prolong eosinophil survival *in vitro*, and therefore the effect of theophylline on eosinophil survival appears to be independent of phosphodiesterase inhibition (Chang *et al.*, 2000; Peacock *et al.*, 1999; Yasui *et al.*, 1997). The crucial aspect of these studies may be previous cytokine exposure and therefore activation state, as Hallsworth *et al* (1996) have shown that elevation of intracellular cAMP has contrasting effects on eosinophil survival dependent on exposure to GM-CSF. Theophylline may indirectly induce apoptosis by inhibiting degranulation and release of survival cytokines, such as GM-CSF and IL-8, in a PKA dependent manner (Shute *et al.*, 1998).

Leukotrienes are involved in mediating chemotaxis, smooth muscle proliferation and bronchoconstriction, and as such, the generation of leukotriene synthesis inhibitors and receptor antagonists has been a target for the therapeutic intervention of asthma (Lee *et al.*, 2000). It has recently been reported that in conjunction with the above properties, leukotrienes can also support eosinophil survival *in vitro*, and mast cell and lymphocyte-induced eosinophil survival is mediated by cysteinyl leukotrienes (Lee *et al.*, 2000).

1.5.4. Phagocytic clearance

The engulfment of intact apoptotic cells or bodies by resident professional (macrophages) or non-professional (dendritic cells, smooth muscle cells) phagocytes, without the release of potent inflammatory and immunogenic mediators is pivotal in the limitation of inflammation, for without phagocytosis the end result of apoptosis and necrosis would be effectively indistinguishable. In conjunction with the 'silent' removal of inflammatory cells, engulfment by resident phagocytes, such as macrophages and dendritic cells, also induces production of the anti-inflammatory mediators TGF-β, PGE_2, and IL-10 (Fadok *et al.*, 1998), therefore the importance of phagocytosis in the resolution of inflammation is two-fold. Comparison with the system employed by *C. elegans* and *in vitro* studies of human apoptotic cell/ phagocyte interaction implicates a complex array of receptor-ligand interactions governing clearance. Stern *et al* (1992) observed the phagocytosis of eosinophils with apoptotic morphology, such as condensed nucleus and cytoplasm, by macrophages *in vitro*. However, engulfment can occur in the apparent absence of visible apoptotic features, suggesting the emergence of ingestion signals at a relatively early stage of the apoptotic process. This, coupled with the great capacity of macrophages for apoptotic granulocytes implies a rapid and efficient system for the removal of apoptotic, potentially histotoxic eosinophils.

A number of phagocyte/ apoptotic cell interactions have now been determined (recently reviewed by Savill and Fadok, 2000). During apoptosis, an early redistribution of membrane phospholipids, namely phosphatidylserine (PtdSer) occurs independent of the nature of the apoptotic stimulus (Martin *et al.*, 1995). Peripheral blood cells normally exhibit an asymmetric distribution of plasma membrane phospholipids, with PtdSer restricted to the inner leaflet and sphingomyelin to the outer leaflet. Apoptosis induces externalisation of PtdSer to the outer membrane, due to inhibition of aminophospholipid translocase (Middelkoop *et al.*, 1988) and protein kinase Cδ-dependent activation of a phospholipid

scramblase (Frasch *et al.*, 2000). Redistributed PtdSer is recognised by a recently identified stereospecific PtdSer receptor expressed on macrophage and dendritic cells, and consequently induces anti-inflammatory TGF-β1 release from macrophages, whilst simultaneously inhibiting the LPS-induced production of pro-inflammatory TNF-α (Fadok *et al.*, 2000). The redistribution of PtdSer has been utilised as a marker for apoptotic cells, as it is readily bound by a calcium-dependent phospholipid binding protein, Annexin V. Reorganisation of membrane phospholipids is regulated by the ATP-binding cassette (ABC)-type transporter, possible homologue of the *C. elegans* protein Ced-7, and its function is necessary in the membrane of both the phagocyte and the dying cell, indicating alteration of the phagocyte membrane must also occur to facilitate engulfment (Marguet *et al.*, 1999).

It has been proposed that externalisation of PtdSer is dissociated from other features of the apoptotic programme, specifically caspase activation, indicating that phagocytic recognition is under independent regulatory control (Zhuang *et al.*, 1998). This is supported by the observation that neutrophils transgenic for the expression of Bcl-2 failed to exhibit signs of apoptosis, although macrophage recognition and engulfment were unaffected (Lagasse and Weissman, 1994), and PtdSer redistribution during glucocorticoid-induced apoptosis of a T lymphocyte cell line was unaffected by the broad range caspase inhibitor zVAD.fmk (Verhoven *et al.*, 1999).

It is proposed that the initial 'tethering' of the apoptotic cell to the phagocyte is mediated by β_2 integrins or phagocyte surface CD14. Interestingly, although CD14 also acts as a receptor for bacterial LPS, distinct functional responses are elicited, with induction or inhibition of pro-inflammatory cytokine release dependent upon the binding of LPS or an apoptotic cell respectively (Devitt *et al.*, 1998). The $\alpha_v\beta_3$ vitronectin receptor and thrombospondin receptor (CD36) on the macrophage surface cooperatively bind thrombospondin, and mediate interaction with an as yet undetermined ligand on the surface of the apoptotic cell, and facilitates engulfment and degradation of the target.

In contrast, dendritic cells utilise the $\alpha_v\beta_5$ integrin heterodimer to recognise and ingest apoptotic cells (Albert *et al.*, 1998). $\alpha_v\beta_5$ recruits the signalling complex CrkII-Dock180-Rac1, functionally analogous to the *C. elegans* cell migration and phagocytosis regulating complex Ced2-Ced5-Ced10, and is proposed to mediate the cytoskeletal changes necessary

for apoptotic cell engulfment (Albert *et al.*, 2000; Wu and Horvitz, 1998). The divergence in recognition mechanism employed by macrophages and dendritic cells is proposed to be crucial in the alternative function of this latter phagocyte in its role in antigen presentation (Albert *et al.*, 2000).

It is now apparent that a number of cell types have the ability to recognise and engulf apoptotic cells. Interestingly, a recent report by Walsh *et al* (1999) has implicated a role for epithelial cells in the lectin/ integrin mediated phagocytosis of apoptotic eosinophils. The epithelium is generally regarded as the target for the action of cytotoxic eosinophil-derived mediators in the allergic airway, but this study indicates a potentially important role for epithelial cells in eosinophil clearance and regulation of the inflammatory response.

1.6. Aims

The aims of the project were to investigate and characterise the molecular mechanisms regulating the apoptosis of human peripheral blood eosinophils. Apoptosis and subsequent clearance of eosinophils may provide an efficient mechanism for limiting the inflammatory potential of the cell. Investigating the molecular control of eosinophil survival and elucidating the point in the signalling cascade at which the potent, eosinophil-specific, pro-inflammatory cytokine, IL-5, exerts its protective effect would enable specific inhibitors of growth factor mediated eosinophil survival to be developed. The Bcl-2 homologues and caspases are pivotal regulators of apoptosis in many cell types. It was hypothesised that IL-5 regulated eosinophil survival by modulating the expression/ activity of these apoptosis mediators, therefore the role of these proteins in the control and execution of eosinophil apoptosis in response to diverse apoptotic stimuli was investigated.

Chapter 2

Materials and Methods

2.1. Reagents and cell lines

2.1.1.Antibodies and reagents

Recombinant human IL-5 and GM-CSF were obtained from R&D Systems (Abingdon, UK). Antibodies targeting Bcl-2 homologues are detailed in Table 2.1. Human β-actin-specific mAb from Sigma Chemical Co. (Poole, UK). Isotype-matched control mouse and rabbit IgGs were from DAKO Ltd (High Wycombe, UK). Cell culture media was purchased from GIBCO-BRL (Paisley, UK). zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp-(*O*Me) fluoromethyl ketone) was from Enzyme Systems Inc. (Dublin, CA). Rabbit polyclonal antibodies directed against caspase-3, -7, -8, and –9 were generated as previously described (MacFarlane *et al.*, 1997), and a polyclonal against caspase-6 was purchased from Upstate Biotechnology (Lake Placid, NY). The epitope recognition site of the caspase-6 antibody spanned the prodomain cleavage site, therefore the subunits were not immunoreactive. Anti-tubulin-α was from Oncogene (Cambridge, MA). Anti-mouse and anti-rabbit HRP conjugates were from Sigma (Poole, UK) and DAKO (Ely, UK) respectively. Anti-Fas (CD95/ Apo-1) monoclonal antibodies ZB4 (non-activating, IgG1) and CH11 (activating, IgM) were purchased form Immunotech (Luton, UK) and Upstate Biotechnology (Lake Placid, NY) respectively. Species-specific Alexa 488™ secondary antibodies for immunocytochemistry, tetramethylrhodamine ethyl ester (TMRE), and Mitotracker Red CMXRos were from Molecular Probes (Eugene, OR). FITC-conjugated Annexin V was obtained from Bender Medsystems (Vienna, Austria). Rabbit polyclonal antibodies directed against Erk1/2 (K-23) and p38 MAPK were from Santa Cruz Biotechnology (CA), and Sigma Chemical Co. (Poole, UK) respectively. Phosphorylation state-specific antibodies recognising phosphorylated Thr202/Tyr204 of Erk and phosphorylated Thr180/Tyr182 of p38 MAPK were from Cell Signalling Technology (Beverley, MA). Inhibitors of phosphatidylinositol-3-kinase (PI-3-K), wortmannin and LY294002, and staurosporine were obtained from Calbiochem-Novabiochem (Notts, UK). Specific inhibitor of MAPK kinase (MEK)1/2, U0126 was from Promega (Southampton, UK). All other reagents were from Sigma unless otherwise stated.

2.1.2.Cell lines

Human myeloid cell line HL-60, MCF-7 breast carcinoma cell line, and Jurkat T cells (clone E6-1) were obtained from European Collection of Cell Cultures (Wilts, UK) and maintained in RPMI 1640 supplemented with 10% FCS and 1% Glutamax. Cells were maintained at 37°C in a humidified 95 % air, 5 % CO_2 incubator. Suspension cells (Jurkat, HL-60) were routinely split by diluting 1:12 in pre-warmed culture medium to maintain a culture density of between

5×10^5 and 1×10^6 cells/ml. The adherent MCF-7 cell line was split approximately every three days. Cells were washed in PBS and incubated with trypsin (0.05 % in EDTA/PBS buffer) for 10 min at 37°C to detach cells from the culture dish. The cell suspension was transferred to a centrifuge tube, complete medium added to inactivate the trypsin, and cells pelleted for 5 min at 500 *g*. Cells were resuspended in complete medium at a dilution of 1:10. The apoptosis of Jurkat T cells and MCF-7 cells was induced by treatment with etoposide (50 μM) and TRAIL (500 ng/ml) for 4 h respectively.

Table 2.1. Antibodies targeting Bcl-2 homologues.

Bcl-2 homologue	Clone	Host	Immunogen	Source
Bcl-2	100	M[1]	41-54	Santa Cruz Biotechnology, CA.
Bax	2D2	M	3-16	Sigma Chemical Co., Poole, UK.
	6A7	M	12-24	Pharmingen, Oxford, UK.
	Clone 3	M	55-178	Transduction Laboratories, KY.
	N-20	R	11-30	Santa Cruz Biotechnology, CA.
	43-61	R	43-61	Pharmingen, Oxford, UK.
Bcl-$x_{S/L}$	S-18	R	2-19	Santa Cruz Biotechnology, CA.
Bad (IB)	-	R	~Ser112	New England Biolabs, MA.
Bad (IP)	N-19	G	N terminus	Santa Cruz Biotechnology, CA.
Ser136	Ser136	S	131-pSer-141	Upstate Biotechnology, NY.
Ser155	Ser155	R	147-pSer-160	Upstate Biotechnology, NY.
Bak	G317-2	M	1-50	Pharmingen, Oxford, UK.
Bid	-	R	-	Dr. X. Wang (Luo *et al.*, 1998)
Bik	C33-1	R	40-114	Pharmingen, Oxford, UK.
$Bim_{EL/L/S}$	-	R	22-40	Stressgen Biotech., York, UK.

[1]M, mouse; G, goat; IB, immunoblotting; IP, immunoprecipitation; R, rabbit; S, sheep; Ser, serine. Immunogen described as amino acid residue relative to the N-terminus.

2.2. Isolation of peripheral blood cells

2.2.1. Isolation of peripheral blood eosinophils and cell culture

Heparinised peripheral venous blood was taken from healthy volunteers with peripheral blood eosinophilia of less than 0.5 x 10^6 ml^{-1}. Eosinophils were purified by a two-step method of density gradient centrifugation, followed by negative immunomagnetic selection modified

from (Hansel *et al.*, 1991). Following removal of erythrocytes by sedimentation with 1.25% dextran for 45 min at room temperature, the leukocyte rich supernatant was centrifuged at 100 *g* for 15 min at room temperature. The cell pellet was resuspended in Hanks' balanced salt solution (HBSS), 30 mM Hepes, 5 mM EDTA, without Ca^{2+} and Mg^{2}, supplemented with 1% BSA. The cell suspension was centrifuged on Histopaque 1083 (400 *g* for 25 min at room temperature) and the mononuclear cell layer carefully removed, prior to lysis of erythrocytes contaminating the granulocyte pellet by hypotonic shock using sterile, ice-cold water. Eosinophils ($CD16^{-}$) were separated from neutrophils ($CD16^{+}$) by negative immunomagnetic selection using anti-CD16-coated magnetic beads (Miltenyi Biotec Inc., Auburn, CA), which specifically bind to neutrophils during a 40 min incubation on ice. The granulocyte cell suspension was then passed through a magnetic column, allowing retention of neutrophils and elution of eosinophils. Post-isolation purity and viability was routinely >99% as assessed by morphology after Kimura stain and trypan blue exclusion respectively. Peripheral blood eosinophils were cultured in RPMI 1640 supplemented with 1% BSA and 1% Glutamax.

2.2.2. Isolation of neutrophils

Heparinised peripheral venous blood was taken from healthy volunteers with peripheral blood eosinophilia of less than 0.1 x 10^{6} ml^{-1} and neutrophils were purified by density gradient centrifugation. Following removal of erythrocytes by dextran sedimentation, the leukocyte rich supernatant was centrifuged at 100 *g* for 15 min at room temperature. The cell pellet was resuspended in HBSS supplemented with 2% FCS. The cell suspension was centrifuged on Histopaque 1077 and the mononuclear cell layer carefully removed, prior to lysis of erythrocytes contaminating the predominantly neutrophil pellet by hypotonic shock using sterile, ice-cold water. Neutrophils were washed in HBSS with post-isolation purity routinely >98%.

2.2.3. Isolation of lymphocytes

Lymphocytes were isolated simultaneously during eosinophil or neutrophil preparations and depleted of monocytes by adherence to plastic. The mononuclear cell layer was removed following the density gradient centrifugation on Histopaque 1088, washed once in HBSS without Ca^{2+} and Mg^{2+} supplemented with 2% FCS, and resuspended in RPMI supplemented with 10% FCS. Cells were incubated for 1 h at 37°C in a tissue culture flask to allow adherence of monocytes, thereby allowing retrieval of non-adherent lymphocytes.

2.3. Assessment of eosinophil apoptosis

2.3.1. Apoptotic morphology

Eosinophils were stained with Kimura stain (Kimura *et al.*, 1973) and examined under light microscopy for the characteristic morphological changes associated with apoptosis, namely condensation of the nucleus and cytoplasm. 250 cells were counted per treatment in a blinded fashion.

2.3.2. Assessment of apoptosis by Annexin V binding

Plasma membrane phospholipids are asymmetrically distributed in a viable cell. Normally restricted to the inner, cytoplasmic face, during apoptosis, phosphatidylserine (PtdSer) translocates to the outer leaflet. Externalisation of PtdSer has been shown to be a sensitive marker of apoptosis in many cell types regardless of the initiating stimulus (Martin *et al.*, 1995). Annexin V is a calcium-dependent phospholipid binding protein with specific affinity for PtdSer. Binding of a fluorescent conjugate of Annexin V therefore provides an efficient and sensitive marker of apoptosis in other cell types (Zhang *et al.*, 1997). Cells were washed once in ice-cold PBS, and incubated with Annexin V-FITC (1:650) diluted in binding buffer (10 mM HEPES/ NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8mM $CaCl_2$) at a concentration of 1 x 10^6 cells $ml^{-1}$for 8 min at room temperature. Cells were then incubated with 50 μg/ml propidium iodide (PI) in PBS for 2 min on ice prior to analysis using a fluorescence-activated cell scan (FACScan®, Becton Dickinson, Oxford, UK), excitation at 488 nm and detection between 515 and 550 nm. Non-cell events (debris and cell fragments) were discounted from analysis by excluding small events on the basis of size (forward scatter, FSC) and granularity (side scatter, SSC). 10,000 events were analysed for each sample.

2.3.3. Assessment of apoptosis by mitochondrial transmembrane potential

To measure mitochondrial transmembrane potential ($\Delta\Psi m$), cells were loaded with 75 nM TMRE for 30 min at 37°C. Alterations of $\Delta\Psi m$ can be determined from the fluorescence intensity of TMRE as the transmembrane distribution of this lipophilic, cationic rhodamine derivative is dependent on membrane potential (Scaduto and Grotyohann, 1999). Cells were washed once and resuspended in PBS for FACScan® analysis, excitation at 488 nm and detection at 610 nm. Freshly isolated eosinophils were treated with the mitochondrial membrane uncoupler carbonylcyanide m-chlorophenyl hydrazone (m-CCP) at 1 μM for 15 min at 37°C. m-CCP acts as a protonophore (Felle and Bentrup, 1977), dissipating the proton gradient build-up across the inner mitochondrial membrane and therefore acts as a positive

control for dissipation of $\Delta\Psi m$. Non-cell events (debris and cell fragments) were discounted from analysis by excluding small events on the basis of size (forward scatter, FSC) and granularity (side scatter, SSC). 10,000 events were analysed for each sample.

2.3.4. Assessment of apoptosis by red autofluorescence

Red autofluorescence by flow cytometric analysis was used to distinguish apoptotic and non-apoptotic eosinophils. It has been shown previously that culturing *in vitro*, generated a subpopulation of eosinophils that exhibited both reduced size and increased red autofluorescence. Isolation and morphological examination of this distinct population revealed the highly autofluorescent, condensed eosinophils to be predominantly apoptotic (Stern *et al.*, 1992). Following treatment, eosinophils were briefly washed in ice-cold PBS, gently resuspended in buffer (10 mM HEPES/ NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and analysed by flow cytometry. Non-cell events (debris and cell fragments) were discounted from analysis by excluding small events on the basis of size (forward scatter, FSC) and granularity (side scatter, SSC). 10,000 events were analysed for each sample.

2.3.5. Analysis of internucleosomal chromatin fragmentation

Eosinophils were washed with ice-cold PBS and lysed with extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) containing 1% SDS and RNase A for 1 h at 37°C. 0.2 mg/ml Proteinase K was added for 3 h at 50°C. DNA was extracted by mixing with an equal volume of phenol:chloroform (1:1), centrifugation at 13000 *g* for 1 min and retrieval of the aqueous phase. This was repeated three times, followed by addition of an equal volume of chloroform and spun as before. DNA was precipitated from the aqueous phase with two volumes of ethanol at –20°C, and 1/10 volume of 3 M sodium acetate on ice for 30 min. DNA was pelleted at 13000 *g* for 10 min at 4°C, washed in ice-cold 70% ethanol, prior to resuspension in Tris-EDTA. Samples were electrophoresed through 1.8% agarose containing 0.5 μg/ml ethidium bromide and viewed under UV light.

2.4. Analysis of protein expression

2.4.1. Western blotting

2.4.1.1. Generation of whole cell lysate

5 x 10^5 cells were washed with ice-cold PBS, and snap-frozen in dry ice before resuspension in sample buffer (62.5 mM Tris-HCl, pH 6.8, 15% glycerol, 2% SDS, 500 μg/ml

bromophenol blue) with freshly added 5% β-mercaptoethanol and boiled for 5 min. Alternatively, cells were lysed in ice-cold isotonic lysis buffer containing detergents as indicated, containing freshly added protease inhibitors (100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A) and incubated on ice for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation (12000 *g* for 5 min at 4°C).

2.4.1.2. Cytosolic fractionation

Eosinophils were washed in ice cold PBS, centrifuged at 200 *g* for 8 min at 4°C, and resuspended in hypotonic extraction buffer (220 mM mannitol, 68 mM sucrose, 50 mM Pipes-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT) with freshly added protease inhibitors as above, and allowed to swell on ice for 30 min. Cells were then homogenised by 40 strokes in a glass dounce homogeniser. A sample was retrieved and examined under light microscopy for cell lysis. The cell homogenate was spun at 14000 *g* for 15 min at 4°C to generate a cytosolic supernatant and a pellet containing mitochondria, heavy membrane, nuclei and unbroken cells. The membrane fraction was resuspended in sample buffer and all fractions were stored at –80°C prior to SDS-PAGE. For the detection of cytochrome *c* in subcellular fractions, cytosolic and membrane extract from 1 x 10^{-6} cells was separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with cytochrome *c* monoclonal antibody (7H8.2C12, Pharmingen, CA). In order to control for cross contamination of fractions, extracts were also immunoblotted for the inner mitochondrial membrane component cytochrome *c* oxidase subunit II using the monoclonal antibody 12C4-F12 (Molecular Probes, OR) according to Kluck *et al* (1997).

2.4.1.3. Nuclear/ cytosol fractionation

Fractionation of eosinophil nuclei and cytosol was performed in order to assess potential protein-protein interactions within the nucleus, using a modified version of the method employed by Schreiber *et al* (1989). 5 x 10^{-6} eosinophils were resuspended in 0.5 ml of ice-cold hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT) with freshly added protease inhibitors as above, and incubated on ice for 20 min. Cold 10% Nonidet P-40 (NP-40) in ddH_2O was added to a final concentration of 1% NP-40 and vortexed vigorously for 30 sec, and cell lysis and nuclear integrity assessed under light microscopy. Lysates were spun at 13000 *g* for 30 s at 4°C and pelleted nuclei were resuspended in 0.5 ml of ice cold nuclear lysis buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 2 mM DTT) and incubated on ice for 20 min

with constant agitation. Unlysed debris was removed by spinning at 13000 *g* for 5 min at 4°C and fractions were stored at –80°C prior to immunoprecipitation and SDS-PAGE.

2.4.1.4. Immunoprecipitation

Whole cell lysates and subcellular fractions were precleared with 5µl of normal rabbit serum and 2mg Protein A-Sepharose for 2 h at 4°C, and spun for 45 s. Target antigen was immunoprecipitated with 5 µg of rabbit polyclonal antibody (Bax N-20, Bim 22-40) or rabbit Ig as a control overnight at 4°C with constant agitation. Antigen:antibody complexes were captured with 5 mg of Protein A-Sepharose for 2 h at 4°C and spun for 30 s. Immunoprecipitate was washed four times with ice-cold hypotonic buffer, 1% Chaps and once with ice-cold PBS. Chaps detergent was used as it does not cause aberrant dimerisation of Bax (Hsu and Youle, 1997). Antigen was removed from the beads by resuspension in sample buffer with 5% β-mercaptoethanol and boiling for 5 min, prior to analysis by SDS-PAGE.

2.4.1.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Following generation of whole cell lysates, cellular fractions or immunoprecipitates, proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 10-15% resolving gels were cast (375 mM Tris/ HCl, pH 8.8, 0.1% SDS, appropriate final concentration of 30% (w/v) acrylamide gel stock, 0.05% ammonium persulphate, 0.08% TEMED) and overlaid with stacking gel (125 mM Tris/ HCl, pH 6.8, 0.1% SDS, 4% final concentration of 30% (w/v) acrylamide gel stock, 0.05% ammonium persulphate, 0.08% TEMED) for protein resolution. Proteins were electrophoresed in electrode buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS) at 60 V within the stacking gel and 100 V within the resolving gel.

2.4.1.6. Immunoblotting

Proteins were electrotransferred onto Hybond nitrocellulose membrane (Amersham Life Science Ltd, Bucks., UK) pre-soaked in transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol) for 1 h at room temperature. Non-specific binding sites on the membrane were blocked by incubation (1 h at room temperature) with 5 % non-fat milk in TBS 0.1% Tween-20 (TBS-T). Filters were incubated with specific primary antibody or negative control, diluted to 0.1 µg/ml in blocking buffer (2 h at room temperature). The membrane was then incubated for 1 h at room temperature with species-specific HRP-conjugated secondary antibody, diluted 1 in 3000 in TBS-T. Incubations were performed with constant agitation, followed by

1 x 15 min and 2 x 5 min wash with TBS-T. Membranes were developed by enhanced chemiluminescence according to manufacturers instructions (Amersham Life Science Ltd, Bucks., UK) and exposed to photographic film. Where necessary blots were stripped of primary and secondary antibody by incubating in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol for 40 min at 55°C. The membranes were then re-blocked and re-probed with specific primary antibody as described above.

2.4.2. Intracellular flow cytometric analysis

Cells were washed in PBS, prior to fixation and permeabilisation using a kit according to manufacturers instructions (Bradsure Biologicals, Bucks., UK). 0.5 x 10^6 cells were fixed for 15 min at room temperature in fixing solution A (Bradsure Biologicals) followed by a wash with PBS. The cells were permeabilised in 100 µl of permeabilisation solution B (Bradsure Biologicals) in the presence of 1 µg/ml of primary antibody or isotype-matched negative control antibody for 25 min at room temperature. Cells were then washed in PBS and incubated with species-specific FITC-conjugated secondary antibody, diluted 1 in 100 in PBS for 25 min at room temperature in the dark. After washing in PBS, cells were analysed using the FACScan. Quantitation of the flow cytometry data was performed on the basis of specific mean fluorescence, which was calculated using the following:

Specific Mean Fluorescence (SMF)	= Mean fluorescence of test Ab	-	Mean fluorescence of isotype-matched control

2.5. Analysis of mRNA

2.5.1. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol according to manufacturers protocol (Gibco BRL, Paisley, UK). Poly A^+ RNA was selected with oligo dT (Gibco BRL, Paisley, UK) at 70°C for 10 min and reverse transcribed with *Superscript* RT (Gibco BRL. Paisley, UK) in the presence of 0.2 mM dNTP, 10 mM DTT, ribonuclease inhibitor, RNasin, for 1 h at 37°C followed by heat inactivation for 5 min at 95°C. cDNA product was amplified by polymerase chain reaction (PCR) using primers obtained from Cruachem Ltd (Glasgow, UK) specific for *bcl-2, bax, bcl-x$_{S/L}$, bad,* and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) as a positive control, as described in Table 2.1. The *bcl-x$_{S/L}$* primers corresponded to the 5' and 3' untranslated regions and allowed simultaneous amplification of *bcl-x$_S$* (0.6 kb) and *bcl-x$_L$* (0.8 kb). 50µl PCR reactions were set up containing 5µl cDNA, 10 mM Tris-HCl, 25 pmol each

primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units *BioTaq* DNA polymerase (Bioline, London, UK). Amplification consisted of 30-35 cycles of denaturation at 94°C for 20 s, annealing according to Table 2.1 for 30 s and extension at 72°C for 1 min. Amplification products were electrophoresed on 1.2% agarose gels and visualised by ethidium bromide staining under UV and photographed.

Table 2.2. Properties of PCR primers.

Target	5' primer (5'→3')	3' primer (5'→3')	AT /°C
bcl-2	GATGTCCAGCCAGCTGCACCTG	CACAAAGGCATCCCAGCCTCC	60
bax	GGACCCGGTGCCTCAGGA	CAAAGATGGTCACGGTCTGC	58
bcl-x$_{S/L}$	TTGGACAATGGACTGGTTGA	GTAGAGTGGATGGTCAGTG	58
bad	CCAGAGTTTGAGCCGAGTGAGCA	**p2**-GTCCTGACCTTCTGAGCGCAGGT	65
		p3-GCGTTGCCAATTTGGACCG	65
gapdh	GGGAAGCTCACTGGCATGGCCTTCC	CATGTGGGCCATGAGGTCCACCAC	60

Specific PCR was performed using primer pairs detailed. PCR products were verified by 'nested PCR' (see Appendix 1). PCR reaction annealing temperatures (AT) are indicated.

2.5.2. Semi-quantitative duplex RT-PCR

Semi-quantitative analysis of *bcl-x$_L$* mRNA was performed using the primer dropping method as described previously (Wong *et al.*, 1994). Eosinophil total RNA was isolated using TRIzol reagent as described above and 2 µg of total RNA was used for first strand cDNA synthesis. Poly A^+ RNA was selected with oligo dT (Gibco BRL, Paisley, UK) at 70°C for 10 min and reverse transcribed with *Superscript* RT (Gibco BRL. Paisley, UK) in the presence of 0.2 mM dNTP, 10 mM DTT, ribonuclease inhibitor, RNasin, for 1 h at 37°C followed by 5 min at 95°C. 50 µl PCR reactions were set up containing 5 µl cDNA, 10 mM Tris-HCl, 25 pmol each primer, 0.2 mM dNTP, 2.5 units of *BioTaq* DNA polymerase (Bioline, London, UK), and 1.5mM $MgCl_2$ added as 'hot-start' during the initial denaturation step (94°C, 2 min). Preliminary PCR reactions were performed to determine the saturation point of each individual PCR reaction for target sequence and *gapdh* internal control, as it is necessary for both PCR reactions to be in the exponential phase to be quantitative. In subsequent duplex reactions, the secondary control (*gapdh*) primers were 'dropped' in to the reaction during the

denaturation step of the appropriate cycle, allowing simultaneous exponential amplification of target and control sequence.

2.5.3.Verification of RT-PCR products

RT-PCR products were excised from agarose gels with a clean scalpel, minimising exposure to UV irradiation. The gel slice was dissolved completely in solubilisation buffer (QG, Qiagen Ltd., Sussex, UK.) for 10 min at 50°C, and one volume of isopropanol was added to increase the yield of DNA fragments <500 bp. The DNA was bound to a spin column by centrifugation of the DNA solution for 1 min at 10,000*g*. To remove traces of agarose the column was washed once with solubilisation buffer and once with wash buffer (PE, Qiagen Ltd.). The flow through was discarded prior to a final spin at 10,000*g* for 1 min to remove residual ethanol contained in the wash buffer and the purified DNA was eluted with 10 mM Tris-HCl, pH8.5. The product was then verified by direct automated sequencing or 'nested PCR'. Sequencing reactions were performed using the dRhodamine terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Bucks, UK), which involved incorporation of unlabelled deoxynucleotides (dNTPs) and dye-labelled dideoxynucleotide (ddNTP) terminators, that do not support continued DNA elongation, during a cycling reaction in the presence of a single oligonucleotide primer. In a 20µl reaction, 200 ng of purified PCR product was combined with 5 pmol of primer and sequencing reagent mix. The sequencing reaction consisted of initial denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 15 s and extension at 60°C for 4 min. The reaction products were precipitated with the addition of 2µl of 3 M sodium acetate and automated sequencing was kindly performed by the Protein and Nucleic Acid Chemistry Laboratory (Leicester University, Leicester, UK).

Nested PCR was also performed on the purified RT-PCR products. The primary PCR product was used as a template for a second reaction, involving a third specific primer sequence internal to the initial primer pair. The reduction in size of the product could then be verified by agarose gel electrophoresis. The reaction conditions were as described above, using only 1 µl of purified PCR product as template. Primers were obtained from Cruachem Ltd (Glasgow, UK), and are detailed in Tables 2.2 and Section A1.2.

2.6. Microscopy

2.6.1. Immunocytochemistry and confocal analysis

Eosinophils were incubated with 75 nM Mitotracker Red CMXRos, for 45 min at 37°C, washed in RPMI without BSA, and cytospins were performed (300 *g* for 6 min) on silane-coated slides at $1x10^6$ cells ml^{-1}. Mitotracker Red CMX Ros is a mitochondrion-selective cell permeant dye, with thiol-reactive chloromethyl moieties that, upon accumulation within active mitochondria, reacts with thiol groups on proteins forming aldehyde-fixable conjugates. This facilitates retention of the dye following fixation and permeabilisation steps and visualisation of mitochondria under fluorescent microscopy, with absorbance and emission maxima of 578 and 599 nm respectively, well resolved from the fluorescence profile of fluorescein. MCF-7 cells were incubated in and allowed to adhere to chamber microscope slides (Nalge Nunc, IL) for 24 h at 37°C prior to incubation with Mitotracker as above. Slides were then fixed in 2% (eosinophils, neutrophils) or 4% (lymphocytes, MCF-7) paraformaldehyde for 15 min at room temperature and washed five times in PBS. Cells were permeabilised for 10 min at room temperature in blocking buffer (3% BSA in PBS) + 0.1% Triton X-100 followed by blocking of non-specific binding in blocking buffer for 1 h at room temperature. Cells to be stained with anti-Bax 6A7 were not permeabilised as the recognised Bax conformation has been shown to be sensitive to non-ionic detergent (Hsu and Youle, 1997). Cells were incubated overnight at 4°C with 5 μg/ml primary antibody diluted in blocking buffer. Cells were then incubated for 20 min at room temperature with 0.2% Chromotrope-2R which binds to highly basic eosinophil granules thereby reducing non-specific binding of the secondary antibody. Cells were then incubated with species-specific Alexa 488™ conjugated secondary antibody diluted 1:300 in blocking buffer for 50 min at room temperature in the dark. The nuclei were stained with membrane permeable DNA-binding dye, Hoechst 33258, (250 ng/ml) for 10 min at room temperature in the dark prior to mounting with fluoromount (Dako Ltd., Ely, UK). Images were collected by confocal laser microscopy (model TCS 4D, Leica, Heidelberg, Germany). The 488- and 568-nm lines of the krypton/ argon laser were used for the excitation of Alexa 488™ and Mitotracker Red CMXRos respectively. Excitation of Hoechst 33258 was by ultraviolet laser. Cytochrome *c* release and Bax translocation were quantified on these cytospins by counting at least 300 cells in blinded fashion in four random visual fields and assessing evidence of diffuse cytochrome *c* or punctate Bax staining.

2.6.2. Immunogold labelling and electron microscopy

For immunogold labelling, the eosinophil pellet (~$15x10^6$ cells) was fixed in 4% paraformaldehyde in phosphate buffer (75 mM sodium dihydrogen orthophosphate, 25 mM sodium phosphate, 150 mM NaCl, pH 7.3), for 1 h at room temperature. Cells were washed three times with phosphate buffer and three times in water, prior to sequential dehydration with increasing concentrations of ethanol (70%, 90%, 95%, 100%) for 15 min. Cells were incubated in 100% ethanol/ LR white (1:1) for 2 h, followed by infiltration with LR white for 1 h, and three changes of LR white during a day long incubation. Cells were then embedded in gelatine capsules with air excluded, and chilled at 4°C for 6 h. Blocks were polymerised under UV light for 7 h at 4°C. 1 μm sections were cut with a glass block using an ultracut OM4 ultramicrotome (Leica, Heidelberg, Germany) and stained with toluidene blue to assess gross morphology and ascertain regions of interest. 50 nm sections were taken to smooth the block face prior to ultrathin (75 nm) sectioning with a diamond block. Sections were collected onto the dull side of nickel grids and baked at 37°C overnight to facilitate retention of the sections prior to immunostaining. Non-specific binding was blocked by incubation with 10% normal rabbit serum diluted in blocking buffer (0.1 M PBS, pH 7.3, 1% BSA, 1% Tween-20), for 4 h at room temperature. Grids were blotted dry before and after each antibody step. Sections were incubated in the presence or absence of anti-Bax monoclonal antibody 2D2, diluted 1 in 25 in blocking buffer supplemented with 1% normal serum, overnight at 4°C. Sections were washed three times for 2 h in blocking buffer with 1% normal serum. Sections were then incubated with species-specific gold-conjugated secondary antibody, diluted 1 in 50 in blocking buffer with 1% normal serum, overnight at 4°C. Sections were washed three times for 1 h in blocking buffer with 1% normal serum, followed by three washes of 10 min with water. Sections were then counterstained with 5% urinyl $acetate_{(aq)}$ for 1 min at room temperature. Images were then captured by transmission electron microscopy.

2.7. Treatment of eosinophils with antisense oligonucleotide targeting *bcl-2*

In the absence of a specific peptide inhibitor and eosinophils deficient in the target protein of interest (i.e. eosinophils derived from a *bcl-2*$^{-/-}$ mouse), in order to specifically inhibit an intracellular pathway antisense technology was utilised to prevent translation of *bcl-2*, using a kit from Biognostik GmbH (Göttingen, Germany). Antisense oligonucleotides have previously been used to elucidate various signal transduction pathways within this terminally differentiated cell type. Preliminary experiments were performed to monitor uptake of a

FITC-conjugated oligonucleotide. Eosinophils were isolated and resuspended at 1 x 10^6 ml^{-1} in RPMI 1640 + 1% BSA, and cultured in polyethylene tubes in the presence or absence of IL-5 and the presence or absence of 2 μM fluorescein-labelled phosphorothioate oligonucleotide. The addition of phosphorothioate side chains facilitates passive uptake across the plasma membrane. Over a period of 48 h, cells were retrieved, washed and resuspended in PBS, dropped onto a silane-coated microscope slide, and air-dried. Slides were then incubated with 20 μg/ml PI diluted in permeabilisation buffer (0.1% saponin, 0.1% BSA in PBS) for 10 min, washed in PBS and a coverslip applied. Uptake of the FITC-labelled oligonucleotide was assessed by laser scanning cytometry (LSC™, CompuCyte Corps., Cambridge, MA). Slides were scanned individually using the × 20 objective and 5 mW laser output. The LSC™ captures each cell using WinCyte™ software (CompuCyte Corps., Cambridge, MA) contouring around areas of fluorescence if they are sufficiently distinct from the surrounding background. Using nuclear staining with PI as the threshold parameter, single eosinophils were identified by their nuclear fluorescence as detected by the photomultiplier tube in the red channel. Fluorescence of individual eosinophils due to labelled oligonucleotide uptake was sampled by defining a perimeter around the nucleus, thereby allowing quantification of fluorescence in the green channel. 1000 cells were quantified per treatment.

In order to investigate the effect of antisense oligonucleotides targeting *bcl-2* translation, eosinophils were cultured in the presence or absence of 4 μM *bcl-2* antisense phosphorothioate oligonucleotide or control randomised-sequence phosphorothioate oligonucleotide (Biognostik GmbH), and the presence or absence of IL-5. At various timepoints, eosinophils were assessed for apoptosis and viability by morphology and trypan blue exclusion respectively, and target protein expression by intra-cellular flow cytometry (as described above).

2.8. Eosinophil adhesion assay

A 96 well tissue culture plate was coated with 1 mg/ml of fibrinogen overnight at 4°C. Wells were washed twice with RPMI prior to blocking of non-specific binding sites for 1 h at 37°C with 1% BSA in RPMI. Wells were washed three times with RPMI and 100 μl of pre-treated eosinophils were added per well and incubated for 30 min at 37°C. Wells were filled with RPMI and sealed avoiding air bubbles and the inverted plate spun at 300 *g* for 5 min at room temperature. Unbound cells were tipped out, RPMI was added and adherent cells were counted under light microscopy.

2.9. Statistical analysis

2.9.1. Comparison of two data series

Data are expressed as the mean ± SEM of independent experiments and comparisons between treated and untreated cells were made at individual time-points using the Student's paired *t*-test, with a p value of < 0.05 considered significant.

2.9.2. Correlation between two data sets

Pearsons correlation coefficient (r) between two data sets was calculated as a measure of linear association. A correlation coefficient has a value between –1 and 1, with values close to 1 and -1 indicating a positive and negative correlation respectively, and a value of 0 indicating no relationship. r was calculated using the formula:

$$r = \frac{\sum(x-\tilde{x})(y-\tilde{y})}{\sqrt{[\sum (x-\tilde{x})^2(y-\tilde{y})^2]}}$$

Chapter 3

Characterisation of eosinophil apoptosis

3.1. Introduction

Eosinophils possess a battery of cytotoxic mediators including cytokines, highly basic granule proteins and lipid mediators, and as such are potentially important in the initiation and mediation of the inflammatory response. There is increasing evidence suggesting that modulation of cell survival in response to the prevailing cytokine microenvironment is an important mechanism regulating the expansion of eosinophils during allergic inflammation, with delayed eosinophil apoptosis shown to be associated with the eosinophilia observed in inflamed tissue (Simon *et al.*, 1997). The apoptosis and subsequent phagocytosis without phlogistic component release is proposed to be a crucial mechanism regulating the histotoxic potential of the eosinophil and the resolution of airway inflammation (Woolley *et al.*, 1996). Prolonged eosinophil survival is also associated with asthma, inhalant allergy and atopic dermatitis (Wedi *et al.*, 1997, Kankaanranta *et al.*, 2000), with a negative correlation observed between eosinophil apoptosis and asthma severity (Vignola *et al.*, 1999).

Eosinophils are terminally differentiated cells that undergo spontaneous apoptosis rapidly both *in vitro* and *in vivo* unless exposed to survival enhancing cytokines including IL-3, IL-5 and GM-CSF (Stern *et al.*, 1992, Yamaguchi *et al.*, 1991). In order to investigate the apoptosis of eosinophils it was important to characterise the morphological and biochemical events involved, thereby allowing reliable and sensitive assessment of cell death. The objective of this study was to characterise eosinophil apoptosis and evaluate methods for the assessment of apoptosis *in vitro.* Peripheral blood eosinophils were cultured in the absence of survival-enhancing factors in order to investigate the spontaneous apoptosis of resting eosinophils, using various biochemical and morphological hallmarks of apoptosis, namely, internucleosomal DNA cleavage, nucleic and cytoplasmic condensation, redistribution of membrane phospholipids and alteration of autofluorescence. The effect of survival enhancing (IL-5, GM-CSF) and inhibiting (STS, Fas) factors on the apoptotic phenotype of the eosinophil was also investigated.

3.2. Assessment of eosinophil apoptosis

3.2.1. Gross morphology

During spontaneous apoptosis, eosinophils exhibited the characteristic morphological changes associated with the apoptotic phenotype. Figure 3.1A shows eosinophils cultured in the absence of cytokine for 24 h stained with Kimura stain and viewed under light microscopy. An apoptotic eosinophil (*arrow*) clearly exhibited condensation of the cytoplasm, and compaction of the nucleus resulting in overall cell shrinkage in comparison with the adjacent

non-apoptotic eosinophils pictured. Transmission electron microscopy illustrated the marked nuclear morphological changes associated with apoptosis. A non-apoptotic eosinophil (Fig. 3.1B) exhibited the characteristic bi-lobed nucleus and numerous electron-dense cytoplasmic granules. In contrast, an apoptotic eosinophil exhibited significant nuclear pyknosis, forming a uni-compartmental nucleus of compacted chromatin with evidence of apoptosis-associated cytoplasmic vacuolation (Fig. 3.1C).

Condensation of the cytoplasmic compartment and compaction of chromatin are generally regarded as features of the execution phase of apoptosis, occurring as a result of the action of caspases. Various techniques for assessing eosinophil apoptosis were investigated.

3.2.2. DNA laddering

The nuclear condensation observed during apoptosis (Fig. 3.1) is associated with the endonuclease fragmentation of DNA and ultrastructural reorganisation of the nucleus. Initially, chromatin is cleaved to 200-300 and 30-50 kilobase fragments that are subsequently cleaved to internucleosomal fragments (Brown *et al.*, 1993; Cohen *et al.*, 1994). Such internucleosomal fragmentation generates a DNA ladder after agarose gel electrophoresis and is considered a characteristic marker of apoptotic cell death (Wyllie, 1980). The cleavage of DNA during eosinophil apoptosis was investigated. Peripheral blood eosinophils routinely exhibited <98% apoptosis post-isolation, as assessed by morphological examination, with no detectable evidence of DNA cleavage (Fig. 3.2). Culturing eosinophils in the absence of cytokine for 48 h resulted in marked DNA cleavage into discrete ~200 bp fragments, and was associated with ~60% eosinophils exhibiting evidence of nuclear and cytoplasmic condensation. Therefore, eosinophils spontaneously die *in vitro* by apoptosis as indicated by morphological examination and DNA laddering. However, it has been shown that important morphological changes of apoptosis can be dissociated from DNA fragmentation, indicating internucleosomal cleavage to be a relatively late event in the apoptotic process (Cohen *et al.*, 1992). This was supported by the observation that more than half the eosinophils were required to show morphological evidence of end-stage apoptosis in order for DNA laddering to be clearly detectable.

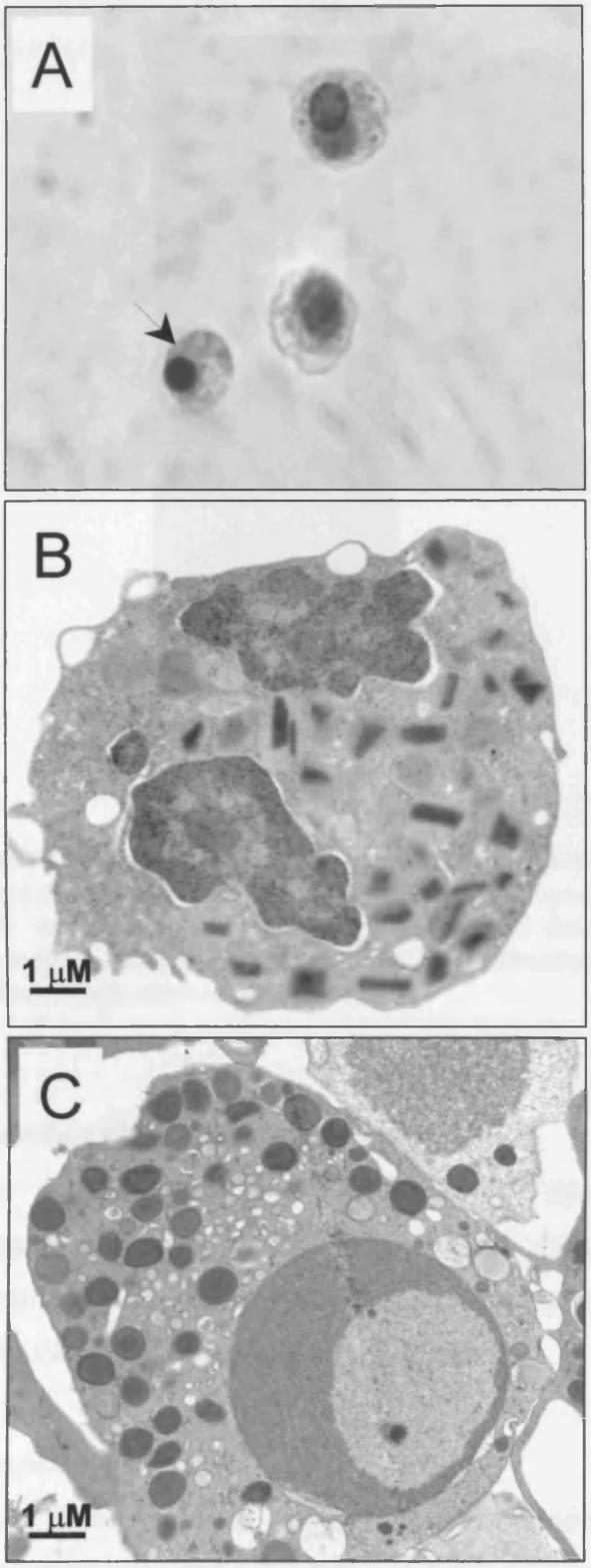

Figure 3.1. Eosinophils exhibit characteristic morphology during apoptosis. A. Peripheral blood eosinophils were cultured in the absence of cytokine for 24 h and viewed under light microscopy after Kimura stain (A, x1000) or images captured by transmission electron microscopy (B and C). An apoptotic eosinophil (A, *arrow*, and C) exhibits characteristic nuclear and cytoplasmic condensation and vacuolation, and nuclear morphology readily distinguishable from the bi-lobed appearance of a non-apoptotic eosinophil (B).

Figure 3.2. Internucleosomal DNA cleavage during spontaneous eosinophil apoptosis. DNA was extracted from freshly isolated peripheral blood eosinophils (*0h*) or eosinophils cultured in the absence of cytokine for 48 h (*48h*) and electrophoresed through agarose as described in *Materials and Methods*. Figures indicate apoptosis quantified by morphological examination under light microscopy. Figure is representative of three independent experiments.

3.2.3. Flow cytometric assessment of eosinophil apoptosis

Flow cytometry provides a rapid and efficient assessment of apoptosis, and lacks the subjectivity of morphological examination. A number of flow cytometric techniques have been developed for the quantitation of apoptosis *in vitro*. Two assays were used to analyse the apoptosis of eosinophils, PtdSer exposure and red autofluorescence.

3.2.3.1. Red autofluorescence

Flow cytometric analysis of freshly isolated peripheral blood eosinophils revealed a single population in terms of size, granularity and red autofluorescence. Culturing *in vitro*, generated a subpopulation of eosinophils that exhibited both reduced size and increased red autofluorescence. Isolation and morphological examination of this distinct population revealed the highly autofluorescent, condensed eosinophils to be predominantly apoptotic (Stern *et al.*, 1992). Therefore the quantitation of eosinophil apoptosis by red autofluorescence was investigated. Eosinophils cultured for 48 h were analysed by flow

cytometry and a representative histogram is shown in Figure 3.3, indicating ~35% eosinophils exhibited increased autofluorescent in the red (FL2) channel.

3.2.3.2. PtdSer externalisation

During apoptosis PtdSer is exposed on the cell surface and is therefore accessible to binding by Annexin V, a phospholipid binding protein with specific avidity for PtdSer. An Annexin V fluorescein conjugate was used to assess eosinophil apoptosis by flow cytometry (Fig 3.4). Freshly isolated eosinophils exhibited little or no binding of Annexin V-FITC. A time-dependent increase in Annexin V binding was observed whilst culturing eosinophils over a 48 h period, with approximately 40-50% manifesting signs of PtdSer externalisation after 24 h.

3.3. Comparison of eosinophil apoptosis assays

Eosinophils were cultured in the absence of cytokine for up to 48 h. At the various time points, eosinophils were assessed for apoptosis by morphology, Annexin V binding and red autofluorescence. Viability was also assessed by PI exclusion by flow cytometry (Fig. 3.5). Annexin V binding proved the most sensitive detecting approximately 60% apoptosis compared with less than 25% as indicated by morphology and red autofluorescence after 18 h (Figure 3.5A). Visual determination of apoptotic morphology was the next most sensitive assay, with red autofluorescence only detecting relatively late changes in the apoptotic population. However, comparison with viability assessed by PI uptake (Fig. 3.5B) indicated membrane integrity was largely maintained up to 48 h, concomitant with the majority of eosinophils exhibiting an apoptotic phenotype.

3.4. Bivariate flow cytometric analysis of eosinophil apoptosis

Comparison of the kinetics of apoptosis and viability assessed by Annexin V binding and PI exclusion respectively (Fig. 3.5) indicate that there is a progression from detectable signs of apoptosis to the loss of plasma membrane integrity associated with late-stage apoptosis and necrosis *in vitro*. This highlights the necessity for bivariate flow cytometric analysis involving Annexin V binding in conjunction with vital dye exclusion, when quantifying spontaneous apoptosis. The loss of membrane integrity associated with necrosis enables both necrotic and

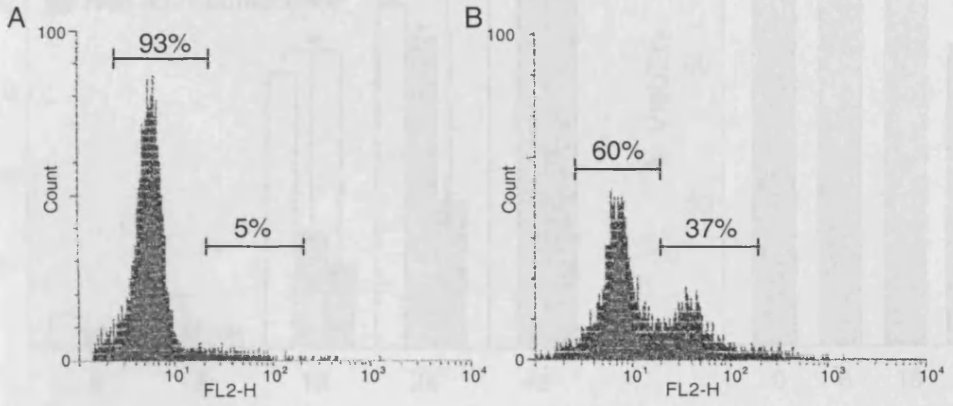

Figure 3.3. Eosinophils exhibit increased red autofluorescence during spontaneous apoptosis. Unlabelled, freshly isolated (A) or eosinophils cultured for 24 h in the absence of cytokine (B) were analysed by flow cytometry. Flow cytometry histograms representative of three independent experiments are shown.

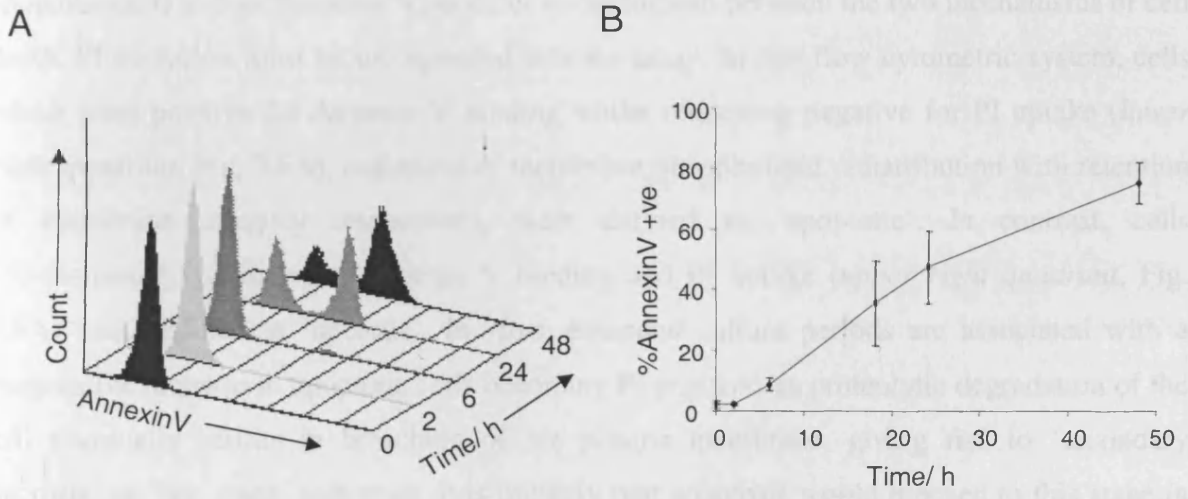

Figure 3.4. Eosinophils bind Annexin V-FITC during spontaneous apoptosis. Peripheral blood eosinophils were cultured in the absence of cytokine for up to 48 h and assessed for Annexin V-FITC binding at the indicated time points. A) Histograms are representative of four independent experiments. B) Shows the mean ± SEM of four independent experiments.

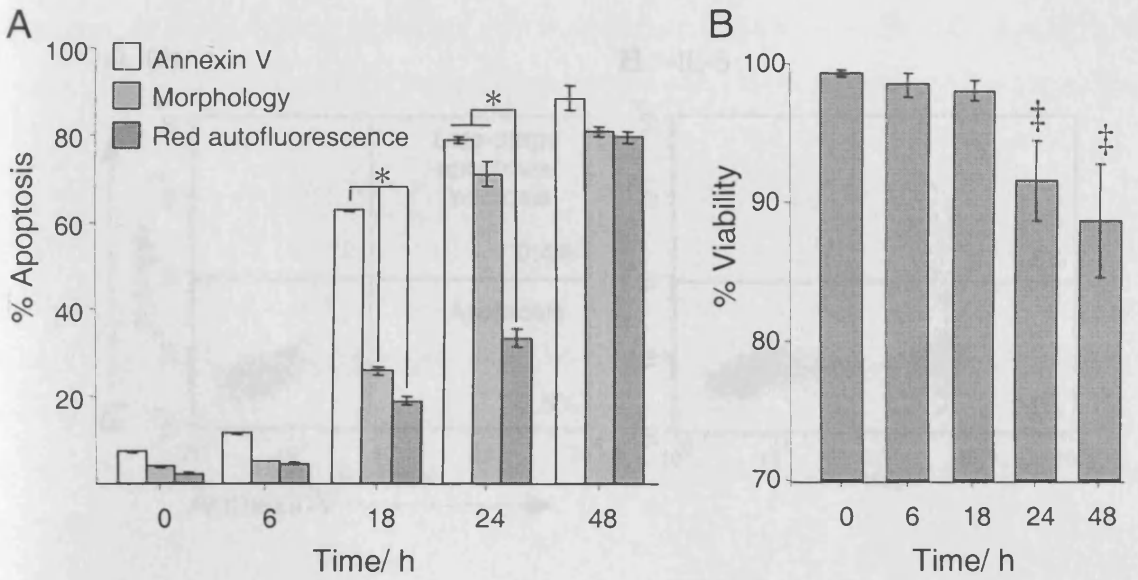

Figure 3.5. Assessment of eosinophil apoptosis and viability. Eosinophils were cultured in the absence of cytokine and at each timepoint apoptosis (A) was assessed by morphology under light microscopy, red autofluorescence and Annexin V binding by flow cytometry, and viability (B) by propidium iodide (PI) uptake. Results are expressed as the mean ± SEM of at least four separate experiments. *$p<0.05$ comparing methods of apoptotic assessment at individual time points (A). ‡$p<0.05$ compared with 0 h (B).

apoptotic cells to bind Annexin V, in order to distinguish between the two mechanisms of cell death, PI exclusion must be incorporated into the assay. In this flow cytometric system, cells which were positive for Annexin V binding whilst remaining negative for PI uptake (*lower right quadrant*, Fig. 3.6A), indicative of membrane phospholipid redistribution with retention of membrane integrity respectively, were defined as 'apoptotic'. In contrast, cells simultaneously positive for Annexin V binding and PI uptake (*upper right quadrant*, Fig. 3.6A) were defined as 'necrotic'. *In vitro*, extended culture periods are associated with a progressive increase in apoptotic cells becoming PI positive, as proteolytic degradation of the cell eventually results in breaching of the plasma membrane, giving rise to 'secondary necrosis' or 'late stage' apoptosis. It is unlikely that apoptosis would proceed to this stage *in vivo* due to the rapid and efficient clearance of apoptotic cells by resident phagocytes, which recognise cells undergoing apoptosis by detecting early membrane changes, including the externalisation of PtdSer (Bennett *et al.*, 1995; Fadok *et al.*, 2000). Culturing eosinophils in the absence of survival-enhancing cytokine induced a progressive increase in eosinophils binding Annexin V whilst simultaneously excluding PI (Fig. 3.6D).

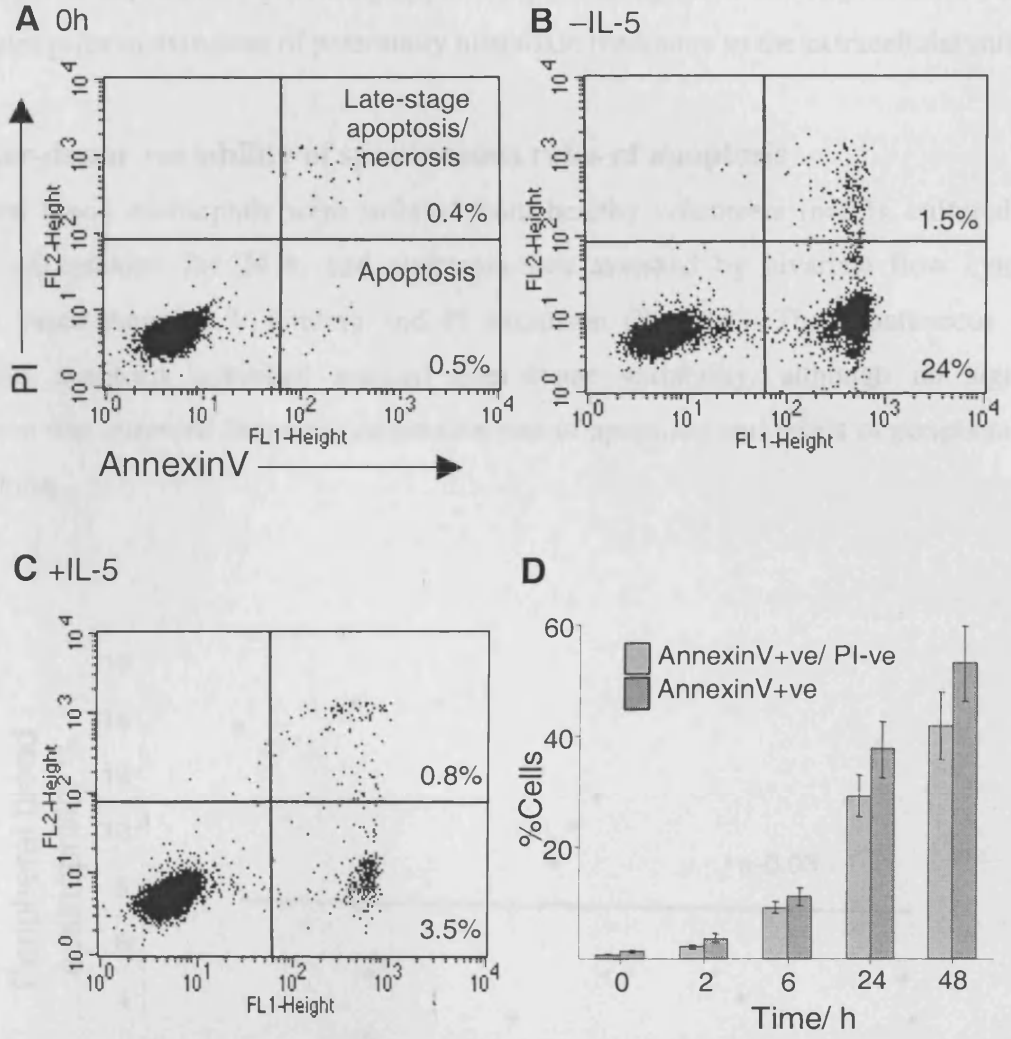

Figure 3.6. Multivariate flow cytometric analysis of eosinophils undergoing apoptosis. Freshly isolated (A) peripheral blood eosinophils were cultured in the absence (B) or presence (C) of IL-5 (10^{-10}M) for 24 h. Multivariate flow cytometry was performed according to *Materials and Methods* with Annexin V and propidium iodide (PI) to assess apoptosis and necrosis/ late-stage apoptosis. Eosinophils positive for Annexin V, but PI negative are defined as apoptotic (*lower right quadrant*). Cells which are Annexin V and PI positive have lost plasma membrane integrity and therefore are defined as necrotic/ late-stage apoptotic (*upper right quadrant*). During eosinophil apoptosis there is a progressive increase in Annexin V positive cells that was inhibited by IL-5. (D) Eosinophils were cultured in the absence of cytokine and at the time points indicated multivariate flow cytometric analysis performed. Results are expressed as the mean ± SEM of three experiments.

Eosinophils rapidly undergo apoptosis *in vitro*, which can be efficiently and sensitively quantified by assessment of PtdSer externalisation. The data indicates that the majority of eosinophils exhibiting evidence of apoptosis retained their plasma membrane integrity even after culturing for 48 h. This may have important implications for the non-phlogistic clearance of apoptotic eosinophils, as there appears to be a significant lag period, at least *in vitro*, (>24 h) between the initial signs of apoptosis and the membrane breaching associated with

secondary necrosis, thereby providing opportunity for recognition and engulfment by resident phagocytes prior to extrusion of potentially histotoxic mediators to the extracellular milieu.

3.5. Inter-donor variability of spontaneous rates of apoptosis

Peripheral blood eosinophils were isolated from healthy volunteers (n=23), cultured in the absence of cytokine for 24 h, and apoptosis was assessed by bivariate flow cytometric analysis based Annexin V binding and PI exclusion (Fig. 3.7). The spontaneous rate of eosinophil apoptosis exhibited marked inter-donor variability, although no significant correlation was observed between constitutive rate of apoptosis and levels of peripheral blood eosinophilia.

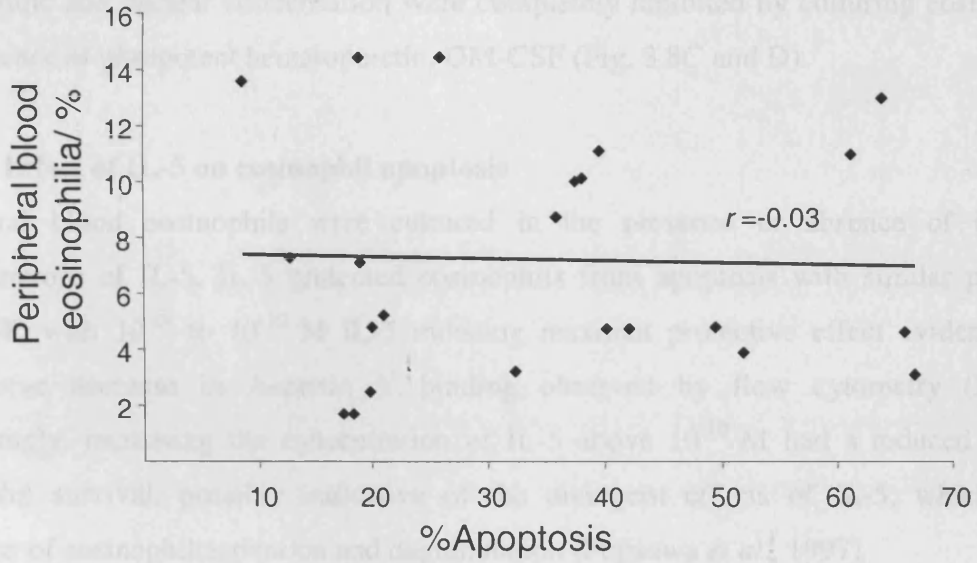

Figure 3.7. Inter-donor variability of spontaneous eosinophil apoptosis. Peripheral blood eosinophils were isolated from healthy volunteers (n=23), cultured for 24 h in the absence of cytokine, and apoptosis was determined on the basis of simultaneous Annexin V binding and PI exclusion. Pearsons correlation coefficient (*r*) indicates no relationship between the two data sets.

Eosinophil apoptosis can be modulated by a number of extracellular factors including cytokines (IL-5, GM-CSF, IFN-γ, TGF-β), chemicals (staurosporine, dexamethasone, theophylline), and ligation of specific cell surface receptors (Fas (CD95/Apo-1), CD69, CD45) (reviewed by Dewson *et al.*, 1996). The effect of exogenous factors GM-CSF, IL-5, STS, and ligation of FasR on the apoptosis of peripheral blood eosinophils was investigated.

3.6. Effect of exogenous factors on eosinophil survival

3.6.1. Pro-survival cytokines

The effect of potent survival-enhancing cytokines GM-CSF and IL-5 on eosinophil apoptosis was investigated.

3.6.1.1. Effect of GM-CSF on eosinophil apoptosis

Peripheral blood eosinophils were cultured in the presence or absence of increasing concentrations of GM-CSF and assessed for apoptosis by Annexin V binding (Fig 3.8A and B). 10^{-11} to 10^{-10} M GM-CSF induced maximal survival effect in eosinophils in terms of both cell viability and apoptosis. Eosinophils cultured for 96 h exhibited detectable DNA fragmentation to multiples of ~200 bp (Fig. 3.8C), corresponding with > 80% apoptosis as assessed by morphology (Fig. 3.8D). Internucleosomal DNA cleavage and the appearance of cytoplasmic and nuclear condensation were completely inhibited by culturing eosinophils in the presence of pluripotent hematopoietin, GM-CSF (Fig. 3.8C and D).

3.6.1.2. Effect of IL-5 on eosinophil apoptosis

Peripheral blood eosinophils were cultured in the presence or absence of increasing concentrations of IL-5. IL-5 protected eosinophils from apoptosis with similar potency to GM-CSF, with 10^{-11} to 10^{-10} M IL-5 inducing maximal protective effect evidenced by a progressive decrease in Annexin V binding observed by flow cytometry (Fig. 3.9). Interestingly, increasing the concentration of IL-5 above 10^{-10} M had a reduced effect on eosinophil survival, possibly indicative of the divergent effects of IL-5, which include induction of eosinophil activation and degranulation (Fujisawa *et al.*, 1997).

Optimal survival enhancing concentrations of GM-CSF and IL-5 (10^{-10}M) were used in subsequent experiments unless otherwise indicated.

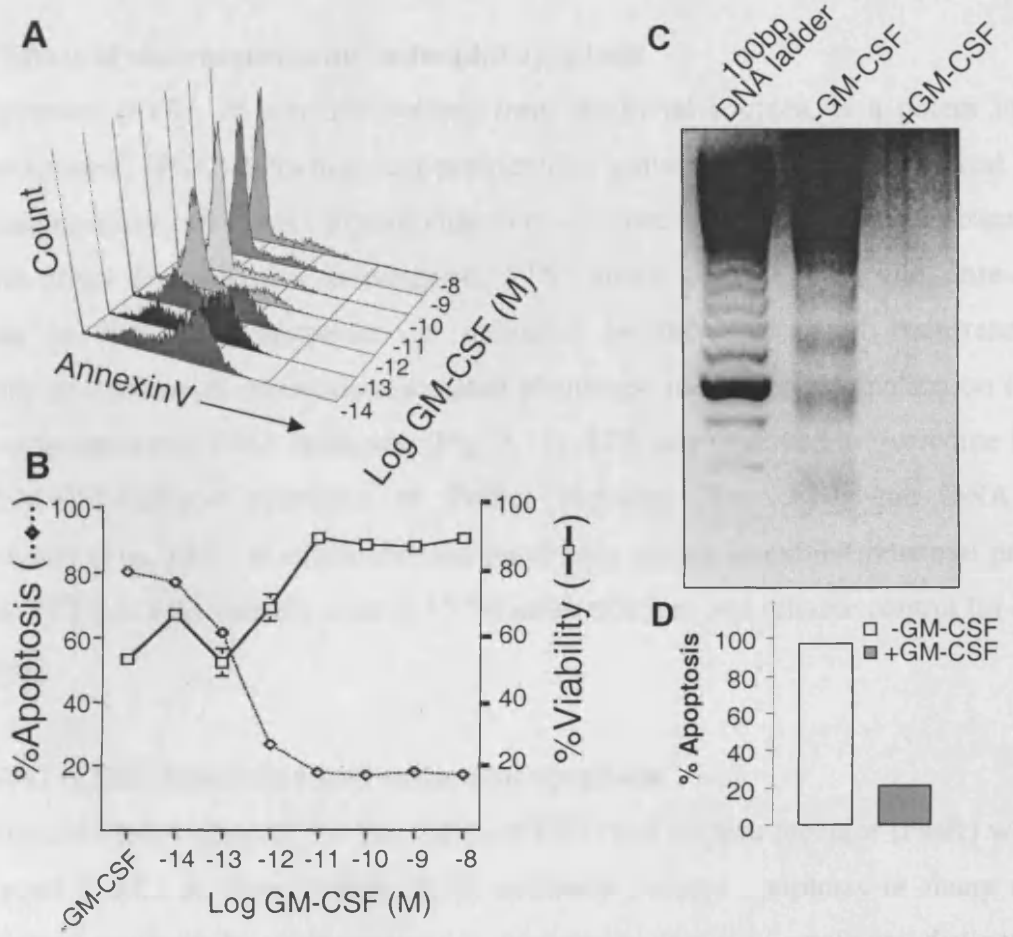

Figure 3.8. Dose-dependent inhibition of eosinophil apoptosis by GM-CSF. Peripheral blood eosinophils were cultured for 18 h in the presence or absence of GM-CSF at the indicated log molar concentrations (A and B). Representative flow cytometry histograms of progressive decrease in Annexin V binding in the presence of increasing concentrations of GM-CSF (A). Apoptosis was assessed by Annexin V staining and viability by trypan blue exclusion and is presented as the mean ± SEM of two independent experiments (B). Eosinophils were cultured in the presence or absence of GM-CSF (10^{-10}M) for 96 h and assessed for internucleosomal DNA fragmentation (C) and apoptotic morphology after Kimura stain (D). Results are representative of three separate experiments.

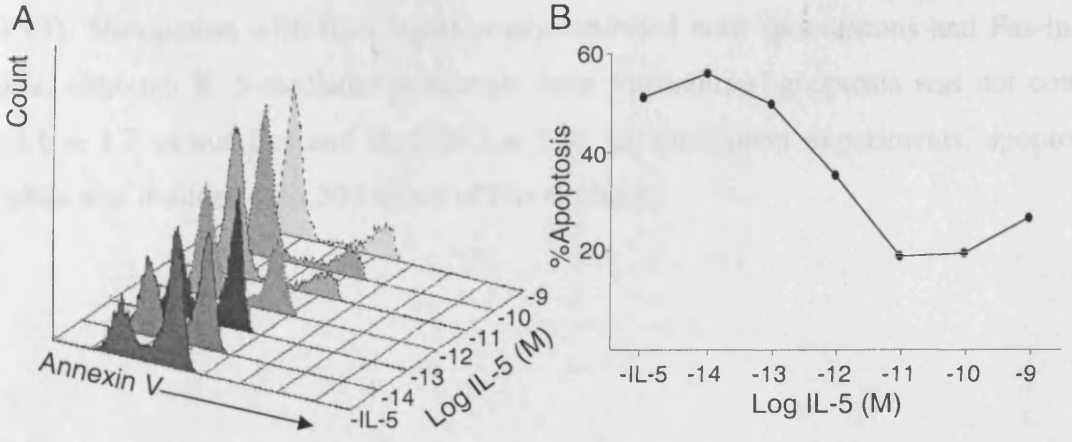

Figure 3.9. Dose-dependent inhibition of eosinophil apoptosis by IL-5. Peripheral blood eosinophils were cultured for 18 h in the presence or absence of IL-5 at the indicated log molar concentrations. A) Representative flow cytometry histograms of progressive decrease in Annexin V binding in the presence of increasing concentrations of IL-5. B) Apoptosis was assessed by Annexin V staining and is presented as the mean of two independent experiments.

3.6.2. Effect of staurosporine on eosinophil apoptosis

Staurosporine (STS), an alkaloid isolated from microbial sources, is a potent inhibitor of protein kinase C (PKC), with high anti-proliferative activity *in vitro* and a classical inducer of apoptosis in many cell types. Eosinophils were incubated in the presence or absence of STS and the effect on apoptosis investigated. STS caused a rapid, time and dose-dependent increase in eosinophil apoptosis, as indicated by the accelerated occurrence of the spontaneous eosinophil apoptosis-associated phenotype of PtdSer externalisation (Fig. 3.10) and internucleosomal DNA laddering (Fig. 3.11). STS was observed to overcome both IL-5- and GM-CSF-induced inhibition of PtdSer exposure (Fig. 3.10) and DNA cleavage respectively (Fig. 3.11), at concentrations previously shown to exhibit maximal pro-survival effects. STS was subsequently used at 10^{-5}M as an efficient and reliable control for eosinophil apoptosis.

3.6.3. Fas (CD95/Apo-1)-induced eosinophil apoptosis

Ligation and trimerisation of the Fas (Apo-1/CD95) cell surface receptor (FasR) with soluble Fas ligand (FasL) or cross-linking (IgM) antibody induces apoptosis in many cell types. Induction of apoptosis by death-receptor is proposed to occur by a pathway distinct from that induced by stress, such as growth factor deprivation (Bratton *et al*, 2001). Peripheral blood eosinophils constitutively expressed cell surface FasR as assessed by flow cytometry (Fig. 3.12) .

Ligation of FasR with cross-linking monoclonal antibody, CH11, induced significant acceleration of eosinophil apoptosis assessed by PtdSer exposure, compared with IgM control (Fig. 3.13). Stimulation with IL-5 significantly inhibited both spontaneous and Fas-induced apoptosis, although IL-5-mediated protection from Fas-induced apoptosis was not complete (IL-5: 8.6 ± 1.7 versus Fas and IL-5:16.2 ± 5.4). In subsequent experiments, apoptosis of eosinophils was induced with 500 ng/ml of Fas antibody.

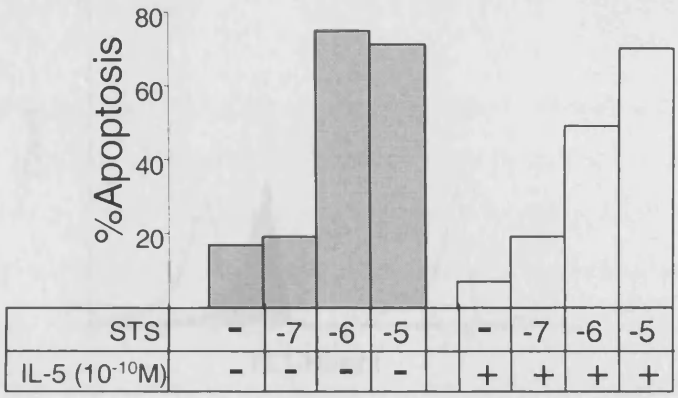

Figure 3.10. Dose-dependent induction of eosinophil apoptosis by protein kinase C inhibitor, staurosporine. Peripheral blood eosinophils were cultured for 18 h in the presence or absence of STS at the indicated log molar concentrations and IL-5. Apoptosis was assessed by Annexin V staining. Results are expressed as mean of two independent experiments.

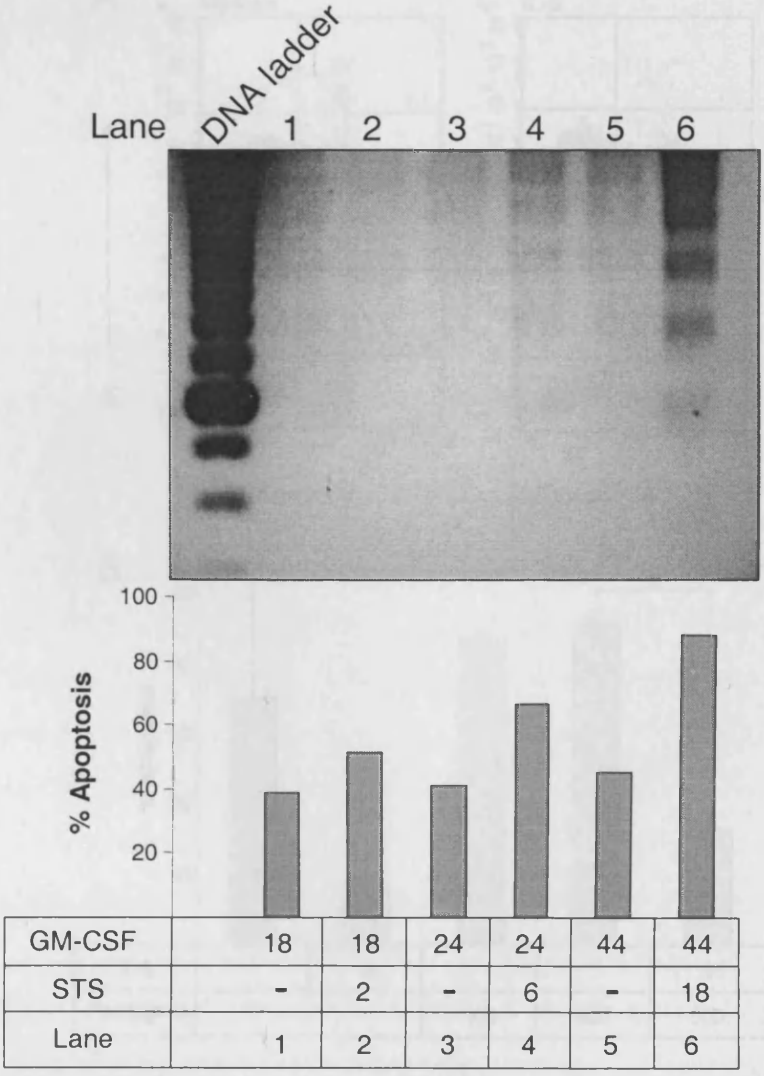

Figure 3.11. Protein kinase C inhibitor, staurosporine, induces internucleosomal DNA laddering in eosinophils. Peripheral blood eosinophils were cultured in the presence or absence of staurosporine (STS, 10^{-5}M) and GM-CSF (10^{-10}M) for the indicated times and assessed for DNA laddering and Annexin V binding. Results are representative of three separate experiments.

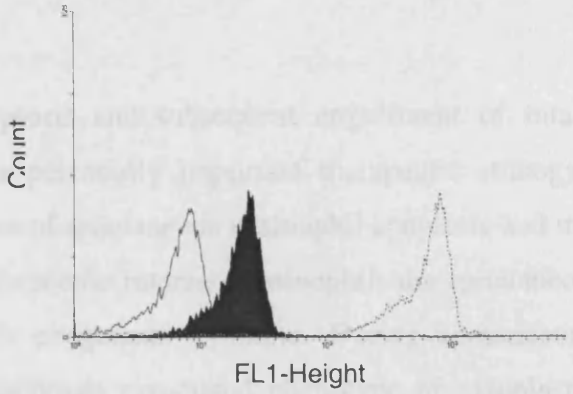

Figure 3.12. Eosinophils constitutively express cell surface FasR (CD95/ Apo-1). Peripheral blood eosinophils were immunostained with anti-Fas mAb (*solid histogram*), anti-CD9 positive control (*dashed histogram*), or isotype-matched negative control (*hollow histogram*) and examined by flow cytometry. Representative of three independent experiments.

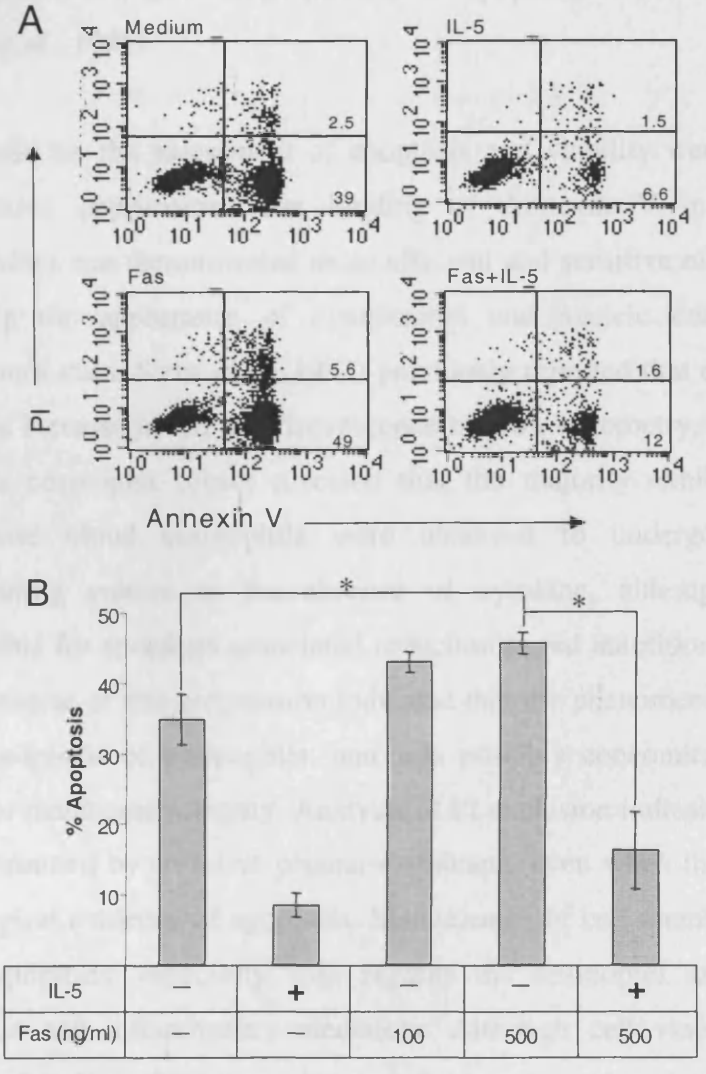

Figure 3.13. Ligation of FasR induces eosinophil apoptosis that is partially inhibited by IL-5. Eosinophils were cultured for 24 h in the presence or absence of IL-5 (10^{-10}M) and anti-Fas mAb (CH11) at the indicated concentration, or IgM control and apoptosis determined by Annexin V binding and exclusion of PI. Plots of 10000 events representative of four independent experiments with percentage of cells in each quadrant indicated (A). Results expressed as mean ± SEM of four independent experiments, *p<0.05 (B).

3.7. Discussion

The induction of apoptosis and subsequent engulfment of intact eosinophils in a non-phlogistic fashion is a potentially important therapeutic strategy for the amelioration of asthma. Characterisation of spontaneous eosinophil apoptosis and modulation with exogenous factors are therefore of specific interest. Eosinophils die spontaneously *in vitro* by apoptosis unless stimulated with exogenous cytokine. During spontaneous apoptosis, eosinophils exhibit the classical apoptosis associated phenotype of cytoplasmic condensation, nuclear pyknosis, internucleosomal DNA fragmentation, as indicated by DNA laddering, and redistribution of membrane phospholipids. The cytoplasmic condensation leading to detectable reduction in cell volume is thought to be due, at least in part, to the rapid efflux of K^+ ions, as eosinophil shrinkage during spontaneous apoptosis is sensitive to K^+ channel blockers (Beauvais *et al.*, 1995).

A number of methods for the assessment of apoptosis and viability were examined under conditions of cytokine deprivation. The binding of Annexin V in response to the externalisation of PtdSer was demonstrated as an efficient and sensitive marker of eosinophil apoptosis, preceding the appearance of cytoplasmic and nucleic condensation readily detectable after Kimura stain. Stern *et al* (1992) previously reported that eosinophils *in vitro* exhibit a progressive increase in red autofluorescence by flow cytometry, and morphological examination of this eosinophil subset revealed that the majority exhibited an apoptotic phenotype. Peripheral blood eosinophils were observed to undergo a shift in red autofluorescence during culture in the absence of cytokine, although the underlying mechanism responsible for apoptosis-associated reduction in red autofluorescence is unclear. However, the time course of this progression indicated that the phenomenon was a relatively late event in the apoptosis of eosinophils, and was possibly concomitant with late-stage apoptosis and loss of membrane integrity. Analysis of PI exclusion indicated that eosinophils retain viability, determined by an intact plasma membrane, even when the majority of cells exhibited morphological evidence of apoptosis. Maintenance of cell membrane integrity is a crucial aspect of apoptosis, especially with regards the eosinophil and its plethora of potentially histotoxic and inflammatory mediators. Although cell viability *in vitro* was observed to decline after 48 h, there was a lag period where ingestion signals, such as PtdSer exposure, were presented by the apoptotic cell, facilitating recognition and engulfment by resident phagocytes of intact cells in a non-phlogistic fashion.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) is a frequently used, alternative technique for the determination of apoptosis on the basis of double strand DNA cleavage and the consequent generation of reactive 3' OH ends. Kern *et al* (2000) developed a system for the flow cytometric discrimination of neutrophils, and apoptotic and viable eosinophils, and reported that cytospins of freshly isolated peripheral blood eosinophils were stained positive by the TUNEL assay. However, the TUNEL staining exhibited a granular distribution predominantly within the cytoplasm, did not colocalise with a PI nuclear counter stain, and was independent of the activity of TdT, indicating a non-specific interaction of FITC with basic granular proteins within eosinophils. Therefore, the use of TUNEL technique, at least with a FITC-conjugate, appears an unsuitable marker of apoptosis in eosinophils.

The constitutive rate of apoptosis was highly donor variable, although had no significant relationship with levels of peripheral blood eosinophilia. Delayed eosinophil apoptosis has been observed in peripheral blood eosinophils derived from asthmatics, and patients with atopic disease (Wedi *et al.*, 1997; Kankaanranta *et al.*, 2000). Such delay is proposed to be in response to circulating cytokines GM-CSF and IL-5. Stimulation of eosinophils *in vitro* with hematopoietins GM-CSF and IL-5 significantly inhibits spontaneous apoptosis (Figs. 3.7 and 3.8; Tai *et al.*, 1991; Yamaguchi *et al.*, 1991). Subsequent investigation concentrated on elucidating IL-5-mediated survival, due to its selectivity for eosinophils, with a more potent survival enhancing effect than GM-CSF and IL-3 (Stern *et al.* 1992).

Inhibition of PKC by STS has been shown to decrease granulocyte viability *in vitro* (Cousin *et al.*, 1997; Hossain *et al.*, 1994). Treatment of peripheral blood eosinophils with STS rapidly induced the morphological changes consistent with those observed during spontaneous apoptosis, namely internucleosomal DNA cleavage, nuclear pyknosis, and PtdSer externalisation. Therefore STS provided an efficient positive control for eosinophil apoptosis, important due to the donor dependent variability of constitutive rates of cell death.

A physiologically relevant mechanism for the induction of apoptosis is via ligation of the FasR by either membrane bound or soluble FasL. FasL is synthesised as a type II transmembrane protein and the soluble form is generated upon proteolytic cleavage of the membrane-bound form. FasL is expressed on a number of cells, including T lymphocytes and bronchial epithelial cells. Eosinophils constitutively express functional cell surface FasR (Fig. 3.13; Hebestreit *et al.*, 1996; Matsumoto *et al.*, 1995), with no detectable expression of FasL

(Liles *et al.*, 1996). Neutrophils also express FasR, but, in contrast, are a potential source of soluble FasL (Liles *et al.*, 1996). Interestingly, expression of FasL on BAL T cells was observed to increase in asthma (Krug *et al.*, 1999), although no concomitant increase in apoptosis was observed. Abrogation of FasR induced eosinophil apoptosis *in vitro*, although the effect observed over and above spontaneous apoptosis was relatively modest in comparison with STS. The data indicate that stimulation with IL-5 can at least partially overcome the pro-apoptotic effect of Fas. A protective effect of IL-5 may be in response to downregulation of the expression of FasR. Although, cell surface FasR expression was not investigated here, previous reports have indicated that levels of FasR remain unchanged *in vitro* in the absence or presence of GM-CSF and IL-5 for 24 h (Matsumoto *et al.*, 1995; Hebestreit *et al.*, 1998). Matsumoto *et al* (1995) suggested that Fas-induced loss of viability of eosinophils was mediated by a pathway independent of the pro-survival effects of IL-5. However, the authors did report an IL-5-induced increase in viability of Fas-treated eosinophils from ~10% (anti-Fas, 100 ng/ml) to ~80% (co-incubation with anti-Fas and IL-5) after 48 h. Similar survival-enhancing concentrations of IL-5 were found to have a 20% protective effect on Fas-treated eosinophils by Hebestreit *et al* (1998). The propensity of eosinophils to undergo apoptosis spontaneously *in vitro* caused difficulty when trying to elucidate the effects of a survival antagonist, such as Fas, whose effects are relatively modest. It cannot be excluded that IL-5 was merely inhibiting the death of the subset of eosinophils (~40%) undergoing spontaneous apoptosis, independent of FasR ligation. Administration of anti-Fas mAb to the lungs following induction of airway eosinophilia by aerosol allergen challenge increased the number of macrophages within the BAL with evidence of eosinophil-derived products, and was followed by a marked reduction in the number of eosinophils in the airways (Tsuyuki *et al.*, 1995). Interestingly, peripheral blood eosinophils isolated from some eosinophilic patients, and nasal polyp-derived tissue eosinophils lack a functional response to FasR ligation (Hebestreit *et al.*, 1996). This was not merely due to lack of surface expression of the receptor, but intriguingly, Fas-induced eosinophil apoptosis was inhibited by NO, the level of which is increased in the airways of asthmatics (Alving *et al.*, 1993) and is potentially generated by eosinophils (del Pozo *et al.*, 1997). Therefore, it is tempting to speculate that dysfunction of Fas-induced eosinophil apoptosis, termed 'Fas resistance', may be partly responsible for the eosinophilia observed in the asthmatic airway.

The molecular regulation of eosinophil cell death in response to diverse apoptotic stimuli, and the protective effect of IL-5 was investigated.

Chapter 4

Bcl-2 homologues in IL-5-mediated eosinophil survival

4.1. Introduction

The *bcl-2* proto-oncogene was first described at chromosomal breakpoint t(14:18) found in B cell lymphomas (Tsujimoto *et al.*, 1986) and was determined to be the mammalian homologue of the *C. elegans* death repressor gene, *ced-9* (Hengartner and Horvitz 1994). A large family of genes with sequence homology to Bcl-2 have emerged which possess important divergent functions in the mediation of apoptosis, being either death antagonists (Bcl-2, Bcl-x_L, Bcl-w) or death agonists (Bax, Bcl-x_S, Bak). The complexity of Bcl-2 homologue activity is highlighted by Bcl-x. Alternative transcription of the *bcl-x* gene results in two distinct mRNA transcripts, the larger, *bcl-x_L*, encoding a death suppressor, and the alternative variant, *bcl-x_S*, smaller due to an internal deletion of 189 bp, encoding a protein capable of antagonising the effects of Bcl-x_L (Boise *et al.*, 1993; Minn *et al.*, 1996). Bcl-2 homologues form a dynamic equilibrium of homo/ heterodimerisation via conserved regions, known as BH domains, and also associate with unrelated proteins, such as protein kinase Raf-1 (Wang *et al.*, 1996), forming a complex life-death rheostat linked to growth factor receptors and kinase signalling cascades. Most have a trans-membrane region allowing localisation to intracellular membranes with the potential to mediate the release of apoptogenic factors, such as cytochrome *c* (Kluck *et al.*, 1997), Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000) and AIF (Susin *et al.*, 1999b) from the mitochondria. Peripheral blood lymphocytes express Bcl-2, Bcl-x and Bax, although neutrophils express only the latter homologue (Ohta *et al.*, 1995; Iwai *et al.*, 1994).

Bcl-2 homologues are classified on the basis of their pro or anti-apoptotic function and their homology within distinct regions, the BH domains. Several pro-apoptotic homologues (Bid, Bik, Bim) have been identified which lack significant homology with other Bcl-2 proteins, with the exception of the BH3 domain, and are therefore termed 'BH3-only proteins'. BH3-only proteins mediate the apoptotic pathway in response to diverse stimuli, interact with anti-apoptotic Bcl-2 and Bcl-x_L, and their pro-apoptotic effect manifests in the induction of cytochrome *c* release from the mitochondria (Shimizu and Tsujimoto, 2000). Bim is transcribed as three alternative splice variants, generating proteins designated Bim_{EL} (extra long, ~33 kDa), Bim_L (long, ~25 kDa) and Bim_S (short, ~15 kDa), with the Bim_S being the most potent inducer of apoptosis. Expressed in hematopoietic, epithelial, neuronal and germ cells, Bim_{EL} and Bim_L are proposed to interact with the microtubule-associated dynein light chain, being released upon initiation of apoptosis to target intracellular membranes via its hydrophobic C terminus and inhibit the anti-apoptotic activity of Bcl-2 (Puthalakath *et al.*, 1999). Bik also forms complexes with, and presumably inhibits Bcl-2 and Bcl-x, although this

oligomerisation is insufficient for its pro-apoptotic activity. Interestingly, Bik expression was observed to decrease during the neutrophilic differentiation of HL-60 cells and was essentially absent from peripheral blood neutrophils (Santos-Beneit and Mollinedo, 2000). Bid is preferentially activated by caspase-8, and is a crucial mediator of mitochondrial perturbation during receptor-induced apoptosis. Caspase-8-dependent cleavage generates tBid that translocates to the mitochondria and cooperates with Bax and Bak to induce cytochrome *c* release (Korsmeyer *et al.*, 2000).

The susceptibility of a cell to undergo apoptosis can be potentially governed by the balance of Bcl-2 proteins, with upregulation of pro-apoptotic homologues, such as Bax and Bak, sensitising the cell to apoptotic stimuli. However, the capacity of Bcl-2 homologues to regulate apoptosis may also be determined by post-translational modification as well as modulating protein levels. The anti-apoptotic Bcl-2 protein can be converted into a pro-apoptotic protein by caspase-dependent cleavage (Cheng *et al.*, 1997). Also, the anti-apoptotic activity of Bcl-2 is regulated by phosphorylation status (Ito *et al.*, 1997), with hyperphosphorylation of Bcl-2 following stimulation with IL-3 and activation of Erk1/2 in an IL-3–dependent cell, possibly maximising its anti-apoptotic activity by stabilising Bcl-2:Bax heterodimerisation (Deng *et al.*, 2000). Similarly, the dimerisation properties and consequent activity of the pro-apoptotic Bcl-2 homologue, Bad, have been shown to be phosphorylation state dependent. Bad interacts with Bcl-2 and Bcl-x_L at the mitochondrial membrane, dissociating them from Bax and abrogating their anti-apoptotic effect (Yang *et al.*, 1995). Phosphorylation of Bad in response to growth factor stimulation facilitates interaction with, and sequestration by, the cytosolic protein, 14-3-3 (Zha *et al.*, 1996), thereby preventing association with and inhibition of Bcl-2 and Bcl-x_L. This provides an attractive mechanism for integration of a proximal growth factor survival signal and the Bcl-2 family-dependent control of apoptosis.

In order to elucidate the regulation of eosinophil apoptosis *in vitro*, the expression of Bcl-2 and its homologues was investigated using various techniques. Potential modulation of these apoptosis-mediators by survival-enhancing concentrations of IL-5 was also investigated.

4.2. Bcl-2 homologue expression in peripheral blood eosinophils

4.2.1. Detection of apoptosis-associated proteins

Bcl-2 was not detectable in unstimulated eosinophils (Fig. 4.1A), compared with HL-60 cell line as a positive control (Fig. 4.1B) as assessed by intracellular flow cytometry. Constitutive expression of Bax (SMF- 26.3± 0.76), and Bcl-x (SMF- 11.3± 1.42) in freshly isolated peripheral blood eosinophils was observed (Fig. 4.1C and D respectively). It was not possible to distinguish between Bax α/ β, and the small and large isoforms of Bcl-x by this method, as the antibodies used targeted epitopes common to both.

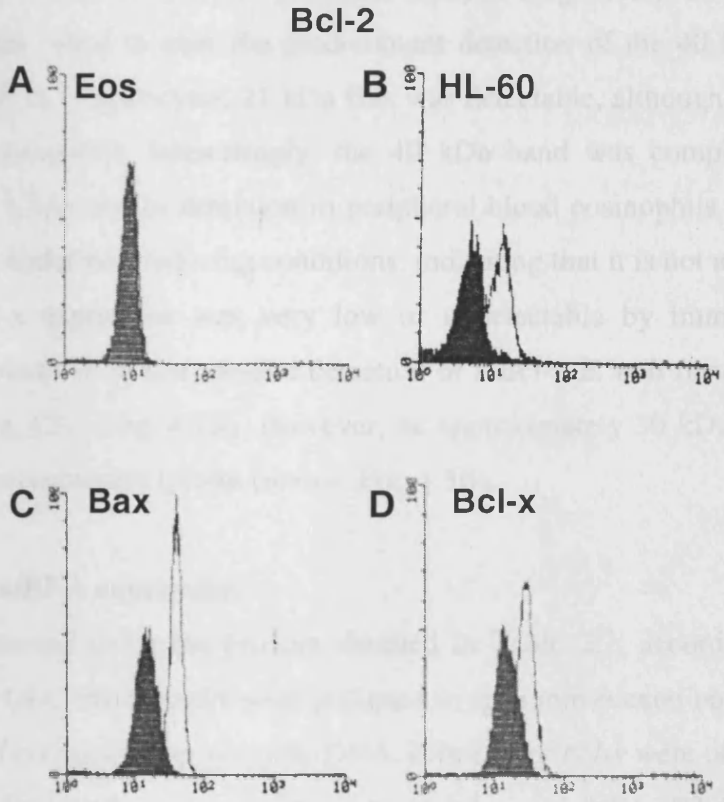

Figure 4.1. Bcl-2 homologue expression in eosinophils by intracellular flow cytometry. Peripheral blood eosinophils were isolated, and intracellular flow cytometry performed according to *Materials and Methods.* Eosinophils were stained with antibody specific for Bcl-2 (A and B), Bax (C) and Bcl-x (D). Test antibody (*hollow histogram*), negative control (*solid histogram*). HL-60 cell line was used a positive control for Bcl-2 expression (B). Results are representative of four independent experiments.

Consistent with the flow cytometry data, immunoblot analysis detected the 26kDa Bcl-2 in Jurkat and HL-60, but the protein was essentially absent from freshly isolated eosinophils (Fig. 4.2A). Bax is expressed as at least two alternative transcripts, *bax*α and *bax*β, encoding proteins of 21kDa and 24kDa respectively (Oltvai *et al.*, 1993). Immunoblot analysis of whole cell lysates revealed that eosinophils expressed only Bax α, with no detectable expression of

Bax β (Fig. 4.2B). Immunoblot positive control for Bax was a purified Bax fusion protein (Santa Cruz, CA). Under stringent reducing conditions, an ~40 kDa band was consistently observed in eosinophil whole cell lysates probed with anti-Bax pAb N-20. Bcl-x could not be clearly detected by immunoprecipitation, or immunoblotting of eosinophil or HL-60 whole cell lysates.

Immunoblotting was performed under various conditions of lysate generation in order to investigate the high molecular weight Bax band (Fig. 4.2C) and detect the constitutive expression of Bcl-x indicated by flow cytometric analysis (Fig. 4.1D). Different preparations of eosinophil lysates failed to alter the predominant detection of the 40 kDa band detected with anti-Bax N-20. In lymphocytes, 21 kDa Bax was detectable, although was not expressed as highly as in eosinophils. Interestingly, the 40 kDa band was completely absent from lymphocytes (Fig. 4.3A) and its detection in peripheral blood eosinophils was not altered by gel electrophoresis under non-reducing conditions, indicating that it is not a di-sulphide bound form of Bax. Bcl-x expression was very low or undetectable by immunoblotting in all preparations of eosinophil lysates, despite detection of a Bcl-x *E. coli* fusion protein positive control (Santa Cruz, CA) (Fig. 4.3B). However, an approximately 30 kDa protein band was faintly detectable in eosinophil lysates (*arrow*, Fig. 4.3B).

4.2.2. Analysis of mRNA expression

RT-PCR was performed using the primers detailed in Table 2.2, according to the scheme depicted in Figure 4.4A. Primer pairs were designed to span intron:exon boundaries to control for amplification of contaminating genomic DNA. Primers for *bcl-x* were designed to span the splice site in order for simultaneous amplification of *bcl-x*$_L$ and *bcl-x*$_S$. RT-PCR indicated that freshly isolated eosinophils do not express message for *bcl-2* (Fig. 4.4B), compared with HL-60 cell line as a *bcl-2* positive control, and *gapdh* amplification as an internal PCR control. Similarly, peripheral blood neutrophils had little or no constitutive *bcl-2* expression, whereas *bcl-2* mRNA was readily detectable in peripheral blood lymphocytes (Fig. 4.4C). *bax* (495 bp) and *bcl-x*$_L$ (0.8 kb) mRNA were readily detectable in unstimulated peripheral blood eosinophils (Fig. 4.4D and E respectively). Co-amplification of *bcl-x*$_S$ (0.6 kb) mRNA was also consistently detectable, but to a lesser degree relative to the longer splice variant (Fig. 4.4E). The PCR products were verified by direct sequencing and/ or 'nested' PCR (see Appendix 1).

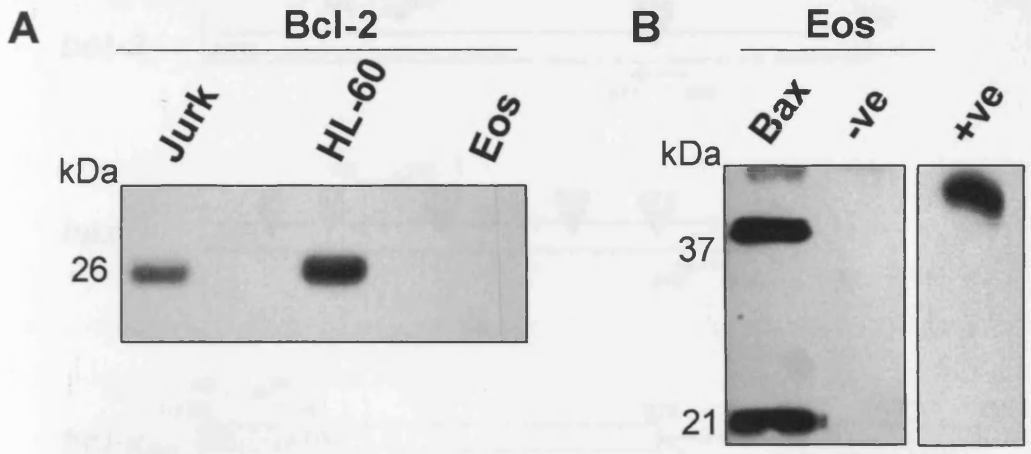

Figure 4.2. Detectable expression of Bax but not Bcl-2 in eosinophils by immunoblotting. Peripheral blood eosinophils were isolated and assessed by immunoblotting for the expression of (A) Bcl-2 and (B) Bax. Eosinophils do not constitutively express Bcl-2 compared with Jurkat and HL-60 positive control cell lines. Eosinophils constitutively express 21kDa Bax, but also readily express a ~40kDa protein detected by immunoblotting. Bax positive control (*+ve*) was an *E. coli* fusion protein and the lysate was probed with rabbit IgG fraction as negative control (*-ve*). Results are representative of four independent experiments.

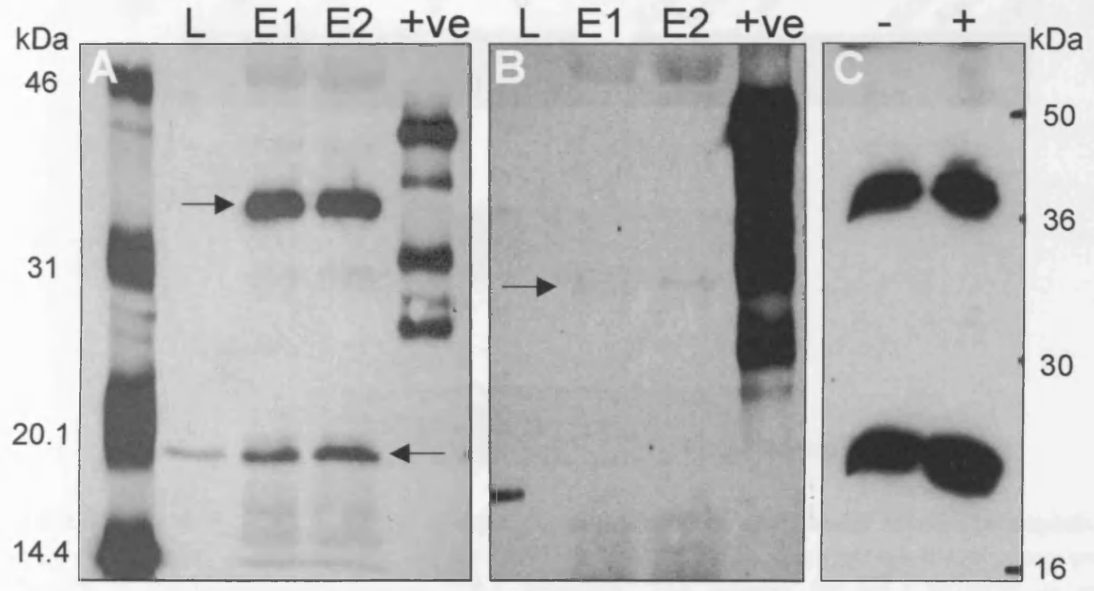

Figure 4.3. Immunoblot analysis of Bax and Bcl-x in lymphocytes and eosinophils. Peripheral blood lymphocytes (*L*) and eosinophils (*E*) were isolated and assessed for Bax (A and C) and Bcl-x (B) by immunoblotting. A and B) Eosinophil lysates were generated by 1% Triton X-100, SDS and sonication (*E1*) or 30 mM octyl glucopyranoside (non-ionic) and sonication (*E2*). Eosinophils and lymphocytes expressed detectable Bax at 21kDa (*arrow*). Bcl-x_S (31kDa) and Bcl-x_L (33kDa) were undetectable in lymphocytes, although very low level of Bcl-x was detected in eosinophil lysates (*arrow*). *E. coli* expressed Bax and Bcl-x fusion proteins were used as positive controls (*+ve*). C) Eosinophils were lysed in the presence (+) or absence of (-) β-mercaptoethanol and Bax detected by immunoblotting. Results are representative of two independent experiments.

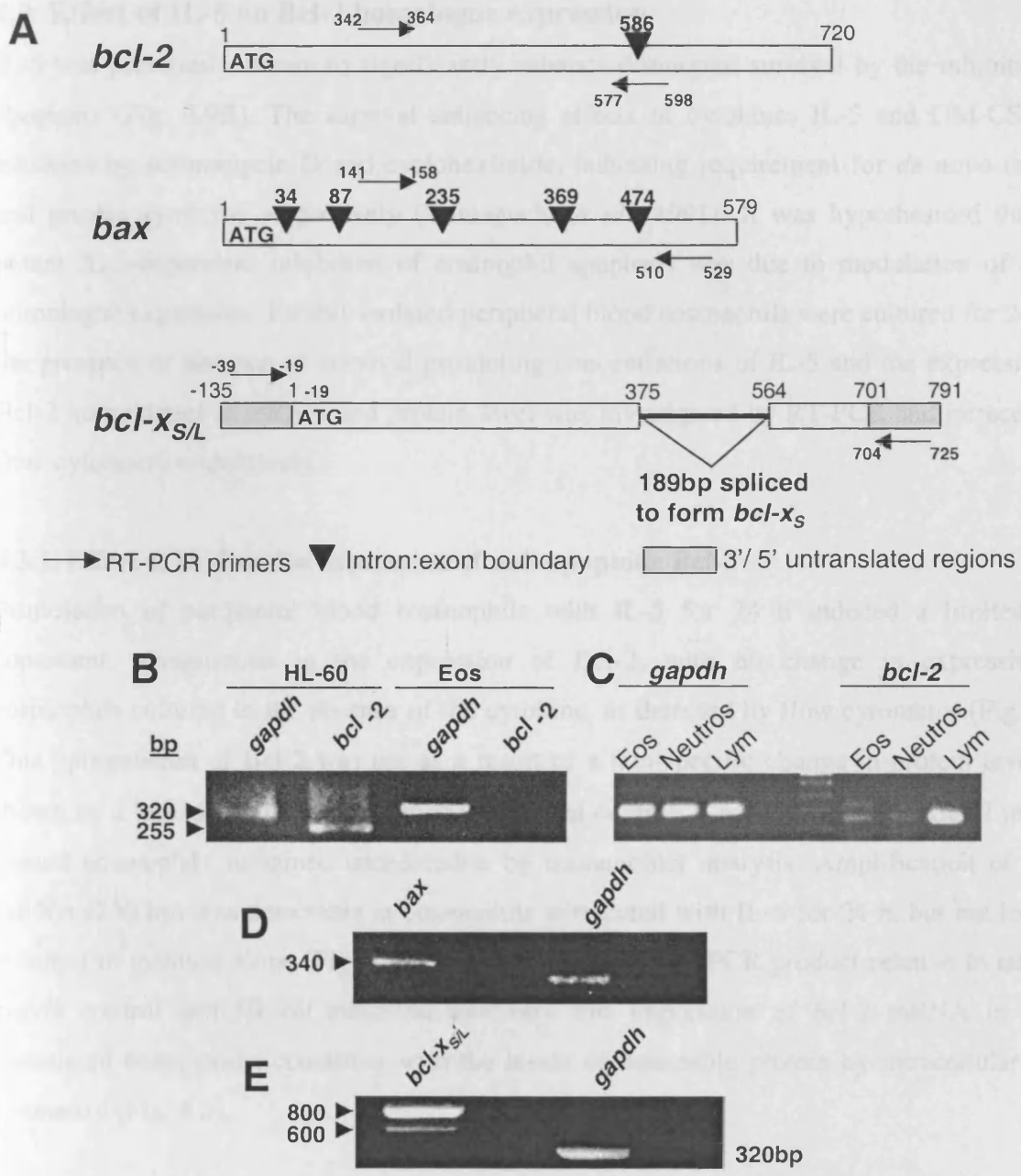

Figure 4.4. RT-PCR analysis of *bcl-2* homologue expression in peripheral blood eosinophils. A) Schematic of RT-PCR primer design. Primer pairs were designed to span introns thereby preventing amplification of contaminating genomic DNA sequence. The primers for *bcl-x* spanned its splice region and therefore allowed simultaneous amplification of *bcl-x*$_S$ and *bcl-x*$_L$ mRNA. Peripheral blood eosinophils, neutrophils and lymphocytes were isolated and analysed by RT-PCR according to *Materials and Methods* for the expression of *bcl-2* (B and C), *bax* (D) or *bcl-x*$_{S/L}$ (E) mRNA. HL-60 cell was used a positive control for *bcl-2* expression and *gapdh* as an internal RT-PCR control. Results are representative of three separate experiments.

4.3. Effect of IL-5 on Bcl-2 homologue expression

IL-5 was previously shown to significantly enhance eosinophil survival by the inhibition of apoptosis (Fig. 3.9B). The survival enhancing effects of cytokines IL-5 and GM-CSF are inhibited by actinomycin D and cycloheximide, indicating requirement for *de novo* mRNA and protein synthesis respectively (Yamaguchi *et al.*, 1991). It was hypothesised that the potent IL-5-dependent inhibition of eosinophil apoptosis was due to modulation of Bcl-2 homologue expression. Freshly isolated peripheral blood eosinophils were cultured for 24 h in the presence or absence of survival promoting concentrations of IL-5 and the expression of Bcl-2 homologues at mRNA and protein level was investigated by RT-PCR and intracellular flow cytometry respectively.

4.3.1. Effect of IL-5 on the expression of anti-apoptotic Bcl-2

Stimulation of peripheral blood eosinophils with IL-5 for 24 h induced a limited, but consistent, upregulation in the expression of Bcl-2, with no change in expression in eosinophils cultured in the absence of the cytokine, as detected by flow cytometry (Fig. 4.5). This upregulation of Bcl-2 was not as a result of a non-specific change in protein levels as shown by a lack of significant variation of internal control, β-actin (Fig. 4.5B). Bcl-2 in IL-5 treated eosinophils remained undetectable by immunoblot analysis. Amplification of *bcl-2* mRNA (250 bp) was detectable in eosinophils stimulated with IL-5 for 24 h, but not in cells cultured in medium alone (Fig. 4.6). The signal intensity of PCR product relative to internal *gapdh* control and HL-60 indicated relatively low expression of *bcl-2* mRNA in IL-5-stimulated eosinophils, consistent with the levels of detectable protein by intracellular flow cytometry (Fig. 4.5).

4.3.2. Effect of IL-5 on the expression of Bax and Bcl-$x_{S/L}$

Incubation of eosinophils with IL-5 for 24 h was found to have no significant effect on the levels of Bax and Bcl-$x_{S/L}$ protein expression as determined by intracellular flow cytometry (Fig. 4.7). Dibbert *et al* (1998) observed expression of Bcl-x_L was downregulated in the absence of cytokine, but maintained in the presence of IL-5 over the 24 h culture period. No clear downregulation of Bcl-x was observed by flow cytometry after 24 h (Fig. 4.7). However, the antibody used failed to distinguish Bcl-x_S and Bcl-x_L, therefore semi-quantitative RT-PCR of *bcl-x_L* was performed by the 'primer-dropping' method in order to determine whether *bcl-x_L* mRNA expression was altered. The expression of *bcl-x_L* was found to markedly decrease from its constitutive level in freshly isolated eosinophils after 24 h *in vitro* (Fig. 4.7). Stimulation with IL-5 over the 24 h culture period maintained the expression

of *bcl-x*$_L$ mRNA expression relative to the *gapdh* internal control (Fig. 4.7). The yield of *bcl-x*$_S$ was too low to perform accurate semi-quantitative RT-PCR.

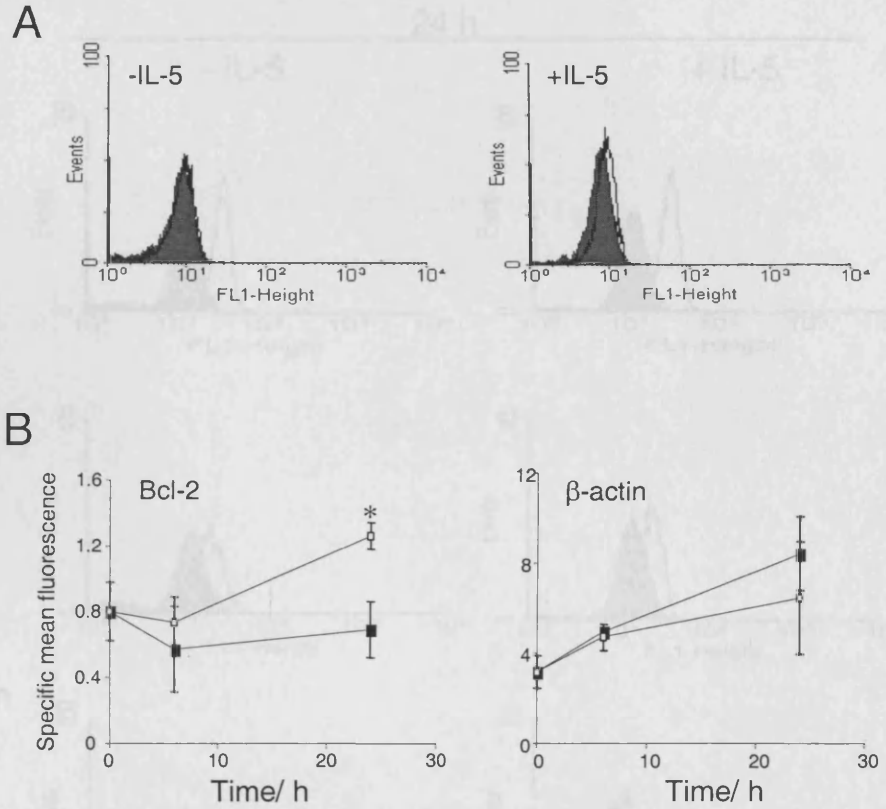

Figure 4.5. IL-5 induces modest upregulation of Bcl-2 expression by intracellular flow cytometry. A) Eosinophils were cultured in the presence or absence of IL-5 for 24 h and the expression of Bcl-2 was assessed by flow cytometry. Shows flow cytometry histograms representative of three separate experiments. α-Bcl-2, *hollow histogram*; negative control, *solid histogram*. B) Eosinophils were cultured in the presence (*open symbols*) or absence (*closed symbols*) of IL-5 over 24 h and the level of Bcl-2 protein expression determined by flow cytometry. Results are expressed as specific mean fluorescence, standardised with an irrelevant negative control. β-actin was used as an internal control. Results are the mean ± SEM of three separate experiments. * P<0.05.

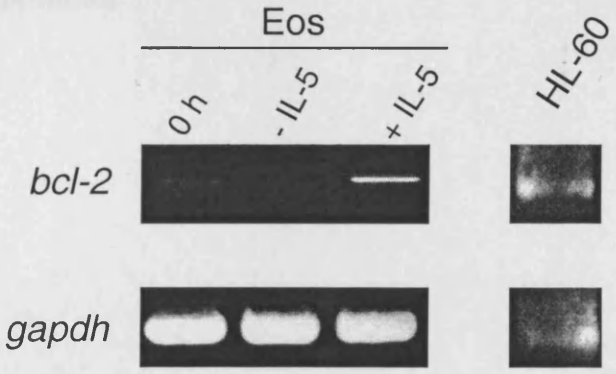

Figure 4.6. IL-5 induces the expression of *bcl-2* mRNA. Expression of *bcl-2* mRNA by RT-PCR in peripheral blood eosinophils cultured in the presence or absence of IL-5 (10^{-10}M). *gapdh* as an internal RT-PCR control, HL-60 as a positive control for *bcl-2* expression. Results representative of two independent experiments.

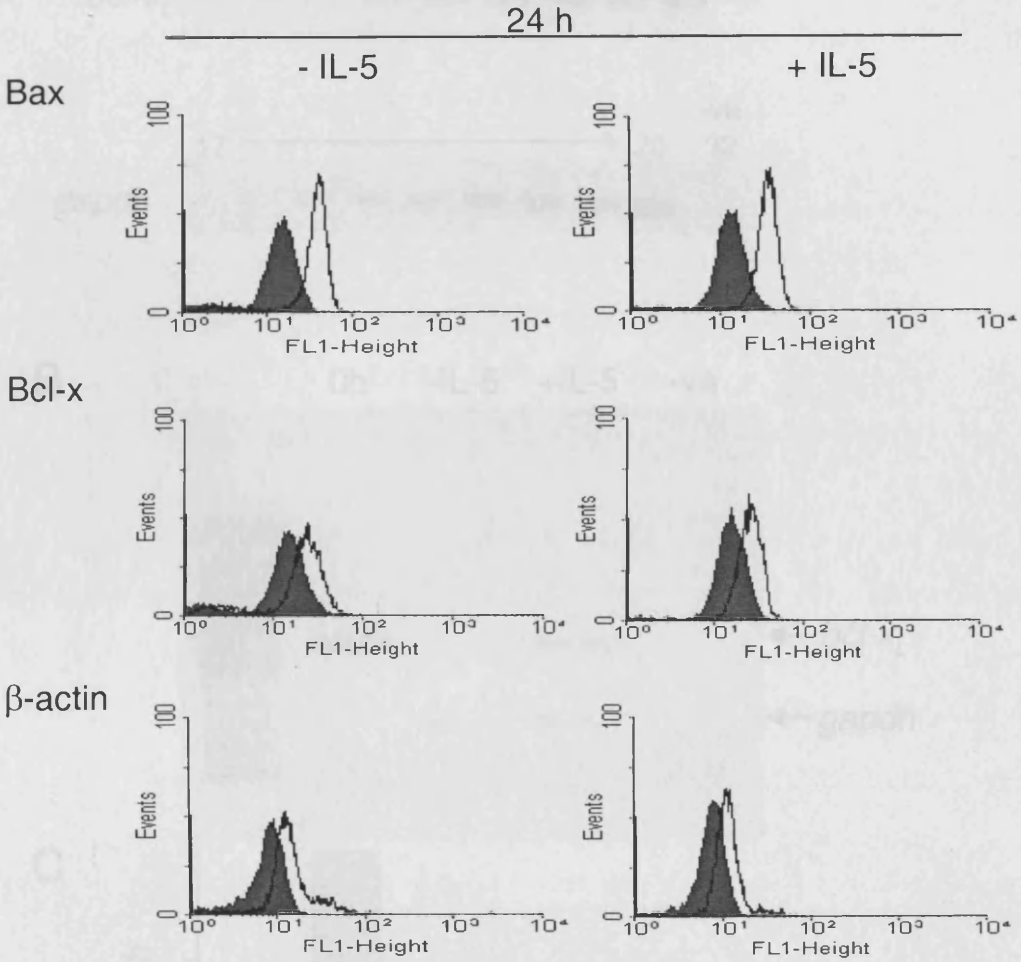

Figure 4.7. Effect of IL-5 on the expression of Bcl-2 homologues by intracellular flow cytometry. Peripheral blood eosinophils were cultured in the presence or absence of IL-5 (10^{-10}M) for 24 h and the expression of Bax and Bcl-x was assessed by flow cytometry. β-actin was used as an internal control. Test antibody, *hollow histogram*; control antibody, *solid histogram*. Results are representative of three independent experiments.

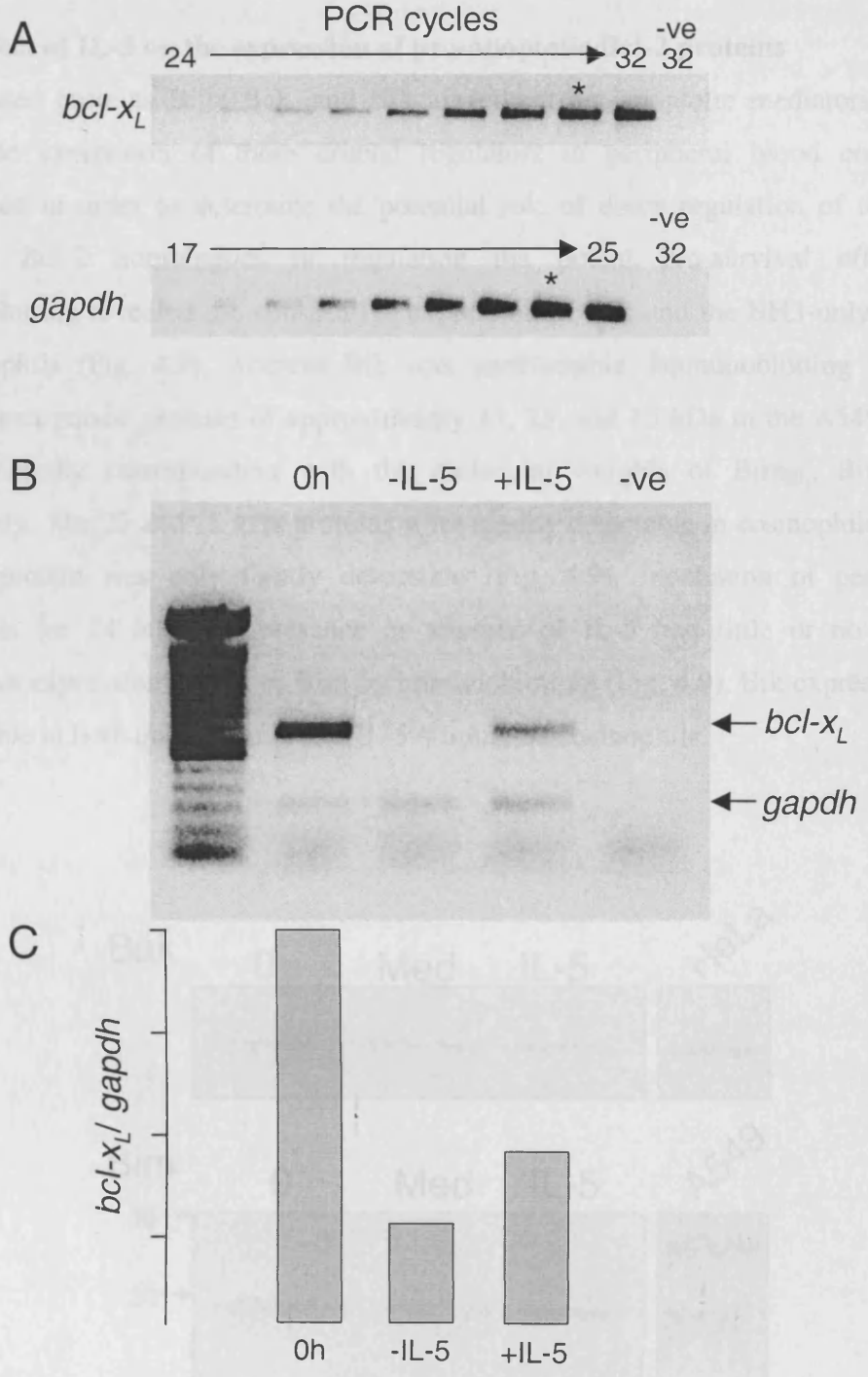

Figure 4.8 Effect of IL-5 on *bcl-x_L* expression in eosinophils by semi-quantitative RT-PCR. A) Monoplex PCR reactions were set up for *bcl-x_L* and *gapdh* for increasing number of cycles of amplification to determine saturation point of each reaction (*). B) mRNA was extracted from freshly isolated (*0 h*) or eosinophils cultured for 24 h in the absence (*-IL-5*) or presence (*+IL-5*) of IL-5 (10^{-10}M) and semi-quantitative duplex RT-PCR for *bcl-x_L* (30 cycles) and *gapdh* (23 cycles) as internal control performed by the primer-dropping method as detailed in *Materials and Methods*. Negative control reactions were set up in parallel without RT product (-ve). C) Band intensities were quantified and expressed as *bcl-x_L* intensity relative to *gapdh*.

4.3.3. Effect of IL-5 on the expression of pro-apoptotic Bcl-2 proteins

Bcl-2-related proteins Bim, Bak, and Bik are potent pro-apoptotic mediators in many cell types. The expression of these crucial regulators in peripheral blood eosinophils was investigated in order to determine the potential role of down regulation of the potent pro-apoptotic Bcl-2 homologues in regulating the potent pro-survival effect of IL-5. Immunoblotting revealed the constitutive expression of Bak and the BH3-only protein, Bim, in eosinophils (Fig. 4.9), whereas Bik was undetectable. Immunoblotting with the Bim antibody recognised proteins of approximately 33, 25, and 15 kDa in the A549 control (Fig. 4.9), potentially corresponding with the molecular weights of Bim_{EL}, Bim_L and Bim_S respectively. The 25 and 15 kDa proteins were readily detectable in eosinophils, although the 33 kDa protein was only faintly detectable (Fig. 4.9). Incubation of peripheral blood eosinophils for 24 h in the presence or absence of IL-5 had little or no effect on the constitutive expression of Bak or Bim by immunoblotting (Fig. 4.9). Bik expression remained undetectable in both unstimulated and IL-5-stimulated eosinophils.

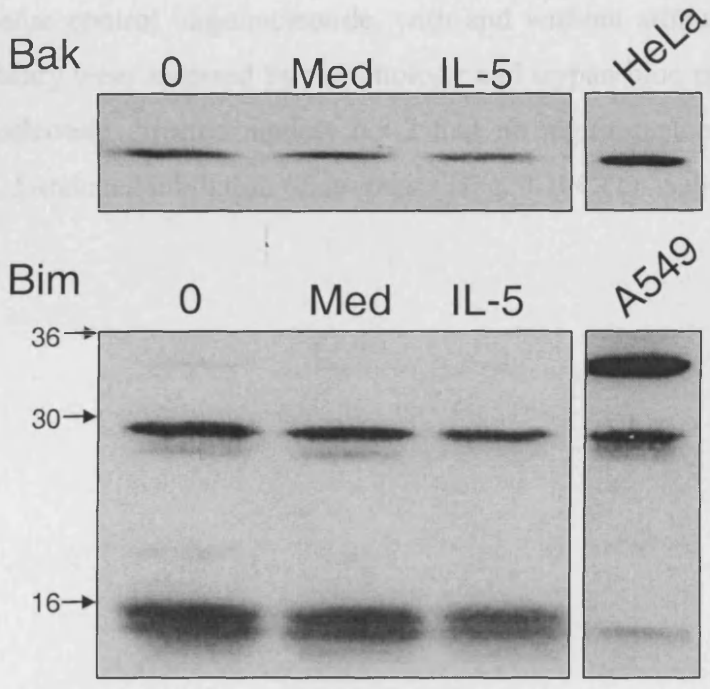

Figure 4.9. Expression of Bak and Bim in eosinophils. Peripheral blood eosinophils were isolated and cultured for 24 h in the absence (*Med*) or presence of IL-5 (10^{-10}M), and the expression of BH3-only Bcl-2 homologues Bak and Bim was examined by immunoblotting. HeLa and A549 cell lines were used as positive controls for Bak and Bim expression respectively. Results are representative of two independent experiments.

4.4. Effect of *bcl-2* antisense oligonucleotides on IL-5-induced eosinophil survival

IL-5 induced modest, but significant upregulation of the anti-apoptotic protein, Bcl-2, in peripheral blood eosinophils. To investigate whether this upregulation was responsible for the potent survival enhancing effects of IL-5, antisense oligonucleotides targeting *bcl-2* were used to prevent its translation. Antisense oligonucleotides have proven an invaluable tool in studies elucidating intracellular pathways of terminally differentiated eosinophils (Yousefi *et al.*, 1997; Pazdrak *et al.*, 1997). A potential problem of antisense technology is ensuring the efficient uptake of the oligonucleotide into the cell, which occurs partially by fluid-phase endocytosis and potentially via the p80 receptor protein that allows uptake of negatively charged molecules. Modification of the oligonucleotides with phosphorothioate side chains facilitates cellular uptake, and was verified in eosinophils by assessing the uptake of a FITC-conjugated *bcl-2* oligonucleotide, in the presence or absence of IL-5, by laser scanning cytometry. Fluorescein alone is not taken up by cells and is rapidly excluded from both nucleus and cytoplasm upon cleavage from the oligonucleotide, thereby providing a reliable marker for cellular uptake of the oligo. Rapid uptake of the oligo was observed within 6 h (Fig. 4.10A), with rate of uptake independent of stimulation with IL-5 (Fig. 4.10B). Eosinophils were then incubated for 24 h in the presence or absence of *bcl-2* antisense or randomised, nonsense control oligonucleotide, with and without stimulation with IL-5, and apoptosis and viability were assessed by morphology and trypan blue exclusion respectively. Antisense oligonucleotide directed against *bcl-2* had no significant effect on the level of spontaneous or IL-5-induced inhibition of apoptosis (Fig. 4.10C) or viability (Fig. 4.10D).

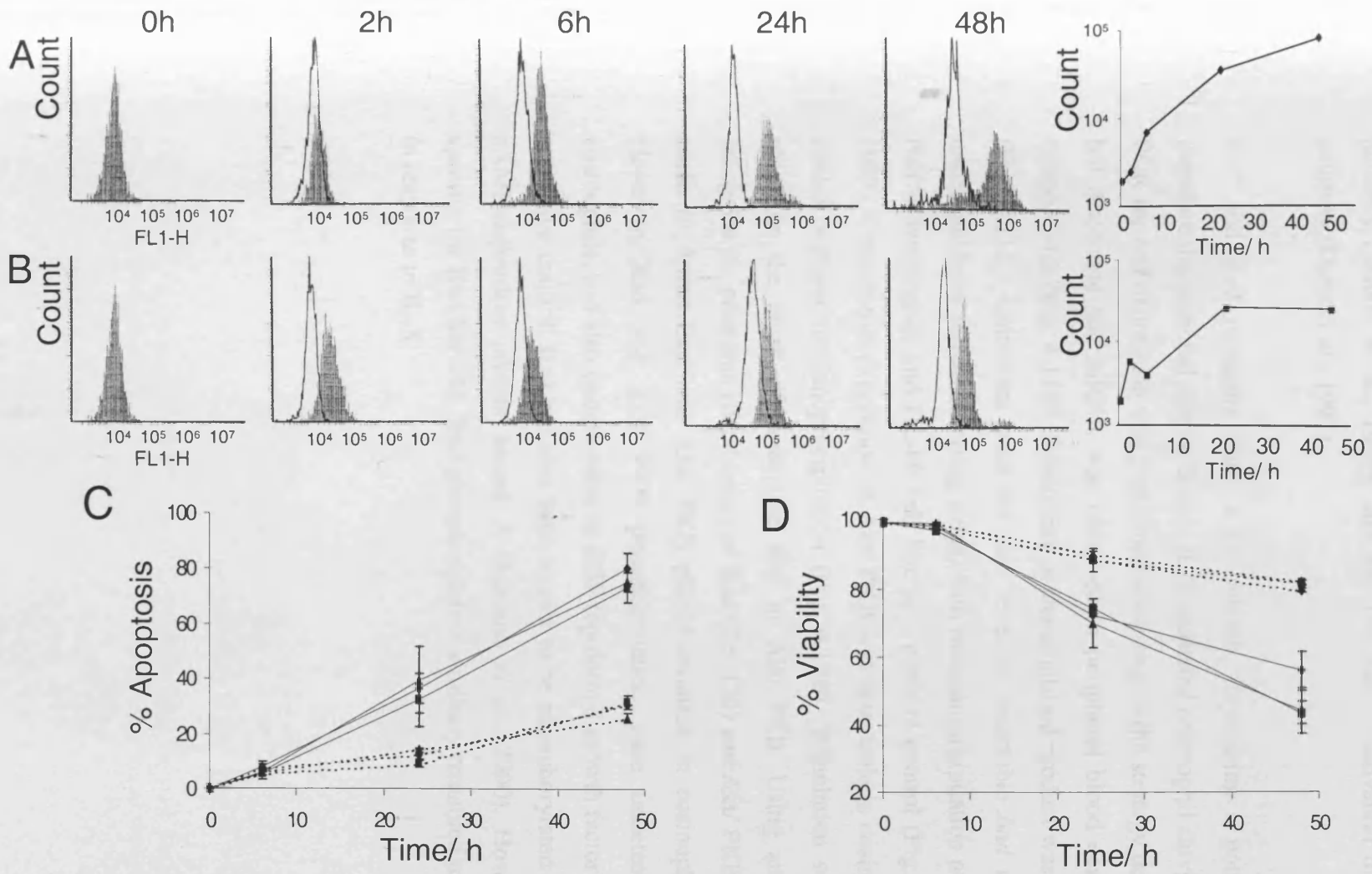

Figure 4.10. Uptake and effect of *bcl-2* antisense oligonucleotide in eosinophils. Eosinophils were cultured in the absence (A) or presence (B) of IL-5 (10^{-10}M) and in the presence (*solid histogram*) or absence (*hollow histogram*) of FITC-labelled antisense oligonucleotide (2 μM). At the times indicated, cell smears were generated and counterstained with propidium iodide according to *Materials and Methods* in order to monitor uptake of fluorescently-labelled oligonucleotide by laser scanning cytometry (A and B). Representative histograms of showing 5000 events; FITC positive, *solid histogram*; autofluroescence, *hollow histogram*. Line chart shows time-dependent increase in the number of cells taking up the FITC-labelled oligonucleotide (A and B). Eosinophils were cultured in the presence (*dashed line*) or absence (*solid line*) of IL-5 and the absence (*squares*) or presence of *bcl-2* antisense (*triangles*) or control (*diamonds*) oligonucleotides for the times indicated and apoptosis (C) and viability (D) assessed by morphology and trypan blue exclusion respectively. Results are expressed as the mean ± SEM of two independent experiments.

4.5. Role of Bad in eosinophil apoptosis

The mechanism by which modulation of intracellular phosphorylation events regulate survival remains unclear. Reception of growth factor, such as IL-3 and insulin-like growth factor (IGF), is proposed to induce phosphorylation of Bad at Ser 112 by the Ras-MAP kinase pathway (Scheid *et al.*, 1999), and Ser 136 due to activation of the PI-3-K/ Akt (PKB) pathway (Datta *et al.*, 1997).

IL-5 and IL-3 receptors share a βc subunit, implicating a potential common pathway, therefore the potential role of Bad in IL-5-mediated eosinophil survival was investigated. RT-PCR for *bad* expression was performed according to the strategy detailed in Fig 4.11A. Low, but detectable *bad* mRNA was observed in peripheral blood eosinophils, neutrophils and lymphocytes (Fig. 4.11B). Specificity of the amplified product was verified by 'nested PCR' (Fig. 4.11C). Consistent with the low level of detectable *bad* mRNA, Bad protein was undetectable by immunoblotting alone, with immunoprecipitation necessary in order to detect Bad in eosinophils and HL-60 cell line as a positive control (Fig. 4.12A; Chatterjee *et al.*, 1997). Constitutive expression of Akt/ PKB was detectable in eosinophils and A431 cell line control without immunoprecipitation (Fig. 4.12B). Stimulation with IL-5 had no marked effect on the levels of detectable Bad or Akt/ PKB. Using antibodies that specifically recognise the phosphorylated forms of Bad (Ser 136) and Akt/ PKB (Ser 473), attempts were made to detect Bad and Akt/ PKB phosphorylation in eosinophils in response to IL-5. However, Bad and Akt/ PKB phosphorylation were undetectable in IL-5-stimulated eosinophils, and also undetectable in EGF (epidermal growth factor)-stimulated A431 cells as a positive control. Bad has also been shown to be phosphorylated at Ser 155 in response to cAMP-dependent protein kinase A (Lizcano *et al.*, 2000). However, using an antibody specific for Bad Ser 155, Bad phosphorylation similarly remained undetectable in eosinophils in response to IL-5.

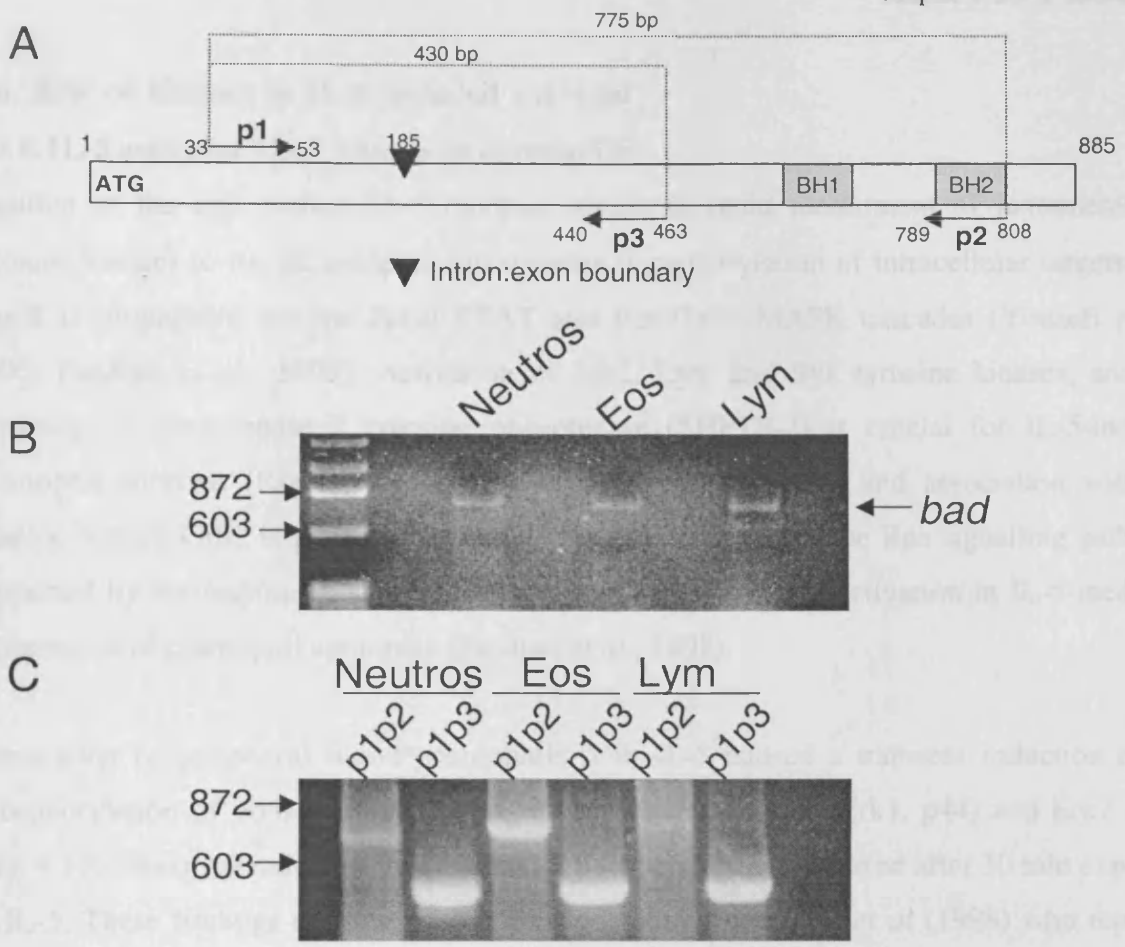

Figure 4.11. RT-PCR analysis of *bad* mRNA expression in peripheral blood cells. A) Schematic of *bad* primer design. Primer pairs were designed to span introns thereby preventing amplification of contaminating genomic DNA sequence. B) Eosinophils, neutrophils and lymphocytes were purified from peripheral blood and mRNA extracted as described in Materials and Methods. RT-PCR was performed with primers p1 and p2 to generate a product of 775 bp. C) Verification of bad amplification by nested PCR with primers p1 and p3, generating a product of 430 bp.

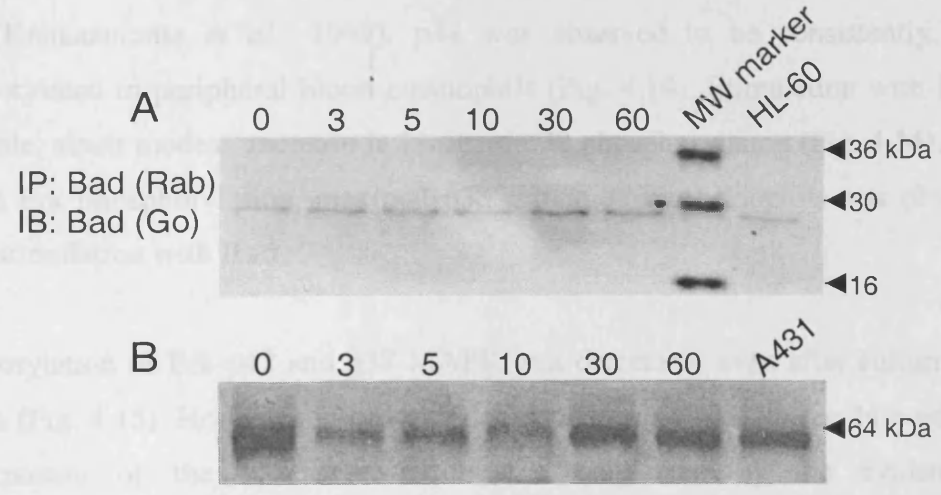

Figure 4.12. Immunoprecipitation and immunoblotting of Bad and Akt/ PKB in eosinophils. Peripheral blood eosinophils were incubated with IL-5 for the indicated times. A) Cell lysates were generated and Bad was immunoprecipitated with rabbit polyclonal antibody (NEB) according to Materials and Methods. Bad was similarly immunoprecipitated from HL-60 cells as a positive control. Immunoprecipitates were immunoblotted with goat anti-Bad polyclonal antibody (Santa Cruz). B) Akt/ PKB was detected by immunoblotting with a goat polyclonal antibody (NEB). Results are representative of two independent experiments.

4.6. Role of kinases in IL-5 induced survival

4.6.1. IL-5 activates MAP kinases in eosinophils

Ligation of the cell surface IL-5 receptor results in rapid recruitment of juxtamembrane tyrosine kinases to the βc receptor and tyrosine phosphorylation of intracellular targets. The signal is propagated via the Jak2/ STAT and Ras-Raf-1-MAPK cascades (Yousefi *et al.*, 1996; Pazdrak *et al.*, 1998). Activation of Jak2, Lyn, and Syk tyrosine kinases, and Src homology 2 phosphatase 2 tyrosine phosphatase (SHPTP-2) is crucial for IL-5-induced eosinophil survival (Pazdrak *et al.*, 1997). SHPTP-2 activation and association with the adaptor protein Grb2 is proposed to couple the IL-5 receptor to the Ras signalling pathway, supported by the requirement for Raf-1 serine/ threonine kinase activation in IL-5-mediated suppression of eosinophil apoptosis (Pazdrak *et al.*, 1998).

Stimulation of peripheral blood eosinophils with IL-5 caused a transient induction in the phosphorylation of both extracellular signal-regulated kinase 1 (Erk1, p44) and Erk2 (p42) (Fig. 4.13). Maximal induction of Erk1 and 2 phosphorylation occurred after 30 min exposure to IL-5. These findings contrast with a previous study by Coffer *et al* (1998) who reported phosphorylation of Erk2 with no detectable phosphorylation of Erk1, but are consistent with two subsequent reports (Bates *et al.*, 2000; Adachi *et al.*, 2000).

p38 MAPK has been implicated in the regulation of spontaneous eosinophil apoptosis, with eosinophils exhibiting high constitutive p38 phosphorylation being less susceptible to cell death (Kankaanranta *et al.*, 1999). p38 was observed to be consistently, constitutively phosphorylated in peripheral blood eosinophils (Fig. 4.14). Stimulation with IL-5 induced a detectable, albeit modest, increase in levels of p38 phosphorylation (Fig. 4.14). As with IL-5-induced Erk phosphorylation, maximal p38 activation in eosinophils was observed after 30 min of stimulation with IL-5.

Phosphorylation of Erk p42 and p38 MAPK was detectable even after culturing eosinophils for 24 h (Fig. 4.15). However, in order to detect phosphorylated kinase 24 h post-stimulation, over-exposure of the blot was required as indicated by the evident constitutive phosphorylation of Erk in freshly isolated eosinophils (Fig. 5.15, *0h*), compared with the low levels previously detected (Fig. 4.13, Lane 1). Culturing for 24 h was associated with a reduction in detectable phosphorylation compared with the constitutive levels exhibited by freshly isolated eosinophils.

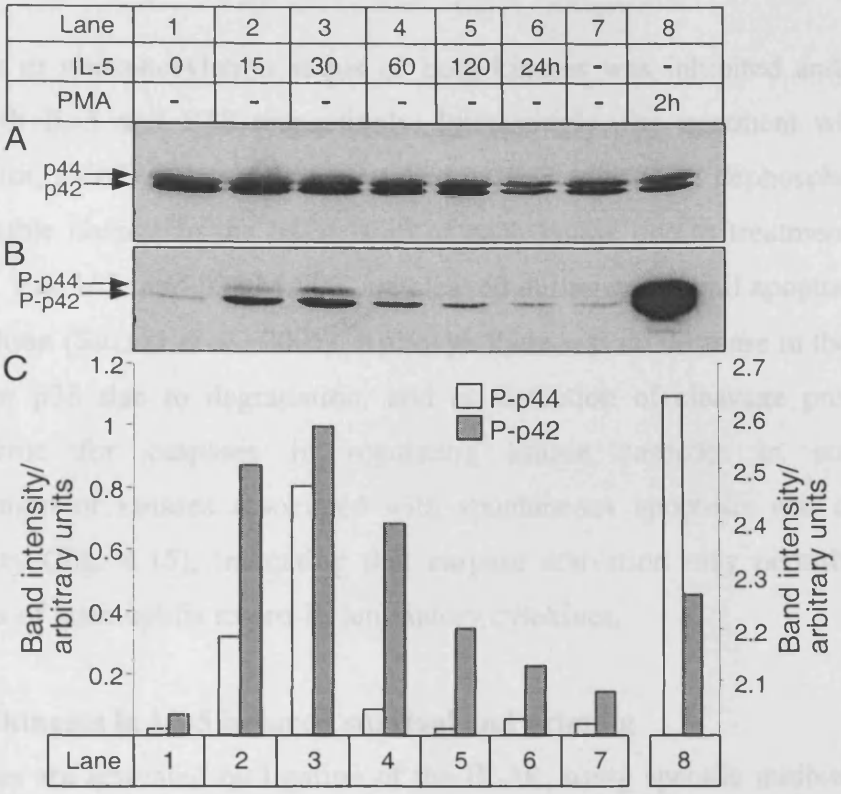

Figure 4.13. IL-5 induces phosphorylation of p44 and p42 Erk kinase. Eosinophils were stimulated in the presence or absence of IL-5 (10^{-10}M) or PMA as control for the indicated times, or cultured medium alone for 24 h (*Lane 7*). Immunoblotting was performed according to *Materials and Methods* and probed with antibodies directed against p44/42 Erk kinase (A) and phosphorylated p44/42 (P-p44/42, B). (C) Graphical representation of P-p44/42 band intensity, normalised for variation in basal p44/42. Representative of two independent experiments.

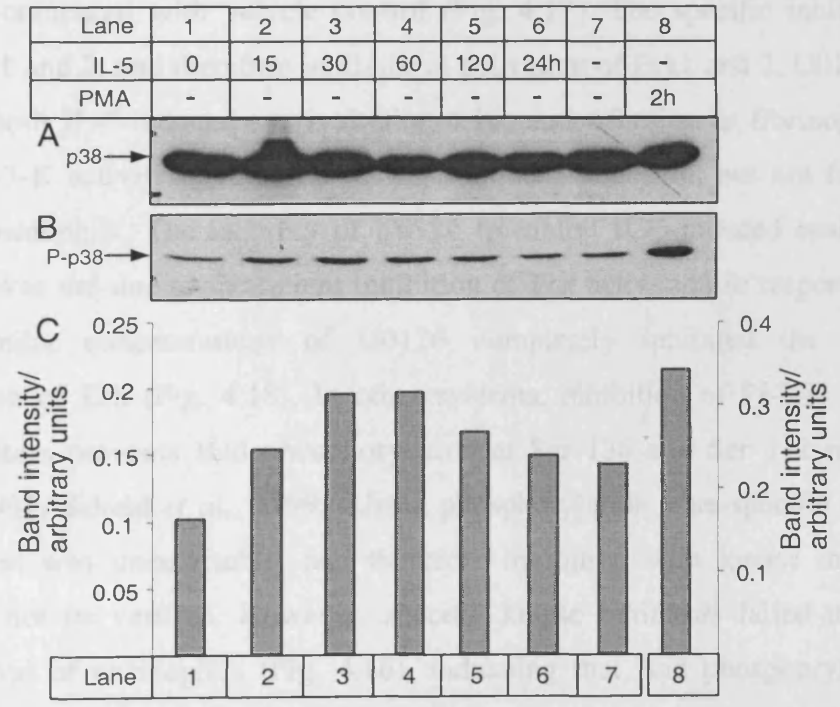

Figure 4.14. IL-5 induces phosphorylation of p38 MAP kinase. Eosinophils were stimulated in the presence or absence of IL-5 (10^{-10}M) or PMA as control for the indicated times, or cultured medium alone for 24 h (*Lane 7*). Immunoblotting was performed according to *Materials and Methods* and probed with antibodies directed against p38 MAP kinase (A), and phosphorylated p38 (P-p38, B). (C) Graphical representation of P-p38 band intensity, normalised for variation in basal p38. Representative of two independent experiments.

This reduction in phosphorylation status of both kinases was inhibited and accelerated by incubation with IL-5 and STS respectively. Interestingly, the treatment with broad range caspase inhibitor, zVAD.fmk, was most marked in the inhibition of dephosphorylation. There was no detectable change in the basal level of each kinase due to treatment over the 24 h culture period. Erk 1/ 2, and p38 MAPK are cleaved during neutrophil apoptosis in a caspase-dependent fashion (Suzuki *et al.*, 2001). Although there was no decrease in the basal levels of Erk1 and 2 or p38 due to degradation, and no detection of cleavage products, the data suggests a role for caspases in regulating kinase cascades in eosinophils. The dephosphorylation of kinases associated with spontaneous apoptosis was dependent upon caspase activity (Fig. 4.15), indicating that caspase activation may perturb the functional responsiveness of eosinophils to pro-inflammatory cytokines.

4.6.2. Role of kinases in IL-5 induced survival and priming

Various kinases are activated by ligation of the IL-5R, using specific inhibitors, the roles of kinase cascades in IL-5-dependent eosinophil survival were investigated. PI-3-K inhibitors, wortmannin and LY294002, had no significant effect on IL-5-induced eosinophil survival compared with vehicle (DMSO) control (Fig. 4.16). However, both wortmannin and LY294002, with wortmannin the most potent, inhibited IL-5-induced adhesion of eosinophils to fibrinogen compared with vehicle control (Fig. 4.17). The specific inhibitor of MAPK kinase (MEK)1 and 2, and therefore inhibitor of activation of Erk1 and 2, U0126, had little or no effect on both IL-5-induced survival (Fig. 4.16) and adhesion to fibrinogen (Fig. 4.17). Therefore, PI-3-K activity is required for IL-5-induced adhesion, but not for IL-5-induced survival of eosinophils. The inability of U0126 to inhibit IL-5-induced eosinophil survival and adhesion was not due to inefficient inhibition of Erk activation in response to IL-5 (Fig. 4.13), as similar concentrations of U0126 completely inhibited the IL-5-dependent phosphorylation of Erk (Fig. 4.18). In other systems, inhibition of PI-3-K and MEK with specific inhibitors prevents Bad phosphorylation at Ser 136 and Ser 112 respectively (del Peso *et al.*, 1997; Scheid *et al.*, 1999). Using phosphorylation state-specific antibodies, Bad phosphorylation was undetectable, and therefore inhibition with kinase inhibitors in this system could not be verified. However, specific kinase inhibitors failed to prevent IL-5-induced survival of eosinophils (Fig. 4.16), indicating that Bad phosphorylation via these pathways was not the mode of action for IL-5-dependent antagonism of apoptosis. The inability of specific inhibitors of both PI-3K and MEK to prevent IL-5-induced eosinophil survival suggests that activation of these kinase cascades are not requisite for survival, although PI-3-K activity is implicated in IL-5-mediated adhesion.

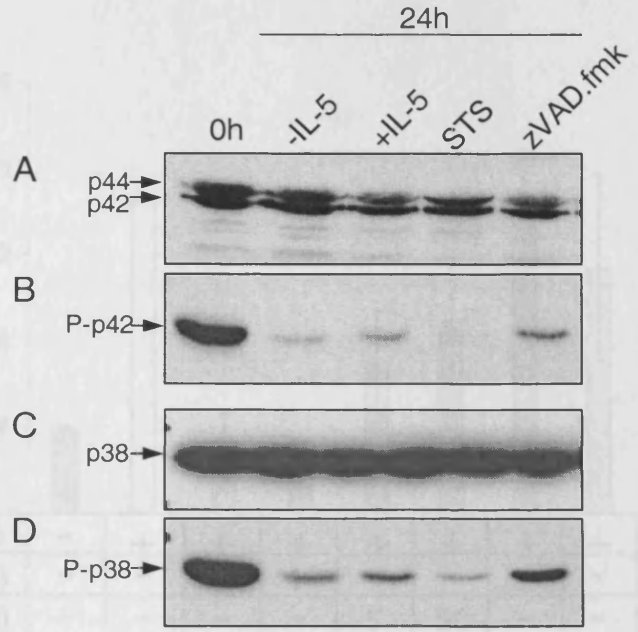

Figure 4.15. Erk and MAP kinase phosphorylation in cultured eosinophils. Eosinophils were freshly isolated (*0h*) or cultured in the presence or absence of IL-5 (10^{-10}M), staurosporine (STS, 10^{-5}M), or zVAD.fmk (100 μM) for 24 h. Immunoblotting was performed according to *Materials and Methods* and probed with antibodies directed against p44/42 Erk kinase (A), phosphorylated p44/42 (P-p44/42, B), p38 MAP kinase (C), and phosphorylated p38 (P-p38, D). Representative of two independent experiments.

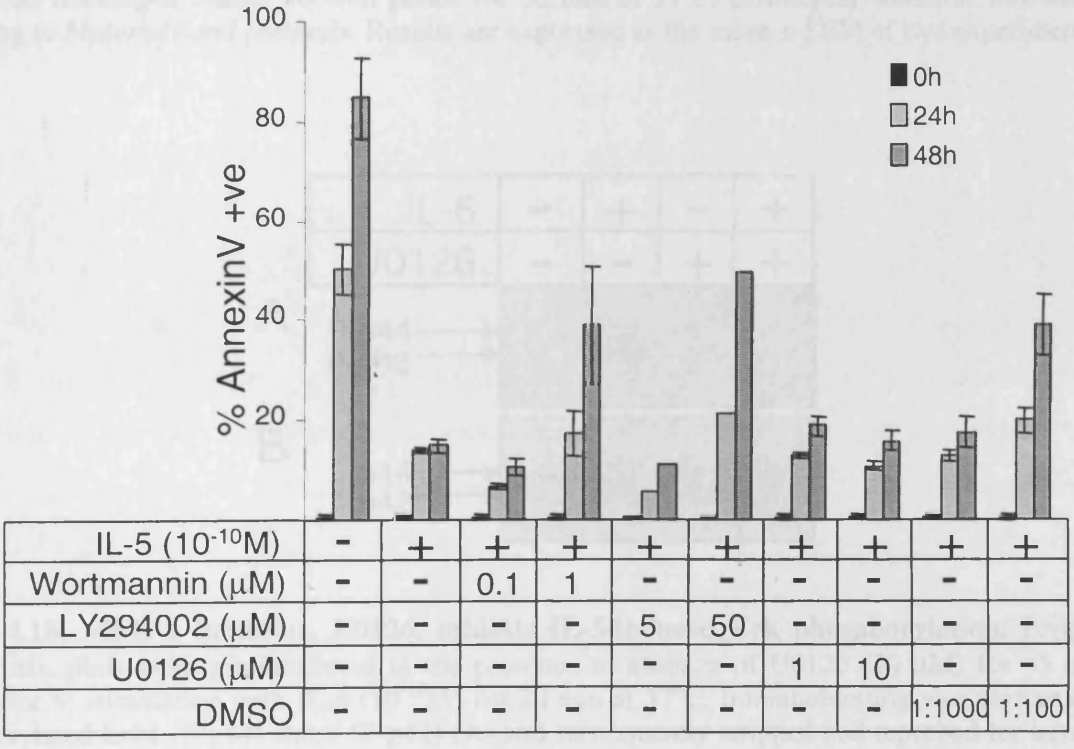

Figure 4.16. Effect of kinase inhibitors on eosinophil apoptosis. Eosinophils were pre-treated with the indicated concentrations of PI-3-K inhibitors (wortmannin or LY294002) or MEK1/2 inhibitor, (U0126) for 45 min at 37°C. Eosinophils were then cultured in the presence of absence of IL-5 and eosinophil apoptosis was assessed by Annexin V binding at the indicated times. Results are expressed as the mean ± SEM of three separate experiments.

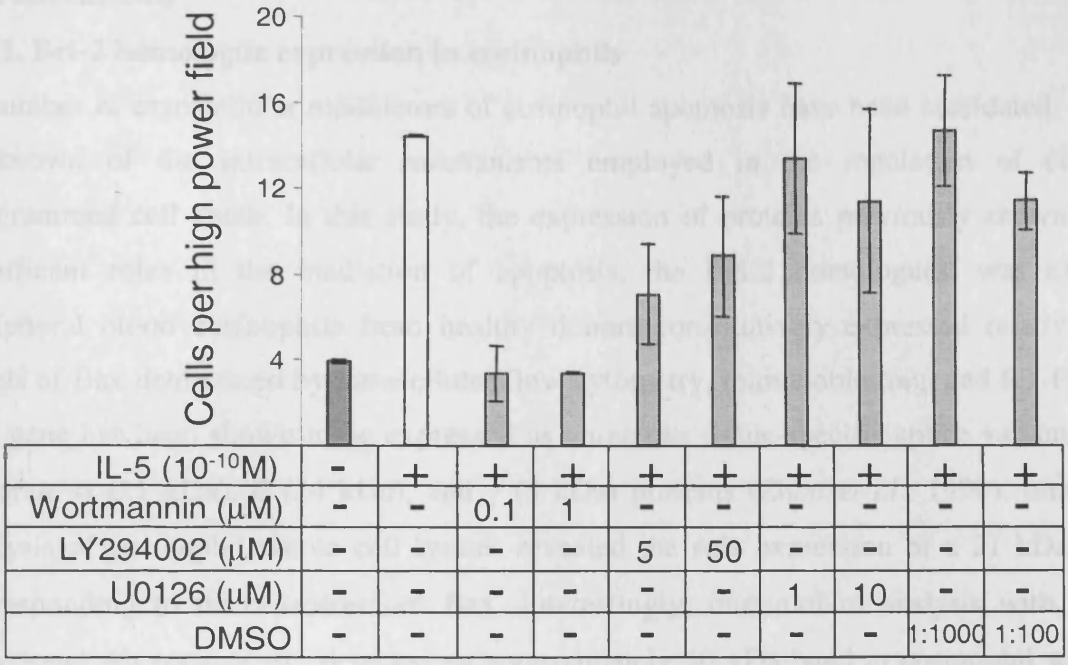

Figure 4.17. Effect of kinase inhibitors on eosinophil adhesion to fibrinogen. Eosinophils were pre-treated with the indicated concentrations of PI-3-K inhibitors (wortmannin or LY294002) or MEK1/2 inhibitor, (U0126) for 45 min at 37°C. Eosinophils were incubated in the presence or absence of IL-5 on fibrinogen coated 96 well plates for 30 min at 37°C. Eosinophil adhesion was assessed according to *Materials and Methods*. Results are expressed as the mean ± SEM of two experiments.

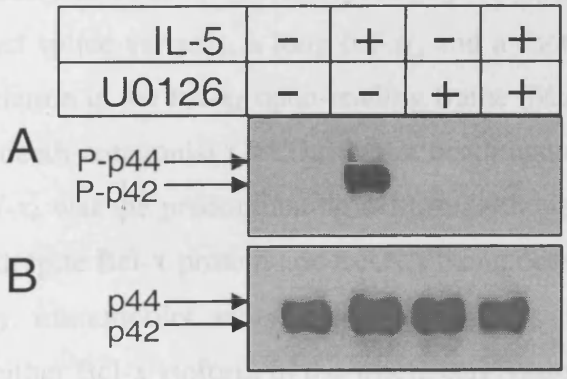

Figure 4.18. MEK-1 inhibitor, U0126, inhibits IL-5-induced Erk phosphorylation. Peripheral blood eosinophils were pre-incubated in the presence or absence of U0126 (10 μM) for 45 min at 37°C, prior to stimulation with IL-5 (10^{-10}M) for 30 min at 37°C. Immunoblotting was performed for phosphorylated Erk1 (P-p44) and 2 (P-p42) (A) and subsequently stripped and reprobed for basal Erk p44and p42 (B). Representative of two independent experiments.

4.7. Discussion

4.7.1. Bcl-2 homologue expression in eosinophils

A number of extracellular modulators of eosinophil apoptosis have been elucidated, but little is known of the intracellular mechanisms employed in the regulation of eosinophil programmed cell death. In this study, the expression of proteins previously shown to have significant roles in the mediation of apoptosis, the Bcl-2 homologues, was examined. Peripheral blood eosinophils from healthy donors constitutively expressed relatively high levels of Bax determined by intracellular flow cytometry, immunoblotting, and RT-PCR. The *bax* gene has been shown to be expressed as numerous tissue-specific splice variants, which generate α (21 kDa), β (24 kDa), and γ (5 kDa) proteins (Zhou *et al.*, 1998). Immunoblot analysis of eosinophil whole cell lysates revealed the sole expression of a 21 kDa protein, corresponding to the α isoform of Bax. Interestingly, immunoblot analysis with anti-Bax polyclonal Ab consistently detected an approximately 40 kDa band in eosinophil whole cell lysates. The nature of this protein is as yet undetermined, but it is unlikely to be a result of the homo/ heterodimerisation of Bax observed in the regulation of apoptosis, as the protein was resistant to reducing agents. Further investigation is required to confirm whether this band represents a novel Bax splice variant or an unrelated cross-hybridising protein.

Bcl-x protein was also readily detectable by flow cytometry. Alternative splicing of the bcl-x gene generates two distinct splice variants, a long *bcl-x*$_L$, and a short *bcl-x*$_S$. *bcl-x*$_S$ is created due to a 63 amino acid deletion in the *bcl-x*$_L$ open-reading frame (Minn *et al.*, 1996), with *bcl-x*$_L$ and *bcl-x*$_S$ encoding a death antagonist (28kDa) and a death agonist (17kDa) respectively. RT-PCR revealed that *bcl-x*$_L$ was the predominating isoform, although *bcl-x*$_S$ mRNA was also detectable. In this study, despite Bcl-x protein and mRNA being detectable by flow cytometry and RT-PCR respectively, immunoblot analysis consistently revealed absence or very low detectable expression of either Bcl-x isoform in the whole cell lysates of eosinophils and HL-60 cell line. The reason for this is unclear as a fusion protein positive control was readily detectable, possibly suggesting a lack of solubilisation of the protein during cell lysis. Considerable effort to solubilise Bcl-x, including pellet homogenisation, sonication, freeze fracture and multiple detergent lysis proved unsuccessful. Attempts to concentrate the protein by immunoprecipitation prior to immunoblotting also failed to specifically detect Bcl-x. Lack of solubilisation may be due to compartmentalisation within the cell, as complex interaction and membrane localisation are features of Bcl-x. Druilhe *et al* (1998) reported a profile of apoptosis-associated proteins in peripheral blood eosinophils by immunoblot analysis, similarly failing to detect expression of Bcl-x isoforms by immunoblotting alone. However,

intracellular flow cytometry and analysis of mRNA with respect to Bcl-x were not performed in this study. Peripheral blood eosinophils were observed not to constitutively express Bcl-2 protein or mRNA, supporting the findings of Ochiai *et al* (1997). Immunoblot analysis has previously shown that peripheral blood neutrophils express Bax, with Bcl-2 and Bcl-x essentially absent (Ohta *et al.*, 1995). The lack of expression of the major death suppressor, Bcl-2, may reflect the relatively short *in vivo* lifespan of these granulocytes, and the *in vitro* persistence of eosinophils relative to neutrophils (Stern *et al.*, 1992) may potentially be afforded by the expression of anti-apoptotic Bcl-x_L. There is disagreement within the literature regarding constitutive expression of Bcl-2 in eosinophils. Detectable expression of Bcl-2 protein in freshly isolated human peripheral blood eosinophils has been reported (Chung *et al.*, 2000). This contrasts with the observations of other investigators (Dibbert *et al.*, 1998; Druilhe *et al.*, 1998; Zangrilli *et al.*, 2000) and the data reported here.

The BH3-only proteins are potent inducers of apoptosis in many cell types. Eosinophils exhibited detectable constitutive expression of Bim, with the molecular weights of the proteins detected implicating potential expression of Bim_L and Bim_S isoforms in eosinophils, with Bim_{EL} absent or expressed at relatively low levels. O'Reilly *et al* (2000) have previously shown Bim isoform expression in numerous cell types including hematopoietic and neuronal cells, although in contrast no detectable expression of Bim_S was observed. Further investigation, involving RT-PCR, is required to provide conclusive evidence of the Bim isoform expression profile in eosinophils. Interestingly, *bim*$^{-/-}$ mice have abnormally high levels of granulocytes, lymphocytes and macrophages, possibly due to their increased resistance to growth factor deprivation (Bouillet *et al.*, 1999), suggesting a potentially important role for Bim in granulocyte apoptosis.

Bak, is capable of potently enhancing apoptosis by inducing the release of cytochrome *c*, and was consitutively expressed by eosinophils. A synergistic model has been postulated in which Bax and Bak have partially redundant roles within the apoptotic pathway. Similarly to *bim*$^{-/-}$ mice, animals deficient in both Bax and Bak have markedly increased numbers of granulocytes (Lindsten *et al.*, 2000).

4.7.2. Modulation of Bcl-2 homologue expression by IL-5

It has been reported that a requisite of IL-5-dependent abrogation of eosinophil apoptosis is the *de novo* synthesis of mRNA and protein (Yamaguchi *et al.*, 1991). It has also been shown recently that Bcl-2 and Bcl-x_L can act as substrates for caspase-3, activated during apoptosis

(Cheng *et al.*, 1997; Clem *et al.*, 1998). Therefore, the potential modulation of expression of Bcl-2 members during spontaneous apoptosis and in the presence of survival enhancing concentrations of IL-5 was investigated. Spontaneous apoptosis was observed when culturing eosinophils in the absence of cytokines for 24 h, but no variation in the expression of Bcl-2, Bax or Bcl-x was detected at protein level. Culturing peripheral blood eosinophils in the presence of IL-5 for 24 h maintained cell survival, and was found to induce detectable *bcl-2* mRNA, but at low level relative to *gapdh* internal control and HL-60 *bcl-2* positive control. Flow cytometry revealed a modest, but consistent increase in Bcl-2 expression, without significant variation in β-actin control, consistent with the observed increase in message. However, the increase in Bcl-2 protein remained undetectable by immunoblotting.

The upregulation of Bcl-2 by IL-5 supported the findings of Ochiai *et al* (1997). However, a conflicting report (Druilhe *et al.*, 1998) suggested that despite an increase in anti-apoptotic protein, Mcl-1, in IFN-γ-treated umbilical-cord-blood eosinophils, no modulation of Bcl-2 in either IL-5 or IFN-γ-treated peripheral blood eosinophils was detectable, although immunoblot analysis was the only technique employed. Dibbert *et al* (1998) also failed to observe any increase in detectable Bcl-2 expression by flow cytometry and immunohistochemistry. The reason for the conflicting evidence regarding both constitutive Bcl-2 expression and cytokine-induced upregulation of Bcl-2 expression is unclear, although may be due to different methodologies employed. The low levels of Bcl-2 expression reported may be below the limits of detectability by immunoblotting, and intracellular flow cytometry is prone to misinterpretation without thorough and appropriate controls due to the high non-specific antibody binding to eosinophil basic proteins (Mahmudi-Azer *et al.*, 1998). Also, the fixing and permeabilisation protocol utilised may also affect the target antigenicity during intracellular flow cytometry and, as a result, the observed expression profile.

Interestingly, the expression of Bcl-x_L protein has been shown to decrease in the absence of cytokine, and stimulation with GM-CSF maintained constitutive levels of Bcl-x_L expression, with antisense oligonucleotides against *bcl-x_L* inhibiting cytokine-induced eosinophil survival (Dibbert *et al.*, 1998). Although no detectable down regulation of Bcl-x_L protein was observed after 24 h by intracellular flow cytometry in this study, semi-quantitative RT-PCR indicated that *bcl-x_L* mRNA expression was reduced, and this down regulation was inhibited by IL-5. As there was consistently no detectable alteration in Bcl-x protein after 24 h, the data would

suggest that modulation of Bcl-x_L expression alone is not responsible for the survival enhancing effect of IL-5 that manifests before 24 h *in vitro*.

Despite the consistent upregulation of Bcl-2 at mRNA and protein level observed in IL-5 stimulated eosinophils, antisense oligonucleotides directed against Bcl-2 failed to have any effect on IL-5-induced survival compared with control oligonucleotide. This contrasts with the observations of Ochiai *et al* (1997) who report a modest, but significant effect of *bcl-2* antisense oligonucleotide, indicating a contribution of Bcl-2 upregulation in mediating IL-5-dependent eosinophil survival. The susceptibility of a cell to death signals is proposed to be determined, at least in part, by the ratio of death agonist: death antagonist, and their subsequent interaction via homo and heterodimerisation, forming a 'life-death rheostat'. Overexpression of Bax, leading to predomination of Bax homodimers encourages apoptosis (Oltvai *et al.*, 1993), whereas prevalence of Bcl-2 affords protection for the cell (Rossé *et al.* 1998). The modest IL-5-dependent Bcl-2 increase, coupled with the relatively high constitutive expression of Bax in eosinophils would suggest that upregulation of Bcl-2 within such a stoichiometric framework is unlikely to be solely responsible for the potent protective effects of IL-5. This may implicate a role for other death antagonists in the regulation of eosinophil apoptosis, such as Bcl-x_L, determined to be the predominating Bcl-x isoform in freshly isolated eosinophils, or Mcl-1, shown to be upregulated in neutrophils following stimulation with GM-CSF (Epling-Burnette *et al.*, 2001).

Interestingly, (Plotz *et al.*, 1998) observed tissue and blood eosinophils derived from a patient with hypereosinophilia exhibited increased Bcl-2 expression and enhanced *in vitro* survival compared with normal eosinophils. Bcl-2 expression has also been reported in sputum eosinophils from patients with acute asthma, correlating with detectable levels of IL-5 (Jang *et al.*, 2000). The data would suggest that Bcl-2 does contribute to prolonged eosinophil survival both *in vitro* and *in vivo*. The growth factor-induced phosphorylation of Bcl-2 is required to facilitate its anti-apoptotic effect (Ito *et al.*, 1997). As the modest upregulation of Bcl-2 in response to IL-5 is predicted to be insufficient to be responsible for the potent survival enhancing effect of IL-5 in such a background of high constitutive Bax expression, it would be interesting to determine whether the phosphorylation status of Bcl-2 is modulated in eosinophils following stimulation with IL-5.

The constitutive expression of neither Bak nor Bim was altered *in vitro* after 24 h in the absence or presence of IL-5. The observed phenotype of mice deficient for these Bcl-2

homologues implicates potentially important roles for these proteins in mediating the apoptosis of hematopoietic cells (Bouillet *et al.*, 1999), and therefore warrants further investigation. Bim is proposed to be sequestered by an interaction with the microtubule skeleton in a non-apoptotic cell, and following an apoptotic stimulus, dissociates and translocates to the mitochondria to elicit its pro-apoptotic effect (Puthalakath *et al.*, 1999). It is intriguing to speculate that such a Bim-dependent mechanism functions during the growth factor deprivation induced apoptosis of eosinophils.

4.7.3. Role of Bad in eosinophil survival

The pro-apoptotic protein, Bad, has been shown to be phosphorylated in response to cytokine in IL-3 dependent cell lines, and is sequestered in the cytoplasm preventing interaction with anti-apoptotic proteins at the mitochondrial surface (Yang *et al.*, 1995; Datta *et al.*, 1997; Scheid *et al.*, 1999). Some authors have debated the validity of the PI-3-K/ Akt/ Bad pathway as Bad is only one of many possible targets of Akt/ PKB, and the affinity of interaction between Bad and Akt/ PKB is relatively low compared with other substrates, such as FKHRL1, a member of the Forkhead family of transcription factors (Brunet *et al.*, 1999). The crucial Bad phosphorylation site has also been debated, with a third candidate, Ser 155, phosphorylated in response to cAMP-dependent PKA (Lizcano *et al.*, 2000), implicated in mediating Bad/ Bcl-x_L heterodimerisation and cell survival (Tan *et al.*, 2000). Lizcano *et al* (2000) have recently questioned the relevance of phosphorylation of Bad at Ser 136, as IGF, a potent activator of the PI-3-K pathway, failed to induce phosphorylation at this residue. Interestingly, Ser 155 resides within the BH3 domain of Bad (Zhou *et al.*, 2000), which is critical in regulating the dimerisation of Bcl-2 homologues. Although *bad* mRNA was detectable within peripheral blood eosinophils, protein expression was low or undetectable, and there was no conclusive evidence of Bad phosphorylation in response to stimulation with IL-5. Furthermore, incubation of eosinophils with specific inhibitors of PI-3-K (wortmannin and LY29004) and MEK1/2 (U0126) had little or no effect on IL-5-induced survival. Current evidence suggests growth factor-dependent phosphorylation of pro-apoptotic Bad is regulated by PI-3-K/ Akt(PKB) and MAPK pathways. The data presented here indicates that Bad phosphorylation, at least mediated by these pathways, is not required for IL-5-dependent survival. However, problems with the phosphorylation state-specific antibodies and lack of reliable positive controls, mean that a potential role for Bad phosphorylation by a pathway independent of PI-3-K and MEK1/2, such as cAMP-dependent kinase mediated phosphorylation (Lizcano *et al.*, 2000), cannot be ruled out.

4.7.4. Functional divergence downstream of IL-5 receptor

IL-5 induced the activation of Erk1/ 2 and to a lesser extent p38 MAPK, implicating a role for these kinases in the eosinophil functional response to IL-5 stimulation. Specific inhibition of PI-3-K and MEK1/2 indicated that the activity of neither kinase is required for IL-5-induced survival signalling, whereas PI-3-K activity is seemingly essential for IL-5-mediated adhesion events. Similarly, GM-CSF-induced eosinophil survival was shown to be independent of PI-3-K and MAPK pathways (Miike *et al.*, 1999). Interestingly, an IL-5 point mutant, E12K, has previously been shown capable of enhancing eosinophil survival *in vitro*, although with reduced potency compared with the wild-type, but incapable of effecting eosinophil adhesion to immobilised IgG (McKinnon *et al.*, 1997). Also, Jak2, Lyn and Raf-1 are all required for IL-5-mediated survival, whereas only Raf-1 activation is essential for IL-5-dependent upregulation of the adhesion molecule, CD11b, and eosinophil degranulation as determined by ECP release (Pazdrak *et al.*, 1998). Therefore, there seems to be a certain degree of functional divergence downstream of the IL-5 receptor, with distinct kinase cascades transducing IL-5-dependent survival and priming signals. Figure 4.19 illustrates the kinases activated in eosinophils in response to IL-5 stimulation based on the evidence in the current literature and the data reported here. Although numerous kinases are activated in response to IL-5, the activation of only a few has been shown to be requisite for the transduction of IL-5-dependent survival signals. Although the IL-5-stimulated activation of p38 MAPK is not thought to be involved in cytokine-mediated survival, a report by Kankaanranta *et al* (1999) suggests that the basal level of p38 phosphorylation determines the spontaneous rate of eosinophil apoptosis in the absence of cytokine, with constitutively phosphorylated p38 correlating with reduced spontaneous rate of cell death.

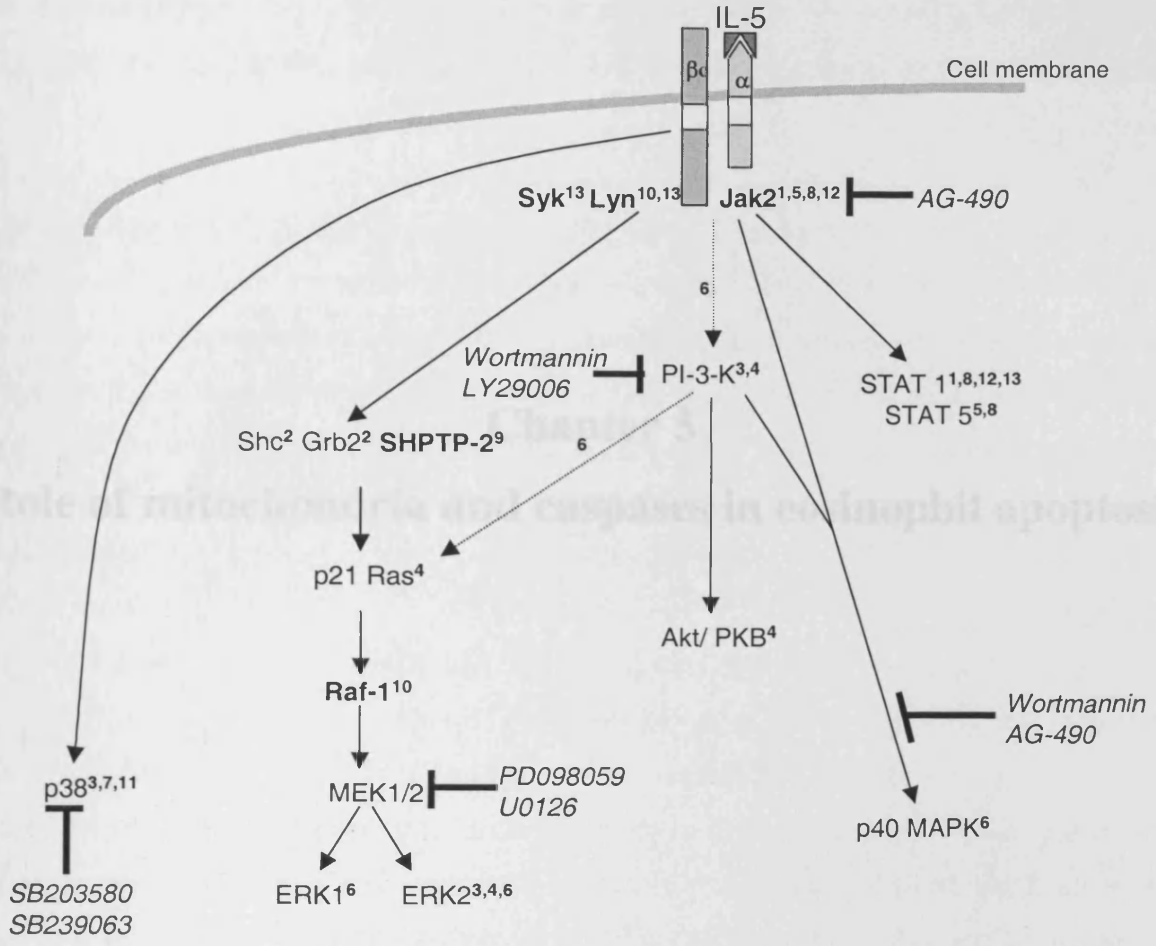

Figure 4.19. Role of kinases in cytokine-mediated eosinophil survival. Depicts the kinases activated in eosinophils following stimulation with IL-5, and the relevant inhibitors (*italic*). Activation of the kinases in bold are crucial for IL-5-mediated survival. Activation of kinases in normal type is not required for survival, but are required for other IL-5-dependent functions including degranulation and adhesion. [1]Alam *et al.*,1995; [2]Bates *et al.*, 2000; [3]Bracke *et al.*, 2000; [4]Coffer *et al.*, 1998; [5]Du *et al.*, 2000; [6]Hiraguri *et al.*, 1997; [7]Kankaanranta *et al.*, 1999; [8]Ogata *et al.*, 1997; [9]Pazdrak *et al.*, 1997; [10]Pazdrak *et al.*, 1998; [11]Underwood *et al.*, 2000; [12]van der Bruggen *et al.*, 1995; [13]Yousefi *et al.*, 1996. See text for details.

Chapter 5

Role of mitochondria and caspases in eosinophil apoptosis

5.1. Introduction

At present, two hypotheses have developed with regards the identity of the true effectors of the execution of apoptosis, mitochondria or caspases. Until recently, mitochondria were solely regarded as the 'powerhouses' of the cell, due to their role in essential generation of ATP by oxidative phosphorylation, maintenance of pHi, REDOX and Ca^{2+} homeostasis. Subsequent, pioneering studies have revealed that mitochondria are not only necessary for the life, but also the death and degradation of a cell. The mitochondrion plays a major role in the execution of apoptosis in response to diverse stimuli, including DNA damage, activation of the tumour suppressor gene p53, and chemotherapeutic agents, being responsible for apoptogenic factor release and coordinating the activation of caspases due to cytochrome *c* efflux (Green and Reed, 1998).

Major advances in our understanding of the apoptotic pathway have come from studies performed in *C. elegans*, with many of the molecules involved in regulating the apoptosis of the nematode finding homologues within the mammalian system. Interestingly, there is no evidence for a role for mitochondria in the apoptosis of *C. elegans* apoptosis, and mutations in the caspase homologue, Ced-3, prevents apoptosis during development. Such an *in vivo* system is believed by some researchers to be of greater relevance, than the largely *in vitro* evidence supporting an integral role for mitochondria. In contrast, a recent study by Wei *et al* (2001) has shown that hepatocytes derived from mice deficient in Bax and Bak, were incapable of inducing mitochondrial dysfunction and cytochrome *c* release, and were resistant to Fas-induced apoptosis, providing significant *in vivo* evidence that 'extrinsic' apoptosis is mitochondria-dependent, at least in some cell types. Further evidence supporting a central role for mitochondria comes from the use of peptide caspase inhibitors. Although caspase inhibition, for example with zVAD.fmk, maintains the viability of neurons following growth factor withdrawal, prevents apoptosis in *Drosophila*, and significantly reduces tissue damage after ischaemic injury, there are many systems where the cell cannot be rescued. These include glucocorticoid treated thymocytes (Hirsch *et al.*, 1997) and hematopoietic cells following growth factor withdrawal (Ohta *et al.*, 1997), where apoptosis proceeds, albeit at a reduced rate, implicating an alternative apoptotic pathway that is not caspase-dependent.

As discussed previously, distinct caspases are activated depending on the apoptotic stimulus, with 'stress'/ chemical-induced and death receptor-induced apoptosis involving apical activation of caspase-9 and –8 respectively (Sun et al., 1999; Bratton et al., 2000). In concert with being stimulus-dependent, there is evidence that caspase activation in response to death

receptor-induced apoptosis is cell type dependent. Two types of cell have been identified, determined by their functional response to Fas (CD95/ Apo-1)-induced apoptosis, and the role of mitochondria in the execution of the consequent death (Scaffidi *et al.*, 1998). Type I cells, rapidly recruit and activate large amounts of caspase-8 to the DISC, in contrast Type II cells activate only small mounts of caspase-8 with reduced kinetics of effector caspase activation, despite kinetics of apoptosis and loss of $\Delta\Psi m$ being similar in both cell types. Caspase-8 activation in Type II cells occurs predominantly after perturbation of the mitochondrial membrane, and was inhibited by overexpression of Bcl-2 and Bcl-x_L. Although the receptor-proximal events appear similar in the two cell types, with both signalling via FADD and caspase-8, the level of caspase-8 recruitment to the receptor complex determines whether a cell undergoes death receptor-induced apoptosis in a mitochondria-independent fashion (Type I) or dependent fashion (Type II). In Type II cells, limited amounts of caspase-8 are recruited to and activated at the DISC, but may be insufficient for direct induction of the caspase cascade. However, the activation of caspase-8 may be sufficient for the cleavage of Bid and consequent perturbation of the outer mitochondrial membrane, forming an amplification step to compensate for the low constitutive caspase activity at the DISC (Scaffidi *et al.*, 1998).

The expression and activation of caspases, and the potential role of the mitochondria during eosinophil apoptosis induced by a number of stimuli was investigated.

5.2. Caspases in eosinophil apoptosis

5.2.1. Caspases are expressed and processed during eosinophil apoptosis

Initial investigation explored the role of caspases in eosinophil apoptosis. Peripheral blood eosinophils endogenously expressed both the 'initiator' caspase-8 and -9, and the 'effector' caspase-3, -6 and-7 (Figs. 5.1 and 5.2). Treatment of eosinophils for 2 h with the protein kinase C inhibitor, staurosporine (STS, 10^{-5}M), was sufficient to induce activation of caspase-3, -6, -7, -8 and -9, as evidenced either by the generation of the immunoreactive, catalytically active, large subunits resulting from processing of the caspase proforms, or direct loss of the proform itself (Figs. 5.1 and 5.2). As a positive control, Jurkat T cells were treated with DNA topoisomerase II inhibitor, etoposide, which induces apoptosis in a number of cell types (Sun *et al.*, 1992; Zhu *et al.*, 1995).

Procaspase-3 is expressed as an inactive 32 kDa zymogen, which on activation is cleaved initially at Asp175 generating a p20 subunit, followed by further proteolytic cleavage at Asp9 and Asp28 to generate p19 and p17 subunits respectively (Fernandes-Alnemri *et al.*, 1996). In

freshly isolated eosinophils, caspase-3 was present as a 32 kDa proform (Fig 5.1A, Lane 1). Exposure to STS for 2 h resulted in the formation of 20 kDa (p20), 19 kDa (p19) and 17 kDa (p17) immunoreactive fragments (Fig. 5.1A, Lane 4). After 6 h the proform was almost completely processed (Fig. 5.1A, Lane 7). Jurkat T cells exposed to etoposide for 4 h exhibited similar caspase-3 processing (Fig. 5.1A, Lane 12).

Procaspase-6 was expressed in eosinophils as its 34 kDa zymogen (Fig. 5.1B, Lane 1). Processing of caspase-6 generates p18 and p11 subunits due to sequential cleavage at Asp179 and Asp193 (Srinivasula *et al.*, 1996). Due to lack of immunoreactivity of these subunits, processing of caspase-6 was indicated by the time-dependent loss of the proform after 6 h STS treatment (Fig 5.1B, Lane 7). A slight loss of caspase-6 proform was observed in Jurkats treated with etoposide for 4 h (Fig. 5.1B, Lane 12).

In eosinophils, caspase-7 was expressed as its 35 kDa proform (Fig. 1C, Lane 1). Treatment with STS for 2 h resulted in formation of the immunoreactive 19 kDa (p19) fragment (Fig. 5.1C, Lane 4), corresponding to the large catalytically active subunit generated due to cleavage at Asp198 and removal of the prodomain (MacFarlane *et al.*, 1997). After 6 h STS treatment, procaspase-7 was almost completely lost (Fig. 5.1C, Lane 7). Similar caspase-7 processing was observed in etoposide-treated Jurkat T cells (Fig. 5.1C, Lane 12).

Caspase-8 was detectable in freshly isolated eosinophils and Jurkat T cells as the 55 kDa proform (Fig. 5.1D, Lanes 1 and 12). However, shorter exposure revealed that caspase-8 was expressed as a doublet of 55 and 53 kDa, corresponding to the proposed occurrence of two isoforms, caspase-8a and caspase-8b (Scaffidi *et al.* 1997). After STS treatment for 2 h, cleavage of the proform resulting in the formation of 43 kDa (p43) and 41 kDa (p41) subunits was observed corresponding to cleavage between the large and small subunits (Fig. 5.1D, Lane 4). After 6 h STS treatment, an 18 kDa (p18) fragment was observed due to removal of the death effector domains from p43 and p41 (Fig. 5.1D, Lane 7). Generation of the p41 and p43 subunits was observed in Jurkats T cells treated with etoposide for 4 h, although the p18 subunit was not marked (Fig. 5.1D, Lane 12).

Caspase-9 is the apical caspase of stress-induced apoptosis. Activated upon association with Apaf-1 in the presence of cytochrome *c*, caspase-9 activates downstream 'effector' caspase-3, -6, and –7 (Srinivasula *et al*, 1998; Slee *et al.*, 1999). Procaspase-9 was detectable in eosinophils as its 46 kDa zymogen (Fig. 5.2E, Lane 1), and time-dependent formation of 35

kDa and 37 kDa (p35 and p37) subunits was observed after 2 and 6 h STS treatment respectively, due to cleavage at Asp315 and Asp330 (Fig. 5.2E, Lanes 4 and 8).

Detection of caspase processing in eosinophils after treatment with STS for 2 h was commonly associated with approximately 35-50% apoptosis assessed by PtdSer exposure and 10-15% cells showing evidence of nuclear condensation by morphology after Kimura stain (Fig. 5.1).

5.2.2. IL-5 inhibits eosinophil apoptosis upstream of caspase processing

In the absence of IL-5 for 24 h, approximately 65% apoptosis assessed by PtdSer externalisation was associated with processing of caspase-3, -7, -8 and -9 in eosinophils (Figs. 5.1 and 5.2). After 2 and 6 h of IL-5 deprivation, subunit detection was difficult due to low levels of caspase processing. However, longer exposure revealed the presence of caspase-3 subunits p20, p19 and p17 after 6 h in the absence of IL-5 (approximately 15% apoptosis by PtdSer exposure). Processing of caspase-9 was less marked compared with caspase-3, -7 and -8, but the p37 subunit was detectable after 24 h IL-5 deprivation, but was inconsistent (Fig. 5.2E, Lane 10). Processing of caspase-3, -7, -8 and -9 was completely inhibited by IL-5 (10^{-10} M) over the 24 h culture period (Fig. 5.1A, C and D, Lane 9; Fig. 5.2E, Lane 11). Lack of immunoreactivity of caspase-6 subunits made determination of processing in the absence of IL-5 difficult. A slight loss of proform in IL-5 deprived eosinophils compared with IL-5 stimulated after 24 hours was observed, but was not marked (Fig. 5.1B, Lanes 8 and 9). The broad-spectrum cell-permeable caspase inhibitor, zVAD.fmk, inhibited the processing of each caspase in IL-5 deprived eosinophils after 24 h (Fig. 5.2A, B, C, and D, Lane 13). However, inhibition was not absolute as caspase-9 processing after 24 h in the presence of zVAD.fmk was observed, evidenced by the detection of the p35 subunit (Fig. 5.2E, Lane 13). Also, although zVAD.fmk prevented the processing of caspase-3 to the p19 and p17 subunits, the p20 subunit was still detectable after 24 h (Fig. 5.2A, Lane 13).

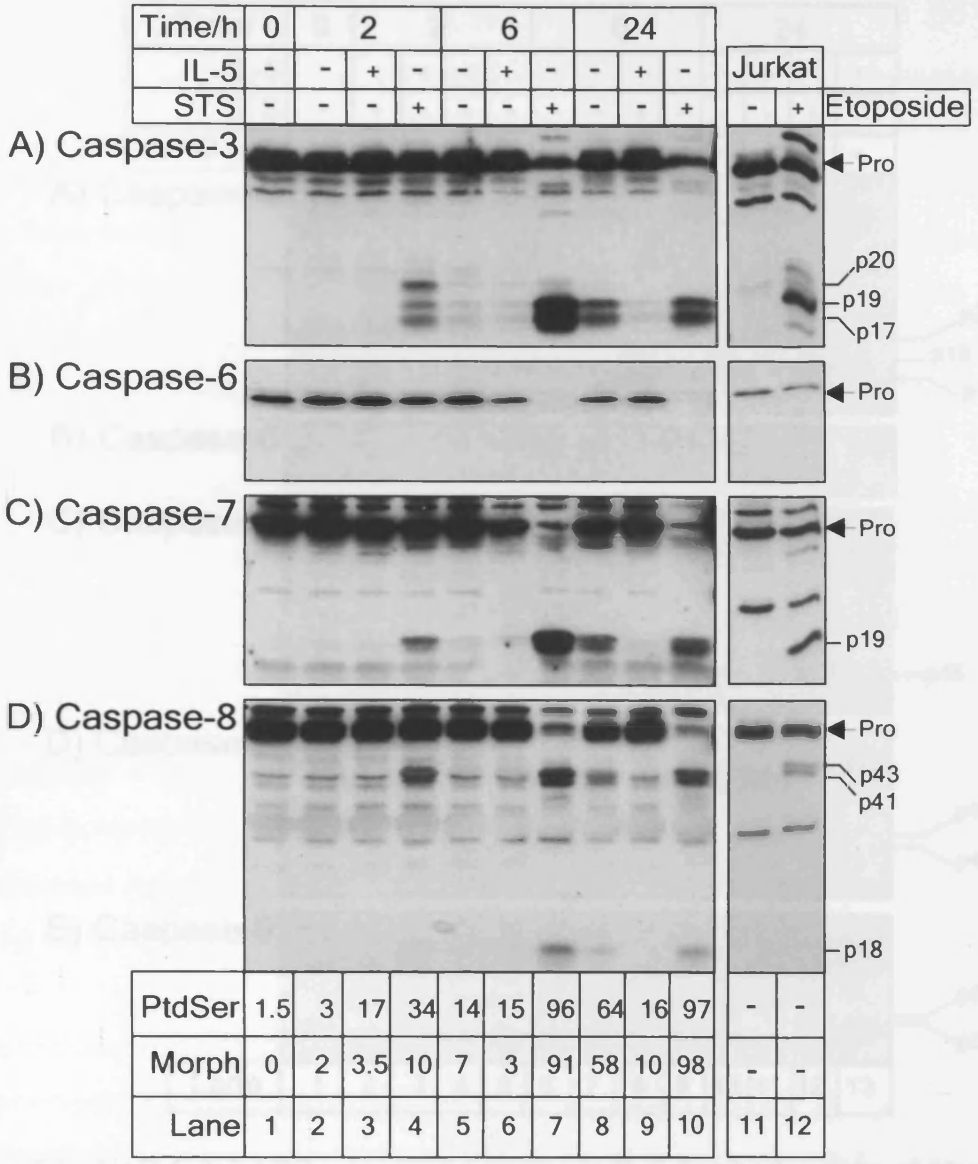

Figure 5.1. IL-5 inhibits caspase processing in the spontaneous apoptosis of peripheral blood eosinophils. Peripheral blood eosinophils were cultured in the presence or absence of IL-5 (10^{-10} M) or STS (10^{-5} M) and analysed at the times shown for processing of caspase-3 (A), -6 (B), -7 (C), and –8 (D) by immunoblotting. Jurkat T cells treated with or without etoposide (50 μM) for 4 h were used as controls. The proforms and processed subunits are indicated. Apoptosis was assessed by AnnexinV binding to determine percentage cells with externalised PtdSer and apoptotic morphology after Kimura stain as described in *Materials and Methods*. Eosinophils constitutively expressed caspase-3, -6, -7, and -8. Culturing in the absence of IL-5 and treatment with STS resulted in activation of all of the caspases. IL-5 prevented caspase activation over the 24 h culture period. Longer exposure of the caspase-3 (A) blot revealed the presence of p20 and p19 subunits after 6 h in untreated eosinophils. Shorter exposure of the caspase-8 (D) blot revealed the proform to be a doublet of 53 and 55 kDa. Results are representative of three separate experiments.

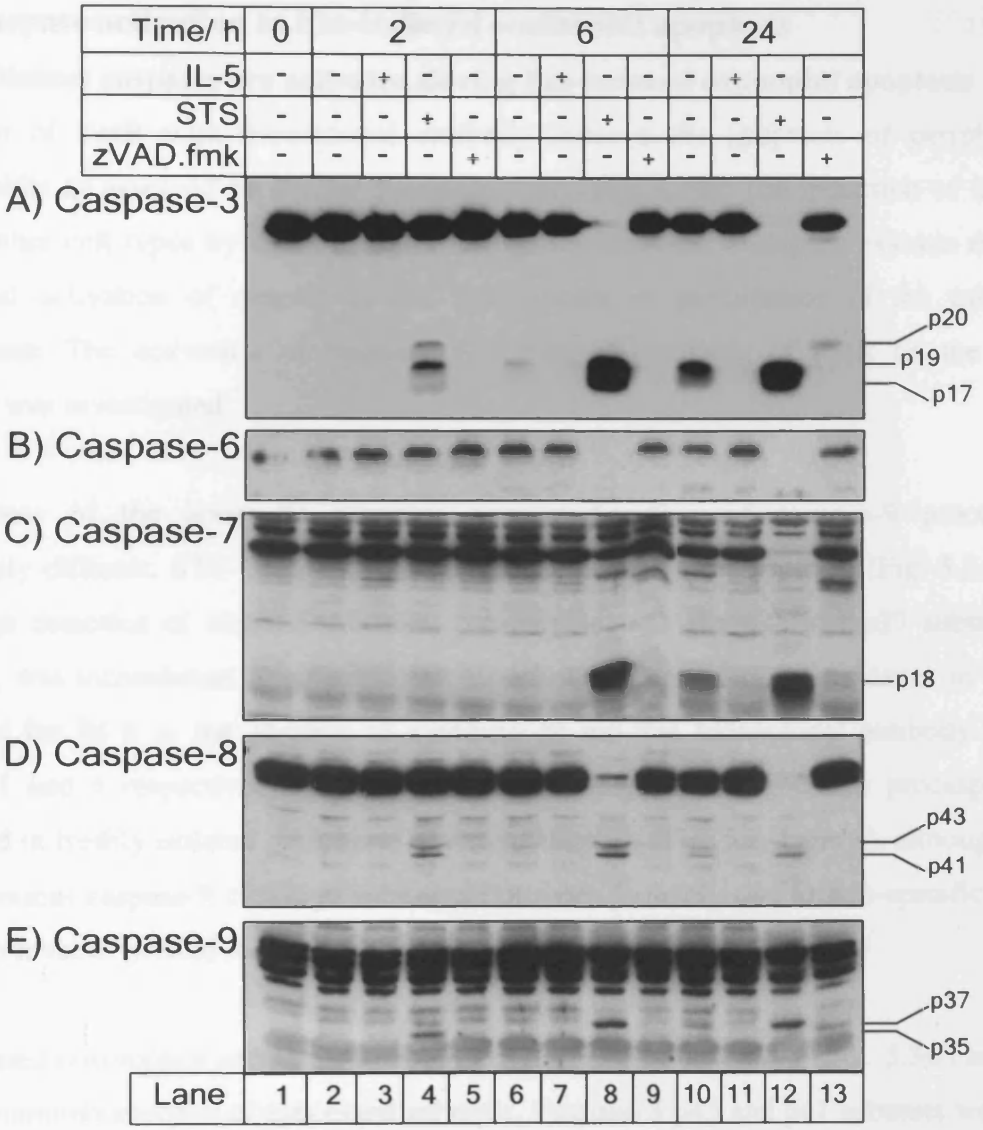

Figure 5.2. zVAD.fmk inhibits caspase processing in IL-5 deprived peripheral blood eosinophils. Cells were incubated with IL-5 (10^{-10} M), STS (10^{-5} M) or zVAD.fmk (100 μM), harvested at the times indicated and analysed for caspase-3 (A), -6 (B) –7 (C), -8 (D), and –9 (E) processing by immunoblotting. Eosinophils constitutively expressed caspase-9, which was activated during staurosporine-induced and spontaneous apoptosis. IL-5 prevented the processing of caspase-9 observed during spontaneous apoptosis over the 24 h culture period. zVAD.fmk inhibited caspase activation in IL-5 deprived eosinophils. Results are representative of three separate experiments.

5.3. Caspase activation in Fas-induced eosinophil apoptosis

5.3.1. Distinct caspases are activated during Fas-induced eosinophil apoptosis

Ligation of FasR with monoclonal antibody induced the apoptosis of peripheral blood eosinophils as assessed by PtdSer externalisation (Fig. 3.13). The induction of apoptosis in many other cell types by death-receptor abrogation activates a caspase cascade mediated by proximal activation of caspase-8, and independent of perturbation of the mitochondrial membrane. The activation of caspases following engagement of FasR on the eosinophil surface was investigated.

Regardless of the apoptotic stimulus, immunodetection of caspase-9 processing was extremely difficult. STS treatment did result in the loss of the proform (Fig. 5.3A, Lane 3), although detection of cleaved subunits, comparable with the p35 and p37 subunit positive control, was inconsistent. Processing of caspase-9 was very difficult to detect in eosinophils cultured for 24 h in the absence of cytokine or anti-Fas monoclonal antibody (Fig. 5.3A, Lanes 1 and 4 respectively). Proteins of lower molecular weight than procaspase-9 were detected in freshly isolated peripheral blood eosinophils (Fig. 5.3, Lane 8), although these did not represent caspase-9 cleavage subunits, but were probably due to non-specific binding of the high level of protein loading of this sample.

Fas-treated eosinophils exhibited detectable processing of caspase-8 (Fig. 5.3B) as evidenced by the immunodetection of processed subunits. Caspase-8 p43 and p41 subunits were detected in Fas-treated eosinophils, but also were detectable in untreated eosinophils due to spontaneous apoptosis (Fig. 5.3A; Lanes 2 and 4). However, the p18 subunit was more evident in the Fas treated cells than untreated, indicating induction of caspase-8 activation by Fas. zVAD.fmk completely inhibited the Fas-induced processing of caspase-8 (Fig. 5.3B, Lane 7). Interestingly, stimulation of eosinophils with IL-5 also inhibited Fas-dependent caspase-8 processing, with the p18 subunit barely detectable (Fig. 5.3B, Lane 5).

Fas-treated eosinophils expressed processed caspase-3, evidenced by the detection of the p19 and p17 subunits (Fig. 5.3C, Lane 4), indicative of processing of caspase-3 to its active form, although there was no detectable loss of the proform as was observed in eosinophils treated with STS for just 8 h (Fig. 5.3C, Lane 3). Eosinophils undergoing spontaneous apoptosis after 24 h also exhibited processing of caspase-3 to the p19 and p17 subunits (Fig. 5.3C, Lane 1), although processing was slightly decreased from that observed in Fas treated eosinophils. Supporting the data in Figure 5.1, IL-5 inhibited the processing of caspase-3 observed during

spontaneous apoptosis (Fig. 5.3C, Lane 2), and similarly to caspase-8, co-culture with IL-5 and anti-Fas mAb (Fig. 5.3C, Lane 5) markedly inhibited the caspase-3 processing detectable in eosinophils treated with anti-Fas alone (Fig. 5.3C, Lane 4). Treatment with zVAD.fmk prevented processing of caspase-3 during Fas-induced apoptosis, with complete prevention of processing to p19 and p17, although the p20 subunit remained detectable (Fig. 5.3C, Lanes 6 and 7), as was observed during spontaneous apoptosis (Fig. 5.2A, Lane 13 and Fig. 5.3C, Lane 6).

5.3.2. FasR ligation induces Bid cleavage in eosinophils

Bid is a 23 kDa BH3-only Bcl-2 homologue, which when cleaved by caspase-8, generates tBid (truncated Bid, 15 kDa) which translocates to the mitochondria and facilitates the release of cytochrome *c*, possibly by interacting with Bax and Bak (Desagher *et al.*, 1999; Wei *et al.*, 2000). This is proposed to form an amplification loop, due to the consequent activation of the caspase-9-mediated cascade. The constitutive expression and apoptosis-associated cleavage of Bid in eosinophils was investigated. Bid was observed to be constitutively expressed in freshly isolated peripheral blood eosinophils (Fig. 5.4). Induction of apoptosis with STS was associated with Bid cleavage indicated by the loss of the 23 kDa protein and concomitant detection of immunoreactive cleavage product (15 kDa; Fig. 5.4, Lane 3). Cleaved Bid was also detectable in eosinophils exposed to anti-Fas mAb for 24 h although not to the extent observed in STS treated eosinophils, and with no evidence of loss of full length Bid (Fig. 5.4, Lanes 4 and 5). Eosinophils undergoing spontaneous apoptosis for 24 h exhibited little or no detectable cleavage of Bid (Fig. 5.4, Lane 2). However, lack of detection of cleaved Bid in untreated eosinophils does not rule out its occurrence during spontaneous apoptosis, due to the rapid turnover and potentially low levels of tBid required for induction of mitochondrial perturbation (Scaffidi *et al.*, 1998).

The results demonstrate that caspases were activated during spontaneous and Fas-induced eosinophil apoptosis, and that IL-5 acted at a point prior to caspase activation in inhibiting spontaneous eosinophil apoptosis. The events that precede the activation of caspases in the apoptotic pathway were then investigated. The mitochondria are pivotal in the regulation of apoptotic cell death, with apoptogenic factor release required for the activation of caspase-9. The role of mitochondria in eosinophil apoptosis was investigated.

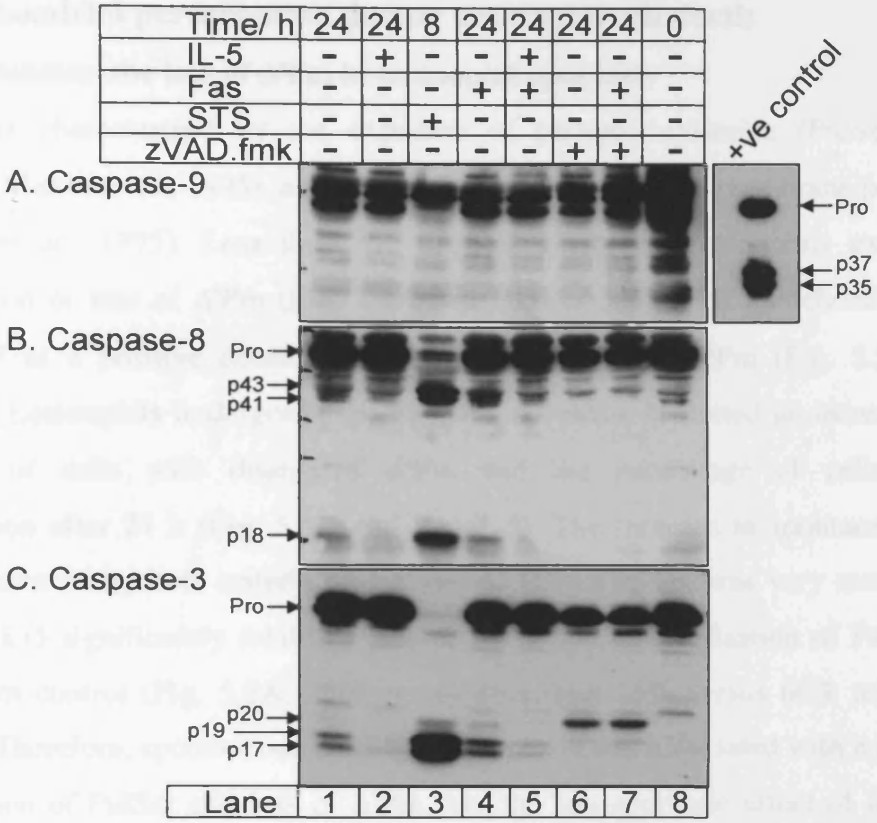

Figure 5.3. Ligation of FasR induces activation of specific caspases. Freshly isolated eosinophils or eosinophils cultured in the presence or absence of IL-5 (10^{-10}M), anti-Fas mAb (CH11, 500 ng/ml), STS (10^{-5}M) and zVAD.fmk (100 μM) for the indicated times, were analysed for caspase-9 (A), -8 (B) and –3 (C) processing by immunoblotting. Recombinant proform and p35 and p37 subunits of caspase-9 were run as a positive control for caspase-9 processing. Results are representative of three separate experiments.

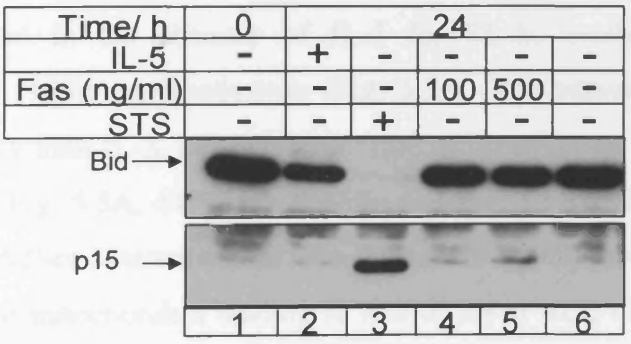

Figure 5.4. Eosinophils express Bid which is cleaved during STS and Fas-induced apoptosis. Freshly isolated eosinophils or eosinophils cultured in the presence or absence of IL-5 (10^{-10}M), anti-Fas mAb (CH11, 500 ng/ml) or STS (10^{-5}M) were analysed for expression and processing of Bid by immunoblotting. Results are representative of two independent experiments.

5.4. Mitochondrial perturbation during eosinophil cell death

5.4.1. IL-5 inhibits the loss of ΔΨm in eosinophil apoptosis

Apoptosis is characterised by the exposure of phosphatidylserine (PtdSer) at the cell membrane (Martin *et al.*, 1995), and a reduction in mitochondrial membrane potential (ΔΨm) (Zamzami *et al.*, 1995). Less than 5% of freshly isolated eosinophils exhibited PtdSer externalisation or loss of ΔΨm (Fig. 5.5A and B). Uncoupling of mitochondrial respiration with mCCP as a positive control caused a marked loss of ΔΨm (Fig. 5.5A, 0 h, *open histogram*). Eosinophils undergoing spontaneous apoptosis exhibited an increase in both the percentage of cells with dissipated ΔΨm and the percentage of cells with PtdSer externalisation after 24 h (Fig. 5.5A and B, *-IL-5*). The increase in spontaneous eosinophil apoptosis assessed by both criteria was observed after 6 h, but was very marked after 24 h (Fig. 5.6). IL-5 significantly inhibited loss of ΔΨm and externalisation of PtdSer compared with medium control (Fig. 5.5A, 25% versus 48%, and 13% versus 66% respectively; and Fig. 5.5B). Therefore, spontaneous eosinophil apoptosis was associated with a time-dependent externalisation of PtdSer and loss of ΔΨm, and the anti-apoptotic effect of IL-5 acted at or prior to the loss of ΔΨm. There was no significant difference between the progression of PtdSer externalisation and dissipation of ΔΨm during spontaneous eosinophil apoptosis (Fig. 5.6). Apoptosis assessed by both these criteria was significantly increased as early as 2 h exposure to STS and was almost maximal after 6 h (Fig. 5.6).

5.4.2. zVAD.fmk inhibits eosinophil PtdSer exposure after loss of ΔΨm

In eosinophils cultured in the absence of IL-5 for 24 h, incubation with zVAD.fmk significantly inhibited PtdSer externalisation (Fig. 5.5A, 46% versus 66%; and Fig. 5.5B), although less efficiently than IL-5, but exhibited little or no effect on loss of ΔΨm compared with medium control (Fig. 5.5A, 44% versus 48%; and Fig. 5.5B). Thus during spontaneous eosinophil apoptosis PtdSer externalisation was dependent upon caspase activation, whereas the perturbations of the mitochondria leading to loss of ΔΨm were independent of caspases. Prevention of caspase activation inhibited apoptosis assessed by PtdSer externalisation, thereby implicating caspases as major effectors of the execution of spontaneous eosinophil apoptosis. To further elucidate the role of the mitochondria in eosinophil apoptosis, cytochrome *c* release was investigated. Cytochrome *c* release from mitochondria is an early, pivotal event in the apoptosis of many cell types (Green and Reed, 1998).

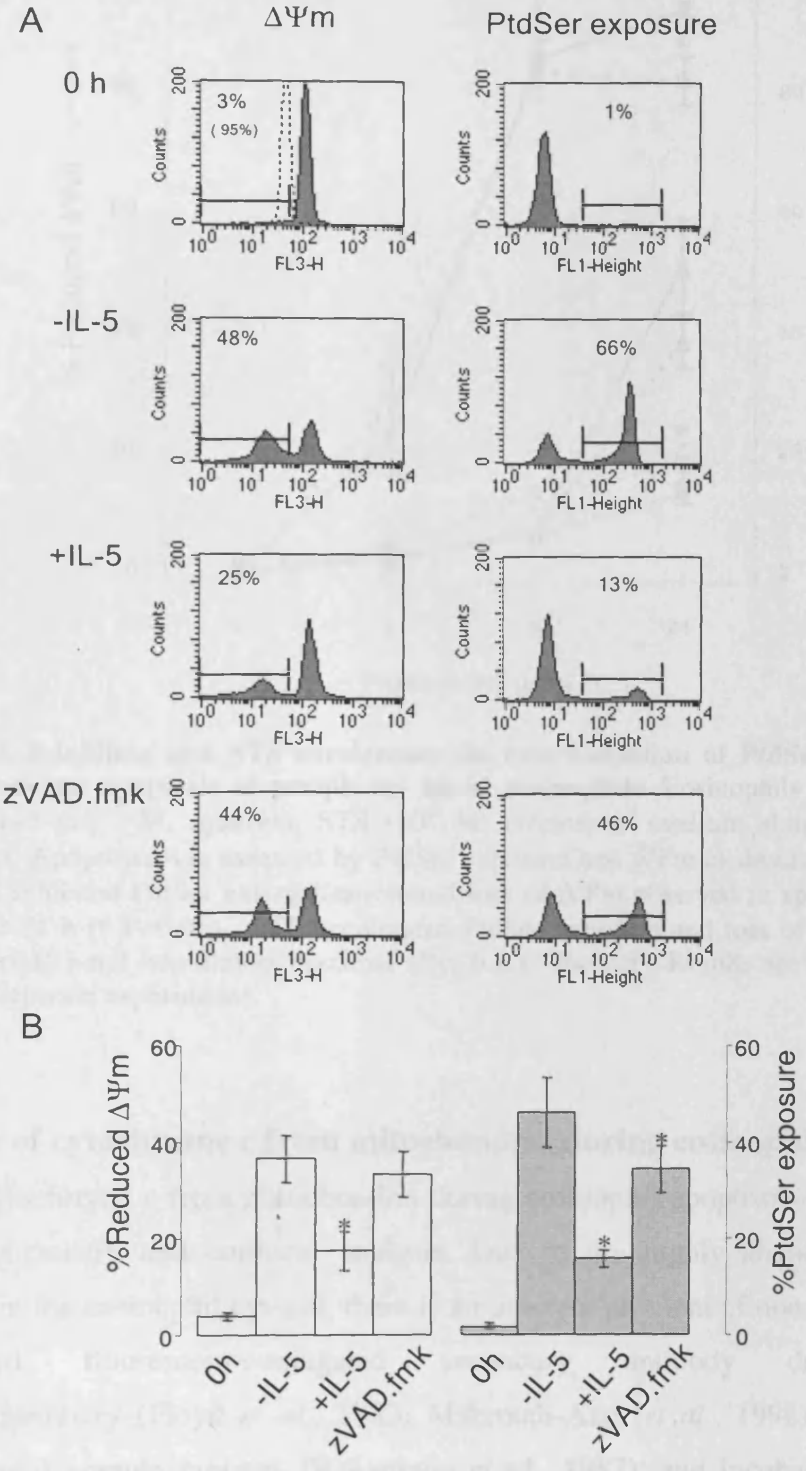

Figure 5.5. IL-5 and zVAD.fmk prevent phosphatidylserine externalisation, but only IL-5 prevents loss of mitochondrial membrane potential in eosinophils. Freshly isolated eosinophils (*0 h*) or eosinophils incubated in the absence (*-IL-5*) or presence of IL-5 (10^{-10} M) or zVAD.fmk (100 μM) for 24 h were assessed for mitochondrial membrane potential (ΔΨm) and phosphatidylserine (PtdSer) exposure as described in *Materials and Methods*. (A) Flow cytometry histograms of 10,000 events showing number of cells exhibiting reduced ΔΨm or increased PtdSer exposure. At 0 h, eosinophils were also treated with the mitochondria uncoupling agent, mCCP, as a positive control for loss of ΔΨm (*open histogram*, figure in parentheses). Results are representative of six separate experiments. (B) Shows percentage eosinophils with reduced ΔΨm (*open bars*) or PtdSer externalisation (*filled bars*). Results are expressed as mean ± SEM of six separate experiments. * IL-5 and zVAD.fmk significantly inhibited PtdSer exposure compared with medium control (-IL-5), but only IL-5 inhibited loss of ΔΨm ($P<0.05$).

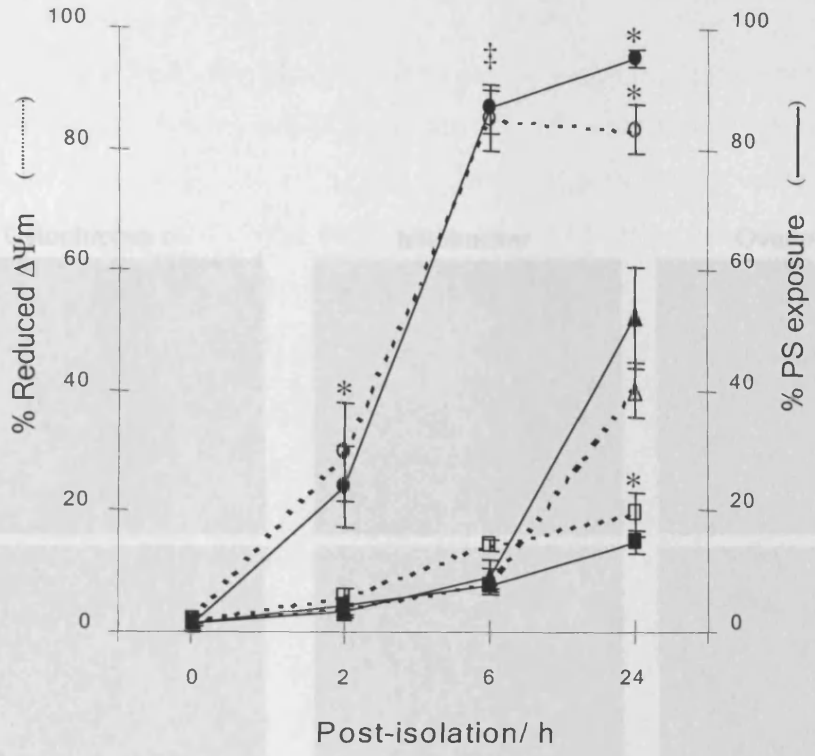

Figure 5.6. IL-5 inhibits and STS accelerates the externalisation of PtdSer and loss of ΔΨm during spontaneous apoptosis of peripheral blood eosinophils. Eosinophils were cultured in the presence of IL-5 (10^{-10} M, *squares*), STS (10^{-5} M, *circles*) or medium alone (*triangles*) for the indicated times. Apoptosis was assessed by PtdSer exposure and ΔΨm as described in *Materials and Methods*. IL-5 inhibited PtdSer externalisation and loss of ΔΨm observed in spontaneous eosinophil apoptosis after 24 h (* $P<0.05$). STS accelerated PtdSer exposure and loss of ΔΨm in eosinophils after 2 h (‡ $P<0.01$) and was almost maximal after 6 h (* $P<0.05$). Results are expressed as mean ± SEM of three separate experiments.

5.5. Release of cytochrome *c* from mitochondria during eosinophil apoptosis

Release of cytochrome *c* from mitochondria during eosinophil apoptosis was investigated by immunocytochemistry and confocal analysis. Due to the highly abundant basic granule proteins within the eosinophil cytosol, there is an inherent problem of non-specific binding of the charged, fluorescein-conjugated secondary antibody during eosinophil immunocytochemistry (Floyd *et al.*, 1983; Mahmudi-Azer *et al.*, 1998). Chromotrope 2R stains eosinophil granule proteins (Hakansson *et al.*, 1987), and incubation of eosinophils prior to secondary antibody markedly reduced non-specific binding in a dose-dependent manner, allowing detection of the specific punctate cytochrome *c* distribution pattern (Fig. 5.7). For subsequent immunostaining, 0.2% Chromotrope 2R was used as this concentration was optimal for reducing non-specific antibody binding whilst allowing visualisation of mitochondria with Mitotracker Red CMXRos.

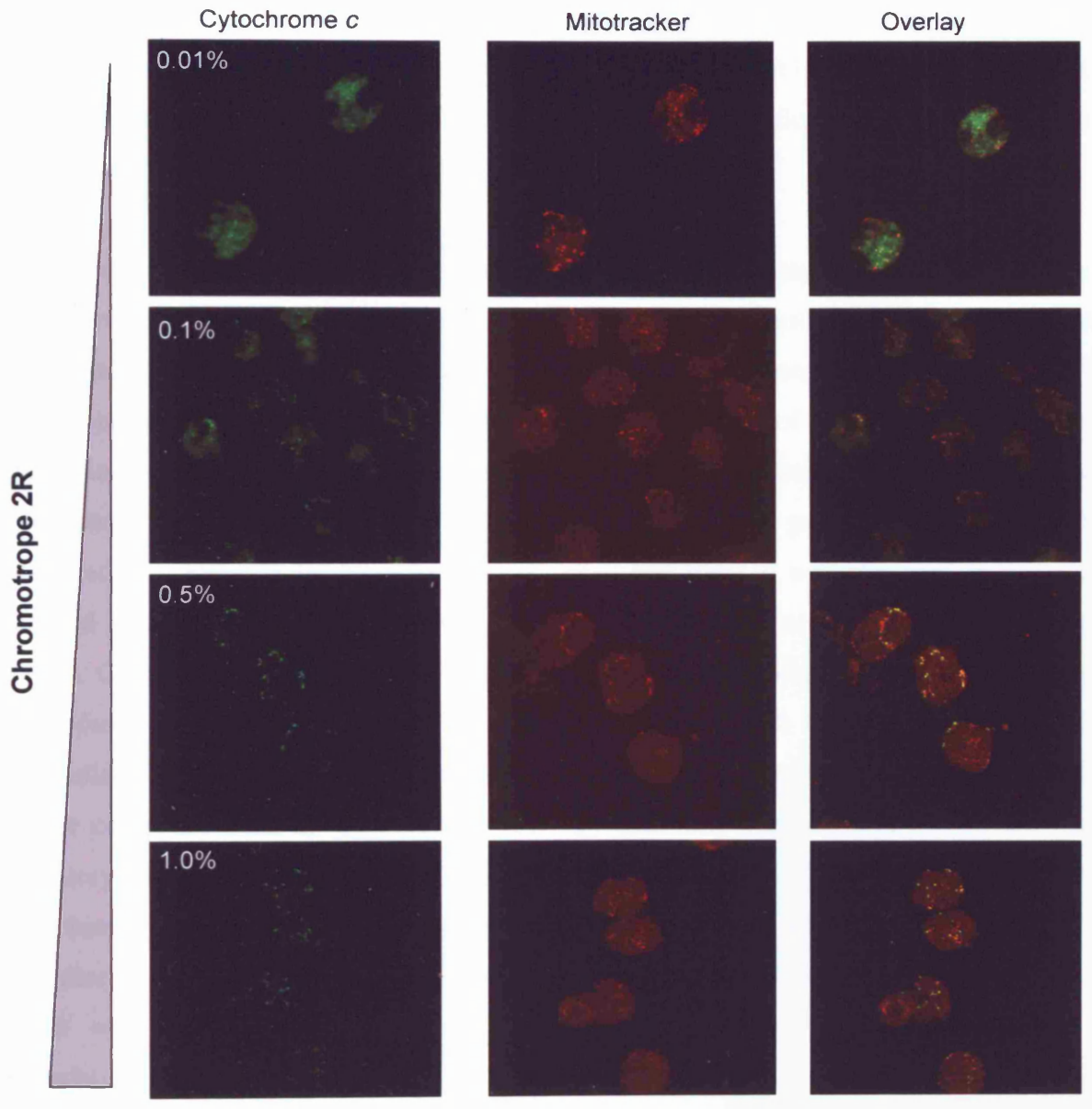

Figure 5.7. Inhibition of non-specific staining of eosinophils with Chromotrope-2R. Cytospins of freshly isolated eosinophils stained with Mitotracker were generated and probed with antibody directed against cytochrome *c* according to *Materials and Methods*. Prior to incubation with fluorescent conjugate secondary antibody, the cytospins were incubated with the indicated concentration of Chromotrope 2R and confocal analysis was performed.

Cytochrome *c* normally resides within the mitochondrial inter-membrane space. Incubation of peripheral blood eosinophils with the Mitotracker CMXRos generated a punctate, cytosolic staining, which was dissipated with treatment with the mitochondrial membrane uncoupler, mCCP (Fig. 5.8A). Mitotracker retained its affinity for mitochondria in the presence of Chromotrope 2R, whereas Chromotrope 2R alone produced a diffuse cytosolic staining (Fig. 5.8B). Therefore, Mitotracker CMXRos was verified as a specific marker for mitochondria in eosinophils.

Spontaneous eosinophil apoptosis was associated with caspase-dependent release of mitochondrial cytochrome *c*, which was inhibited by IL-5. In freshly isolated peripheral blood eosinophils cytochrome *c* exhibited a punctate distribution, which colocalised with mitochondria (Fig. 5.9A). Due to the relatively low level of constitutive cytochrome *c* expression and number of mitochondria in eosinophils, cells which had undergone cytochrome *c* release generally appeared diffuse or devoid of green staining entirely (Figs. 5.9B and D, indicated by arrows). Release of cytochrome *c* was observed in eosinophils cultured in the absence of IL-5 for 6 h (Fig. 5.9B), and was marked after 24 and 48 h (Fig. 5.10A). Cytochrome *c* release was commonly associated with eosinophils exhibiting evidence of apoptotic nuclear condensation (Fig. 5.9B). Stimulation with IL-5 for 6 h maintained the association of cytochrome *c* with the mitochondria (Fig. 5.9C), and inhibited cytochrome *c* release compared with medium control after 24 and 48 h (Fig. 5.10A). zVAD.fmk had no inhibitory effect on cytochrome *c* release compared with medium control (Fig. 5.10A). Cytochrome *c* release was also observed in eosinophils treated with STS for 2 h (Fig. 5.9D), and after 6 h STS treatment almost 100% of eosinophils exhibited cytochrome *c* release, which was not inhibited by zVAD.fmk (Fig. 5.10B). Due to downstream proteolytic degradation of the cell, accurate determination of cytochrome *c* release beyond 6 h STS treatment was not possible. Therefore spontaneous and STS-induced eosinophil apoptosis involved caspase-independent cytochrome *c* release. IL-5 inhibited spontaneous eosinophil apoptosis at or prior to release of cytochrome *c*. The release of cytochrome *c* was also observed by subcellular fractionation and immunoblotting. Little or no cytochrome *c* was detectable within the cytosol of freshly isolated eosinophils, but was released during spontaneous apoptosis over 24 h, and rapidly after treatment with STS for 2 h (Fig. 5.11A and B). Incubation with IL-5 inhibited the release of cytochrome *c*, although it was detectable (Fig 5.11A and B). Cytochrome *c* oxidase subunit II, a mitochondrial inner membrane protein, was used to verify purity of the cytosolic fractions, and was readily detectable in the heavy membrane fractions, but undetectable in the cytosolic fractions in all treatments. As a control,

Jurkat T cells were cultured in the presence or absence of etoposide to induce apoptosis. Etoposide induced marked release of cytochrome *c* into the cytosolic fraction of Jurkat T cells (Fig. 5.11C).

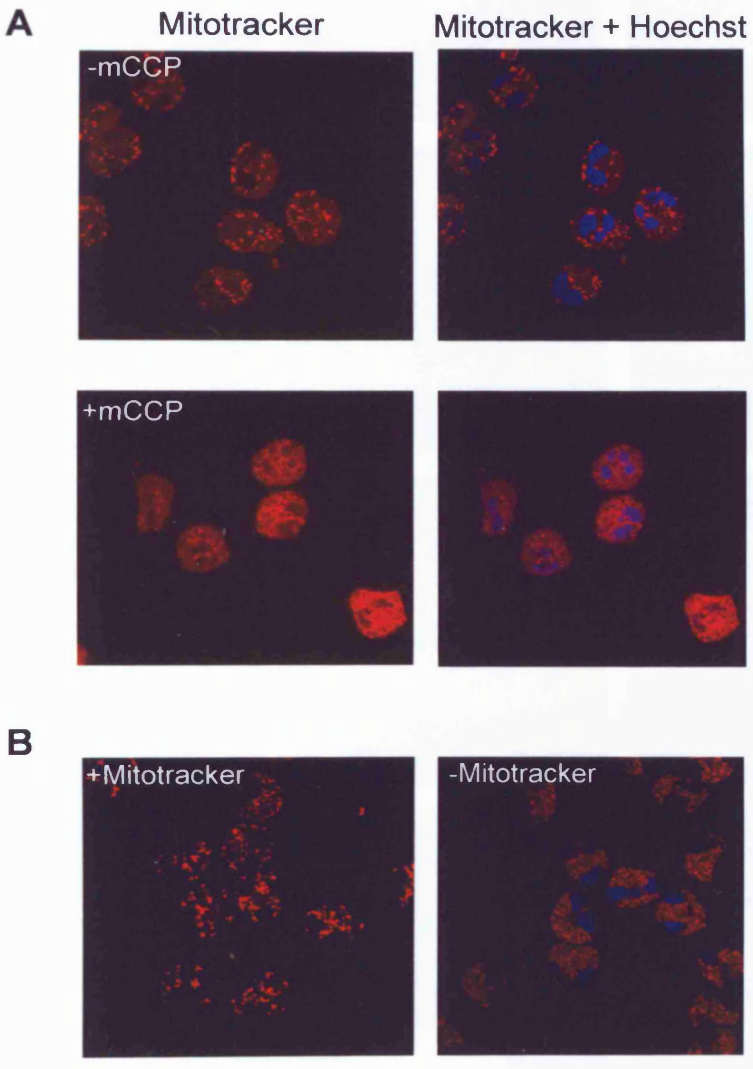

Figure 5.8. Mitotracker specifically labels mitochondria in eosinophils. A) Eosinophils were incubated with Mitotracker CMXRos (75 nM) for 30 min at 37°C in the presence or absence of the mitochondrial membrane uncoupler carbonylcyanide m-chlorophenyl hydrazone (mCCP, 1 μM) for 15 min at 37°C. B) Eosinophils were incubated in the presence (*right panel*) or absence (*left panel*) of Mitotracker CMXRos (75 nM) for 30 min at 37°C. Cytospins were prepared according to *Materials and Methods*, stained with Chromotrope-2R (B) and examined by confocal laser microscopy.

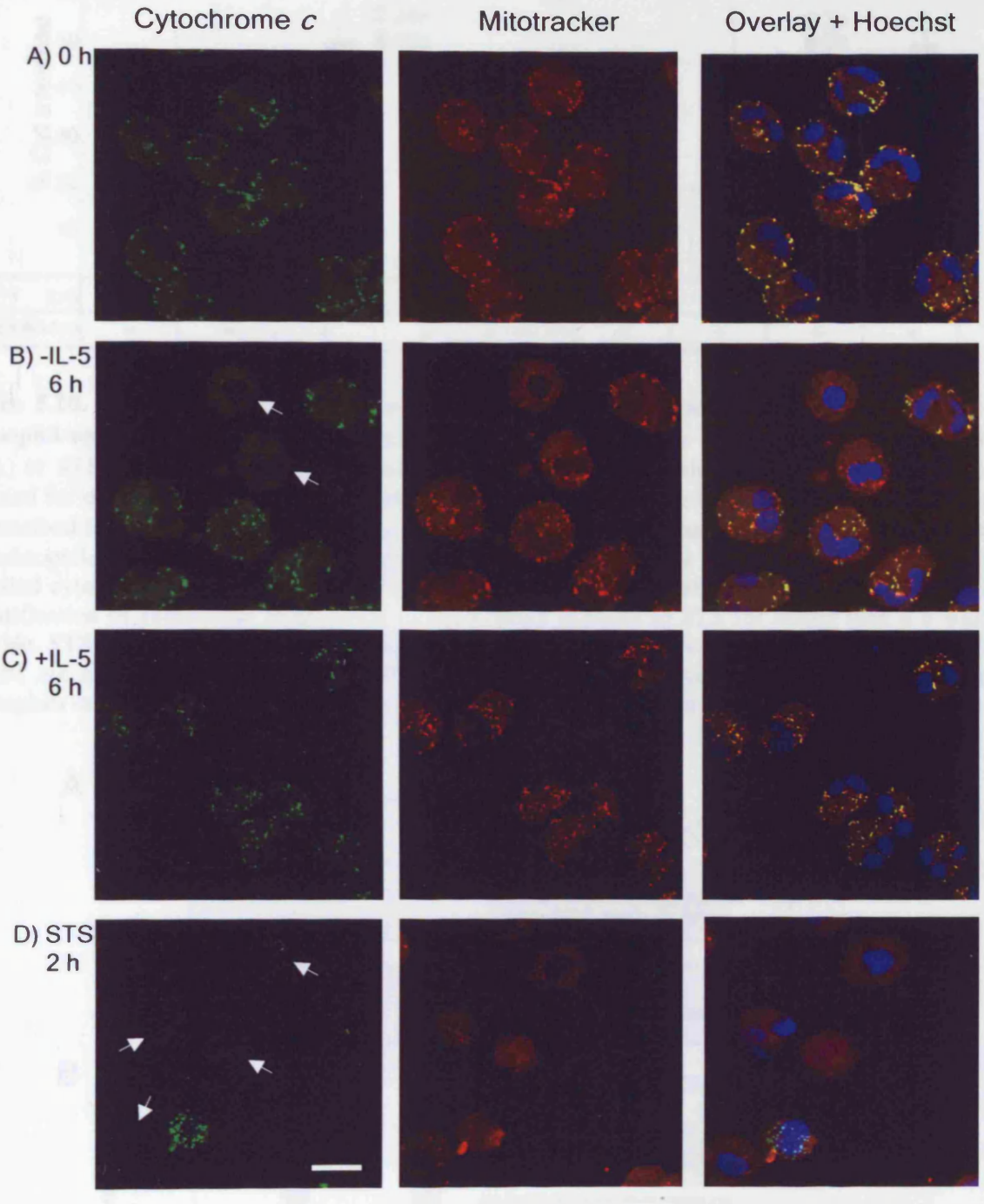

Figure 5.9. Cytochrome *c* is released from the mitochondria during spontaneous and STS-induced eosinophil apoptosis. Immunohistochemistry was performed, as described in *Materials and Methods*, on fixed cytospins of freshly isolated eosinophils (A) or eosinophils cultured in the absence (B) or presence of IL-5 (10^{-10} M) for 6 h (C) or STS (10^{-5} M) for 2 h (D). Images were captured by confocal microscopy at x1000 magnification under oil immersion. Cytochrome *c* was detected using a monoclonal antibody 6H2.B4 and the mitochondria and nuclei stained with Mitotracker CMXRos and Hoechst 33258 respectively. Co-localization (*yellow*) of cytochrome *c* (*green*) and mitochondria (*red*) was observed in single sections. Eosinophils exhibiting evidence of cytochrome *c* release are indicated (B and D, *arrow*). Culturing in the absence of IL-5 for 6 h and treatment with STS for 2 h induced cytochrome *c* release from the mitochondria. Bar represents 10 μM. Results are representative of experiments performed on four separate donors.

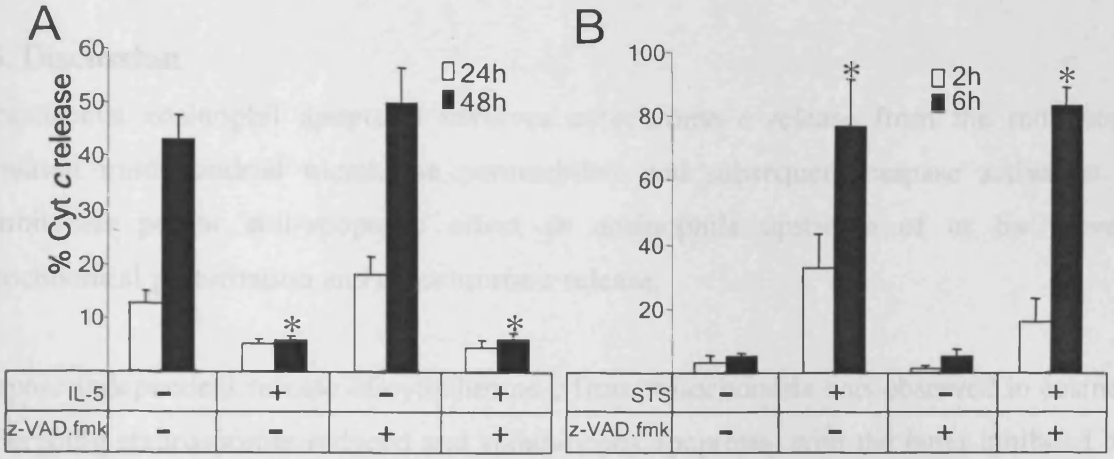

Figure 5.10. IL-5 prevents cytochrome *c* release from the mitochondria during spontaneous eosinophil apoptosis. Eosinophils were co-incubated in the presence or absence of either IL-5 (10^{-10} M, A) or STS (10^{-5} M, B) and zVAD.fmk (100 μM, A and B) for the indicated times and the cells assessed for evidence of cytochrome *c* release. Cytochrome *c* was detected by immunocytochemistry as described in *Materials and Methods*, using the 6H2.B4 monoclonal antibody, and the percentage of eosinophils exhibiting diffuse cytochrome *c* was quantified under fluorescence microscopy. IL-5 inhibited cytochrome *c* release observed during spontaneous eosinophil apoptosis after 24 and 48 h. Quantification of subcellular localisation in eosinophils exposed to STS for longer than 6 h was not possible. STS rapidly induced cytochrome *c* release after 2 h, which was not inhibited by zVAD.fmk. Results are expressed as the mean ± SEM of three separate experiments. * IL-5 or STS treated eosinophils compared with the relevant untreated or zVAD.fmk treated control, $P<0.05$.

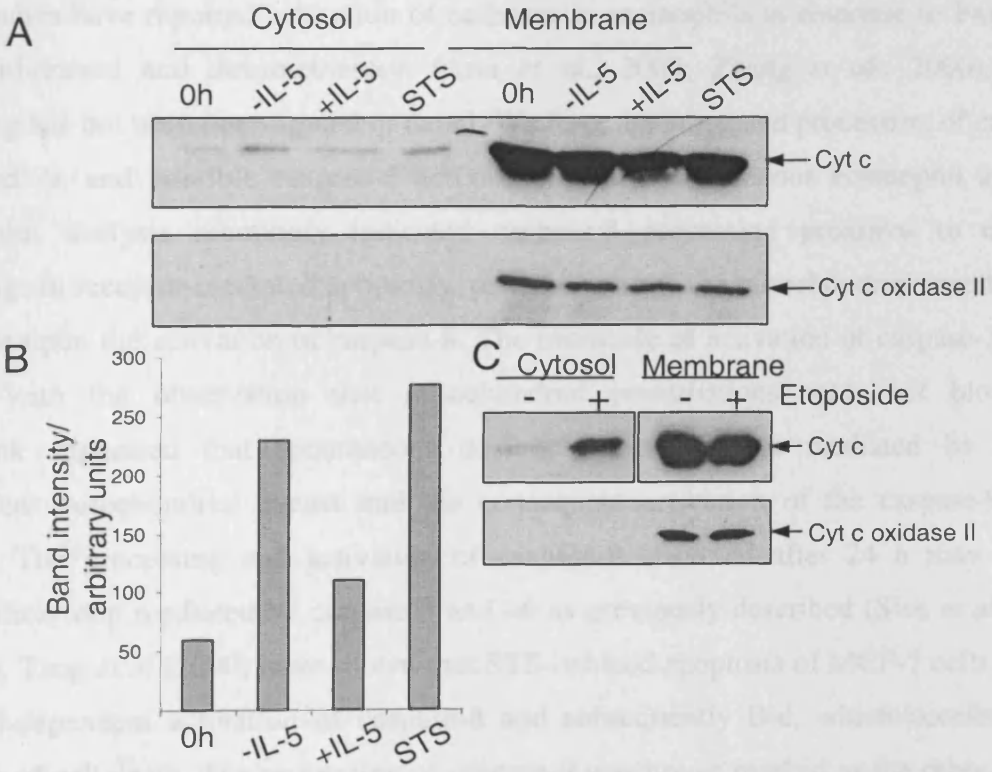

Figure 5.11. Cytochrome *c* release in eosinophils undergoing spontaneous and staurosporine-induced apoptosis by subcellular fractionation. Eosinophils were cultured in the presence or absence of IL-5 (10^{-10}M) for 24 h or staurosporine (STS, 10^{-5}M) for 2h. Subcellular fractionation was performed by Dounce homogenisation according to *Materials and Methods* and immunoblotted for cytochrome *c* or cytochrome *c* oxidase subunit II as a control for the fractionation process (A), and band densitometry performed (B). Etoposide-induced apoptosis of Jurkats was used as a control for cytochrome *c* release (C). Results are representative of two independent experiments.

5.6. Discussion

Spontaneous eosinophil apoptosis involves cytochrome *c* release from the mitochondria, increased mitochondrial membrane permeability and subsequent caspase activation. IL-5 exhibits its potent anti-apoptotic effect in eosinophils upstream of or by preventing mitochondrial perturbation and cytochrome *c* release.

Caspase-independent release of cytochrome *c* from mitochondria was observed in eosinophils undergoing staurosporine-induced and spontaneous apoptosis, with the latter inhibited by IL-5. The mechanism by which cytochrome *c* is released from the mitochondria remains unclear. It has been proposed that Bax induces the opening of the permeability transition pore complex via interaction with the ANT, resulting in mitochondrial membrane depolarisation and cytochrome *c* release (Marzo *et al.*, 1998a). Spontaneous eosinophil apoptosis clearly involved caspase-independent loss of $\Delta\Psi m$, which was also inhibited by IL-5.

Elucidation of receptor-mediated and chemical/ stress-induced caspase activation in cell lines or *in vitro* have implicated apical processing of caspase-8 and –9 respectively. Although recent studies have reported activation of caspases in eosinophils in response to Fas, growth factor withdrawal and dexamethasone (Arai *et al.*, 2000; Zhang *et al.*, 2000), caspase processing has not been investigated in detail. We have demonstrated processing of caspase-3, -7, -8 and -9, and possible caspase-6 activation during spontaneous eosinophil apoptosis. Immunoblot analysis seemingly indicated caspase-3 processing proximal to caspase-8 processing. In receptor-mediated apoptosis, perturbations of the mitochondrial membrane are dependent upon the activation of caspase-8. The timescale of activation of caspase-3 and –8, coupled with the observation that mitochondrial perturbations were not blocked by zVAD.fmk suggested that spontaneous eosinophil apoptosis is mediated by caspase-independent mitochondrial events and the consequent activation of the caspase-9/ -3/ -7 pathway. The processing and activation of caspase-8 observed after 24 h may form an amplification loop mediated by caspase-3 and –6 as previously described (Slee *et al.*, 1999). Similarly, Tang *et al* (2000) have shown that STS-induced apoptosis of MCF-7 cells involves caspase-3-dependent activation of caspase-8 and subsequently Bid, which accelerated the execution of cell death. The processing of caspase-9 was not as marked as the other caspases tested, and was often undetectable even in the presence of STS. This may be due to the relative efficacy of the antibodies used, although it must be noted that caspase-9 can be activated without proteolytic cleavage (Stennicke *et al.*, 1999). Incubation with the broad range caspase inhibitor, zVAD.fmk prevented detectable processing of caspase-3, -7, -8, and –

9 during spontaneous apoptosis. Interestingly, despite preventing the complete processing of caspase-3 to the active p19 and p17 subunits, zVAD.fmk did not prevent processing to the p20 subunit. Similarly, some evidence of caspase-9 processing, albeit reduced, was observed in the presence of 100 μM zVAD.fmk. The caspase-9 zymogen possesses inherent catalytic activity, which is increased ten-fold (see Table 1.2) during the autocatalytic processing by induced proximity upon oligomerisation with Apaf-1 and cytochrome *c* in the presence of dATP. zVAD.fmk is inefficient at preventing caspase-9 transcatalysis at the apoptosome complex, but inhibits the activity of the processed caspase. Initial caspase-9-dependent processing of caspase-3 occurs at Asp175 between the large and small subunits, thereby generating the p20 subunit, followed by autocatalysis to generate the p19 and p17 subunits (Martin *et al.*, 1996). It is proposed that caspase-3 is cleaved by the limited activity of caspase-9 zymogen when oligomerised with Apaf-1, and therefore is not efficiently blocked by zVAD.fmk, generating the p20 immunoreactive subunit. The autocatalytic activity of caspase-3 is inhibited by zVAD.fmk, and therefore prevents subsequent processing to p19 and p17. We have demonstrated that caspases are major effectors of the execution phase of spontaneous eosinophil apoptosis, and IL-5 induces eosinophil survival by inhibiting apoptosis prior to caspase activation. Zangrilli *et al* (2000) have reported processing of caspase-3 and –8 by immunoblotting during the spontaneous apoptosis of human eosinophils. Contrastingly, using caspase-specific substrates, two studies have reported increased caspase-3 activity, but no increase in caspase-8 activity during spontaneous eosinophil apoptosis (Arai *et al.*, 2000; Zhang *et al.*, 2000). However, specific peptide inhibitors often failed to inhibit caspase activity, raising questions regarding either the specificity or cell permeability of the peptide inhibitors, or the specificity of the caspase assays used.

Fas-induced apoptosis was associated with detectable processing and activation of both caspase-8 and caspase-3, although the processing of both was only modestly increased over that observed during spontaneous apoptosis, with little or no detectable cleavage of caspase-9. Cross-linking of the FasR with monoclonal antibody has been shown by others to reduce eosinophil viability due to induction of apoptosis, by a mechanism seemingly independent of IL-5 (Matsumoto *et al.* 1995; Hebestreit *et al.*, 1998). The data presented here indicates that IL-5 does inhibit, although not completely, Fas-induced processing of caspase-3 and –8, and supports the findings of Zangrilli *et al* (2000), who reported partial inhibition of Fas-induced cleavage of caspase-3 and –8 by IL-5 in eosinophils. The high levels of spontaneous eosinophil apoptosis coupled with the fairly modest, although consistent, effect of FasR ligation made it difficult to discriminate between the two simultaneous pathways of apoptosis.

It should be noted that co-treatment with IL-5 failed to completely block Fas-induced apoptosis, and therefore IL-5 may simply be protecting those cells destined to die due to growth factor deprivation, having little or no effect on eosinophils responding to FasR ligation. Fas-induced eosinophil apoptosis was also associated with cleavage of the caspase-8 substrate, Bid, although not to the degree observed in STS-treated cells, implicating a potential link of death-receptor-induced apoptosis and mitochondria. Peripheral blood eosinophils are heterogeneous in terms of their rate of constitutive apoptosis (Fig. 3.7), therefore delineating the caspase cascade in response to Fas treatment is difficult. The evidence supports a potentially caspase-8-mediated cascade downstream of FasR ligation in eosinophils, which induces cleavage of Bid, subsequent perturbation of the mitchondrial membrane and activation of the mitochondria-dependent pathways. Bid facilitates the release of cytochrome *c* by inducing as conformational change and insertion of Bax into the mitochondria (Korsmeyer *et al.*, 2000).

The Bcl-2 homologues are proposed to determine susceptibility to apoptotic stimuli by modulating the permeability of the outer mitochondrial membrane, thereby regulating the efflux of cytochrome *c*. The potential role of Bax in eosinophil apoptosis was investigated, as Bax translocation to the mitochondrial membrane facilitates the release of cytochrome *c* in many models of apoptosis (Rossé *et al.*, 1998; Jurgensmeier *et al.*, 2000; Korsmeyer *et al.*, 2000).

Chapter 6

Subcellular distribution of Bax during eosinophil apoptosis

6.1. Introduction

The mechanism by which cytochrome *c* translocates the outer mitochondrial membrane is at present unclear. It is proposed that Bcl-2 homologues regulate the permeability of the outer mitochondrial membrane, and consequently mediate the release of apoptogenic factors from the mitchondrial intermembrane space. Overexpression of Bax induces cytochrome *c* release from mitochondria in the absence of an apoptotic stimulus, and addition of Bax to isolated mitochondria also results in the release of cytochrome *c* (Eskes *et al.*, 1998; Antonsson *et al.*, 2000). Bcl-2 is considered an integral membrane protein in contrast to a number of pro-apoptotic proteins such as Bax and Bid that undergo translocation from the cytosol to the mitochondria during apoptosis. Bax resides in the cytoplasm of non-apoptotic cells, in a soluble, monomeric form, with N and C termini that interact within a hydrophobic groove, proposed to mediate heterodimer formation. Upon reception of a death stimulus, Bax undergoes a conformational change involving exposure of its helix α9 hydrophobic C-terminus, and exposure of its BH3 binding domain, facilitating membrane insertion and oligomerisation respectively (Suzuki *et al.*, 2000). Deletion of the amino terminus from monomeric Bax allows C-terminus-dependent insertion into the mitochondrial membrane, providing evidence that the N and C termini interact in its soluble, monomeric form (Goping *et al.*, 1998). Exposure of the N-terminus also unmasks the epitope recognised by the monoclonal antibody, 6A7, thereby allowing specific detection of the conformationally active form of Bax (Nechushtan *et al.*, 1999). Hsu and Youle (1997) have shown that the quaternary structure of monomeric Bax is detergent-sensitive, with non-ionic detergents such as Triton X-100 and NP-40 capable of inducing the apoptosis-associated conformational change, and consequently, dimerisation of Bax.

The mechanism responsible for the induction of Bax activation is unclear. Elucidation of the structure of Bax indicated that the interaction of C-terminus and BH3 domain-binding pocket was unlikely to be disrupted simply by competition from the BH3 domain of another Bcl-2 protein (Suzuki *et al.*, 2000). Instead, the authors suggest that an energy-dependent step is requisite for dissociation of the C-terminus and subsequent dimerisation via the BH3 binding domain. Alteration in pHi has been reported during the apoptosis of a number of cell types, with acidification of the cytosol during the execution phase, in response to increased lactate production as ATP is generated by glycolysis following dysfunction of mitchondrial respiration (Li and Eastman, 1995), possibly preceded by a cytosolic alkalinisation phase (Dai *et al.*, 1998; Tsao and Lei, 1996). Interestingly, alteration of Bax conformational state has been observed in response to intracellular alkalinisation during growth factor withdrawal in an

IL-7-dependent cell line (Khaled *et al.*, 1999). However, Suzuki *et al* (2000) reported no direct association between Bax conformation and pH. Enforced dimerisation of a chimeric Bax molecule with a bivalent ligand was sufficient to induce translocation, dissipation of mitchondrial membrane potential and ultimately apoptosis in an IL-3 dependent hematopoietic cell line (Gross *et al.*, 1998). Although in this system there was no detectable efflux of cytochrome *c*, it supports an important role for Bax translocation in the induction of apoptosis.

Numerous models have been proposed for the Bax-mediated induction of cytochrome *c* release following its translocation to mitochondria. The majority of Bcl-2-related proteins have the ability to target intracellular membranes via a 21 residue C-terminal trans-membrane region. Bcl-x_L consists of two central hydrophobic a helices surrounded by five amphipathic helices, which were observed to be similar to the pore forming bacterial colicins and diphtheria toxin (Muchmore *et al.*, 1996). Similar pore-forming structures can be predicted for both Bcl-2 and Bax, due to their significant sequence homology. Interestingly, although the pro-apoptotic protein Bid only shares sequence similarity within the 16 amino acid BH3 domain, its structure is similar to that of Bcl-x_L (Schendel *et al.*, 1999). These Bcl-2 homologues have since been shown to form channels in lipid vesicles with distinct multiple conductance states, pH sensitivity and low ion selectivity (Schlesinger *et al.*, 1997). The three-dimensional structure of Bcl-2 homologues reveals only the two central hydrophobic helices are of sufficient length to span a membrane bilayer, and as such, in monomeric form is incapable of forming an aqueous channel. Indeed monomeric Bax cannot form pores within lipid bilayers or induce the release of cytochrome *c* from mitochondria. However, the oligomerisation of Bax into 96 and 260 kDa complexes, within the outer mitochondrial membrane and induction of cytochrome *c* efflux in cells induced to undergo apoptosis by STS, UV irradiation or ectopic expression of tBid has been described (Antonsson *et al.* 2001). The lumen diameter of a Bax tetramer is sufficient to allow passage of 14 kDa cytochrome *c* (Saito *et al.*, 2000), therefore Bax oligomers may constitute the structural entirety of a cytochrome *c*-conducting channel, or the channel may involve as yet undetermined proteins. Oligomerisation of Bax was also inhibited by overexpression of Bcl-2 (Mikhailov *et al.*, 2001), and therefore provides a potential mechanism for Bcl-2 protein regulation of trans-membrane cytochrome *c* redistribution.

Yeast-two hybrid studies and immunoprecipitation analysis have revealed an association of Bax with components of the PTP, the outer mitochondrial membrane protein, VDAC

(Shimizu *et al.*, 1999), and the inner mitochondrial membrane protein, ANT (Marzo *et al.*, 1998a). An unselective channel that spans the outer and inner mitochondrial membranes, the opening of the PTP results in an increase in the permeability of the inner mitochondrial membrane, rapid depolarisation of the $\Delta\Psi m$, osmotic swelling of the high solute matrix and eventual rupture of the outer mitochondrial membrane. Both Bax and Bak have been shown to induce PTP opening and cytochrome *c* conductance (Narita *et al.*, 1998). In contrast, Bcl-2 and Bcl-x_L inhibit PTP opening in isolated mitochondria and PTPs reconstituted in pure liposomes (Marzo *et al.*, 1998b). Therefore, Bcl-2 homologues may regulate cytochrome *c* release by altering the conductance state of the PTP, and initiating the mitochondrial permeability transition. Contrastingly, there are a number of examples of apoptogenic cytochrome *c* release prior to loss of $\Delta\Psi m$, which were not prevented by the PTP inhibitors, bongkrekic acid (antagonising ligand of ANT) and cyclosporin A (ligand of cyclophilin D) (Bossy-Wetzel *et al.*, 1998; Eskes *et al.*, 1998). Also, no association of either VDAC or ANT was observed with the recently reported high molecular weight Bax oligomers within the mitochondrial membrane of an apoptotic cell (Antonsson *et al.* 2001), and therefore throws into question the role of the PTP in cytochrome *c* efflux.

Alternatively, both Bax and the truncated form of Bid (tBid) have been shown to destabilise phospholipid bilayers (Basanez *et al.*, 1999), leading to the suggestion that pro-apoptotic Bcl-2 homologues facilitate the formation of lipidic pores within the outer mitochondrial membrane sufficient to allow release of intermembrane proteins.

Peripheral blood eosinophils constitutively expressed relatively high levels of Bax, the expression of which was not markedly altered by stimulation with IL-5. The lack of clear evidence supporting modulation of Bcl-2 homologue expression as a major factor governing the pro-survival effects of IL-5, possibly implicates a role for post-translational regulation. During spontaneous apoptosis, cytochrome *c* is released into the cytosol, and caspases are activated downstream of perturbation of the mitchondrial membrane. The role of Bax activation and translocation during eosinophil apoptosis, and potential modulation by IL-5 was investigated.

6.2. Cytosol to mitochondria translocation of Bax during eosinophil apoptosis

6.2.1. Bax translocation during spontaneous eosinophil apoptosis

Spontaneous eosinophil apoptosis was associated with a caspase-independent redistribution of Bax from the cytosol to the mitochondria, which was inhibited by IL-5. Anti-Bax 6A7 detected diffuse cytosolic and nuclear Bax distribution in the majority of freshly isolated eosinophils (Fig. 6.1), indicating constitutive expression of the conformationally altered form of Bax. Interestingly, Bax expression was observed to localise predominantly to the nucleus compared with the cytosol in freshly isolated eosinophils (Fig. 6.1A). Isotype-matched negative control showed essentially no evidence of non-specific staining (Fig. 6.1B). Punctate Bax distribution that colocalised with the mitochondria was observed in eosinophils deprived of IL-5 for 6 h (Fig. 6.1C). This punctate distribution was commonly perinuclear and consistently associated with cells exhibiting characteristic apoptotic morphology of condensed nucleus and cytoplasm. Stimulation of eosinophils with IL-5 for 6 h maintained the cytosolic and nuclear distribution observed in the freshly isolated eosinophils (Fig. 6.1D), and prevented Bax translocation to the mitochondria observed during spontaneous apoptosis after 24 and 48 h (Fig. 6.2A). In contrast, although zVAD.fmk inhibited spontaneous apoptosis assessed by PtdSer externalisation, it failed to prevent Bax translocation to the mitochondria, indeed zVAD.fmk seemingly increased the percentage of eosinophils exhibiting punctate Bax distribution compared with untreated cells (Fig. 6.2A). Treatment of eosinophils with STS for 2 h also resulted in the marked appearance of punctate Bax distribution (Fig. 6.1E), and similarly to cytochrome *c* release, nearly 100% of eosinophils exhibited translocated Bax after 6 h (Fig. 6.2B). STS-induced Bax redistribution was not inhibited by zVAD.fmk (Fig. 6.2B).

6.2.2. Fas-induced Bax translocation is caspase-dependent

Treatment with monoclonal antibody targeting Fas (CD95/ Apo-1) accelerated spontaneous eosinophil apoptosis as assessed by Annexin V binding, which was incompletely inhibited by co-incubation with IL-5 (Fig. 3.13). Fas-induced eosinophil apoptosis was associated with translocation of Bax to the mitochondria (Fig. 6.3A and B), indicating that such an 'extrinsic' stimuli initiates apoptosis via mitochondrial perturbation in eosinophils. In contrast with spontaneous apoptosis, co-incubation with zVAD.fmk modestly, but significantly, reduced the percentage of cells exhibiting evidence of punctate Bax distribution following Fas-induced apoptosis (32.1% ± 3.7 versus 42.1% ± 4.1; Fig. 6.3). IL-5 stimulation inhibited Fas-induced Bax translocation, but similarly to PtdSer externalisation, the inhibition was not complete (Fig. 6.3A and B).

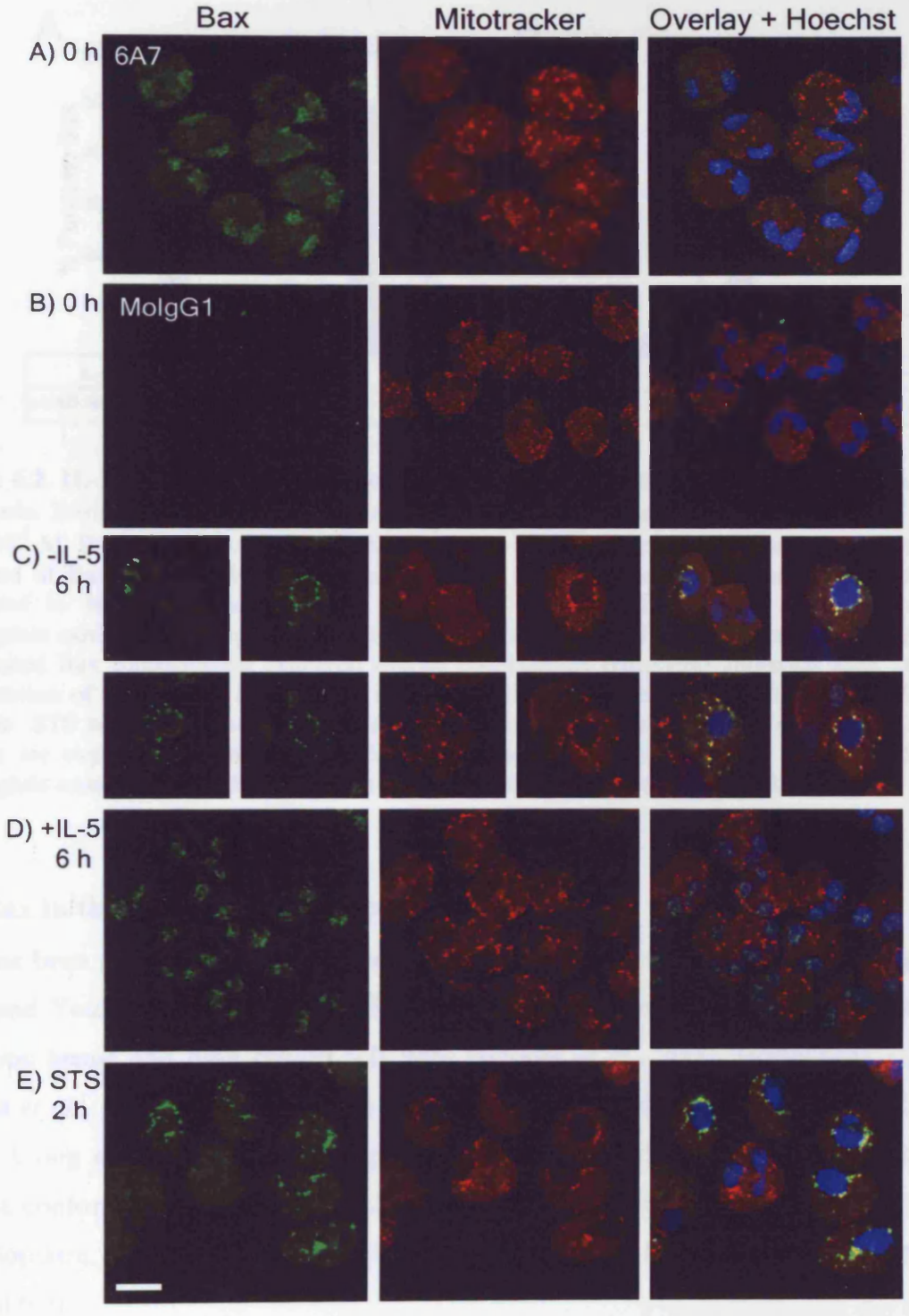

Figure 6.1. Bax translocates to the mitochondria during spontaneous and STS-induced eosinophil apoptosis. Immunohistochemistry was performed, as described in *Materials and Methods,* on fixed cytospins of freshly isolated eosinophils (A and B) or eosinophils cultured in the absence (C) or presence of IL-5 (10^{-10} M) for 6 h (D) or STS (10^{-5} M) for 2 h (E). Images were captured by confocal microscopy at x100 magnification under oil immersion. Eosinophils were stained with monoclonal anti-Bax 6A7 (A, C-E) or isotype-matched control (B), and the mitochondria and nuclei stained with Mitotracker CMXRos and Hoechst 33258 respectively (A-E). Co-localisation (*yellow*) of Bax (*green*) and mitochondria (*red*) was observed in single sections. Culturing in the absence of IL-5 for 6h and treatment with STS for 2 h induced Bax translocation to the mitochondria. Bar represents 10 μM. Results are representative of experiments performed on four separate donors.

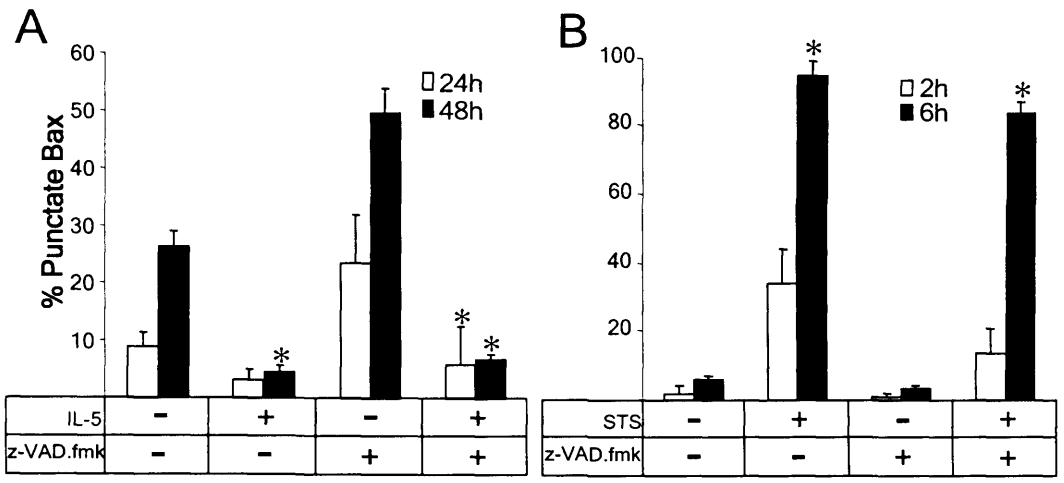

Figure 6.2. IL-5 prevents Bax translocation to the mitochondria during spontaneous eosinophil apoptosis. Eosinophils were co-incubated in the presence or absence of either IL-5 (10^{-10} M, A) or STS (10^{-5} M, B) and zVAD.fmk (100 μM, A and B) for the indicated times and the cells assessed for evidence of Bax translocation to the mitochondria. Bax was detected by immunocytochemistry as described in *Materials and Methods,* using 6A7 monoclonal antibody, and the percentage of eosinophils exhibiting aggregated Bax distribution was quantified under fluorescence microscopy. IL-5 inhibited Bax translocation observed during spontaneous eosinophil apoptosis after 24 and 48 h. Quantitation of subcellular localisation in eosinophils exposed to STS for longer than 6 h was not possible. STS rapidly induced Bax redistribution after 2 h, which was not inhibited by zVAD.fmk. Results are expressed as the mean ± SEM of three separate experiments. * IL-5 or STS treated eosinophils compared with the relevant untreated or zVAD.fmk treated control, $P<0.05$.

6.3. Bax in the nucleus of eosinophils

Bax has been previously shown to be a cytosolic, monomeric protein in non-apoptotic cells (Hsu and Youle, 1998). However, Bax has been shown to localise to the nucleus in non-apoptotic breast and lung cancer cell lines (Bedner *et al.*, 2000; Hoetelmans *et al.*, 2000; Nishita *et al.*, 1998), and also during the apoptosis of two melanoma cell lines (Raffo *et al.*, 2000). Using anti-Bax 6A7, which specifically recognises the conformationally active form, and the conformation-insensitive 2D2 monoclonal Bax antibodies, Bax was observed within the cytoplasm, although predominated within the nucleus of freshly isolated eosinophils (Figs. 6.1 and 6.3).

To further investigate the subcellular localisation of Bax in freshly isolated and apoptotic eosinophils, immunogold labelling with Bax 2D2 and transmission electron microscopy were performed. Bax was predominantly detected within the nucleus, although labelling was also observed within the cytosol (Fig. 6.4A-D). Bax in the cytoplasm of non-apoptotic eosinophils was not observed to definitely associate with a specific organelle, with no Bax associated with the mitochondria (Fig. 6.4A, *arrow*). Very little Bax was detected localising with the highly basic granule proteins (Fig. 6.4E, *arrowheads*). Isotype-matched mouse control antibody

exhibited essentially no non-specific staining (Fig. 6.4F). Attempts to detect Bax with mAb 6A7 under EM proved unsuccessful. Analysis by EM failed to elucidate specific nuclear structures with which Bax was associated, although Bax did not appear to associate with heterochromatin. Similarly, Bax exhibited a diffuse distribution in the cytoplasm under EM, with no obvious localisation to specific organelles or cytosolic structures. Therefore, in non-apoptotic peripheral blood eosinophils, Bax is observed diffusely within the cytoplasm, but predominates within the nucleus by immunocytochemistry and EM.

Bim_L and Bim_{EL} are proposed to interact with the dynein light chain of the microtubule system and translocate to the mitochondria during apoptosis. However, immunocytochemistry detected Bim within the nucleus of peripheral blood eosinophils, and no obvious redistribution was observed during spontaneous or STS-induced apoptosis (Fig. 6.5). Interestingly, the nuclear distribution exhibited by Bim was similar to that observed for Bax in freshly isolated eosinophils (Figs. 6.1 and 6.5). Therefore eosinophil cytospins were double stained with monoclonal Bax and polyclonal Bim antibodies. Co-localisation of Bax (*green*) and Bim (*red*) was observed in single, independently captured, sections (Fig. 6.5). Cells were incubated without Mitotracker, with the high cytoplasmic background observed in the red channel (Bim and RabIg control, Fig. 6.5B) due to the fluorescence of Chromotrope 2R. Therefore immunocytochemistry indicated colocalisation of Bax and Bim within the eosinophil nucleus. Attempts to determine potential association and interaction of these Bcl-2 homologues by co-immunoprecipitation analysis proved unsuccessful.

Nuclear Bax expression was detected with monoclonal antibodies 2D2 and 6A7 that target a similar region of the Bax N-terminus (amino acid residues 3-16 and 12-24 respectively). Further confocal analysis was performed with various antibodies directed against different domains of Bax in order to rule out the possibility of cross-reactivity with an unrelated nuclear antigen (Fig. 6.6). Two mouse monoclonal (2D2 and 6A7) and two rabbit polyclonal antibodies (N-20 and 43-61) clearly detected Bax predominantly within the nucleus of eosinophils, despite having different epitope specificities. However, monoclonal anti-Bax Clone 3, failed to detect nuclear Bax despite retaining affinity for the aggregated form of Bax present in an apoptotic eosinophil (Fig. 6.6), possibly suggesting the nuclear antigenicity was independent of Bax. However, this does not exclude the possibility that Clone 3 is conformation sensitive, and so immunoreacts with the active mitochondria-associated form, whereas the nuclear conformation lacks antigenicity.

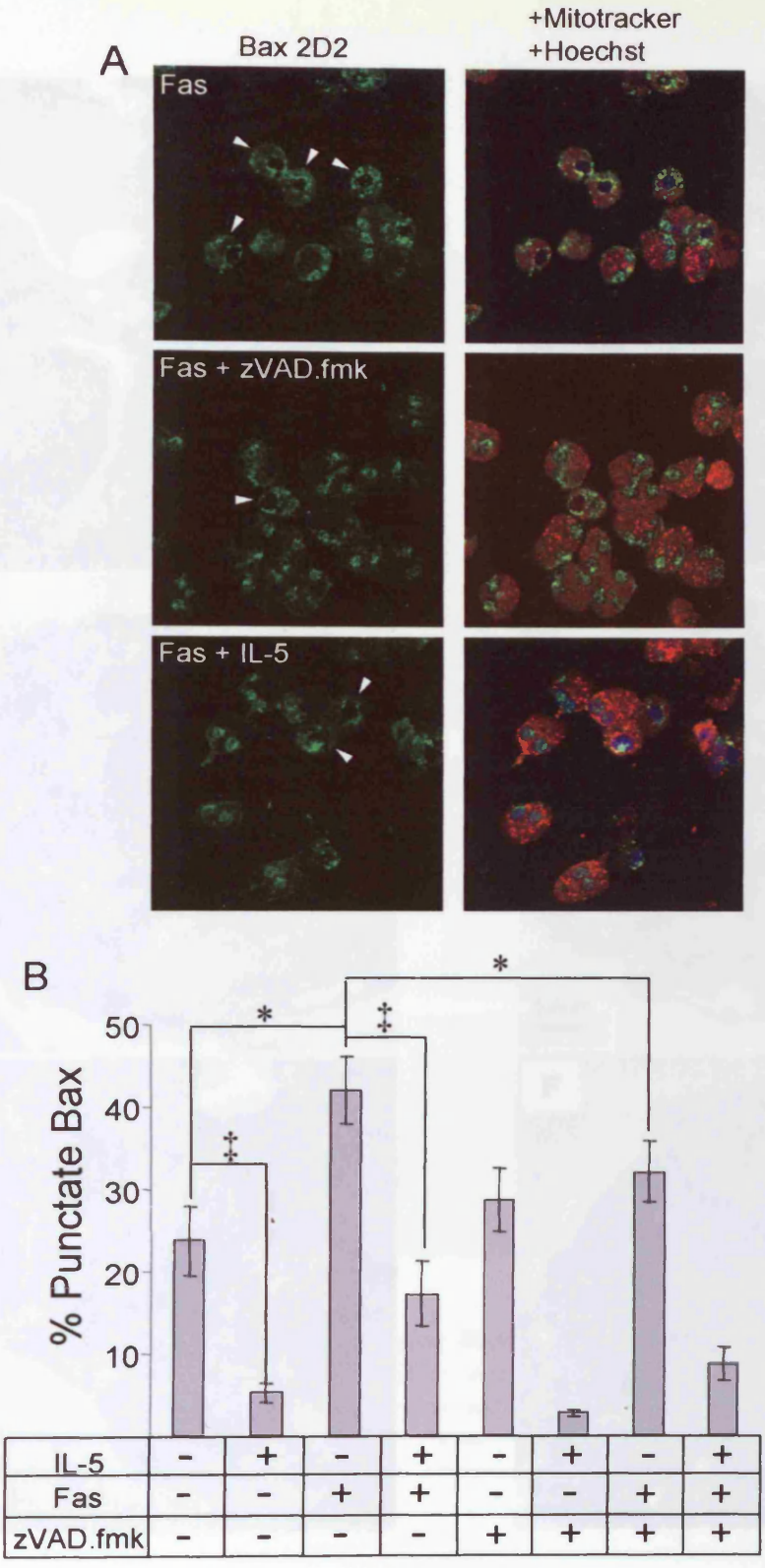

Figure 6.3. Ligation of FasR induces translocation of Bax during eosinophil apoptosis. Eosinophils were cultured in the presence or absence of IL-5 (10^{-10} M), anti-Fas mAb (CH11, 500 ng/ml) and zVAD.fmk (100 μM) for 24 h. A) Eosinophils treated with anti-Fas mAb and stained with Bax 2D2, and mitochondria and nuclei counterstained with Mitotracker and Hoechst respectively. Eosinophils exhibiting evidence of punctate Bax distribution are indicated (*arrowhead*). Images are representative of three independent experiments. B) Quantitation of Bax translocation was performed on immunostained cytospins using anti-Bax mAb 2D2 as described in *Materials and Methods* and is presented as mean ± SEM of n=3; *P<0.05, ‡P<0.01.

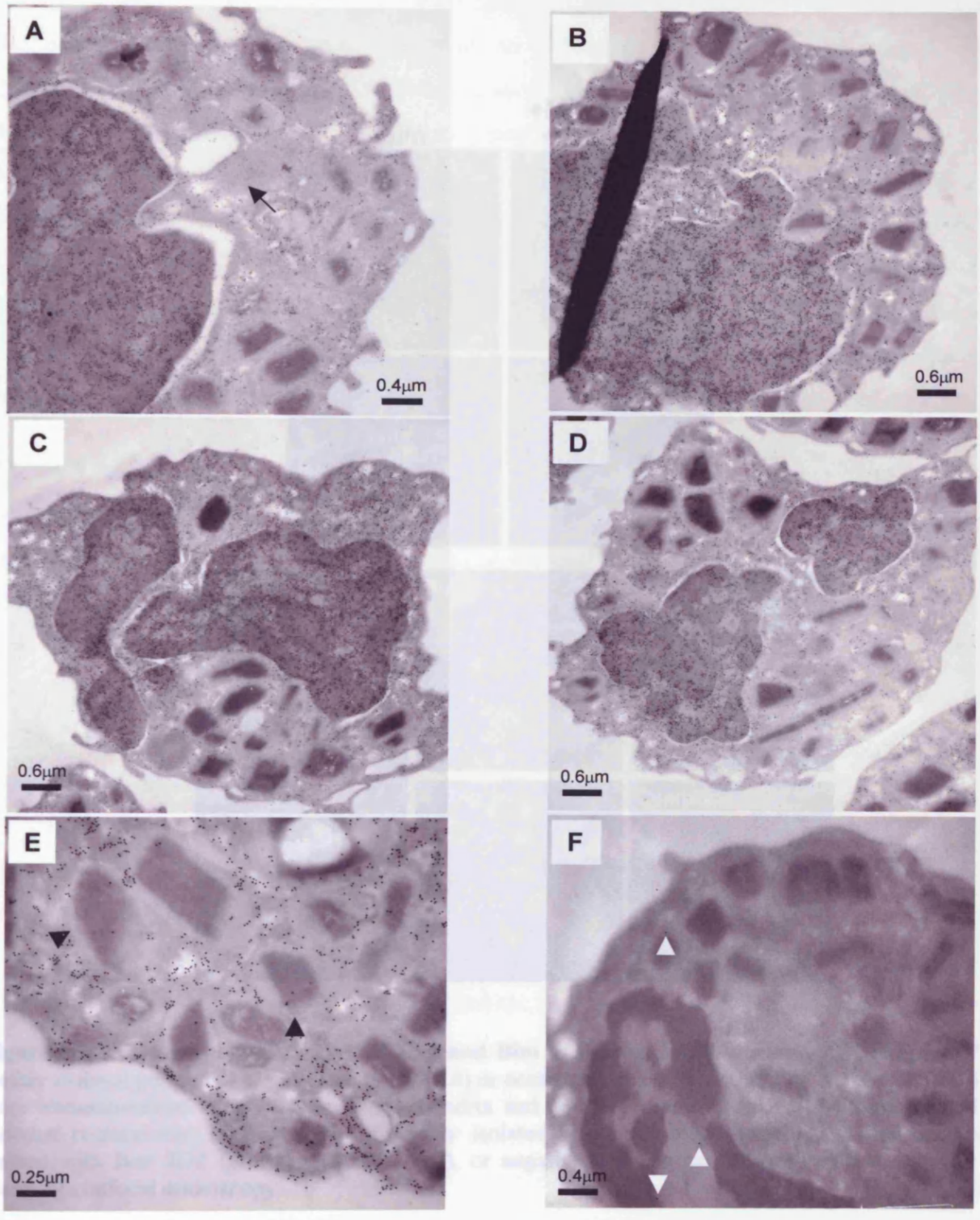

Figure 6.4. Electronmicrographs of immunogold labelled Bax in eosinophils. Peripheral blood eosinophils were prepared for immunogold labelling as described in *Materials and Methods*, stained with monoclonal antibody against Bax (2D2, A-E) or isotype matched IgG control (F) and transmission electron microscopy performed. Immunogold labelled Bax reveals predominant expression in the nucleus, but also detectable within the cytoplasm of non-apoptotic eosinophils (A-E). Within the cytoplasm, little immunogold labelling of the basic granules was observed (E). A mitochondrion (A, *arrow*) and cytoplasmic granules (E, *black arrowhead*) are indicated. Virtually no non-specific binding of isotype matched negative control was observed (F, *white arrowhead*).

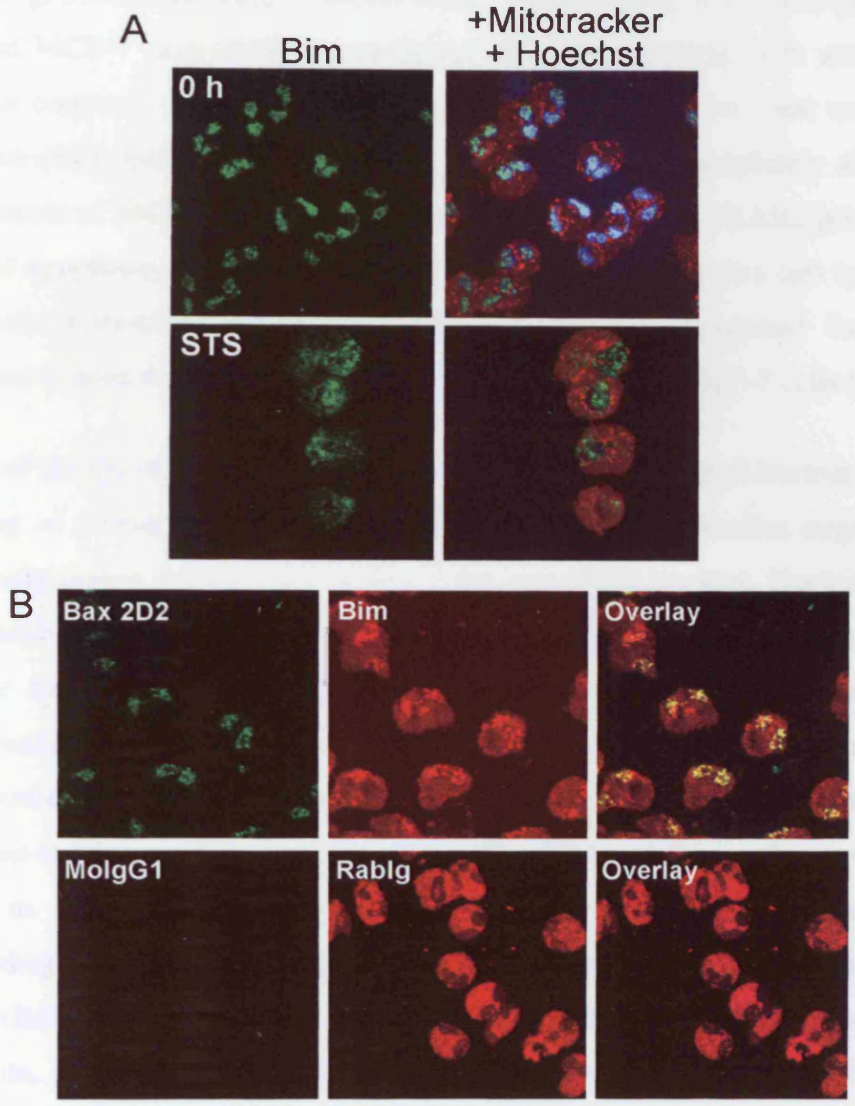

Figure 6.5. Potential co-localisation of Bax and Bim in the eosinophil nucleus. A) Cytospins of freshly isolated peripheral blood eosinophils (*0 h*) or eosinophil treated with STS (10^{-5}M) for 6 h (*STS*) were immunostained with Bim, and mitochondria and nuclei counterstained with Mitotracker and Hoechst respectively. B) Cytospins of freshly isolated peripheral blood eosinophils were double stained with Bax 2D2 (*green*) and Bim (*red*), or negative controls and images captured by laser scanning confocal microscopy.

To establish whether such a Bax distribution was specific to eosinophils, immunocytochemistry was also performed on peripheral blood neutrophils (Fig. 6.7B), lymphocytes (Fig. 6.7C), and MCF-7 breast cancer cell line (Fig. 6.8). Both peripheral blood neutrophils and MCF-7 cells exhibited evidence of nuclear staining with antibody directed against Bax. In contrast, peripheral blood lymphocytes exhibited no clear nuclear staining. Similar to eosinophils, nuclear Bax staining of MCF-7 cells was completely absent with Bax Clone 3. Apoptosis of MCF-7 cells was induced by treatment with TRAIL, previously shown to induce rapid apoptosis and perturbations of the mitochondria in this cell type (Roberts *et al.*, 2001). Both monoclonal antibodies 2D2 and Clone 3 recognised the translocated, mitochondria-associated form of Bax in TRAIL treated apoptotic MCF-7 cells (Fig. 6.8).

The exclusion of isotype-matched negative control from the eosinophil nucleus ruled out non-specific binding of primary or secondary antibody. However, antibodies targeting Bax may still be cross-hybridising with a component of the eosinophil nucleus. Nuclear staining was absent in eosinophils exhibiting evidence of apoptotic morphology .i.e. nuclear condensation, with detectable staining of mitochondria-associated Bax, providing further support for the specificity of nuclear Bax staining. However, the nucleus is a major site of proteolytic activity and translocation during apoptosis, and therefore it does not exclude the possibility that the potentially cross-reacting nuclear protein is translocated from the nucleus or is processed, thereby losing its antigenicity, in an apoptotic cell. Immunocytochemistry was performed on eosinophils undergoing spontaneous apoptosis after 24 h, with Bax 6A7 previously incubated with specific (Bax N-terminal amino acids 12-24; immunogen for 6A7) or non-specific blocking peptide. Bax 6A7 or antibody pre-incubated with a non-specific blocking peptide similarly recognised structures within the nucleus, and punctate mitochondrially-targeted Bax in non-apoptotic and apoptotic eosinophils respectively (Fig. 6.9A and C). In contrast, Bax 6A7 pre-incubated with amino acids 12-24 of Bax failed to detect Bax associated with mitochondria in eosinophils exhibiting apoptotic nuclear morphology, whereas the nuclear staining was retained (Fig. 6.9B). This suggests that, in the case of Bax 6A7, nuclear staining is due to cross-reaction with an alternative nuclear antigen, independent of the N-terminal epitope of Bax.

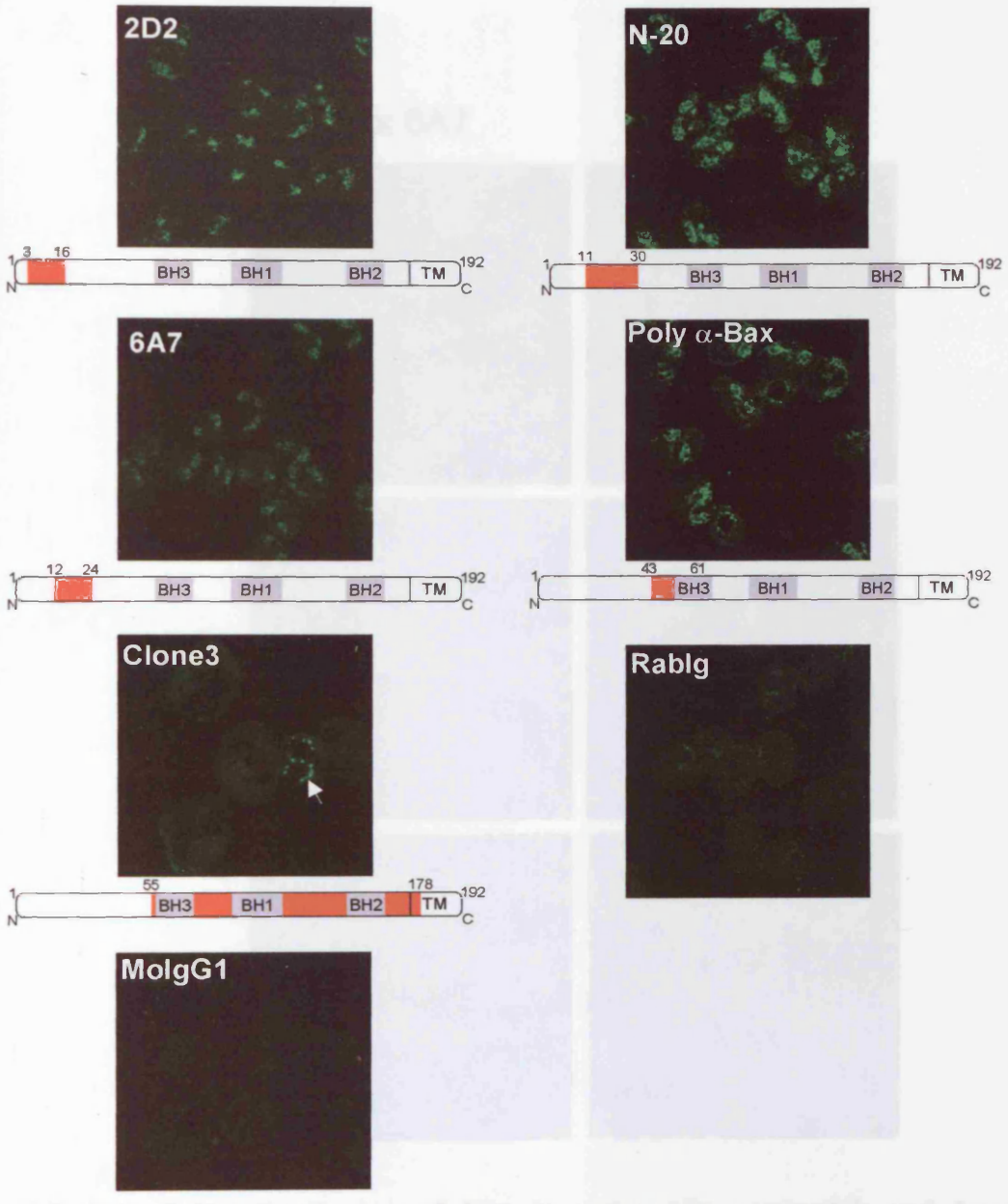

Figure 6.6. Immunocytochemistry of eosinophils with different Bax antibodies. Cytospins of peripheral blood eosinophils were generated and stained according to *Materials and Methods* with antibodies targetting different epitopes of Bax or rabbit and mouse negative control. Epitope specificity of each antibody is indicated. Arrow indicates an apoptotic eosinophil with evidence of aggregated Bax distribution. Results are representative of at least three independent experiments.

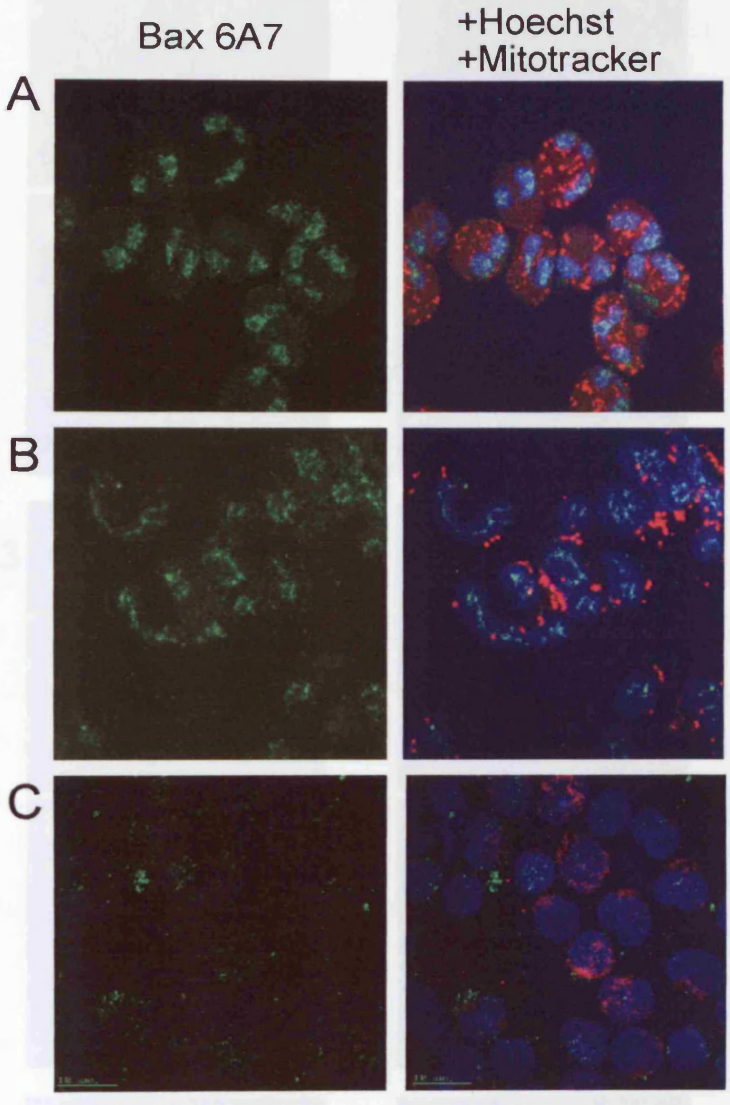

Figure 6.7. Subcellular distribution of Bax in eosinophils, neutrophils and lymphocytes. Cytospins of freshly isolated peripheral blood eosinophils (A), neutrophils (B) and lymphocytes (C) were stained with Bax 6A7 monoclonal antibody as described in *Materials and Methods*. Mitochondria and nuclei were counter-stained with Mitotracker and Hoeschst respectively and images were independently captured by laser scanning microscopy. Representative of at least two separate experiments.

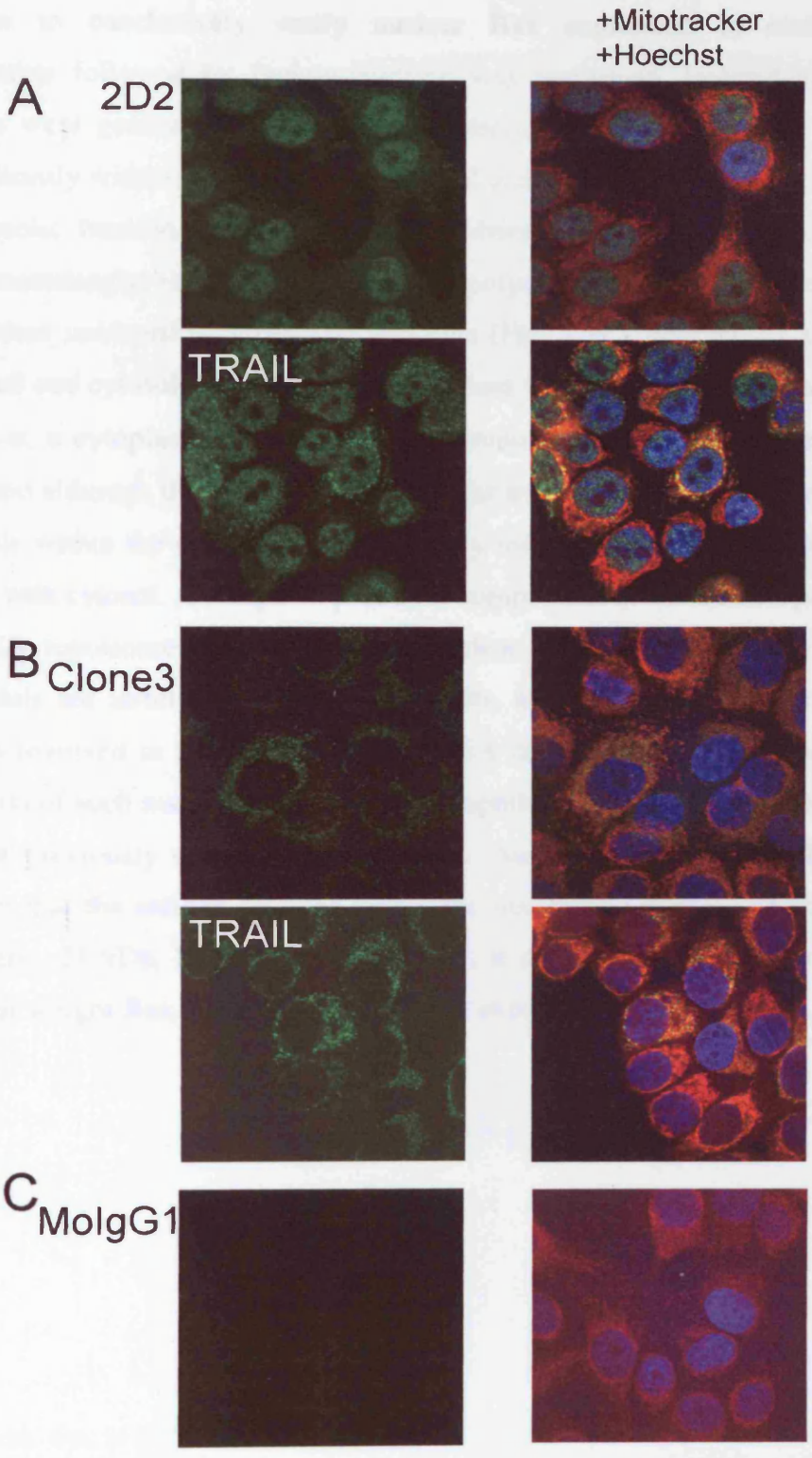

Figure 6.8. Bax expression in MCF-7 cells. MCF-7 cells were grown on slides to 75% confluency and incubated in the presence or absence of TRAIL to induce apoptosis for 4 h. Cells were stained with either Bax 2D2 mAb (A), Bax Clone 3 mAb (B) or an isotype-matched negative control (C) according to *Materials and Methods* and images captured by confocal laser scanning microscopy. Results are representative of three separate experiments.

In order to conclusively verify nuclear Bax expression in eosinophils, subcellular fractionation followed by immunoblotting was performed. Isolated nuclear and cytosolic fractions were generated and probed with monoclonal antibody 2D2, which detected Bax predominantly within the nucleus by confocal analysis and EM. 21 kDa Bax was restricted to the cytosolic fraction and was completely absent from the enriched nuclear fraction (Fig. 6.10). Interestingly, reprobing the blot with polyclonal N-20, which detects 21 kDa Bax and an abundant unidentified protein of ~40 kDa (Fig. 4.3C), detected 21 kDa Bax restricted to whole cell and cytosol, whereas the 40 kDa band was predominant within the nuclear fraction. Tubulin-α, a cytoplasmic component was immunoblotted as a positive control for fraction purity, and although the 60 kDa protein was far more abundant within the cytosol, it remained detectable within the nuclear fraction, thereby indicating minor contamination of the nuclear fraction with cytosol. Attempts to probe for components of the DNA repair machinery, PARP and DNA topoisomerase II, to verify nuclear fractionation integrity were unsuccessful. Eosinophils are terminally differentiated cells, and as such their proposed requirement for enzymes involved in the maintenance of DNA during cell cycling is reduced. Therefore the expression of such nuclear enzymes in eosinophils would be predicted to be low or absent, as has been previously reported in neutrophils (Sanghavi *et al.*, 1998). The data support the argument that the antigen detected within the nucleus by immunocytochemistry was not the monomeric, 21 kDa, form of Bax. However, it does not exclude the possibility of a higher molecular weight Bax variant, predominantly expressed within the nuclei of eosinophils.

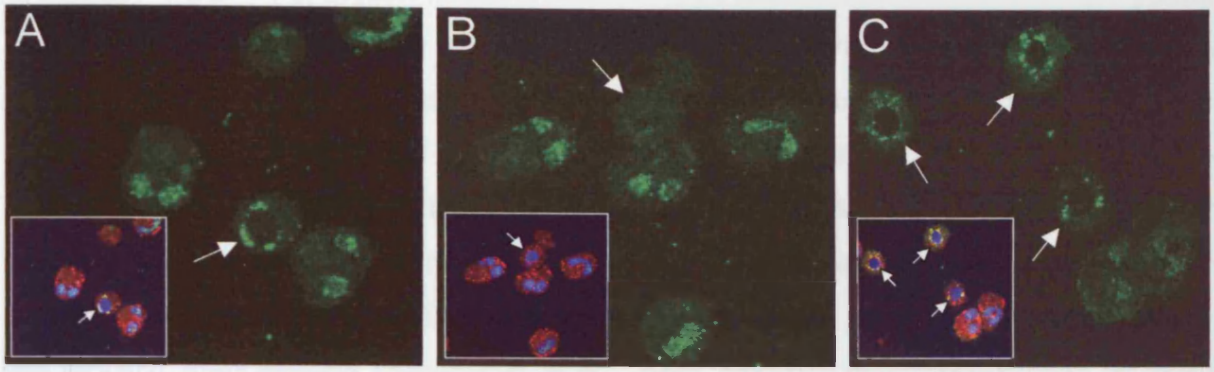

Figure 6.9. Detection of nuclear and mitochondria-associated targets with Bax 6A7 monoclonal antibody are independent. Monoclonal Bax 6A7 was pre-incubated in the absence (A) or presence of specific (B) or non-specific (C) blocking peptide and immunocytochemistry was performed as previously described on eosinophils cultured in the absence of cytokine for 24 h. Inlays are overlaid images of Hoechst and Mitotracker. Cells with evidence of apoptotic nuclear morphology are indicated (*arrows*).

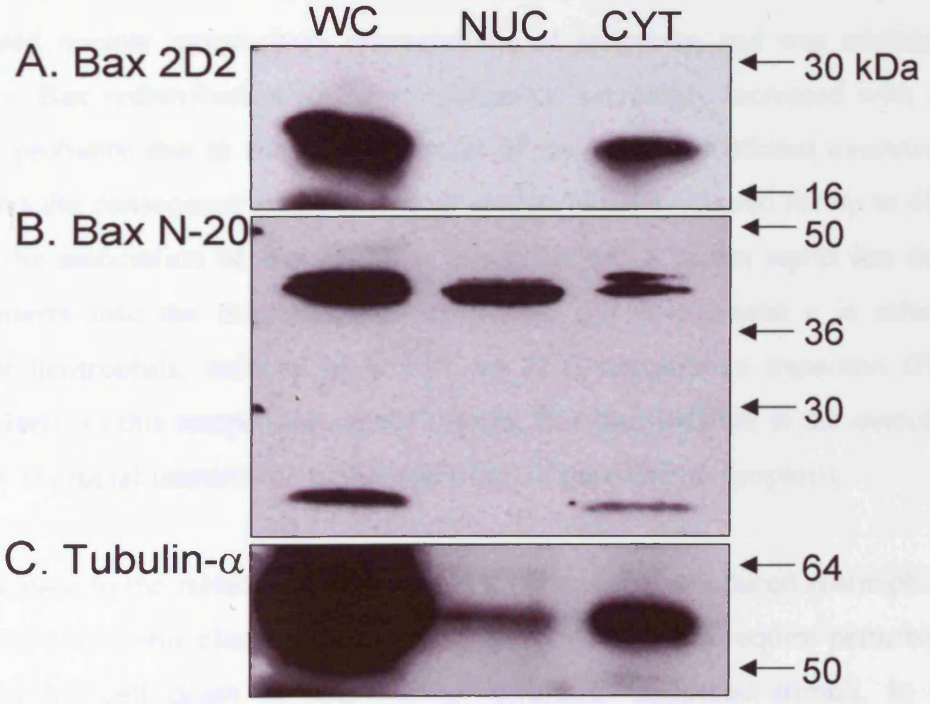

Figure 6.10. Bax is undetectable in the nuclear fraction of eosinophils. Whole cell (WC), nuclear (NUC), and cytosolic (CYT) subcellular fractions of freshly isolated peripheral blood eosinophils were generated as described in *Materials and Methods*, and immunoblotting was initially performed with anti-Bax 2D2 (A), and blots were subsequently stripped and reprobed with anti-Bax N-20 (B) and anti-tubulin-α (C) as a control for fraction purity.

6.4. Discussion

In non-apoptotic cells, Bax is a soluble, cytosolic monomer, with an N terminus proposed to mask its hydrophobic C terminus. During the apoptosis of murine thymocytes, COS-7, and murine fibrosarcoma cell lines, it has been shown that a conformational change exposes the BH3 domain and C terminus, facilitating oligomerisation of Bax and translocation from the cytosol to the mitochondrial membrane respectively (Wolter *et al.*, 1997). Bax oligomers have channel-forming activity, and trigger the release of cytochrome *c* from human melanoma cells and isolated mitochondria (Antonsson *et al.*, 2000; Eskes *et al.*, 1998). Human peripheral blood eosinophils express high levels of Bax, and the data demonstrates that freshly isolated eosinophils constitutively express the conformationally altered form of Bax, detected by the monoclonal antibody 6A7 that specifically recognises an N-terminal epitope inaccessible in soluble, cytosolic Bax (Nechushtan *et al.*, 1999). During both spontaneous and staurosporine-induced apoptosis, Bax underwent a caspase-independent translocation to the mitochondria in human eosinophils. This colocalisation was consistently observed in eosinophils exhibiting the condensed nuclear morphology characteristic of apoptosis, and was inhibited by IL-5. Interestingly, Bax redistribution to the mitochondria seemingly increased with zVAD.fmk, (Fig. 6.2A) probably due to the delayed onset of the caspase-mediated execution phase of apoptosis and the consequent proteolytic degradation of cells cultured for up to 48 h, thereby prolonging the association of Bax with the mitochondria. A recent report has demonstrated that Bax inserts into the mitochondrial membrane and cytochrome *c* is released during apoptosis of neutrophils, induced by a 15°C to 37°C temperature transition (Pryde *et al.*, 2000). Similarly, in this temperature arrest system, Bax translocation to the mitochondria was shown to be of crucial importance in the execution of granulocyte apoptosis.

Bax translocation to the mitochondria was also a feature of Fas-induced eosinophil apoptosis. This supports eosinophil classification as a Type II cell, which require perturbation of the mitochondria for cell death in response to 'extrinsic' apoptotic stimuli. In contrast to spontaneous eosinophil apoptosis, where Bax translocation markedly increased after inhibition of caspase activity, Fas-induced Bax translocation was inhibited by treatment with the broad range caspase-inhibitor zVAD.fmk. This indicated that the redistribution of Bax in response to Fas was caspase-dependent, and implicates stimulus-dependent divergent pathways of eosinophil apoptosis, with spontaneous apoptosis and Fas-induced apoptosis involving post and pre-mitochondrial caspase activation respectively. However, convergence of the two pathways was observed at the mitochondria, indicating that although initiation of cell death is distinct, the execution mechanism is common and independent of apoptotic

stimulus. IL-5 significantly reduced the Bax translocation observed during both spontaneous and Fas-induced apoptosis, possibly indicating convergence of growth factor deprivation and receptor-mediated apoptosis in eosinophils. However, potent pro-survival concentrations of IL-5 failed to completely inhibit the relatively modest apoptotic effect of FasR ligation on both PtdSer exposure, caspase activation and Bax translocation. Therefore the data supports the hypothesis that IL-5 inhibited the spontaneous apoptotic events initiated by growth factor deprivation, with little or no effect on Fas-induced apoptosis. This would indicate that IL-5 does not inhibit the conformational change and subsequent mitochondrial targeting of Bax directly, but effects upstream events which culminate in Bax 'activation'. Contrastingly, IL-5 was also observed to inhibit caspase-8 processing in response to FasR ligation, seemingly indicating an inhibitory role of IL-5 that manifests proximal to the death receptor, upstream of mitochondria in 'extrinsic' apoptosis. In Type II cells, caspase-8 recruitment and activation at the receptor complex is limited, with the majority of caspase-8 activated downstream of mitochondrial perturbation (Scaffidi *et al.*, 1998). It may be the case that IL-5 prevents this post-mitochondrial activation of caspase-8, with no effect on the pre-mitochondrial, receptor-coupled activation, which was potentially too low for detection by immunoblotting, but sufficient to induce alteration of the mitochondrial membrane permeability via processing of Bid. Independent of the initiating stimulus, and regardless of the upstream mechanisms, the data implicate the mitochondria as the central executioners regulating the apoptosis of eosinophils.

Eosinophil apoptosis in response to growth factor deprivation, STS treatment and ligation of FasR was consistently associated with a striking redistribution of the mitochondria to the nuclear periphery. During TNF-induced apoptosis of murine fibrosarcoma cell line, L929, microtubule-dependent translocation of mitochondria from a dispersed distribution to perinuclear clustering occurs in response to defective mitochondria-associated motor protein, kinesin (De Vos *et al.*, 1998). It is possible that the observed redistribution of the organelle during eosinophil apoptosis is merely a consequence of the pronounced condensation of the cell. However, mitochondrial translocation was observed to precede evidence of nuclear fragmentation, correlate with sensitivity to TNF-induced apoptosis of L929 cells, and inhibition of mitochondrial redistribution delayed apoptosis, indicating a potential functional role for the translocation in cell death (De Vos *et al.*, 1998). Overexpression of pro-apoptotic Bax was also observed to induce mitochondrial redistribution in HeLa cells in the absence of an apoptotic stimuli, and independent of the activation of caspases (Eskes *et al.*, 1998). The functional reasons for mitochondrial association with the nuclear periphery during apoptosis

are intriguing to speculate. Perhaps aggregation prior to mitochondrial dysfunction simply brings the ATP-producing organelle in close vicinity to the nucleus, a major site of ATP-dependent apoptotic events. Alternatively, mitochondria have recently been shown to house apoptotic effector molecules that are active within the nucleus. AIF and endonuclease G are released from the mitochondria during apoptosis and translocate to the nucleus where they functionally manifest, inducing DNA fragmentation (Daugas *et al.*, 2000; Li *et al.*, 2001). Redistribution of mitochondria upon initiation of apoptosis may facilitate such intracellular translocations.

Interestingly, despite diffuse Bax expression being detectable throughout the entire cell, expression was predominantly observed in the nucleus. Lack of nuclear and cytosolic staining in the isotype-matched negative control would suggest that the staining was not non-specific. There is evidence of an association of Bax with the nuclear matrix of non-apoptotic breast carcinoma cell line, MCF-7 (Bedner *et al.*, 2000; Hoetelmans *et al.*, 2000), and the glioblastoma cell line, U343 (Wang *et al.*, 1999). However, the nuclear antigenicity of Bax reported by Hoetelmans *et al* (2000) was dependent upon the antibody used. Bax translocation to the nucleus during apoptosis has also been reported. Downregulation of epidermal growth factor receptor and consequent induction of apoptosis in human colorectal cancer cells was associated with a translocation of Bax from the cytosol to the nucleus (Mandal *et al.*, 1998). Similarly, (Raffo *et al.*, 2000) have reported a nuclear association of Bax in human melanoma cell lines following induction of apoptosis with the chemotherapeutic agent, cisplatin. Interestingly, Bax was found complexed with the tumour suppressor protein, p53. Formation of these Bax/ p53 complexes was concomitant with caspase-3 processing, and preceded PtdSer externalisation and hypodiploidy (Raffo *et al.*, 2000). Also, electron microscopy revealed Bax immunoreactivity associated with intermediate filaments of the karyoskeleton and lamina-pore complex of the nuclear matrix of the human colon adenocarcinoma COLO 205 cell line induced to undergo apoptosis by camptothecin (Gajkowska *et al.*, 2001). A potential role for nuclear Bax in cell cycle progression as opposed to apoptosis has also been implicated. Nishita *et al* (1998) investigated Bax expression in a number of human lung cancer cell lines and observed an increase in nuclear Bax as a result of hyperthermia-induced cytostasis.

The specificity of Bax detection within the nucleus is supported by the nuclear detection observed with a number of antibodies raised against different epitopes of Bax (Fig. 6.6). The nuclear antigenicity was also lost during apoptosis, with staining completely excluded from

the nucleus, concomittant with Bax translocation to the mitochondria at the nuclear periphery. However, the expression and potential role of nuclear Bax may be questioned due to the observed independence of detection of nuclear Bax and mitochondrial Bax by the monoclonal antibody 6A7 (Fig. 6.9) and the lack of support from the subcellular fractionation evidence (Fig. 6.10). The ideal control experiment would involve immunocytochemistry of Bax in eosinophils derived from a *bax*$^{-/-}$ mouse. Previously, immunocytochemistry revealed nuclear Bax staining in HeLa cells, which was retained in Bax-deficient cell lines LS180 and LoVo when using a polyclonal antibody, directed against the N-terminal amino acids 1-21 of Bax (Desagher *et al.*, 1999), suggesting cross-reactivity with an alternative nuclear antigen. However, it should be noted that the Bax staining observed using polyclonal anti-Bax 1-21 was predominantly nucleolar, whereas Bax staining in MCF-7 cells with monoclonal antibody 2D2 was totally excluded from the nucleoli (Fig. 6.8; Bedner *et al.*, 2000). An endogenous association of Bax with PML bodies within the nucleus, with a proposed role in caspase-independent cell death has been reported (Quignon *et al.*, 1998). However, the antibody used to recognise the N-terminal amino acids 1-19 of Bax was subsequently observed to cross-react with a conformation–specific epitope of the nuclear body protein Sp100 (Quignon, 1998). These findings highlight the necessity for caution when interpreting immunocytochemistry analysis of Bax expression, but also raise intriguing questions as to the potential role of nuclear Bax, which warrant further investigation. Bax lacks a nuclear localisation signal and therefore the mechanism facilitating its presence within the nucleus is intriguing. Bax may interact with proteins that possess a nuclear localisation signal, thereby facilitating import, such as p53 (Raffo *et al.*, 2000) or the Bcl-2 homologue, Bag-1L (Takayama *et al.*, 1999). It will be interesting to determine the identity of the 40 kDa protein predominantly detected in eosinophils and neutrophils by a polyclonal antibody targeting Bax (N-20), and whether this protein represents the nuclear antigen detected by immunocytochemistry. Immunoblotting suggests that this antigen is predominant within the nuclear fraction of eosinophils, and intriguingly, lymphocytes which lacked this 40 kDa protein (Fig. 4.3) were observed to have little detectable nuclear Bax staining by immunocytochemistry compared with eosinophils and neutrophils (Fig. 6.7).

Chapter 7
General Discussion

7.1. *In vivo* relevance of eosinophil apoptosis and phagocytosis

Delayed apoptosis may provide a significant mechanism determining the prevalence of eosinophils in the asthmatic airway and at sites of allergic inflammation. Clinical relevance of eosinophil apoptosis in asthma was provided by Woolley *et al* (1996), who reported an association of corticosteroid treatment for asthma exacerbation with a significant increase in sputum eosinophils with morphological evidence of apoptosis and eosinophil-derived products within macrophages. *In vitro* studies have supported a role for delayed eosinophil apoptosis in asthma and atopic disease, although the principal cytokine implicated in determining the survival of eosinophils *in vivo* is debated. Kankaanranta *et al* (2000) observed neutralising antibody directed against GM-CSF modestly increased the survival of eosinophils from asthmatics, whereas neutralisation of IL-5 had no significant effect, indicating that eosinophil cell death in asthma is predominantly inhibited by the production of GM-CSF. Wedi *et al* (1997) also reported that eosinophils derived from patients with inhalant allergy and atopic dermatitis had a reduced rate of apoptosis compared with nonatopic subjects. Similarly, the authors implicate autocrine generation of GM-CSF, but also IL-5, as being responsible for the delayed apoptosis. As an *ex vivo* system, nasal polyps provide a suitable model for eosinophilic inflammation, and it was observed that eosinophils within nasal polyps exhibited prolonged survival in the absence of exogenous cytokine compared with similarly cultured peripheral blood eosinophils derived from the same donor (Simon *et al.*, 1997). Contrastingly, treatment of nasal polyp explants with neutralising antibody against IL-5, but not GM-CSF, was reported to increase the number of apoptotic eosinophils, indicating that specific inhibition of apoptosis in response primarily to IL-5 contributes to the eosinophilia observed in this model of inflammation. Davidsson *et al* (2000) also report the presence of apoptotic eosinophils and engulfment by infiltrating macrophages within sinonasal polyps. In a murine model, administration of anti-Fas antibody induced the apoptosis of infiltrated eosinophils and abolished the augmentation of airway hyperresponsiveness in response to ovalbumin sensitisation, indicating apoptosis as a potential mechanism *in vivo* for inhibiting eosinophilic inflammation (Ohta *et al.*, 2001).

However, the *in vivo* relevance of eosinophil apoptosis has been questioned, due to the inconclusive evidence of tissue-residing eosinophils exhibiting apoptotic morphology within the airway (Erjefält and Persson, 2000). Erjefält and Persson (2000) stress that the majority of examples of apoptotic eosinophils are derived from sputum and BAL, and therefore, by definition, the eosinophils have already been extruded into the airway lumen. They suggest that cells undergoing apoptosis are unlikely to actively translocate across the epithelium,

rather that apoptosis is a secondary event occurring after expulsion from the lung tissue, and therefore an alternative pathway of luminal extrusion has been suggested for the clearance of eosinophils infiltrating the lung interstitium. Vignola *et al* (1999) provide evidence of eosinophil apoptosis in bronchial biopsies, although the data is complicated by the relatively high apoptotic background obtained by the TUNEL technique (previously reported by Kern *et al.*, 2000), that detects relatively late-stage apoptosis on the basis of DNA strand breaks. Apoptotic eosinophils have been reported in the lung of mice following allergen challenge and electron micrographs show macrophages engulfing apoptotic bodies within the tissue. Although the authors could not conclusively determine that the apoptotic cells were eosinophils due to problems with dual staining, apoptosis was spatially and temporally associated with significant eosinophil infiltration (Kodama *et al.*, 1998). Lloyd *et al* (2001) have recently shown that IL-3-dependent inhibition of leukocyte apoptosis results in the resolution of BHR, providing evidence of leukocytes undergoing apoptosis within the lung interstitium. Similarly, the authors admit that they were unable to determine the exact nature of the cells undergoing apoptosis, due to the nature of the TUNEL assay employed, although they do report that the major infiltrating cells at equivalent timepoints were eosinophils, and that abrogation of the effect of IL-3 significantly reduced eosinophilia, and therefore may be linked to apoptotic cell clearance. *In vivo* evidence has been reported for the clearance of apoptotic neutrophils from the lung (Grigg *et al.*, 1991), contributing to the amelioration of pulmonary inflammation (Cox *et al.*, 1995). Eosinophil apoptosis and phagocytosis has been observed in extrapulmonary tissues, such as the corticosteroid-treated intestine and in the skin following local allergen challenge (Kawabori *et al.*, 1991; Ying *et al.*, 1997). Erjefält and Persson (2000) suggest that apoptosis and subsequent phagocytosis does indeed occur when there is no alternative 'silent' clearance mechanism, such as luminal extrusion in the lung. The capacity of resident pulmonary macrophages to clear senescent, intact cells in a non-phlogistic fashion, coupled with the evidence of eosinophil apoptosis *in vivo*, suggests that elimination of eosinophils from the airway by sequential apoptosis and phagocytosis is not unlikely. However, *in vivo* evidence of apoptosis of airway tissue-residing eosinophils remains inconclusive.

Prolonged culture of eosinophils *in vitro* results in the marked condensation of nuclei and cytoplasm. The paucity of eosinophils exhibiting such apoptotic morphology *in vivo* may be due to the rapid and efficient clearance by resident phagocytes. It has been shown that recognition of PtdSer exposure on the surface of an apoptotic cell facilitates phagocytosis (Fadok *et al.*, 2000; Fadok *et al.*, 2001). PtdSer externalisation is an early apoptotic event

relative to cytoplasmic and nucleic condensation, and therefore cell clearance *in vivo* may occur prior to the end-stage apoptotic phenotype. Also, in certain circumstances, the mechanisms governing cell clearance and caspase activation are independent (Zhuang *et al.*, 1998), and therefore recognition may potentially occur in the absence of detectable caspase-dependent morphological changes.

Eosinophilic inflammation of the lung in asthmatics is associated with a microenvironment of survival enhancing factors such as IL-5 and GM-CSF. Prolonged survival in conjunction with additional cytokine-dependent effects (IL-5 has been shown to induce eosinophil activation and degranulation) markedly increase the inflammatory potential of the cell. Under such circumstances, eosinophils may more readily undergo cytolysis and degranulation as has been reported in allergic airways (Erjefält *et al.*, 1999) as opposed to apoptosis. Evidence suggests that the delay of eosinophil apoptosis does have a role in mediating the accumulation of eosinophils in the asthmatic airway and sites of allergic inflammation, and that clearance by resident phagocytes in a non-phlogistic fashion may provide a mechanism for limitation of the inflammatory response.

Alexis *et al* (2001) have observed an association between phagocytic dysfunction of sputum derived macrophages, determined by zymosan A engulfment, and sputum eosinophilia, possibly indicating that failure of macrophages to ingest eosinophils contributes to the airway eosinophilia in asthmatics. Eosinophil apoptosis was not assessed by Alexis *et al* (2001), but one may hypothesise a situation whereby dysfunction of phagocytosis would lead to a prevalence of granulocytes within the airway, whose eventual cell fate would be apoptosis followed by secondary necrosis or alternatively activation-induced degranulation. A cytolytic result and release of histotoxic mediators is consistent with either scenario, and may therefore exacerbate airway inflammation. It has been shown in *C. elegans* that in the absence of efficient cell clearance, a reversion of the apoptotic process can occur (Hoeppner *et al.*, 2001; Reddien *et al.*, 2001), facilitating long term survival and increasing the inflammatory potential of a cell previously destined to die 'silently'. However, it remains to be seen whether such a mechanism functions within mammalian cells.

Phagocytosis is a crucial aspect of the apoptotic process, as it ensures the non-phlogistic removal of inflammatory cells. However, phagocytosis of apoptotic cells or bodies also stimulates the preferential release of anti-inflammatory cytokines including TGF-β and IL-10 (Fadok *et al.*, 1998; Voll *et al.*, 1997), in contrast with the pro-inflammatory mediator release

upon ingestion of necrotic material and bacteria. Brown and Savill (1999) have also shown that engulfment by monocyte-derived macrophages stimulates the release of soluble FasL, capable of inducing the apoptosis of resident leukocytes. Therefore, phagocytosis can promote resolution of inflammation by the efficient removal of apoptotic cells and their histotoxic and immunogenic components, stimulating the production of anti-inflammatory cytokines (TGF-β), whilst suppressing release of pro-inflammatory cytokines (TNF-α), and potentially accelerating the cell death of bystander pro-inflammatory cells. Although macrophages are the predominant phagocytes, numerous cells have phagocytic capacity, including fibroblasts and dendritic cells (Savill and Fadok, 2000). Interestingly, apoptotic eosinophils are also recognised and phagocytosed by bronchial epithelial cells in a lectin and integrin-dependent fashion, and therefore may play a significant role in the resolution of airway inflammation (Walsh *et al.*, 1999).

7.2. Mitochondrial control of eosinophil apoptosis

Mitochondria play an integral role in the 'intrinsic' (growth factor deprivation and STS-treatment) and 'extrinsic' (ligation of FasR) apoptosis of eosinophils, with Bax-translocation induced mitochondrial dysfunction resulting in the cytochrome *c*–dependent activation of caspases. Peachman *et al* (2001) have recently reported that eosinophil mitochondria play an important role in mediating eosinophil apoptosis, although, interestingly, mitochondrial respiration has little or no role in the generation of ATP. Evidence suggests that eosinophils derive the majority of their ATP from glycolysis as opposed to respiration, as eosinophil function is enhanced or reduced by activation and inhibition, respectively, of glycolysis, whereas inhibitors of oxidative phosphorylation had little effect (David *et al.*, 1977; Sher *et al.*, 1983; Peachman *et al.*, 2001). Therefore during eosinophil differentiation the role of the mitochondria in respiration-dependent ATP generation is lost, although its role as an important regulator of apoptosis is retained. It is intriguing to speculate that the propensity of terminally differentiated cells, such as eosinophils and neutrophils to undergo apoptosis maybe due to their main source of ATP being from glycolysis as opposed to respiration, thereby allowing maintenance of essential ATP levels during apoptosis-associated dysfunction of mitochondrial respiration, and facilitating the ATP-dependent execution processes. Contrast this with, for example, neurons, which generate the majority of their ATP by mitochondrial respiration and have a tendency to undergo necrotic cell death as opposed to apoptosis.

Trimerisation of FasR with monoclonal antibody accelerates eosinophil apoptosis by a mechanism involving Bax translocation to the mitochondria. Interestingly, caspase-8-mediated mitochondrial dysfunction and subsequent caspase-3 activation has been reported during the Fas-induced apoptosis of neutrophils (Watson *et al.*, 1999). This supports categorisation of both eosinophils and neutrophils as Type II cells, whereby Fas-induced apoptosis is mediated by the mitochondria and requires cytochrome *c* efflux for successful execution of the death programme (Scaffidi *et al.*, 1998). Activation of the FasR induces the apoptosis of lung eosinophils, resulting in depletion of eosinophils within the bronchial subepithelium (Tsuyuki *et al.*, 1995). Eosinophils obtained from hypereosinophilic patients, or isolated from inflammatory tissues exhibit NO-dependent 'Fas resistance' (Hebestreit *et al.* 1999). The lack of functional response to FasR ligation was not dependent upon downregulation of the receptor, but proposed to be due to disruption of the signal transduction pathway downstream of the receptor (Hebestreit *et al.*, 1998). Oligomerisation of FasR in response to monoclonal antibody or FasL causes formation of the DISC, which facilitates the activation of caspase-8. It will be interesting to determine if the formation of such a caspase-activating complex occurs in eosinophils undergoing Fas-induced apoptosis, and whether NO-induced Fas resistance is manifest in a dysfunction in the molecular scaffold of the DISC. The common molecular target for the action of NO is soluble guanylate cyclase (sGC), resulting in the elevation of cytosolic cyclic GMP (cGMP). NO donors (azide and hydroxylamine) and dbcGMP also inhibit spontaneous eosinophil apoptosis *in vitro* (Beauvais *et al.*, 1995). This raises the question whether NO has similar effects on eosinophil apoptosis regardless of the initiating stimulus. As mentioned above, both growth factor deprivation and Fas-induced apoptosis signal through the mitochondria in eosinophils. It has been reported that NO donors inhibit the activation of caspase-3 following cross-linking of the FasR receptor on Jurkat T cells (Bernassola *et al.*, 2001) and synovial cells (Migita *et al.*, 2001), whereas activation of caspase-8 and cytochrome *c* release were unaffected (Migita *et al.*, 2001). NO is proposed to directly inhibit the function of caspases due to the S-nitrosylation of the cysteine thiol groups (Chung *et al.*, 2001), and therefore implicates specific inhibition of caspase-3 is at least partly responsible for the NO-dependent delay of eosinophil apoptosis, and its downstream role as an effector caspase suggest that that inhibition of cell death by NO is independent of the apoptotic stimulus.

Spontaneous and Fas-induced apoptosis was associated with a detectable increase in the intracellular levels of reactive oxygen species (ROS), and apoptosis was inhibited by thiol antioxidants such as glutathione (GSH) and N-acetylcysteine (Wedi *et al.*, 1999), indicating

that eosinophil apoptosis is regulated by oxidative stress. The principal route of ROS generation is mitochondrial oxidative metabolism, due to the premature donation of electrons from the electron transport chain to singlet oxygen, resulting in the production of reactive superoxide anion. PTP opening results in the uncoupling of the electron transport chain and dissipation of the $\Delta\Psi m$ due to the release of cytochrome *c* from the intermembrane space and consequent overproduction of superoxide anion. Conversely, low dose oxidants can induce PTP opening that is inhibited by intracellular antioxidants such as GSH. It is unclear whether the generation of ROS in eosinophils upon induction of apoptosis occurs simply as a result of the perturbation of the outer mitochondrial membrane, or whether disruption of the redox balance early in the apoptotic process actually initiates the permeability transition. The reported reduced oxidative metabolic capacity of eosinophil mitochondria (Peachman et al., 2001) would suggest that generation of ROS during eosinophil apoptosis was a consequence of mitochondrial permeability. The findings of Wedi *et al* (1999) provide further support for an essential role for the mitochondria in apoptosis induced by growth factor deprivation and FasR ligation.

7.3. Pivotal role for Bax in eosinophil apoptosis

IL-5 prolongs eosinophil survival by inhibiting Bax-dependent mitochondrial perturbation (Fig. 7.1). However, the N-terminus seemingly regulates Bax conformation and has several potential sites for serine/ threonine phosphorylation, and residue Ser 184 within the C-terminus is absolutely required for membrane targeting (Wolter *et al.*, 1997). The conformation change and activation of Bax during detachment induced apoptosis (anoikis) of mammary epithelial cells is dependent upon the activation of the tyrosine kinase, pp125FAK (focal adhesion kinase), possibly involving PI-3-K (Gilmore *et al.*, 2000). Similarly, NO-induced neuronal apoptosis is associated with p38 MAPK-dependent Bax translocation (Ghatan *et al.*, 2000). The evidence implicates that phosphorylation events may regulate Bax conformation and intracellular redistribution. However, the mechanism remains obscure, as there is no evidence supporting the direct phosphorylation of Bax (Nechushtan *et al.*, 1999). Alternatively, phosphorylation and dissociation of a Bax-binding partner may provide the controlling mechanism and thereby link Bax 'activation' with growth factor receptor kinase cascades.

The pro-apoptotic protein, Bad, has been shown to be phosphorylated in response to cytokine in IL-3 dependent cell lines, and is sequestered in the cytoplasm preventing interaction with anti-apoptotic proteins at the mitochondrial surface (Yang *et al.*, 1995; Datta *et al.*, 1997;

Scheid *et al.*, 1999). Although Bad mRNA was detectable within peripheral blood eosinophils, protein expression was low or undetectable, and there was no conclusive evidence of Bad phosphorylation in response to stimulation with IL-5. Furthermore, incubation of eosinophils with specific inhibitors of PI-3-K (wortmannin and LY29004) and MEK-1/2 (U0126) had little or no effect on IL-5-induced survival, indicating that the PI-3-K/ Akt (PKB) and MAPK pathways are not required for the pro-survival effect of IL-5. However, problems with the phosphorylation state-specific antibodies and lack of a suitable positive control, meant that a potential role for Bad phosphorylation by an alternative pathway, such as cyclic AMP-dependent kinase-mediated phosphorylation (Lizcano *et al.*, 2000), cannot be ruled out. Interestingly, elevators of cytosolic cAMP, such as prostaglandins, dibutyryl-cAMP, and phosphodiesterase (PDE) inhibitor, rolipram, have been reported to inhibit eosinophil apoptosis *in vitro* (Hallsworth *et al.*, 1996; Momose *et al.*, 1998; Peacock *et al.*, 1999). This evidence possibly suggests a role for PKA-dependent Bad phosphorylation in the mediation of eosinophil survival. However, in the absence of GM-CSF stimulation, reduction in eosinophil apoptosis is seemingly independent of PKA (Hallsworth *et al.*, 1996). In contrast, the non-specific PDE inhibitor, theophylline, used for the treatment of asthma due to its bronchodilator effects, has been shown to abrogate eosinophil survival *in vitro*, by a mechanism independent of PDE (Yasui *et al.*, 1997). Therefore, cAMP appears to have an important role in governing the susceptibility of eosinophils to apoptotic stimuli, although the mechanisms involved are not fully understood.

Recent reports have indicated a synergistic mechanism, whereby Bax and Bak have partially redundant roles, as cells deficient in Bax or Bak retain a functional apoptotic pathway, whereas cells deficient in both pro-apoptotic proteins are resistant to many diverse apoptotic stimuli (Wei *et al.*, 2001). Eosinophils express Bak protein as revealed by immunoblotting, and interestingly, $bax^{-/-}bak^{-/-}$ mice suffered numerous developmental abnormalities, including the inappropriate elevation of granulocytes (Lindsten *et al.*, 2000), indicating that Bax, Bak or both, are potentially important regulators of granulocyte apoptosis. Similarly, Bax has also been implicated in regulating the survival of neutrophils, with Bax translocation crucial for apoptosis of neutrophils following temperature transition *in vitro* (Pryde *et al.*, 2001), and cytokine-induced Bax deficiency contributing to the delayed apoptosis of neutrophils at inflammatory sites (Dibbert *et al.*, 1999). Nechushtan *et al* (2001) have recently reported that the insertion of Bax into the outer mitochondrial membrane during apoptosis is transient, with a rapid translocation from the membrane to form large clusters in the presence of Bak proximal to the mitochondria. This caspase-independent process was also crucial for efficient

execution of cell death, although it was not clear whether Bax and Bak functions within these clusters were complementary. Evidence suggests that the pro-apoptotic protein Bax is a potentially critical regulator of the apoptosis of terminally-differentiated granulocytes.

The data demonstrate that the mechanisms regulating eosinophil apoptosis are dependent upon the initiating stimulus (Fig. 7.1). Growth factor deprivation induces, at present unknown, intracellular signals culminating in the caspase-independent translocation of Bax to the mitochondria, perturbation of the mitochondrial outer membrane, consequent release of cytochrome *c* and activation of the caspase-9-mediated caspase cascade. IL-5 prevents the Bax translocation requisite for spontaneous apoptosis by this pathway, although the mechanism by which the IL-5-induced kinase cascades mediate this molecular transition remains unclear. Optimal survival enhancing concentrations of IL-5 failed to completely prevent Bax translocation induced by FasR ligation, indicating that IL-5 acts apical to Bax and does not inhibit Bax translocation directly. Induction of apoptosis by ligation of FasR is proposed to involve caspase-dependent Bax translocation, possibly mediated by caspase-8 recruitment and activation at the DISC, although further investigation in the eosinophil is required to validate this hypothesis. Although the upstream initiation of cell death is distinct, downstream pathways of apoptotic execution induced by growth factor deprivation and FasR cross-linking apparently coincide at, and indicate a crucial role for, mitochondria during eosinophil apoptosis.

7.4. Future directions

Although a number of reports have implicated delayed apoptosis as a contributory factor in the selective accumulation of eosinophils in the asthmatic airway and at sites of allergic inflammation (Woolley *et al.*, 1996; Simon *et al.*, 1997; Wedi *et al.*, 1997; Kodama *et al.*, 1998; Kankaanranta *et al.*, 2000), substantive evidence of eosinophil apoptosis and subsequent non-phlogistic phagocytic clearance within the bronchial tissues, as has been demonstrated for neutrophils (Grigg *et al.*, 1991; Cox *et al.*, 1995), has yet to be reported.

The data demonstrate that the mitochondria are pivotal regulators of eosinophil apoptosis with Bax translocation a common event following growth factor deprivation, STS treatment, and ligation of FasR. IL-5 inhibits spontaneous apoptosis upstream of Bax translocation and mitochondrial perturbation. It remains to be seen if an alternative, physiologically relevant survival factor, GM-CSF, induces a similar response in eosinophils, although IL-5 and GM-CSF share a βc receptor subunit, and similar cytokine concentrations elicit maximal survival

effects, implying a common underlying mechanism. Elucidating the mechanism by which the IL-5-induced kinase cascade integrates into the apoptotic machinery, thereby regulating mitochondrial permeability and the execution phase of apoptosis may provide potential for therapeutic intervention.

In conjunction with cytochrome *c*, a number of other apoptogenic factors are reported to escape from the mitochondria during apoptosis, such as AIF, endonuclease G and Smac/DIABLO. AIF and endonuclease G translocate from the mitochondria to the nucleus and induce chromatinolysis and apoptosis independent of the activation of caspases (Daugas *et al.*, 2000; Li *et al.*, 2001). Smac/ DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000) is released from the mitchondrial intermembrane space and potentiates apoptosis by interacting with IAPs (inhibitors of apoptosis), and preventing the inhibition of caspase-9, -3, and –7 (reviewed by Deveraux and Reed, 1999). Considering the implicated importance of mitochondria in eosinophil apoptosis in response to diverse stimuli, it will be interesting to determine whether such crucial mitochondrial apoptotic mediators function during eosinophil cell death.

Although the data presented here highlight the necessity for absolute verification of Bax localisation within the nucleus, a number of reports have implicated a functional role for Bax with this distinct subcellular distribution. Nishita *et al* (1998) reported increased detection of Bax within the nucleus of lung carcinoma cell lines undergoing hyperthermia-induced cytostasis, independent of its proven role in apoptosis. A link with nuclear expression and cell cycle is intriguing, due to the observed Bax localisation in terminally differentiated eosinophils and neutrophils. Bax may be sequestered within the nuclei as a result of cytostasis or terminal differentiation, in order to inhibit spontaneous apoptosis. Alternatively, Bax translocation to the nucleus may facilitate an active role in regulating elements of the cell cycle machinery, as Bax and Bcl-2 have been shown to modulate the activity of cyclin-dependent kinase (cdk)2 in thymocyte apoptosis (Gil-Gómez *et al.*, 1998). Verification of nuclear Bax expression and investigation of its potential functional relevance within granulocytes would be of significant interest.

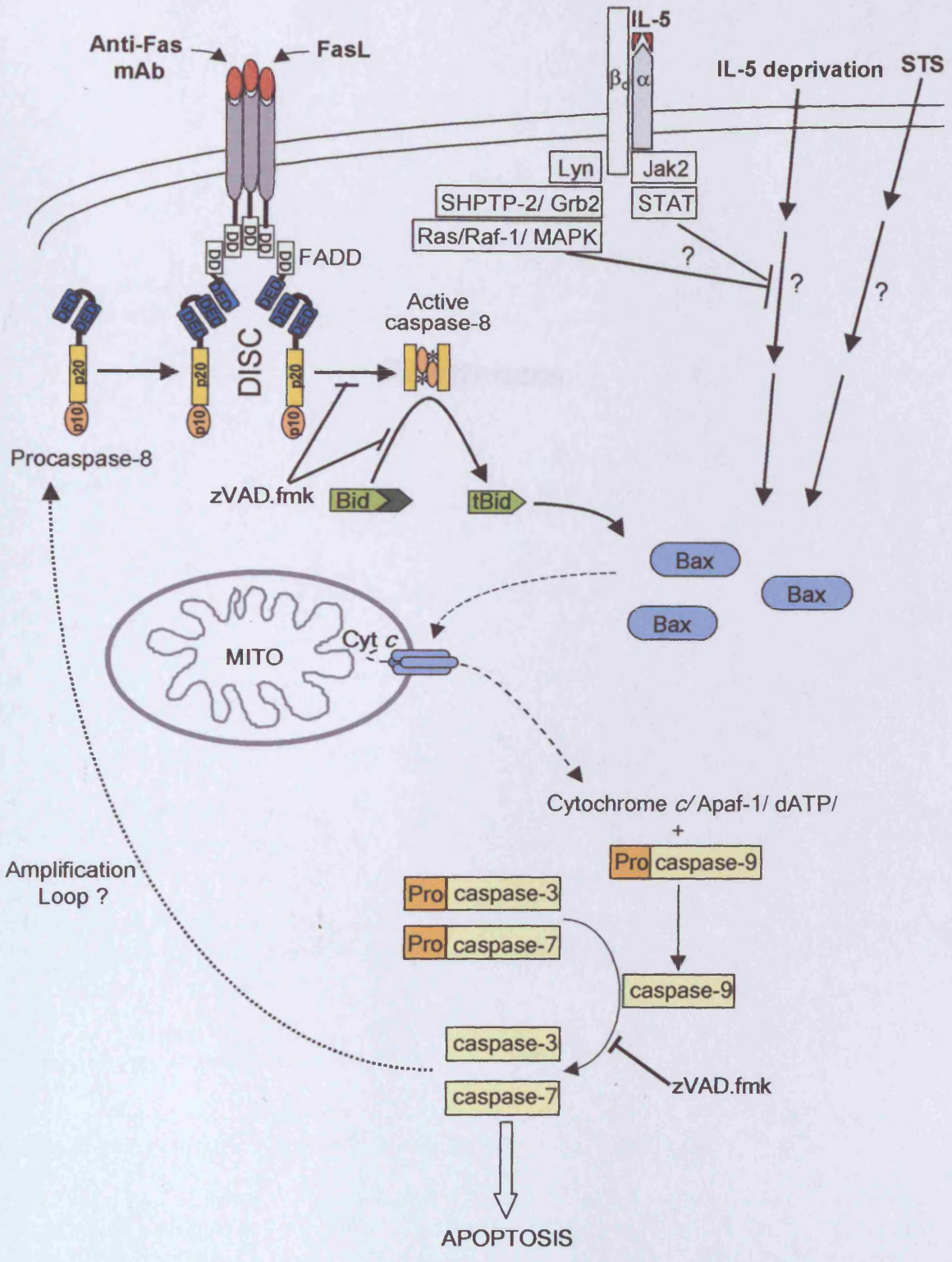

Figure 7.1. Proposed scheme for spontaneous and Fas-induced apoptotic pathway in eosinophils. Spontaneous eosinophil apoptosis involves caspase-independent Bax translocation to the mitochondria, cytochrome *c* release, and perturbation of the mitochondrial membrane followed by activation of caspases-9, -3 and -7, similar to the pathway of chemical-induced apoptosis. Caspase-3 activation of caspase-6 followed by activation of caspase-8 is proposed to form an amplification loop. IL-5 inhibits eosinophil apoptosis at an as yet undetermined site upstream of Bax translocation, cytochrome *c* release and caspase activation. The IL-5 anti-apoptotic signal is transduced by recruitment and activation of Jak2 and Lyn tyrosine kinases, and SHPTP-2 to the receptor, resulting in activation of Jak/ STAT and Ras-Raf-MAPK pathways (see text for details). Dashed arrows represent protein translocation.

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Appendix 1

Verification of PCR products

A1.1. Direct sequencing

A

GNTGTGNNTCTCACAAGAACTGAGCGAGTGTCTCAAGCGCATCGGGGACGAACTGGACAGTAAC
ATGGAGCTGCAGAGGATGATTGCCGCCGTGGACACAGACTCCCCCCGNAGAGGTCTTTTTCCGA
GTGGCAGCTGACATGTTTTCTGACGGCAACTTCAACTGGGGCCCGGGTTGTCGCCCTTTTCCACT
TTGCAANCAAACTGGGTGCTCAAAGGCCCTGTTNACCAAAGGGCCCGGAACTGGTCCAGAANCA
CCATGGGCTGGAATTTCCNNNAANNNNNNNNNNT

N-ambiguous base

B

Highest scoring segment pair from BLAST search of highlighted sequence:

1. gb:HUMBAXA Human Bax alpha mRNA, complete cds
   Length = 579

Score = 63.9 bits (32), Expect = 3e-09
Identities = 32/32 (100%)
Strand = Plus / Plus

```
Query: 1   ggggacgaactggacagtaacatggagctgca 32
           ||||||||||||||||||||||||||||||||
Sbjct: 199 ggggacgaactggacagtaacatggagctgca 230
```

Figure A1.1. Verification of *bax* amplification by sequencing. The *bax* PCR product from human eosinophil mRNA was purified and sequenced according to *Materials and Methods* (A). A BLAST search of genomic database was performed using the highlighted sequence to determine sequence homology (B).

A1.2. 'Nested' PCR

Table A1.1. Properties of 'nested' PCR primers.

***bcl-2* homologue**	**FWD PCR primer sequence (5'→3')**	**R&C PCR primer sequence (5'→3')**
bax	p1- GGACCCGGTGCCTCAGGA	p2- CAAAGATGGTCACGGTCTGC p3- AGGAAGTCCATGTCCAG
bcl-2	p1- GATGTCCAGCCAGCTGCACCTG	p2- CACAAAGGCATCCCAGCCTCC p3- GACGCTCTCCACACACACATGACC p4- CGGTTCAGGTACTCAGTCATCCAC
bcl-x$_L$	p1- TTGGACAATGGACTGGTTGA p3- CCACAAAAGTATCCTGTTCAAAGC	p2- GTAGAGTGGATGGTCAGTG p4- CCAAGCTGCGATCCGACTCAC
bcl-x$_S$	p1- TTGGACAATGGACTGGTTGA p3- CCACAAAAGTATCCTGTTCAAAGC	p2- GTAGAGTGGATGGTCAGTG p5- CAGAAAGGATACAGCTGGAG

FWD, forward; R&C, reverse & complementary.

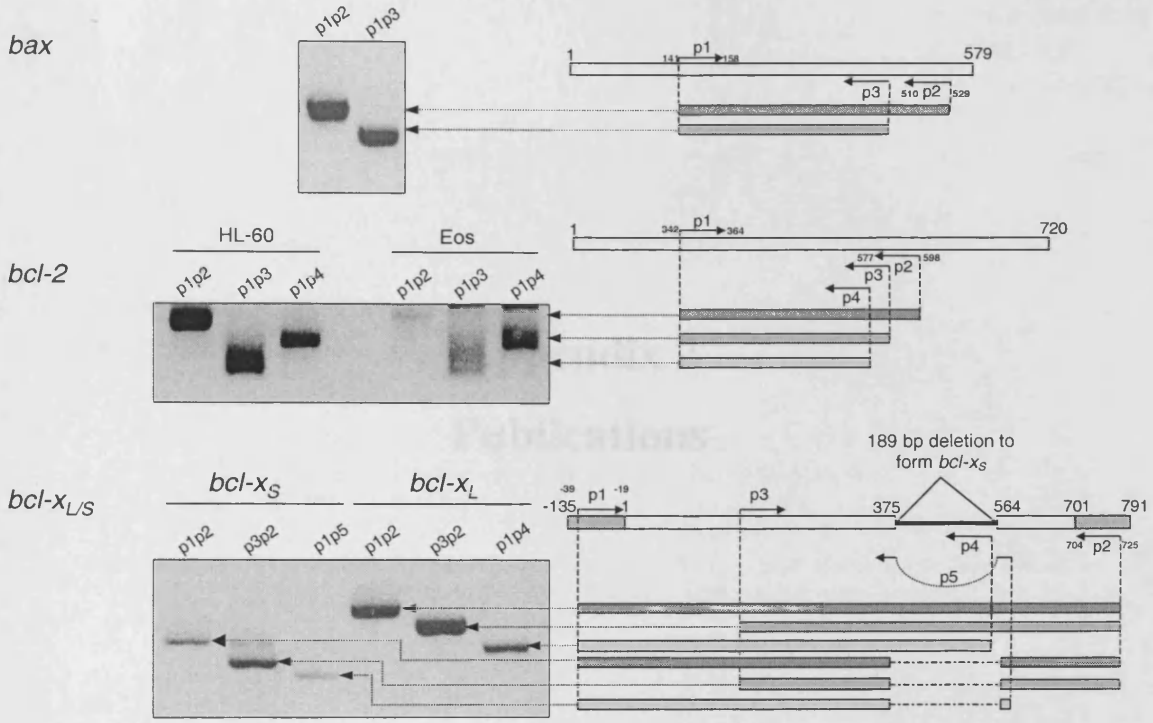

Figure A1.2. Nested PCR of *bcl-2* homologues from human eosinophil mRNA. Original PCR products (p1p2) were excised and purified from agarose gels and used as template for 'nested' PCR reactions with internal primer sequences according to *Materials and Methods* and the scheme shown.

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A comparative study of different methods for the assessment of apoptosis and necrosis in human eosinophils

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Abstract

Eosinophils, prominent cells in asthmatic inflammation, undergo apoptosis or programmed cell death following deprivation of contact with survival-promoting cytokines such as IL-5 and GM-CSF. The aim of this study was to assess a number of techniques for the quantification of apoptosis in human eosinophils cultured with or without IL-5 or GM-CSF and following staurosporine treatment. The relationship between apoptosis and necrosis in eosinophils was also determined. Eosinophils 'aged' in vitro for 48 h exhibited endonuclease DNA degradation, apoptotic morphology, increased red autofluorescence and externalisation of phosphatidylserine (PS) as assessed by binding of FITC-labelled annexin V. Annexin V-FITC binding was first detectable in eosinophils maintained at 37°C for 5 h post-purification. This method proved to be the most sensitive marker of apoptosis. Morphological assessment of wet preparations of eosinophils by Kimura staining was found to be the next most-sensitive marker followed by increased red autofluorescence. The latter was a relatively insensitive method for the detection of apoptosis. At 5, 20 and 24 h of culture trypan blue exclusion indicated that eosinophil viability was high (85–90% viable cells). However, propidium iodide (PI) staining and flow cytometry revealed that, by 24 h, approximately 75% of cells had compromised membrane integrity. Eosinophils maintained in IL-5 or GM-CSF exhibited a non-apoptotic morphology and levels of annexin V-FITC binding and PI uptake similar to that of freshly isolated cells. Staurosporine (10^{-5} M) treatment of eosinophils maintained in IL-5 or GM-CSF resulted in significant levels of apoptotic morphology at 2 h (23.8% ± 6.9, $p < 0.025$) which was associated with negligible annexin binding. At 6 h post-staurosporine treatment significant annexin-FITC binding (38% ± 1.5, $p < 0.025$) was observed compared with 93% ± 1.2 of eosinophils displaying apoptotic morphology. Exclusion of PI demonstrated membrane integrity at all time points up to 6 h. Thus, eosinophils aged in vitro in the absence of viability-promoting cytokines exhibit evidence of both apoptosis and necrosis simultaneously. In contrast, staurosporine-treated eosinophils exhibited both membrane integrity and rapid apoptosis-associ-

Abbreviations: PI, propidium iodide; PCD, programmed cell death; PS, phosphatidylserine; GMSF, granulocyte macrophage-colony stimulating factor; IL-5, interleukin-5; PKC, protein kinase C

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ated morphological changes detected by single step Kimura staining which preceded externalisation of PS.

Keywords: Eosinophil; Apoptosis; Necrosis; Annexin V; Kimura stain

1. Introduction

Apoptosis or programmed cell death (PCD) is an ordered and fundamental biological process designed to safely dispose of surplus, aged or damaged cells (Wyllie et al., 1980; Raff, 1992). Apoptotic cells are phagocytosed whole or as discrete fragments bound by an intact membrane, thus ensuring their disposal without release of their contents. In contrast, necrosis results in the loss of cell membrane integrity and the release of the dying cell's contents in an uncontrolled and often harmful manner. Cell necrosis, therefore, promotes inflammation while apoptosis is associated with its resolution. There is now a consensus that eosinophil-derived mediators are major contributors to the tissue damage underlying the airway inflammation responsible for many aspects of asthma pathogenesis (Bousquet et al., 1990; Gleich, 1990; Wardlaw et al., 1995). While our knowledge concerning the complex mechanisms involved in their accumulation is now considerable (Bochner and Schleimer, 1995; Walsh, 1997) less well understood are the mechanisms controlling eosinophil apoptosis induction. Indeed, it has been proposed that inhibition of, or defects in, eosinophil apoptosis might contribute to a blood or tissue eosinophilia (Simon and Blaser, 1995). Thus, a better understanding of the mechanisms responsible for the induction and control of eosinophil apoptosis might enable the development of therapies designed to induce apoptosis in these cells in situ. In turn, this will allow their rapid and safe removal by phagocytes, prevent their accumulation and thus limit their toxic potential.

When 'aged' in vitro, eosinophils exhibit the classical changes in morphology associated with apoptosis including cytoplasmic condensation, internucleosomal cleavage of DNA by endogenous endonucleases and increased red autofluorescence (Stern et al., 1992). A number of different methods are now available for the detection of apoptosis in diverse cell types. Most of these rely on measurement of the degradation stage of apoptosis. These include nuclear chromatin fragmentation, cytoplasmic shrinking, the incorporation of fluorescent nuclear stains, dUTP nick-end labelling, etc. A major problem with such assays is that the end stages of apoptosis often overlap with necrosis. During these late stages of apoptosis, cells in culture lose their membrane integrity and take up vital dyes such as propidium iodide (PI). This process is often referred to as 'secondary necrosis', and is distinct from 'primary necrosis' which does not involve the apoptotic pathway. There is therefore much interest in generating rapid assays which measure the early stages of apoptosis while plasma membrane integrity is still intact and capable of excluding vital dyes.

Apoptotic cells appear to be recognised by macrophages via a thrombospondin/$\alpha v\beta 3$/CD36 complex interaction with an as yet undefined charge sensitive moiety on the apoptotic cell (Savill et al., 1993). The recognition and ingestion of apoptotic eosinophils by monocyte-derived macrophages is similar to that described for the neutrophil. This often occurs before any manifest signs of the morphological changes associated with apoptosis (Stern et al., 1996). Indeed, changes in the plasma membrane of cells undergoing PCD appears to be an early event in apoptosis. Phosphatidylserine (PS) is normally confined to the inner leaflet of the cell membrane. Early in apoptosis, PS is relocated to the outer layer of the plasma membrane (Fadok et al., 1992) and this appears to be a general feature of apoptosis regardless of the initiating stimulus (Martin et al., 1995). Annexin V is a Ca^{2+} dependent phospholipid binding protein with a high affinity for PS. FITC-labelled annexin V has been shown to provide a sensitive and rapid probe for apoptosis in a number of cell types including B cells (Koopman et al., 1994), neutrophils (Homburg et al., 1995), cell lines (Vermes et al., 1995) and vascular smooth muscle cells (Bennet et al., 1995).

We have therefore examined the relationship between PCD and necrosis in purified peripheral blood human eosinophils cultured alone or in the presence

of the survival-promoting cytokines IL-5 or GM-CSF. Annexin V-FITC binding was compared with apoptosis quantification by DNA laddering and red autofluorescence while apoptotic morphology was assessed by one-step Kimura staining of wet preparation eosinophils. Eosinophil viability was also assessed by trypan blue or PI exclusion. Though culturing eosinophils for long periods in the absence of IL-5 or GM-CSF resulted in significant apoptosis, this was also associated with considerable secondary necrosis. Staurosporine, a potent inhibitor of protein kinase C (PKC), reduces the viability of eosinophils cultured in IL-5 (Hossain et al., 1994) by apoptosis induction (Cousin et al., 1997). We have therefore compared the binding of annexin V-FITC, PI-uptake and morphological assessment of eosinophil apoptosis following culture with or without IL-5 or GM-CSF and also in the presence or absence of staurosporine.

2. Methods

2.1. Eosinophil purification

Blood (100 ml) was obtained from normal donors or individuals with a history of mild allergic-disease with an eosinophilia not greater than 0.5×10^6 eosinophils/ml who were not taking any medication at the time of venesection. Eosinophils were purified under sterile conditions using a modification of the method of Hansel et al. (1991) which has been described elsewhere (Walsh et al., 1995). Briefly, after removal of red cells using dextran sedimentation followed by a slow centrifugation ($100 \times g$, 15 min at RT) to remove platelets, the leukocytes were resuspended in Hanks BSS without Ca^{2+} or Mg^{2+} supplemented with 2% FCS, antibiotics and 0.02 M EDTA and then centrifuged on Histopaque 1083 (Sigma, Poole, UK) cushions at $400 \times g$ for 20 min at RT. Any remaining contaminating red cells in the granulocyte pellet were removed by hypotonic shock with sterile endotoxin-free distilled water. Granulocytes were incubated with micromagnetic beads coated with anti-CD16 for 40 min on ice before passage through the magnetic-activated separation column (Miltenyi, Bisley, UK). Using this method, eosinophils with a purity of at least 99% were obtained. No more than 5% of the freshly isolated eosinophil preparations displayed any morphological evidence of apoptosis and less than 5% of cells displayed positive staining with either annexin V-FITC or PI.

2.2. Eosinophil culture

Purified eosinophils were washed in RPMI 1640 supplemented with 10% foetal calf serum, antibiotics and L-glutamine and resuspended in the same medium at a concentration of 2×10^6 cells/ml. Cells were cultured in flat-bottom 12 well plates (Life Technologies, Paisley, UK) which had been previously coated with BSA (1 mg/ml) as described (Walsh et al., 1996) in a humidified atmosphere with 5% CO_2 in the presence or absence of IL-5 or GM-CSF (R&D Systems, Oxon, UK) at a final concentration 10^{-11} M. Cells were removed at various time intervals and apoptosis and viability assessed as detailed below. In some experiments, staurosporine was added to eosinophils which had been cultured overnight with either IL-5 or GM-CSF to give a final concentration of 10^{-5} M. Preliminary experiments determined that this concentration induced the most rapid apoptosis without evidence of necrosis.

2.3. Chromatin fragmentation assay

Eosinophils were washed with cold PBS and lysed with NTE buffer, pH 8.0 (100 mM NaCl, 10 mM Tris, 1 mM EDTA) containing 1% SDS and 0.2 mg/ml proteinase K (Sigma). Following a 12-h incubation at 37°C, extraction with phenol/chloroform/isoamylalcohol (25:24:1) was performed twice. Samples were precipitated with two volumes of 80% ethanol at −20°C and the DNA resuspended in water. The samples were electrophoresed through a 1% agarose gel containing 0.5 μg/ml ethidium bromide and viewed under UV light.

2.4. Eosinophil viability and morphological assessment

Eosinophils were counted in a haemocytometer and their viability assessed by trypan blue exclusion. Cells were also stained with Kimura prepared as described (Kimura et al., 1973). In each case at least 200 cells were counted by a blinded investigator.

The use of Kimura and light microscopy to distinguish the morphological differences between wet preparations of viable and apoptotic eosinophils has been previously validated by us as being both accurate and highly reproducible (Walsh et al., 1996).

2.5. Flow cytometry

Eosinophils cultured in the presence or absence of IL-5 or GM-CSF were washed in cold PBS and resuspended in staining buffer (HEPES buffered PBS supplemented with 2.5 mM $CaCl_2$) prior to the addition of FITC-labelled annexin V (Bradsure Biologicals, Loughborough, UK) for 15 min at 4°C as described (Homburg et al., 1995). Propidium iodide (PI) was added at a final concentration of 2 μg/ml 5 min before a final wash in PBS and immediate analysis of the cells on the flow cytometer (Becton Dickinson, Oxford, UK). Flow cytometry was performed on eosinophils gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. The flow cytometric analyser was also used as a measure of eosinophil apoptosis by determining differences in the degree of red autofluorescence in the two populations, as described (Stern et al., 1992).

2.6. Statistical analysis

All data are presented as means ± SEM and where n is given this represents the number of experiments each performed in duplicate. Statistical analysis was by the unpaired two tailed Student's t-test where a p value of < 0.05 was considered significant.

3. Results

Fig. 1 is a representative experiment showing an electrophoretic gel of endonuclease DNA degradation by eosinophils cultured in RPMI supplemented with GM-CSF in the presence and absence of staurosporine. The typical DNA 'laddering' indicative of apoptosis can be seen in lanes 6 and 8 which represent staurosporine treatment for 6 and 20 h, respec-

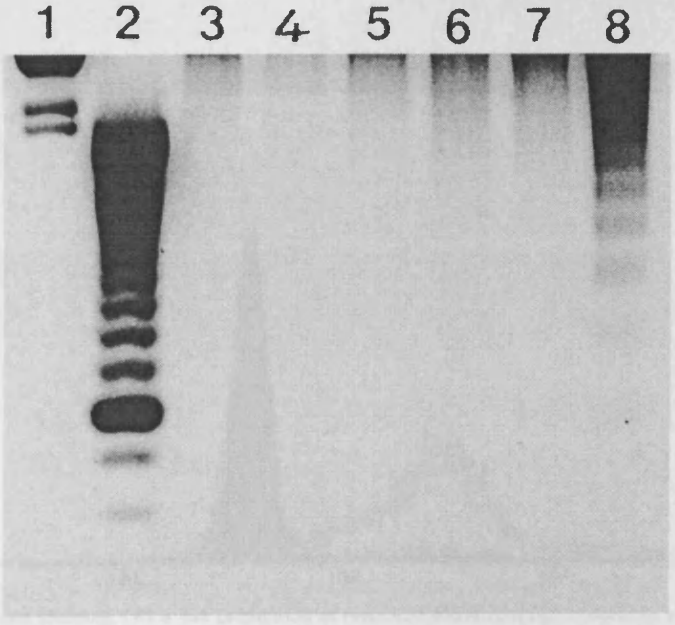

Fig. 1. Representative experiment showing DNA degradation in eosinophils cultured with GM-CSF (10^{-11} M) at various time points in the presence and absence of staurosporine (10^{-5} M). Lane (1) I *Hin*dIII, (2) 100 bp ladder (3) GM-CSF 18 h (4) GM-CSF 18 h + staurosporine 2 h (5) GM-CSF 24 h (6) GM-CSF 24 h/staurosporine 6 h (7) GM-CSF 48 h (8) GM-CSF 48 h + staurosporine 20 h.

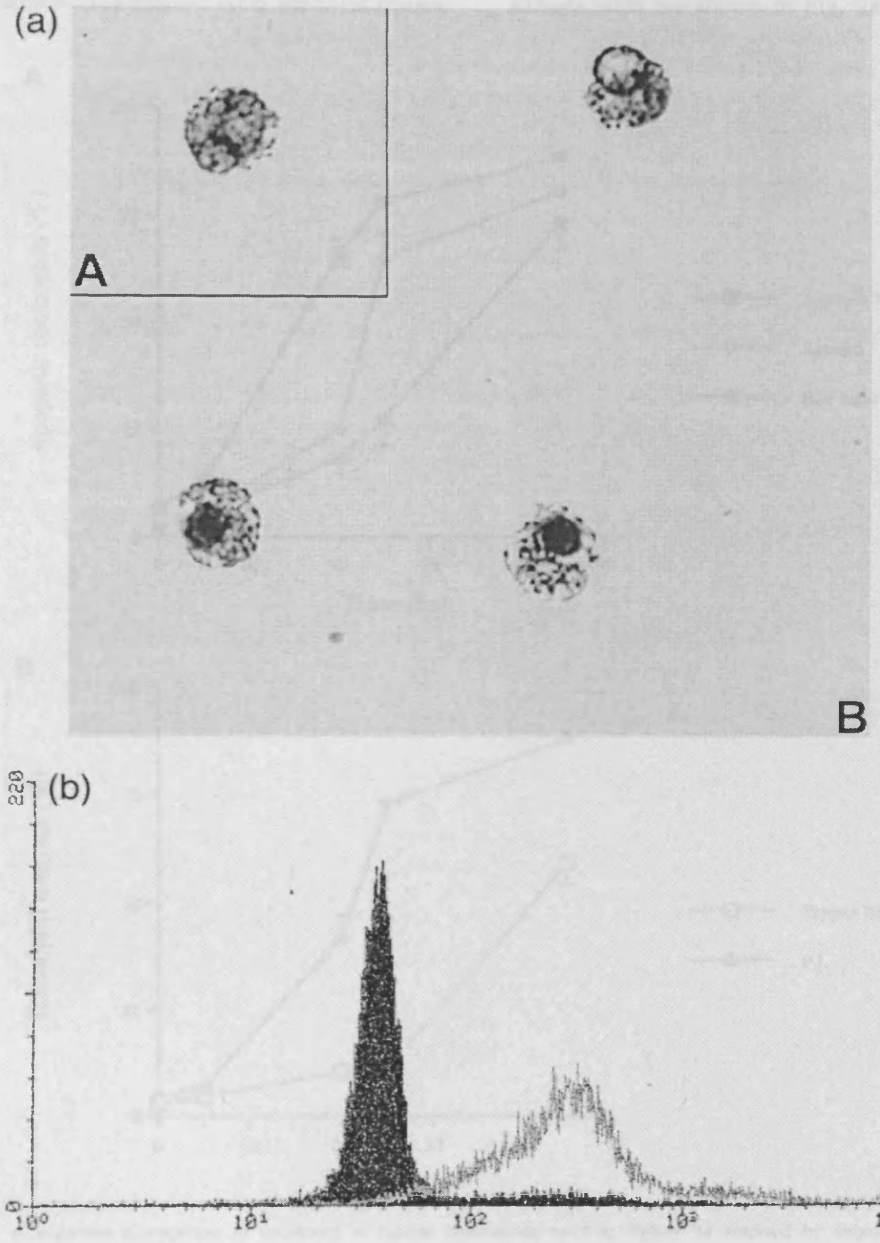

Fig. 2. Representative experiment showing: (A) a wet preparation of peripheral blood eosinophils showing viable (A) and three apoptotic (B) eosinophils stained with Kimura stain (×1000). (B) Representative flow cytometric experiment illustrating the increased red autoflouresence associated with apoptosis in cells aged for 48 h in culture (open trace) compared with that given by freshly isolated eosinophils (filled trace).

tively. No DNA degradation was detectable at 2 h post-staurosporine treatment although significant apoptosis at this time-point was detected by morphological assessment (see below). Near identical results were obtained with eosinophils cultured in IL-5 (data not shown).

Viable and apoptotic eosinophils stained with Kimura stain are shown in Fig. 2a. Apoptotic cells

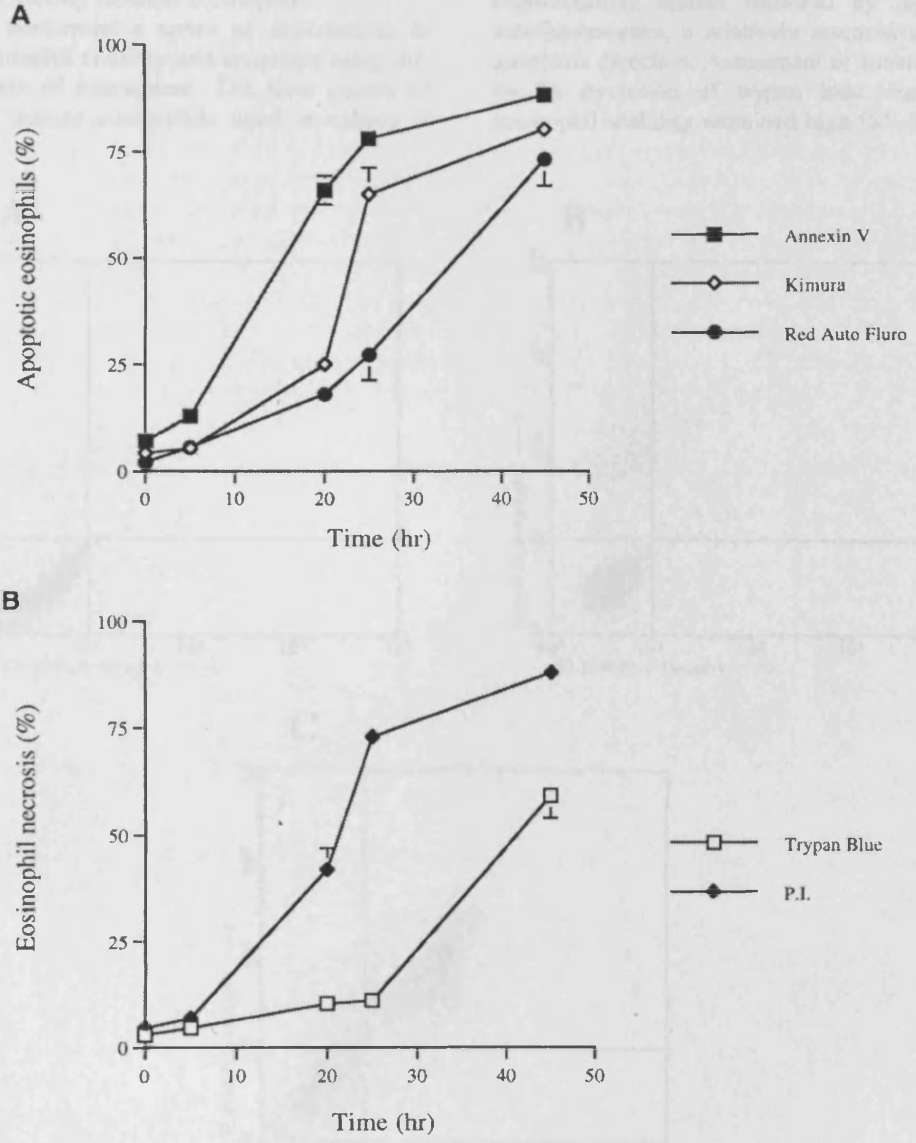

Fig. 3. (A) A time-course comparison of apoptosis in human eosinophils aged in culture as assessed by annexin V-FITC binding, morphological assessment by Kimura staining and increased red autofluorescence. Each point represents the mean ± SEM of four experiments. (B) A time course of the viability of purified eosinophils maintained in culture as assessed by exclusion of trypan blue and light microscopy or propidium iodide (PI) exclusion and flow cytometric assessment. Each point represents the mean ± SEM of four experiments.

had a typical morphology, i.e., decreased cell size and nuclear condensation. Fig. 2b shows a representative flow cytometric experiment illustrating the increased red autofluorescence associated with apoptosis in cells aged for 48 h in culture compared with that given by freshly isolated eosinophils.

We next performed a series of experiments to compare eosinophil viability and apoptosis using different methods of assessment. The time course of apoptosis of human eosinophils aged in culture is shown in Fig. 3a. Annexin V was found to be the most sensitive marker of apoptosis with positive staining in purified eosinophils detected following ageing for 5 h in culture at 37°C. Morphological assessment by Kimura staining proved to be the next most-sensitive marker followed by increased red autofluorescence, a relatively insensitive method of apoptosis detection. Assessment of eosinophil viability by exclusion of trypan blue suggested that eosinophil viability remained high (85–90%) for up

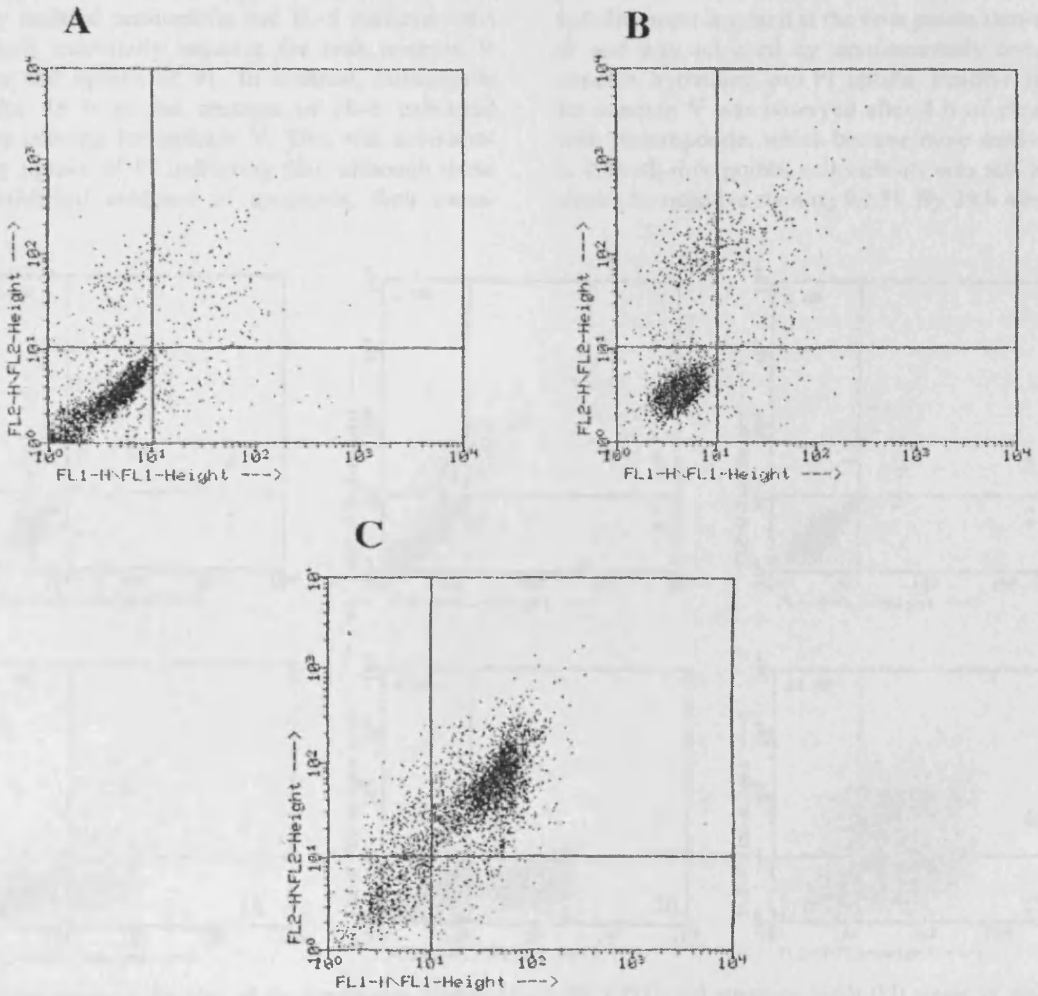

Fig. 4. Flow cytometry dot plots showing the simultaneous binding of annexin V-FITC (FL-1) and propidium iodide uptake (FL2) by: (A) freshly isolated eosinophils, (B) eosinophils aged for 48 h in culture in the presence of IL-5 (10^{-11} M) and (C) eosinophils aged for 48 h in culture in the absence of IL-5. This experiment is representative of three others which gave almost identical results.

to 24 h in culture (Fig. 3b). Thereafter, viability declined to around 60% at 48 h and was associated with high levels of apoptosis as assessed by annexin V binding or morphological assessment (Fig. 3a). However, staining with PI followed by flow cytometry revealed that at least 75% of eosinophils had lost their membrane integrity after 24 h of culture, a feature associated with necrosis (Fig. 3b).

We next assessed the simultaneous uptake of annexin V-FITC and PI by freshly isolated eosinophils and compared them with those aged in culture for 48 h in the presence and absence of IL-5. Freshly isolated eosinophils and IL-5 cultured cells were both essentially negative for both annexin V binding and uptake of PI. In contrast, eosinophils aged for 48 h in the absence of IL-5 exhibited positive staining for annexin V. This was accompanied by uptake of PI indicating that, although these cells exhibited evidence of apoptosis, their membrane integrity had been breached (Fig. 4). Similar results were observed when eosinophils were cultured with GM-CSF (data not shown).

We used the PKC inhibitor staurosporine to induce rapid apoptosis in eosinophils cultured with IL-5. This was an attempt to exclude the possibility that eosinophil-derived granule proteins such as major basic protein or eosinophil cationic protein released from necrotic/apoptotic eosinophils were having a significant cytotoxic effect in our cultures. Purified eosinophils were cultured overnight in IL-5 prior to adding staurosporine. Apoptosis and cell viability were assessed at the time points shown (Fig. 5) and was achieved by simultaneously comparing annexin V binding and PI uptake. Positive staining for annexin V was observed after 4 h of incubation with staurosporine, which became more marked at 6 h. At both time points, cell viability was still high as shown by negative staining for PI. By 24 h almost all

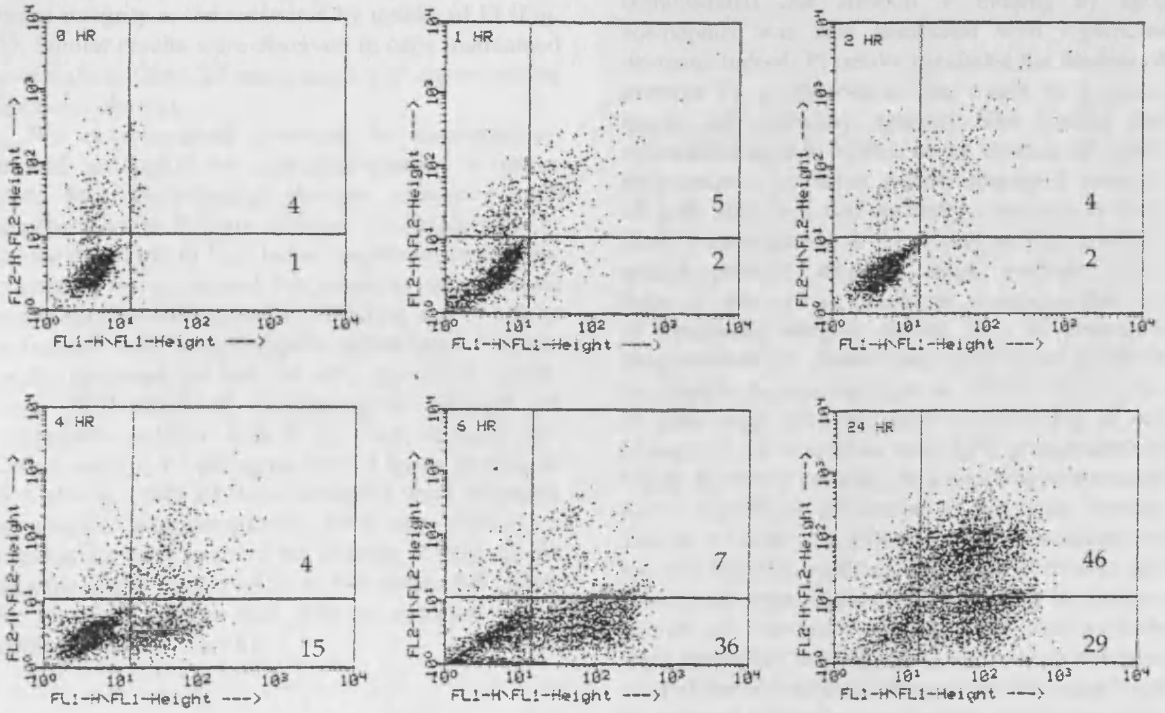

Fig. 5. Flow cytometry dot plots of the simultaneous binding of annexin V-FITC and propidium iodide (PI) uptake by eosinophils maintained for 18 h in IL-5 (10^{-11} M) before addition of staurosporine (10^{-5} M). Eosinophils were assessed for annexin V-FITC binding (FL1) and PI uptake (FL2) at the time points shown. The numbers represent the percentage (%) cells present in the upper and lower right quadrants. This experiment is representative of three others which gave almost identical results.

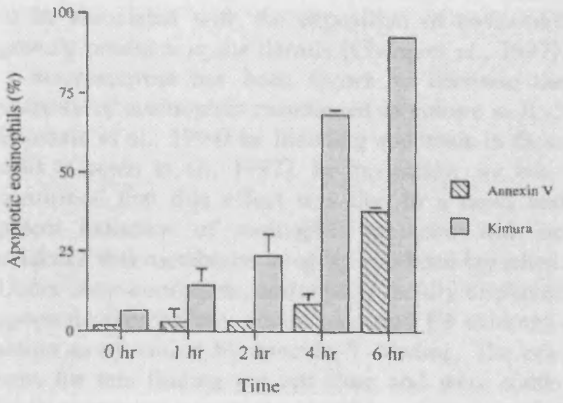

Fig. 6. Time course of apoptosis induction by staurosporine (10^{-5} M) in eosinophils maintained for 18 h in IL-5 (10^{-11} M) before addition of staurosporine. Apoptosis was assessed by annexin V-FITC binding or by morphology (Kimura staining). Each bar represents the mean ± SEM of four experiments.

of the eosinophils were positive for annexin V, but this was associated with a significant loss of membrane integrity as demonstrated by uptake of PI (Fig. 5). Similar results were observed in cells maintained overnight in GM-CSF and treated with staurosporine (data not shown).

We next assessed apoptosis in staurosporine-treated eosinophils by comparing annexin V uptake with the morphological changes associated with apoptosis using Kimura staining. Eosinophils maintained overnight in IL-5 before incubation with staurosporine were assessed for apoptosis and necrosis using simultaneous annexin V binding and PI uptake compared with morphological assessment. Surprisingly, staurosporine induced very rapid and significant PCD-associated morphological changes in eosinophils cultured with IL-5. These changes preceded annexin V binding by several hours. Indeed, at 6 h almost 100% of the eosinophils were apoptotic as assessed morphologically, while only 38% ± 1.5 eosinophils were positive for annexin V (Fig. 6). At all time points examined up to 6 h eosinophil viability remained greater than 90% as assessed by PI uptake (data not shown).

4. Discussion

The safe removal of intact tissue eosinophils from in and around the airways by inducing apoptosis and their subsequent phagocytosis by resident cells or macrophages is a potentially important therapeutic strategy for asthma. Thus, analysis of the mechanisms by which eosinophils can be induced to become apoptotic is a growing area of interest. In this study, we compared a number of methods for their sensitivity in quantifying eosinophil apoptosis under different in vitro conditions. The relationship between eosinophil apoptosis and necrosis was also examined. We demonstrated that eosinophils aged in culture became apoptotic as judged by internucleosomal cleavage of DNA, morphological criteria, increased autofluorescence in the red spectrum and the binding of annexin V-FITC. The latter appeared to be the most sensitive marker of eosinophil apoptosis followed closely by morphological assessment by a one-step staining method with Kimura. Under these conditions of apoptosis induction, eosinophil viability appeared high when judged by the exclusion of trypan blue. However, the use of PI as a vital dye, demonstrated that annexin V binding by aged eosinophils was also associated with significant necrosis. Indeed, PI uptake paralleled the binding of annexin V, an observation that might be a consequence of secondary necrosis. The finding that eosinophils aged in culture in the absence of viability-promoting cytokines rapidly displayed evidence of both apoptosis and secondary necrosis is most likely a consequence of the release of their cytotoxic granule products into the culture medium. Under these in vitro culture conditions, it appears that loss of membrane integrity occurs early in eosinophil programmed cell death. Early removal of apoptotic eosinophils by macrophages or resident tissue cells in vivo might thus be vital in preventing in situ leakage of the ubiquitous eosinophil granule-derived highly cytotoxic proteins. It is tempting to speculate that a significant proportion of the tissue damage seen in the asthmatic airway is not a consequence of specific receptor-mediated eosinophil activation and subsequent degranulation. Instead, it may be a consequence of non-specific leakage of toxic proteins from apoptotic/necrotic cells which have not been cleared due to a defect in recognition and engulfment by relevant infiltrating or resident phagocytic cells. Indirect evidence for this notion is provided by the recent observation that dermal eosinophils in patients with atopic dermatitis do not become apoptotic but

undergo cytolytic degeneration and this was shown to be associated with the deposition of eosinophil granule products in the dermis (Cheng et al., 1997).

Staurosporine has been shown to decrease the viability of eosinophils maintained in culture in IL-5 (Hossain et al., 1994) by inducing apoptosis in these cells (Cousin et al., 1997). In this study, we have confirmed that this effect was due to a rapid and potent induction of eosinophil apoptosis with no evidence that membrane integrity had been breached. Under these conditions, eosinophils rapidly displayed apoptotic morphology which preceded PS externalisation as measured by annexin V binding. The reasons for this finding are not clear and were somewhat surprising given the number of reports that externalisation of PS and annexin V binding in several different cell types precedes other manifestations of apoptosis, including morphological changes (Koopman et al., 1994; Bennet et al., 1995; Homburg et al., 1995; Vermes et al., 1995). However, the rapid reduction in volume characteristic of apoptotic eosinophils was shown to be a result of both nuclear and cytoplasmic condensation. Furthermore, this was an active process linked to the loss of internal ions since it could be inhibited by addition of K^+ channel blockers (Beauvais et al., 1995). Thus, it is possible that the inhibition of PKC by staurosporine results in profound reductions in internal ion levels which precedes the externalisation of PS to the outer membrane leaflet. An alternative explanation is that staurosporine induced these profound changes in eosinophil volume via an additional pharmacological effect which was not necessarily associated with apoptosis induction. Thus, different methods of apoptosis induction in human eosinophils might preferentially induce different parameters thought to be associated with programmed cell death.

In summary, our results demonstrate that annexin V-FITC binding represents a convenient and reproducible early marker of eosinophil apoptosis. However, its use should be combined with PI uptake to permit simultaneous assessment of necrosis. Moreover, staurosporine treatment of eosinophils resulted in the rapid induction of apoptotic morphology. Indeed, this event preceded PS externalisation by several hours. These data also confirm our previous findings (Walsh et al., 1996) that a simple single-step Kimura staining of wet preparation cells represents an accurate, reproducible and relatively rapid method for assessing apoptosis in human eosinophils by morphological criteria. This method also has the added advantages of being cheap and of not requiring access to a flow cytometer. Finally, it would appear that, compared with PI uptake, assessment of eosinophil viability by exclusion of trypan blue appears to be an insensitive and misleading measure of eosinophil membrane integrity and should only be used with this limitation in mind.

Acknowledgements

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Expression of Bcl-2 and Its Homologues in Human Eosinophils
Modulation by Interleukin-5

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The Bcl-2 family has been shown to be vital regulators of programmed cell death in numerous systems. To investigate the role of such proteins in the regulation of apoptosis of eosinophils, the expression of Bcl-2 and homologues Bcl-x_L (death antagonists), Bax, and Bcl-x_S (death agonists) were examined by immunoblot, flow cytometry, and reverse transcriptase–polymerase chain reaction analysis. Potential modulation of apoptosis-associated molecules during spontaneous apoptosis and in the presence of interleukin (IL)-5 was also investigated. Peripheral blood eosinophils were found to express constitutively Bax and Bcl-x, but Bcl-2 was absent. Analysis of mRNA revealed that the *bcl-x_L* isoform predominated, although *bcl-x_S* was also detectable. Spontaneous apoptosis due to culturing in the absence of cytokines for 24 h did not result in modulation of any of the Bcl-2 homologues examined. Culturing eosinophils in the presence of 100 pg/ml IL-5 for 24 h significantly reduced apoptosis ($P < 0.01$) to 10.7 ± 2.6% compared with 46.8 ± 7.4% in the absence of IL-5, and induced Bcl-2 mRNA and protein expression, with no detectable change in Bax, Bcl-x, or β-actin as a control. This investigation indicates a specific profile of apoptotic molecules in eosinophils distinct from that of neutrophils, and indicates that survival-enhancing IL-5 modulates the expression of Bcl-2 *in vitro*. **Dewson, G., G. M. Walsh, and A. J. Wardlaw. 1999. Expression of Bcl-2 and its homologues in human eosinophils: modulation by interleukin-5. Am. J. Respir. Cell Mol. Biol. 20:720–728.**

Eosinophils have a proposed vital role in the pathogenesis of a number of disease states, with tissue and blood eosinophilia being associated with asthma, atopic allergy, and helminthic parasite infections (1–4). The accumulation and persistence of these granulocytes at sites of inflammation is due to a number of factors, such as selective adhesion and migration (5), as well as increased survival in response to growth factors such as interleukin (IL)-5 (6), IL-3, and granulocyte macrophage colony-stimulating factor (GM-CSF) (7), either from exogenous sources, such as T cells, or as a result of autocrine elaboration by eosinophils themselves (8). These hematopoietins have been proven to abrogate eosinophil apoptosis *in vitro* (9), and *ex vivo* using nasal polyposis as a model of tissue inflammation (10, 11). The observation that protection from apoptosis afforded by IL-5 is inhibitable by cycloheximide and actinomycin D suggests that RNA and protein synthesis is required (12).

The release of eosinophil intracellular mediators has been found to be histotoxic, causing tissue lesions and airway inflammation (reviewed in 13). In contrast to necrosis, apoptosis provides a mechanism whereby membrane integrity is maintained throughout cell death, allowing ingestion and clearance of whole cells or discrete vesicles ("apoptotic bodies") by resident phagocytes without toxic mediator release (6, 14). Apoptosis of eosinophils may therefore provide a crucial mechanism for the limitation of inflammation (15–17).

Eosinophils exhibit the classic apoptosis-associated morphological changes such as internucleosomal nucleic acid cleavage, cytoplasmic condensation (18), and redistribution of membrane phospholipids (19), allowing binding of AnnexinV as an early marker of apoptosis (20, 21). A number of extracellular modulators of eosinophil apoptosis have been elucidated. Monoclonal antibody (mAb)-dependent ligation of the Fas receptor (22, 23) or CD69 (24) and treatment with glucocorticoids (25) have been shown to initiate programmed cell death (PCD). Eosinophil survival is enhanced by adhesion to tissue fibronectin via very late antigen-4 (26, 27) or treatment with lipopolysaccharide (28), leading to elaboration of GM-CSF. However, little is known about the intracellular signaling

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Abbreviations: glyceraldehyde-3-phosphate dehydrogenase, GAPDH; interleukin, IL; monoclonal antibody, mAb; polyclonal antibody, pAb; peripheral blood eosinophils, PBE; phosphate-buffered saline, PBS; reverse transcriptase–polymerase chain reaction, RT–PCR; specific median fluorescence, SMF.

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mechanisms employed in the regulation of eosinophil apoptosis.

The *bcl-2* proto-oncogene was first described at chromosomal breakpoint of t(14:18) found in B-cell lymphomas (29, 30), and was determined to be the mammalian homologue of the *Caenorhabditis elegans* death repressor gene, *ced-9* (31, 32). A large family of genes with a sequence homology to *bcl-2* has emerged that possesses important divergent functions in the mediation of PCD, consisting of death antagonists (*bcl-2*, *bcl-x_L*, *bcl-w*) or death agonists (*bax*, *bcl-x_S*, *bak*) (33). Alternative transcription of the *bcl-x* gene results in two distinct mRNAs, the larger transcript, *bcl-x_L*, encoding a death suppressor, and the second variant, *bcl-x_S*, smaller due to an internal deletion, encoding a protein capable of antagonizing the effects of Bcl-x_L (34, 35). Bcl-2 homologues form a dynamic equilibrium of homo-heterodimerization via conserved regions and associate with unrelated proteins, such as protein kinase Raf-1 (36, 37), to form a complex life-death rheostat linked to growth factor receptors and kinase signaling cascades. Most have a transmembrane region allowing localization to the mitochondrial membrane and the potential to mediate the release of apoptogenic factors such as cytochrome C from the organelle (38–40). Peripheral blood lymphocytes express Bcl-2, Bcl-x, and Bax, although neutrophils express only the latter homologue (41, 42). Also, neutrophils transgenic for the expression of *bcl-2* have inhibited apoptosis but not engulfment by phagocytes *in vitro* (43). The literature is currently unclear regarding the expression of Bcl-2 homologues in eosinophils. Druilhe and colleagues (44) have recently shown by immunoblot analysis the constitutive expression of Bax, with Bcl-x_L and Bcl-2 essentially absent from peripheral blood eosinophils (PBE). The lack of constitutive Bcl-2 expression supports the findings of a previous investigation (45). However, these studies report conflicting observations on the potential role of IL-5 in the regulation of Bcl-2 in PBE. In contrast, Yasui and coworkers (46) indicated constitutive expression of Bcl-2, assessed by flow cytometry, which is downregulated by the potential anti-inflammatory agent theophylline.

In this study, to elucidate the regulation of eosinophil apoptosis *in vitro*, the expression of *bcl-2* and its homologues *bax*, and splice variants *bcl-x_S* and *bcl-x_L*, were investigated by reverse transcriptase–polymerase chain reaction (RT–PCR), immunoblot, and flow cytometric analysis. Potential modulation of these apoptosis mediators by survival-enhancing concentrations of IL-5 was also examined. The results indicate that anti-apoptotic *bcl-2* is absent from unstimulated eosinophils but is upregulated by IL-5. Bax and Bcl-x were constitutively expressed in freshly isolated PBE. Both *bcl-x_L* and *bcl-x_S* splice variants were detectable, but *bcl-x_L* predominated. Expression of Bax and Bcl-x was not modulated by IL-5.

Materials and Methods

Cytokines and Antibodies

Recombinant human IL-5 was obtained from R&D Systems (Abingdon, UK). Murine human Bcl-2-specific mAb 124, and Bax (N-20) and Bcl-$x_{s/l}$ (S-18)-specific rabbit antihuman polyclonal antibodies (pAb) were obtained from Autogen Bioclear UK Ltd. (Wilts, UK for Santa Cruz Biotech, Santa Cruz, CA). Human β-actin-specific mAb was obtained from Sigma Chemical Co. (Poole, UK). Control mouse and rabbit immunoglobulin Gs, fluorescein isothiocyanate (FITC), and biotin-conjugated antimouse/rabbit secondary antibodies were obtained from Dako Ltd. (High Wycombe, UK).

Cell Lines

Human myeloid cell line HL-60 and T-cell line Jurkat were obtained from European Collection of Cell Cultures (Wilts, UK).

Isolation of PBE and Cell Culture

Heparinized peripheral venous blood was taken from volunteers with a normal or slightly raised eosinophil count. Eosinophils were purified by a two-step method of density gradient centrifugation, followed by negative immunomagnetic selection as described previously (58). Briefly, removal of erythrocytes by dextran sedimentation was followed by slow centrifugation (100 × *g*, 15 min, room temperature) of leukocyte-rich supernatant, and resuspension in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} supplemented with 2% heat-inactivated fetal calf serum (FCS), and 0.02 mol/L ethylenediaminetetraacetic acid. Leukocytes were then centrifuged (400 × *g*, 25 min, room temperature) on a Histopaque 1083 (Sigma Chemical Co.). Mononuclear cells were carefully removed, and erythrocytes contaminating the granulocyte pellet were lysed by hypotonic shock using sterile, ice-cold water. Eosinophils were separated from neutrophils by negative immunomagnetic selection on the basis of CD16-coated immunomagnetic beads (Miltenyi Biotec Inc., Auburn, CA). Postisolation purity and viability, assessed by Kimura stain and exclusion of trypan blue, respectively, were routinely > 99%. Freshly isolated PBE were washed and cultured at 3×10^6 cells ml^{-1} in RPMI 1640 L-glutamax (GIBCO-BRL, Paisley, UK) supplemented with 10% FCS and antibiotics. HL-60 and Jurkat cell lines were similarly cultured in media that lacked antibiotics. Eosinophils were stimulated by the presence of 100 pg/ml IL-5 where indicated.

Assessment of Eosinophil Apoptosis and Viability

Eosinophil viability was assessed by trypan blue exclusion counting at least 200 cells by light microscopy. Apoptosis was assessed by light microscopy on the basis of cells exhibiting classic apoptotic morphology, such as nuclear and cytoplasmic condensation in the presence of Kimura stain (21).

Immunoprecipitation and Immunoblotting

Cells were washed in cold phosphate-buffered saline (PBS), lysed in ice-cold 1% Triton X-100 isotonic lysis buffer containing freshly added protease inhibitors (100 μg/ml phenylmethyl sulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A), and incubated on ice for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation (12,000 × *g*, 5 min, 4°C). For immunoprecipitation, the lysate was precleared overnight at 4°C with constant agitation, with 50 μl of 1:1 protein A-Sepharose, which was

removed by centrifugation at 400 × *g* for 2 min. Specific antibody or control were added for 60 min and captured by protein A-Sepharose for 60 min at 4°C. Immunoprecipitates were sequentially washed twice in dilution buffer (0.1% Triton X-100; 0.1% bovine hemoglobin; 0.01 M Tris-Cl, pH 8.0; 0.14 M NaCl), followed by a wash in dilution buffer without Triton X-100, followed by a wash in 0.05 M Tris-Cl, pH 6.8.

Equal volume of Laemmli buffer (5% β-mercaptoethanol) was added to immunoprecipitate or whole-cell lysate and boiled for 4 min prior to electrophoresis through 12.5% sodium dodecyl sulfate polyacrylamide gels. Proteins were electrotransferred onto Hybond nitrocellulose membrane (Amersham Life Science Ltd., Bucks, UK). Nonspecific binding sites on the membrane were blocked by incubation (1 h, room temperature) with 5% nonfat milk (Marvel) in PBS 0.1% Tween-20 (PBS-T). Filters were incubated with specific primary antibody/negative control, diluted to 0.1 μg/ml in blocking buffer (2 h, room temperature). The membrane was then incubated (1 h, room temperature) with species-specific biotinylated secondary antibody (1:3,000), followed by reaction with horseradish peroxidase-streptavidin (1:1,000) (1 h, room temperature). Each incubation was performed with constant agitation and followed by 1 × 15 min and 2 × 5 min wash with PBS-T. Membranes were then developed using enhanced chemiluminescent system according to the manufacturer's instructions and exposed to Hyperfilm (Amersham).

Intracellular Flow Cytometric Analysis

Cells were washed in PBS prior to fixation and permeabilization using a kit according to the manufacturer's instructions (Bradsure Biologicals, Bucks, UK). During the permeabilization step, cells were incubated with 1 μg/ml primary antibody (25 min, room temperature). Cells were then washed in PBS and incubated with species-specific FITC-conjugated secondary antibody, diluted 1:100 in PBS (25 min, room temperature, in dark). After being washed in PBS, cells were analyzed on FACScan (Becton Dickinson, Oxford, UK). Quantification of the flow cytometry data was performed on the basis of specific median fluorescence, which was calculated using the following equation:

Specific median fluorescence =
Median fluorescence of test antibody −
Median fluorescence of isotype-matched control.

Analysis of mRNA

Total RNA was extracted from cells using TRIzol according to manufacturer's protocol (GIBCO-BRL). PolyA$^+$ RNA was primed with oligo dT (GIBCO-BRL) and reverse transcribed with *Superscript* RT (GIBCO-BRL) for 1 h at 37°C. The cDNA product was amplified by PCR using primers obtained from Cruachem Ltd. (Glasgow, UK) specific for *bcl-2* (5′ primer GATGTCCAGCCAG CTGCACCTG; 3′ primer-CACAAAGGCATCCCAGCCTCC), *bax* (5′ primer-GGACCCGGTGCCTCAGGA; 3′ primer-

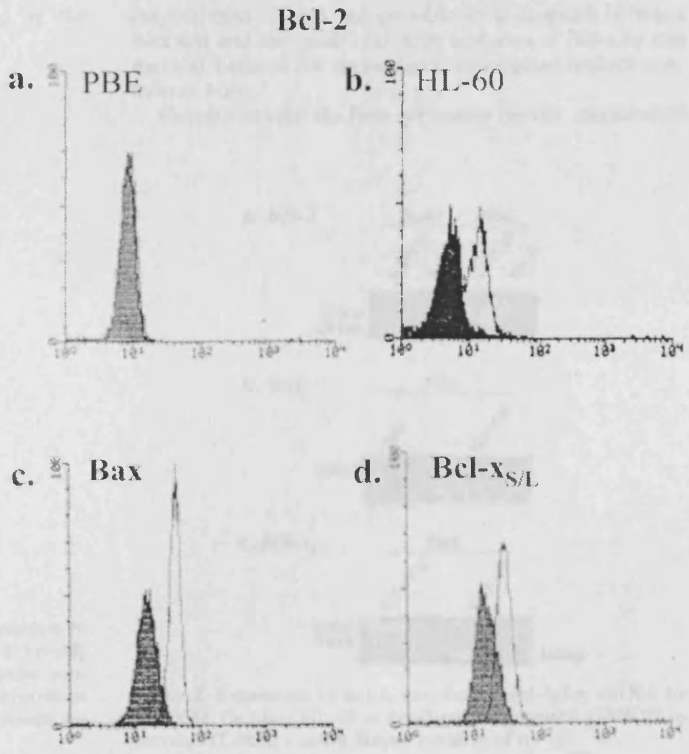

Figure 1. Expression of apoptosis-associated proteins in freshly isolated peripheral blood eosinophils (PBE) assessed by flow cytometry. PBE (*panels a, c,* and *d*) or HL-60 cell line (*panel b*) were fixed, permeabilized and stained with anti-Bcl-2 (*panels a* and *b*), anti-Bax (*panel c*), and anti-Bcl-x (*panel d*) according to MATERIALS AND METHODS. Test antibody (*hollow histogram*); irrelevant control (*solid histogram*). Representative of $n = 4$.

CAAAGATGGTCACGGTCTGC), *bcl-$x_{s/l}$* (5′ primer-TTGGACAATGGACTGGTTGA; 3′ primer-GTAGAG-TGGATGG TCAGTG), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a positive control (5′ primer-GGGAAGCTCACT GGCATGGCCTTCC; 3′ primer-CATGTGGGCCATGAGGTCCACCAC). The *bcl-$x_{s/l}$* primers corresponded to the 5′ and 3′ untranslated regions and allowed simultaneous amplification of *bcl-x_s* (0.6 kb) and *bcl-x_l* (0.8 kb). A 50-μl PCR reaction was set up containing 5 μl cDNA, 10 mM Tris-HCl, 25 pmol each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U BioTaq DNA polymerase (Bioline, London, UK). Amplification consisted of 30 to 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C (*bcl-2*) or 58°C (*bax*, *bcl-$x_{s/l}$*) for 30 s, and extension at 72°C for 1 min. Amplification products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining under ultraviolet light and photographed. Photographs were scanned and imported into Microsoft Power Point 97 (Redmond, WA).

Verification of RT-PCR Products

RT-PCR product was excised from the agarose gel, and the DNA was extracted from the gel using gel extraction columns according to the manufacturer's protocol (Qiagen Ltd., Sussex, UK) and eluted with water. The product was then verified by direct automated sequencing or "nested PCR."

Sequencing reactions were performed using a 30-ng PCR product, with a dRhodamine terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Bucks, UK) according to the manufacturer's protocol. Automated sequencing of the product was kindly performed by the Protein and Nucleic Acid Chemistry Laboratory, Leicester University, Leicester, UK.

Nested PCR was also performed on the purified RT-PCR products. The primary PCR product was used as a template for a second reaction, involving a third specific primer sequence internal to the initial primer pair. The reduction in size of the product could then be verified on an agarose gel. The reaction conditions were as described previously, using only 1 μl of purified PCR product as a template. Nested primers obtained from Cruachem Ltd. (Glasgow, UK), specific for *bcl-2* (3′ primer-GACGCT-CTCCACACACATGACC), *bax* (3′ primer-AGGAAG TCCAATGTC CAG), *bcl-x_s* (3′ primer-CCACAAAAG-TATCCTGTT CAAAGC), and *bcl-x_l* (3′ primer-CCA-AGCTGCGATCCGACTCAC).

Statistical Analysis

Where applicable, values are expressed as mean ± SEM. The mean data from IL-5-treated cells were compared with the untreated cells by paired Student's *t* test. A *P* value of < 0.05 was regarded as significant.

Results

Detection of Apoptosis-Associated Proteins

Bcl-2 was not detectable in unstimulated eosinophils (Figure 1a) compared with HL-60 cell line as a positive control (Figure 1b), as assessed by intracellular flow cytometry. Good constitutive expression of Bax specific median fluorescence ([SMF], 26.3 ± 0.76) and Bcl-x (SMF, 11.3 ± 1.42) in freshly isolated PBE was observed (Figures 1c and 1d, respectively). It was not possible to distinguish between Bax α/β and the small and large isoforms of Bcl-x by this method, because the antibodies used targeted regions common to both.

Consistent with the flow cytometry results, immunoblot

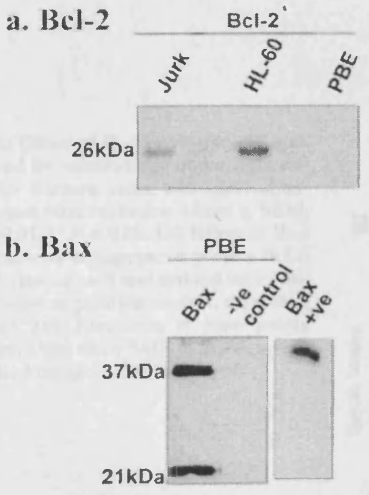

Figure 2. Immunoblot analysis of Bcl-2 and Bax expression in whole-cell lysates under reducing conditions. (*a*) Bcl-2: Freshly isolated PBEs and cell lines Jurkat and HL-60 as positive controls. (*b*) Bax: Freshly isolated PBE and 45 kD fusion protein as positive control. Whole-cell lysate was probed with irrelevant antibody as negative control.

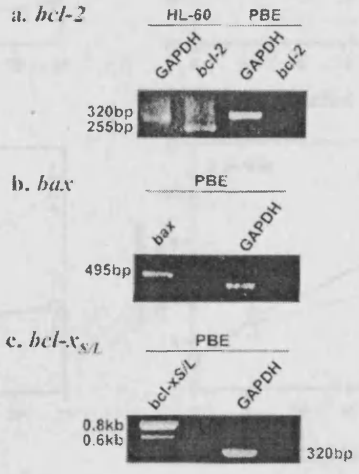

Figure 3. Expression of *bcl-2*, *bax*, *bcl-x_l*, and *bcl-x_s* mRNA by RT-PCR. Cell line HL-60 as *bcl-2*-positive control; GAPDH as internal RT-PCR control. Representative of $n = 3$.

analysis detected the 26-kD Bcl-2 in Jurkat and HL-60, but the protein was absent from freshly isolated eosinophils (Figure 2a). Bax has been shown to be expressed as at least two alternative transcripts, *bax*-α and *bax*-β, encoding proteins of 21 kD and 24 kD, respectively (45). Immunoblot analysis of whole-cell lysates revealed that eosinophils express only Baxα, with no detectable expression of Baxβ (Figure 2b). The immunoblot positive control for Bax was a purified Bax fusion protein. Under stringent reducing conditions, a 37-kD band was consistently observed in eosinophil whole-cell lysates probed with anti-Bax pAb. Bcl-x could not be detected by immunoprecipitation, immunoblotting of eosinophil, or HL-60 whole-cell lysates. However, a Bcl-x fusion protein-positive control (Autogen Bioclear UK Ltd.) was readily detectable (data not shown).

Analysis of mRNA Expression

RT-PCR indicated that freshly isolated eosinophils do not express message for *bcl-2* (Figure 3a) compared with HL-60 as a positive control for *bcl-2* at predicted 255 bp, and GAPDH amplification as an internal PCR control. The *bax* (495 bp) and *bcl-x*$_L$ (0.8 kb) mRNA were readily detectable in unstimulated PBE (Figures 3b and 3c). Coamplification of *bcl-x*$_S$ (0.6 kb) mRNA was also consistently detectable, but to a lesser degree relative to the longer splice variant (Figure 3c). The PCR products were verified by direct sequencing and/or nested PCR (data not shown).

Effect of IL-5 on Eosinophil Survival and Bcl-2 Protein Expression

Culturing eosinophils in the presence of IL-5 caused a significant reduction in apoptosis, assessed by visual examination of morphology, and increased viability, assessed by trypan blue exclusion, compared with medium control (Figure 4A). Occurrence of apoptotic morphology in the presence of 100 pg/ml IL-5 was consistently < 10% after 24 h, compared with 40 to 50% in the absence of IL-5.

Stimulation of PBE with IL-5 for 24 h induced a limited, but significant, expression of Bcl-2, with no change in expression in eosinophils cultured in the absence of the cytokine as detected by flow cytometry (Figure 4B). This upregulation of Bcl-2 was not as a result of a nonspecific change in protein levels as shown by a lack of significant variation of the internal control, β-actin (Figure 4B). Bcl-2 in IL-5–treated eosinophils was undetectable by immunoblot analysis (data not shown).

Incubation of eosinophils with IL-5 for 24 h was found to have no significant effect on the levels of Bax and *bcl-x*$_{s/l}$ expression as determined by flow cytometry (Figure 5).

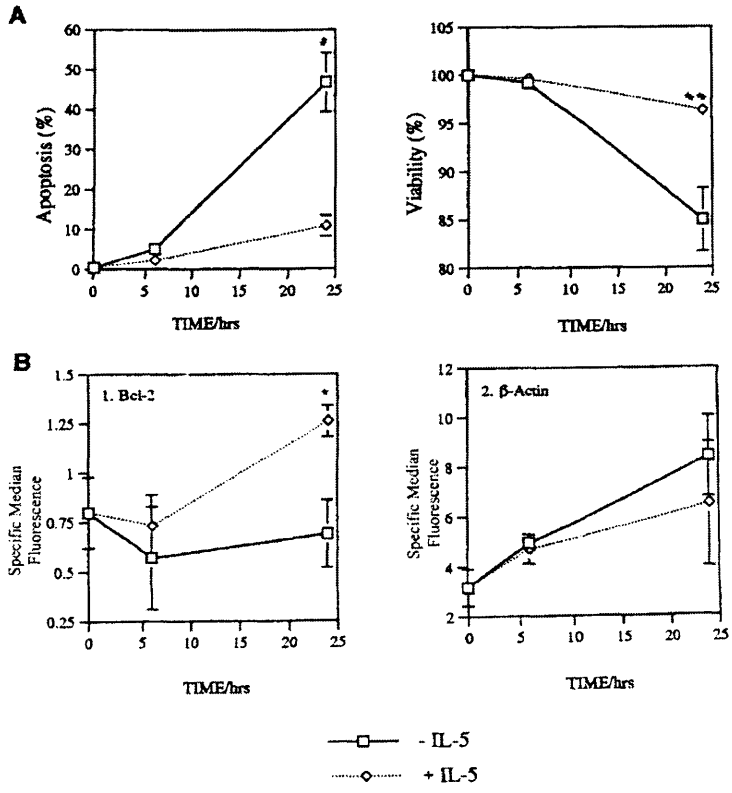

Figure 4. (*A*) Effect of IL-5 on eosinophil apoptosis assessed by morphology under light microscopy after Kimura stain, and survival assessed by trypan blue exclusion. Mean ± SEM, $n = 3$. $^*P < 0.01$; $^{**}P < 0.05$. (*B*) Effect of IL-5 on the expression of antiapoptosis protein Bcl-2. Cells were permeabilized and stained with anti-Bcl-2 and β-actin as positive control, according to MATERIALS AND METHODS, at time points during culture. Data show SMF ± SEM, $n = 3$. $^*P < 0.025$ IL-5 compared with control.

Effect of IL-5 on the Expression of *bcl-2* mRNA

Amplification of *bcl-2* mRNA (250 bp) was detectable in eosinophils stimulated with IL-5 for 24 h, but not unstimulated or cells cultured in medium alone (Figure 6). The signal intensity of PCR product relative to internal GAPDH control and HL-60 *bcl-2* product indicate a low expression of message in stimulated eosinophils, consistent with the levels of detectable protein (Figure 5).

Discussion

A number of extracellular modulators of eosinophil apoptosis have been elucidated, but little is known of the intracellular mechanisms employed in the regulation of eosinophil programmed cell death. In this study, we examined the expression of proteins previously shown to have significant roles in the mediation of apoptosis, the death antagonists Bcl-2 and Bcl-x_L, and the death agonists Bax and *Bcl-*x_S. We observed that the PBE from healthy donors constitutively express relatively high levels of Bax determined by intracellular flow cytometry, immunoblotting, and RT–PCR. The *bax* gene has been shown to be expressed as numerous tissue-specific splice variants, which predict a membrane protein (α, 21 kD) or cytosolic proteins (β, 24 kD; γ, 5 kD) (47). Immunoblot analysis of eosinophil whole-cell lysates revealed the sole expression of a 21-kD protein, corresponding to the α isoform of Bax. Interestingly, immunoblot analysis with anti-Bax pAb consistently detected a 37-kD band in eosinophil whole-cell lysates. The nature of this protein is as yet undetermined, but it is unlikely to be a result of the homo-/heterodimerization of Bax observed in the regulation of apoptosis (36, 47) because of the stringent reducing conditions employed. It is possible that this band represents the newly

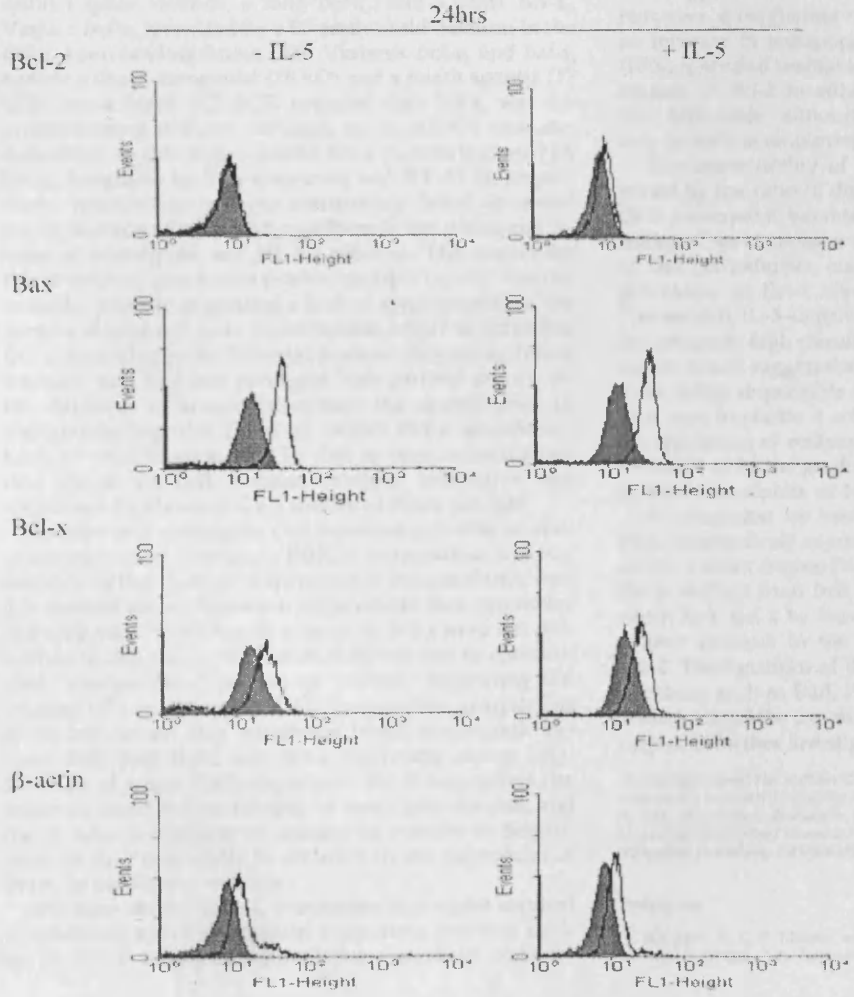

Figure 5. Effect of IL-5 on the expression of apoptosis-associated proteins in PBE assessed by flow cytometry. Freshly isolated PBE (0 h) or PBE cultured in the presence (IL-5) or absence (MED) of 100 pg/ml IL-5 (24 h) were permeabilized and stained as indicated. Test antibody (*hollow histogram*); irrelevant control (*solid histogram*). Representative of $n = 3$.

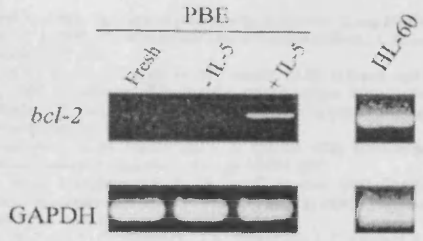

Figure 6. Effect of IL-5 on the expression of *bcl-2* mRNA in PBE. Total RNA was isolated from freshly isolated PBE, eosinophils cultured with IL-5 for 24 h, HL-60 cell line as *bcl-2* positive control, and *bcl-2* amplified by RT-PCR. GAPDH as internal RT-PCR control. Results representative of $n = 3$.

described Bax ω splice variant (48), although further investigation is required to confirm this.

Bcl-x protein was also readily detectable by flow cytometry. Alternative splicing of the *bcl-x* gene generates two distinct splice variants, a long *bcl-*x_L, and a short *bcl-*x_S. Variant *bcl-*x_S is created by a 63-amino acid deletion in the *bcl-*x_L open-reading frame (33). Variants *bcl-*x_L and *bcl-*x_S encode a death antagonist (28 kD) and a death agonist (17 kD), respectively. RT-PCR revealed that *bcl-*x_L was the predominating isoform, although *bcl-*x_S mRNA was also detectable. In this study, despite Bcl-x protein and mRNA being detectable by flow cytometry and RT-PCR, respectively, immunoblot analysis consistently failed to reveal the expression of either Bcl-x isoform in the whole-cell lysates of eosinophils and HL-60 cell line. The reason for this is unclear, as a fusion protein-positive control was detectable, possibly suggesting a lack of solubilization of the protein during cell lysis. Considerable effort to solubilize Bcl-x, including pellet homogenization, sonication, freeze fracture, and multiple detergent lysis proved unsuccessful. Attempts to immunoprecipitate the protein prior to immunoblotting also failed to detect Bcl-x specifically. Lack of solubilization may be due to compartmentalization within the cell, because complex interaction and membrane localization are a feature of Bcl-x (49, 50).

Druilhe and colleagues (44) reported a profile of apoptosis-associated proteins in PBE by immunoblot analysis, similarly failing to detect expression of Bcl-x isoforms with this method alone. However, intracellular flow cytometry and analysis of mRNA with respect to Bcl-x were not performed in this study. PBE were observed not to constitutively express Bcl-2 protein or mRNA, supporting the findings of a recent report (45). Immunoblot analysis has previously shown that peripheral blood neutrophils express Bax, with Bcl-2 and Bcl-x essentially absent (41). The lack of major death suppressor Bcl-2 may reflect the relatively short *in vivo* lifespan of these granulocytes, and the *in vitro* persistence of eosinophils relative to neutrophils (6) may potentially be afforded by the expression of Bcl-x_L in the former cell type.

We have shown that IL-5 increases eosinophil survival by inhibiting apoptosis, thereby supporting previous studies (6, 12). It has been reported that a requisite of IL-5–dependent abrogation of eosinophil apoptosis is the new synthesis of mRNA and protein (12). It has also been shown recently that Bcl-2 and Bcl-x_L can act as substrates for caspase-3, activated during apoptosis (52, 53). Therefore, we investigated the potential modulation of Bcl-2 members during spontaneous apoptosis and in the presence of survival-enhancing concentrations of IL-5. Spontaneous apoptosis was observed when culturing eosinophils in the absence of cytokines for 24 h (46.8 ± 7.4%), but no variation in the expression of Bcl-2, Bax, or Bcl-x was detected at the level of gene or protein. Culturing PBE in the presence of 100 pg/ml IL-5 for 24 h maintained cell survival (10.7 ± 2.6), and was found to induce detectable *bcl-2* mRNA, but at a low level relative to GAPDH internal control and HL-60 *bcl-2*–positive control. Flow cytometry revealed a small but significant increase in Bcl-2 expression, without significant variation in β-actin control, consistent with the observed increase in message. However, the small increase in Bcl-2 protein was undetectable by immunoblotting. There was no observable modulation in the expression of Bax and Bcl-x. This upregulation of Bcl-2 by IL-5 supported the findings of Ochiai and coworkers (45). However, a conflicting report (44) suggested that despite an increase in antiapoptosis protein Mcl-1 in interferon (IFN)-γ–treated umbilical-cord-blood eosinophils, no modulation of Bcl-2 in either IL-5– or IFN-γ–treated PBE was detectable, although immunoblot analysis was the only technique employed.

The susceptibility of a cell to death signals is determined by the ratio of death agonist:death antagonist, and their subsequent interaction via homo- and heterodimerization. Overexpression of Bax, leading to predomination of Bax homodimers, encourages apoptosis (47), whereas prevalence of Bcl-2 affords protection for the cell (53). The modest IL-5–dependent Bcl-2 increase, coupled with the relatively high constitutive expression of Bax in eosinophils, would suggest that upregulation of Bcl-2 is unlikely to be solely responsible for the protective effects of IL-5. This may implicate a role for other death antagonists in the regulation of eosinophil apoptosis, such as Bcl-x_L, determined to be the predominating Bcl-x isoform in freshly isolated eosinophils, or Mcl-1.

In conclusion, we have determined that freshly isolated PBE constitutively express predominantly Bax and Bcl-x_L, and to a lesser degree Bcl-x_S, but Bcl-2 is absent. This profile is distinct from that of peripheral blood neutrophils, which lack Bcl-x by immunoblot analysis. IL-5 induces a modest increase in the expression of death antagonist Bcl-2. The functions of the more recently described Bcl-2 members, such as Bad, Bcl-w, and Bag-1 (54–56), and the pivotal role of the caspases in eosinophil apoptosis are the subject of further investigation.

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Interleukin-5 inhibits translocation of Bax to the mitochondria, cytochrome *c* release, and activation of caspases in human eosinophils

Grant Dewson, Gerald M. Cohen, and Andrew J. Wardlaw

The apoptosis and subsequent clearance of eosinophils without histotoxic mediator release is thought to be crucial in the resolution of airway inflammation in asthma. Interleukin-5 (IL-5) is a potent suppressor of eosinophil apoptosis. The mechanism by which IL-5 inhibits spontaneous eosinophil apoptosis was investigated. Freshly isolated eosinophils constitutively expressed the conformationally active form of Bax in the cytosol and nucleus. During spontaneous and staurosporine-induced apoptosis, Bax underwent a caspase-independent translocation to the mitochondria, which was inhibited by IL-5. Eosinophil apoptosis was associated with the release of cytochrome *c* from the mitochondria, which was also inhibited by IL-5. IL-5 and the cell-permeable caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.fmk), prevented phosphatidylserine (PS) externalization, although only IL-5 inhibited loss of mitochondrial membrane potential ($\Delta\Psi m$). Peripheral blood eosinophils endogenously expressed "initiator" caspase-8 and -9, and "effector" caspase-3, -6, and -7. Spontaneous eosinophil apoptosis was associated with processing of caspase-3, -6, -7, -8, and -9. IL-5 and z-VAD.fmk prevented caspase activation in spontaneous apoptosis. The results suggest that spontaneous eosinophil apoptosis involves Bax translocation to the mitochondria, cytochrome *c* release, caspase-independent perturbation of the mitochondrial membrane, and subsequent activation of caspases. IL-5 inhibits spontaneous eosinophil apoptosis at a site upstream of Bax translocation. (Blood. 2001;98:2239-2247)

Introduction

Eosinophils play a pivotal role in the pathogenesis of asthma and allergic disease.[1,2] Apoptosis and efficient clearance of apoptotic cells without histotoxic mediator release is thought to be crucial in the resolution of airway inflammation,[3] and delayed eosinophil apoptosis has an association with asthma, inhalant allergy, and atopic dermatitis.[4,5] The accumulation and persistence of eosinophils at sites of inflammation are mediated at least in part by extended survival in response to circulating hematopoietins interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Eosinophils rapidly undergo apoptosis unless exposed to these cytokines in vitro[6,7] and in vivo.[8,9] The mechanism by which IL-5 prevents apoptosis in eosinophils is largely unknown.

The Bcl-2 homologues are critical regulators of the apoptotic pathway, with interactions between proapoptotic (Bax, Bik, Bim, Bak) and antiapoptotic (Bcl-2, Bcl-x_L) proteins controlling the release of apoptogenic factors from mitochondria[10,11] and subsequent activation of caspases, the conserved death proteases of the cell. Previous reports investigating the role of Bcl-2 homologues in eosinophils have concentrated on the level of protein expression. Human peripheral blood eosinophils endogenously express relatively high levels of proapoptotic Bax and antiapoptotic Bcl-x_L, with little or no detectable antiapoptotic Bcl-2 expression,[12,13] although there is some evidence of higher Bcl-2 expression in eosinophils derived from patients with asthma and hypereosinophilic syndrome,[14,15] and stimulation in vitro with IL-5 resulted in detectable up-regulation of Bcl-2 expression in some[12,16] but not all studies.[17,18] It has been proposed that susceptibility to cell death is determined by the ratio of death agonist to death antagonist and their subsequent interactions by homodimerization and heterodimerization, via conserved BH3 domains.[11] The, at best, small increase in Bcl-2 in IL-5–stimulated eosinophils,[12] would suggest up-regulation of Bcl-2 alone is unlikely to be solely responsible for the potent survival-enhancing activity of IL-5. This implicates other regulatory mechanisms, possibly involving the well-expressed Bax or Bcl-x_L.[12,18] Bax is a monomeric, cytosolic protein that has been shown in cell lines to undergo a conformational change and translocate from the cytosol to the outer mitochondrial membrane during apoptosis,[19] facilitating the release of cytochrome *c*[20,21] and subsequent activation of caspases. The conformation change allows both oligomerization and membrane insertion of Bax, but also reveals an N-terminal epitope recognized by the monoclonal antibody 6A7.[22]

Caspases, aspartate-specific cysteine proteases, regulate the execution phase of apoptosis, being responsible for most of the biochemical and morphologic changes associated with the apoptotic phenotype.[23] Distinct caspase cascades are initiated dependent on the death stimulus.[24,25] "Receptor-mediated" apoptosis involves ligation of death receptors, such as CD95 (Fas/Apo-1) and tumor necrosis factor receptor-1 within the plasma membrane of many cell types, and direct processing of caspase-8 on recruitment to the receptor complex.[26] A target of caspase-8 is the proapoptotic Bcl-2 homologue, Bid, which on proteolysis translocates from the cytosol to mitochondria, and by a mechanism possibly involving interaction with Bax, potentiates the release of

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cytochrome *c*.[27] In "chemical/stress-induced" apoptosis, cellular signals induce perturbations of the mitochondria and the release of apoptosis mediators including cytochrome *c*,[28] apoptosis-inducing factor (AIF),[29] and Smac/Diablo.[30,31] Release of cytochrome *c* allows formation of the "apoptosome," a caspase-9–activating complex, involving cytochrome *c*, Apaf-1, deoxyadenosine triphosphate (dATP), and procaspase-9.[32,33] The activation of "initiator" caspase-8 and -9, results in direct or indirect activation of "effector" caspases, such as caspase-3, -6, and -7.

In this study, we investigated the mechanism by which IL-5 inhibits spontaneous apoptosis in eosinophils. We report that IL-5 prevented Bax translocation and cytochrome *c* release and mediated eosinophil survival by inhibiting perturbation of the mitochondrial membrane and caspase activation.

Materials and methods

Antibodies and reagents

Cell culture media and recombinant human IL-5 were purchased from Gibco-BRL (Paisley, United Kingdom). Benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.fmk) was from Enzyme Systems (Dublin, CA). Rabbit polyclonal antibodies directed against caspase-3, -7, -8, and -9 were generated as previously described,[24,34] and a polyclonal antibody against caspase-6 was purchased from Upstate Biotechnology (Lake Placid, NY). The epitope recognition site of the caspase-6 antibody spanned the prodomain cleavage site; therefore, the subunits were not immunoreactive. Monoclonal antibodies directed against cytochrome *c* (6H2.B4), Bak (G317-2), Bik (C33-1), and amino acids 12 to 24 of the Bax N-terminus (6A7) were from Pharmingen (Oxford, United Kingdom). Anti-Bim polyclonal antibody was from Calbiochem-Novabiochem (San Diego, CA). Antimouse and antirabbit horseradish peroxidase (HRP) conjugates were from Sigma (Poole, United Kingdom) and Dako (Ely, United Kingdom), respectively. Species-specific Alexa 488 secondary antibodies for confocal analysis, tetramethylrhodamine ethyl ester (TMRE), and Mitotracker Red CMXRos were from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC)–conjugated annexin V was obtained from Bender Medsystems (Vienna, Austria). All other reagents were from Sigma unless otherwise stated.

Cell lines and culture

Jurkat T cells (clone E6-1) were obtained from European Collection of Cell Cultures and maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% Glutamax. Apoptosis was induced by treatment with etoposide (50 μM) for 4 hours.

Isolation of peripheral blood eosinophils and cell culture

Heparinized peripheral venous blood was taken from healthy volunteers with peripheral blood eosinophilia of less than 0.5×10^6/mL. Eosinophils were purified by a 2-step method of density gradient centrifugation, followed by negative immunomagnetic selection as described previously.[35] Briefly, removal of erythrocytes by dextran sedimentation was followed by centrifugation of the leukocyte-rich supernatant (100*g*, 15 minutes at room temperature). The cell pellet was resuspended in Hanks balanced salt solution without Ca^{++} and Mg^{++}, supplemented with 1% bovine serum albumin (BSA) and 20 mM EDTA. The cell suspension was centrifuged on Histopaque 1083 and the mononuclear cell layer carefully removed, prior to lysis of erythrocytes contaminating the granulocyte pellet by hypotonic shock using sterile, ice-cold water. Eosinophils were separated from neutrophils by negative immunomagnetic selection using anti-CD16–coated magnetic beads (Miltenyi Biotec, Auburn, CA). Purity and viability after isolation were routinely more than 99% as assessed by morphology after Kimura stain and trypan blue exclusion, respectively. Peripheral blood eosinophils were cultured in RPMI 1640 supplemented with 1% BSA and 1% Glutamax in the presence of IL-5 (10^{-10} M), staurosporine (STS; 10^{-5} M), or z-VAD.fmk (100 μM) where indicated. Preliminary experiments were performed to determine optimum concentrations.

Immunoblotting

Cells (5×10^5) were washed with ice-cold phosphate-buffered saline (PBS) and snap-frozen in dry ice before storage at −70°C. Samples were resuspended in sample buffer with freshly added 5% β-mercaptoethanol and boiled for 5 minutes. Proteins were then separated on 10%/15% sodium dodecyl sulfate–polyacrylamide gels and electrotransferred onto presoaked Hybond-C nitrocellulose filter (Amersham Life Science, Bucks, United Kingdom). Immunoblotting was performed as described previously[12] and proteins detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Life Science).

Assessment of phosphatidylserine exposure and mitochondrial membrane potential

The exposure of phosphatidylserine (PS) has been shown to be a sensitive marker of apoptosis in eosinophils and other cell types.[36,37] Cultured cells were washed once in PBS and incubated with annexin V-FITC (1:1000) according to the manufacturer's instructions. Cells were then incubated with 50 μg/mL propidium iodide for 2 minutes on ice prior to analysis using the FACScan (Becton Dickinson, Oxford, United Kingdom), excitation at 488 nm and detection between 515 and 550 nm. To measure mitochondrial membrane potential ($\Delta\Psi m$), cells were loaded with 75 nM TMRE for 30 minutes at 37°C. Alterations of $\Delta\Psi m$ can be determined from the fluorescence intensity of TMRE because the transmembrane distribution of this cationic dye is dependent on membrane potential.[38] Cells were washed once and resuspended in PBS for FACScan analysis, excitation at 488 nm and detection at 610 nm. Freshly isolated eosinophils were treated with the mitochondrial membrane uncoupler carbonylcyanide m-chlorophenyl hydrazone (mCCP) at 1 μM for 15 minutes at 37°C as a positive control for dissipation of $\Delta\Psi m$.

Apoptotic morphology

Eosinophils were stained with Kimura stain[39] and examined under light microscopy for the characteristic morphologic changes associated with apoptosis, namely, condensation of the nucleus and cytoplasm.[36] Two hundred and fifty cells were counted per treatment in a blinded fashion.

Immunocytochemistry and confocal analysis

Cells were incubated with 75 nM Mitotracker Red CMXRos for 45 minutes at 37°C and washed in RPMI without BSA, and cytospins were performed (650 rpm for 6 minutes) on silane-coated slides at 1×10^6 cells/mL. Slides were fixed in 2% paraformaldehyde for 15 minutes at room temperature and washed 5 times in PBS. Cells were permeabilized for 10 minutes at room temperature in blocking buffer (3% BSA in PBS) plus 0.1% Triton X-100 followed by blocking of nonspecific binding in blocking buffer for 1 hour at room temperature. Cells to be stained with anti–Bax 6A7 were not permeabilized because the recognized Bax conformation has been shown to be sensitive to nonionic detergent.[40] Cells were incubated overnight at 4°C with 5 μg/mL primary antibody diluted in blocking buffer. Cells were then incubated for 20 minutes at room temperature with 0.2% chromotrope-2R, which binds to highly basic eosinophil granules thereby reducing nonspecific binding of the secondary antibody. Cells were then incubated with species-specific Alexa 488–conjugated secondary antibody diluted 1:300 in blocking buffer for 50 minutes at room temperature in the dark. The nuclei were stained with Hoechst 33258 (250 ng/mL) for 10 minutes at room temperature in the dark prior to mounting with fluoromount (Dako). Images were collected by confocal laser microscopy (model TCS 4D, Leica, Heidelberg, Germany). The 488- and 568-nm lines of the krypton/argon laser were used for the excitation of Alexa 488 and Mitotracker Red CMXRos, respectively. Excitation of Hoechst 33258 was by UV laser. Cytochrome *c* release and Bax translocation were quantified on these cytospins by counting at least 300 cells in a blinded fashion in 4 random visual fields and assessing evidence of diffuse cytochrome *c* or punctate Bax staining.

Statistical analysis

Comparisons between treated and untreated cells were made at individual time points using the Student paired *t* test.

Results

Caspase expression and activation in peripheral blood eosinophils

The primary aim of this project was to identify the point in the apoptotic pathway at which IL-5 exerts its antiapoptotic effect. Initial investigation explored the role of caspases in eosinophil apoptosis. Peripheral blood eosinophils endogenously expressed both the "initiator" caspase-8 and -9, and the "effector" caspase-3, -6, and -7 (Figures 1 and 2). Treatment of eosinophils for 2 hours with the protein kinase C inhibitor, STS (10^{-5} M), was sufficient to induce activation of caspase-3, -6, -7, -8, and -9, as evidenced either by the generation of the immunoreactive, catalytically active, large subunits resulting from processing of the caspase proforms, or direct loss of the proform itself (Figures 1 and 2).

Procaspase-3 is expressed as an inactive 32-kd zymogen, which on activation is cleaved initially at D175 (single-letter amino acid codes) generating a p20 subunit, followed by further proteolytic cleavage at D9 and D28 to generate p19 and p17 subunits, respectively.[41] In freshly isolated eosinophils, caspase-3 was present as a 32-kd proform (Figure 1A, lane 1). Exposure to STS for 2 hours resulted in the formation of 20-kd (p20), 19-kd (p19), and 17-kd (p17) immunoreactive fragments (Figure 1A, lane 4). After 6 hours, the proform was almost completely processed (Figure 1A, lane 7). Jurkat T cells exposed to etoposide for 4 hours exhibited similar caspase-3 processing (Figure 1A, lane 12).

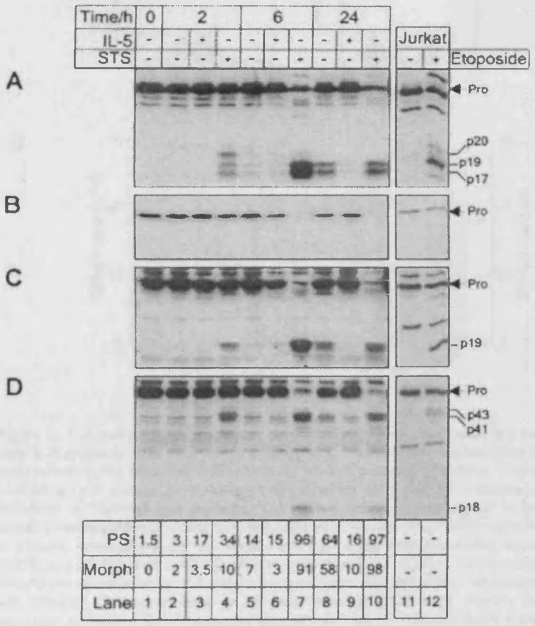

Figure 1. IL-5 inhibits caspase processing in the spontaneous apoptosis of peripheral blood eosinophils. Peripheral blood eosinophils were cultured in the presence or absence of IL-5 (10^{-10} M) or STS (10^{-5} M) and analyzed at the times shown for processing of caspase-3 (A), -6 (B), -7 (C), and -8 (D) by immunoblotting. Jurkat T cells treated with or without etoposide (50 μM) for 4 hours were used as controls. The proforms and processed subunits are indicated. Apoptosis was assessed by annexin V binding to determine percentage cells with externalized PS and apoptotic morphology after Kimura stain, as described in "Materials and methods." Eosinophils constitutively expressed caspase-3, -6, -7, and -8. Culturing in the absence of IL-5 and treatment with STS resulted in activation of all of the caspases. IL-5 prevented caspase activation over the 24-hour culture period. Longer exposure of the caspase-3 (A) blot revealed the presence of p20 and p19 subunits after 6 hours in untreated eosinophils. Shorter exposure of the caspase-8 (D) blot revealed the proform to be a doublet of 53 and 55 kd. Results are representative of 3 separate experiments.

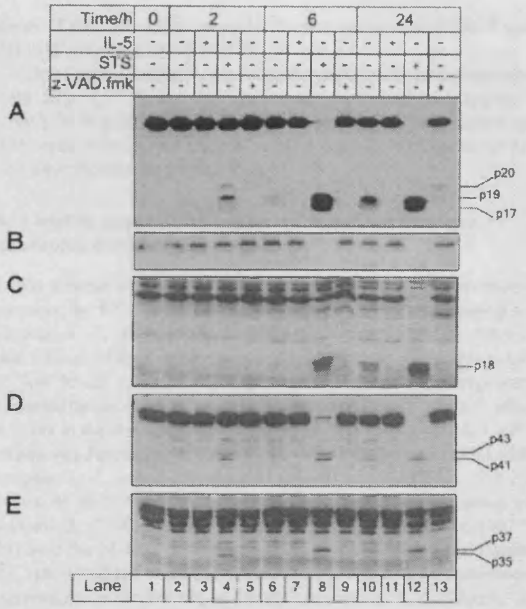

Figure 2. z-VAD.fmk inhibits caspase processing in IL-5–deprived peripheral blood eosinophils. Cells were incubated with IL-5 (10^{-10} M), STS (10^{-5} M), or z-VAD.fmk (100 μM), harvested at the times indicated and analyzed for caspase-3 (A), -6 (B), -7 (C), -8 (D), and -9 (E) processing by immunoblotting. Eosinophils constitutively expressed caspase-9, which was activated during STS-induced and spontaneous apoptosis. IL-5 prevented the processing of caspase-9 observed during spontaneous apoptosis over the 24-hour culture period. z-VAD.fmk inhibited caspase activation in IL-5–deprived eosinophils. Results are representative of 3 separate experiments.

Procaspase-6 was expressed in eosinophils as its 34-kd zymogen (Figure 1B, lane 1). Processing of caspase-6 generates p18 and p11 subunits due to sequential cleavage at D179 and D193.[42] Due to lack of immunoreactivity of these subunits, processing of caspase-6 was indicated by the time-dependent loss of the proform after 6 hours of STS treatment (Figure 1B, lane 7). A slight loss of caspase-6 proform was observed in Jurkat cells treated with etoposide for 4 hours (Figure 1B, lane 12).

In eosinophils, caspase-7 was expressed as its 35-kd proform (Figure 1C, lane 1). Treatment with STS for 2 hours resulted in formation of the immunoreactive 19-kd (p19) fragment (Figure 1C, lane 4), corresponding to the large catalytically active subunit generated due to cleavage at D198 and removal of the prodomain.[34] After 6 hours of STS treatment, procaspase-7 was almost completely lost (Figure 1C, lane 7). Similar caspase-7 processing was observed in Jurkat T cells (Figure 1C, lane 12).

Caspase-8 was detectable in freshly isolated eosinophils and Jurkat T cells as the 55- kd proform (Figure 1D, lanes 1 and 12). However, shorter exposure revealed that caspase-8 was expressed as a doublet of 55 and 53 kd, corresponding to the proposed occurrence of 2 isoforms, caspase-8a and caspase-8b.[43] After STS treatment for 2 hours, cleavage of the proform resulting in the formation of 43-kd (p43) and 41-kd (p41) subunits was observed corresponding to cleavage between the large and small subunits (Figure 1D, lane 4). After 6 hours of STS treatment, an 18-kd (p18) fragment was observed due to removal of the death effector domains from p43 and p41 (Figure 1D, lane 7). Generation of the

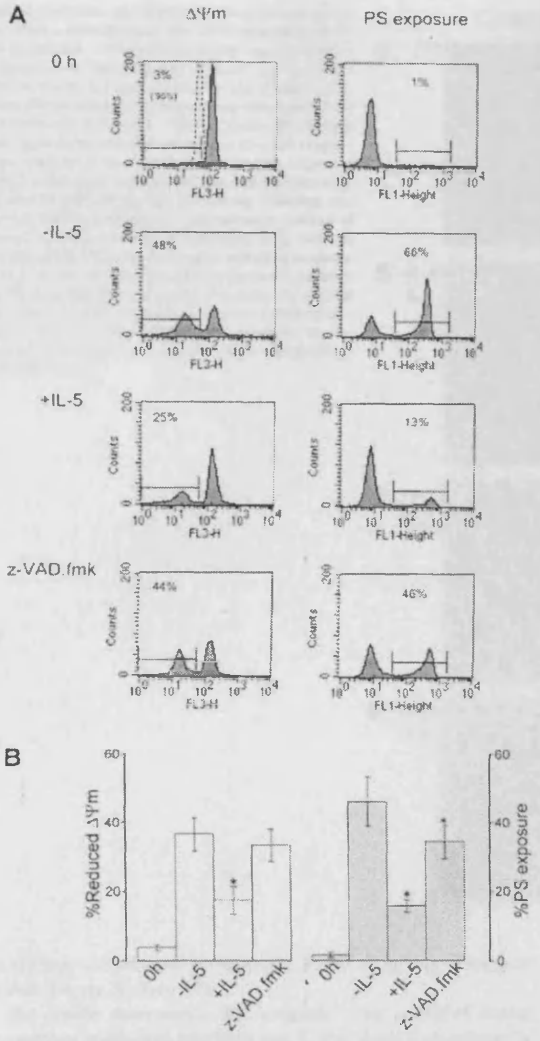

Figure 3. IL-5 and z-VAD.fmk prevent phosphatidylserine externalization, but only IL-5 prevents loss of ΔΨm in eosinophils. Freshly isolated eosinophils (0 hour) or eosinophils incubated in the absence (−IL-5) or presence of IL-5 (10^{-10} M) or z-VAD.fmk (100 μM) for 24 hours were assessed for ΔΨm and PS exposure as described in "Materials and methods." (A) Flow cytometry histograms of 10 000 events showing percentage cells exhibiting reduced ΔΨm or increased PS exposure. At 0 hours, eosinophils were also treated with the mitochondria uncoupling agent, mCCP, as a positive control for loss of ΔΨm (open histogram, figure in parentheses). Results are representative of 6 separate experiments. (B) Percentage eosinophils with reduced ΔΨm (open bars) or PS externalization (filled bars). Results are expressed as mean ± SE of 6 separate experiments. Asterisk indicates that IL-5 and z-VAD.fmk significantly inhibited PS exposure compared with medium control (−IL-5), but only IL-5 inhibited loss of ΔΨm ($P < .05$).

p45 and p43 subunits was observed in Jurkat T cells treated with etoposide for 4 hours, although the p18 subunit was not marked (Figure 1D, lane 12).

Caspase-9 is the apical caspase of stress-induced apoptosis. Activated on association with Apaf-1 in the presence of cytochrome *c*, caspase-9 activates downstream "effector" caspase-3, -6, and -7.[44,45] Procaspase-9 was detectable in eosinophils as its 46-kd zymogen (Figure 2E, lane 1), and time-dependent formation of 35-kd and 37-kd (p35 and p37) subunits was observed after 2 and 6 hours of STS treatment, respectively, due to cleavage at D315 and D330 (Figure 2E, lanes 4 and 8).

Detection of caspase processing in eosinophils after treatment with STS for 2 hours was commonly associated with approximately 35% to 50% apoptosis assessed by PS exposure and 10% to 15% cells showing evidence of nuclear condensation by morphology after Kimura stain (Figure 1).

IL-5 inhibits spontaneous eosinophil apoptosis upstream of caspase processing

In the absence of IL-5 for 24 hours, approximately 65% apoptosis assessed by PS externalization was associated with processing of caspase-3, -7, -8, and -9 in eosinophils (Figures 1 and 2). After 2 and 6 hours of IL-5 deprivation, subunit detection was difficult due to low levels of caspase processing. However, longer exposure revealed the presence of caspase-3 subunits p20, p19, and p17 after 6 hours in the absence of IL-5 (approximately 15% apoptosis by PS exposure). Processing of caspase-9 was less marked compared with caspase-3, -7, and -8, but the p37 subunit was detectable after 24 hours of IL-5 deprivation (Figure 2E, lane 10). Processing of caspase-3, -7, -8, and -9 was completely inhibited by IL-5 (10^{-10} M) over the 24-hour culture period (Figure 1A,C,D, lane 9; Figure 2E, lane 11). Lack of immunoreactivity of caspase-6 subunits made determination of processing in the absence of IL-5 difficult. A slight loss of proform in IL-5–deprived eosinophils compared with IL-5–stimulated eosinophils after 24 hours was observed, but was not marked (Figure 1B, lanes 8 and 9). The broad-spectrum, cell-permeable caspase inhibitor, z-VAD.fmk, inhibited the processing of each caspase in IL-5–deprived eosinophils after 24 hours (Figure 2A-D, lane 13). However, inhibition was not absolute because caspase-9 processing after 24 hours in the presence of

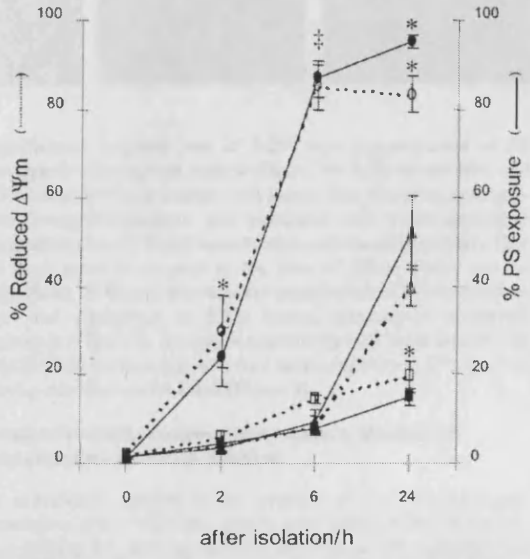

Figure 4. IL-5 inhibits and STS accelerates the externalization of PS and loss of ΔΨm during spontaneous apoptosis of peripheral blood eosinophils. Eosinophils were cultured in the presence of IL-5 (10^{-10} M, □), STS (10^{-5} M, ○), or medium alone (△) for the indicated times. Apoptosis was assessed by PS exposure and ΔΨm as described in "Materials and methods." IL-5 inhibited PS externalization and loss of ΔΨm observed in spontaneous eosinophil apoptosis after 24 hours (*$P < .05$). STS accelerated PS exposure and loss of ΔΨm in eosinophils after 2 hours (‡$P < .01$) and was almost maximal after 6 hours (*$P < .05$). Results are expressed as mean ± SE of 3 separate experiments.

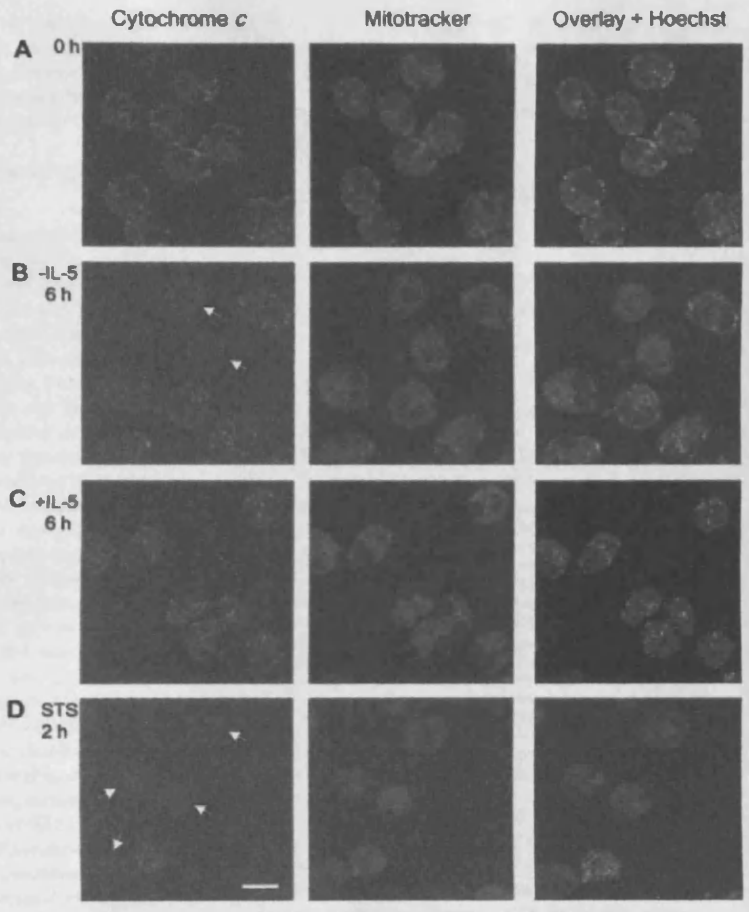

Figure 5. Cytochrome *c* is released from the mitochondria during spontaneous and STS-induced eosinophil apoptosis. Immunocytochemistry was performed, as described in "Materials and methods," on fixed cytospins of freshly isolated eosinophils (A) or eosinophils cultured in the absence (B) or presence of IL-5 (10^{-10} M) for 6 hours (C) or STS (10^{-6} M) for 2 hours (D). Images were captured by confocal microscopy at × 100 magnification under oil immersion. Cytochrome *c* was detected using a monoclonal antibody 6H2.B4 and the mitochondria and nuclei stained with Mitotracker CMXRos and Hoechst 33258, respectively. Colocalization (yellow) of cytochrome *c* (green) and mitochondria (red) was observed in single sections. Eosinophils exhibiting evidence of cytochrome *c* release are indicated (arrows in panels B and D). Culturing in the absence of IL-5 for 6 hours and treatment with STS for 2 hours induced cytochrome *c* release from the mitochondria. Bar represents 10 μm. Results are representative of experiments performed on 4 separate donors.

z-VAD.fmk was observed, evidenced by the detection of the p35 subunit (Figure 2E, lane 13).

The results demonstrate that caspases were activated during spontaneous eosinophil apoptosis and IL-5 acted at a point prior to caspase activation in inhibiting spontaneous eosinophil apoptosis. The events that precede the activation of caspases in the apoptotic pathway were then investigated. The mitochondria are pivotal in the regulation of apoptotic cell death, with apoptogenic factor release required for the activation of caspase-9. The role of mitochondria in eosinophil apoptosis was investigated.

IL-5 inhibits the loss of mitochondrial membrane potential in eosinophil apoptosis

Apoptosis is characterized by the exposure of PS at the cell membrane,[36,37] and a reduction in $\Delta\Psi m$.[46] Less than 5% of freshly isolated eosinophils exhibited PS externalization or loss of $\Delta\Psi m$ (Figure 3A,B). Uncoupling of mitochondrial respiration with mCCP as a positive control caused a marked loss of $\Delta\Psi m$ (Figure 3A, 0 hour, open histogram). Eosinophils undergoing spontaneous apoptosis exhibited an increase in both the percentage of cells with dissipated $\Delta\Psi m$ and the percentage of cells with PS externalization after 24 hours (Figure 3A,B, −IL-5). The increase in spontaneous eosinophil apoptosis assessed by both criteria was observed after 6 hours, but was very marked after 24 hours (Figure 4). IL-5 significantly inhibited loss of $\Delta\Psi m$ and externalization of PS compared with medium control (Figure 3A, 25% versus 48%, and 13% versus 66% respectively; and Figure 3B). Therefore, spontaneous eosinophil apoptosis was associated with a time-dependent externalization of PS and loss of $\Delta\Psi m$, and the antiapoptotic effect of IL-5 acted at or prior to the loss of $\Delta\Psi m$. There was no significant difference between the progression of PS externalization and dissipation of $\Delta\Psi m$ during spontaneous eosinophil apoptosis (Figure 4). Apoptosis assessed by both these criteria was significantly increased as early as 2 hours exposure to STS and was almost maximal after 6 hours (Figure 4).

z-VAD.fmk inhibits eosinophil PS exposure after loss of mitochondrial membrane potential

In eosinophils cultured in the absence of IL-5 for 24 hours, incubation with z-VAD.fmk significantly inhibited PS externalization (Figure 3A, 46% versus 66%, and Figure 3B), although less efficiently than IL-5, but exhibited little or no effect on loss of $\Delta\Psi m$ compared with medium control (Figure 3A, 44% versus 48%, and Figure 3B). Thus during spontaneous eosinophil apoptosis PS externalization was dependent on caspase activation, whereas the perturbations of the mitochondria leading to loss of $\Delta\Psi m$ were independent of caspases. Prevention of caspase activation inhibited apoptosis assessed by PS externalization, thereby

implicating caspases as major effectors of the execution of spontaneous eosinophil apoptosis. To further elucidate the role of the mitochondria in eosinophil apoptosis, cytochrome *c* release was investigated. Cytochrome *c* release from mitochondria is an early, pivotal event in the apoptosis of many cell types.[47]

Release of cytochrome *c* from mitochondria during eosinophil apoptosis

Spontaneous eosinophil apoptosis was associated with caspase-dependent release of mitochondrial cytochrome *c*, which was inhibited by IL-5. In freshly isolated peripheral blood eosinophils, cytochrome *c* exhibited a punctate distribution, which colocalized with mitochondria (Figure 5A). No staining was observed with the isotype-matched negative control (data not shown). Due to the relatively low level of constitutive cytochrome *c* expression and number of mitochondria in eosinophils, cells that had undergone cytochrome *c* release generally appeared diffuse or devoid of green staining entirely (Figure 5B,D, indicated by arrows). Release of cytochrome *c* was observed in eosinophils cultured in the absence of IL-5 for 6 hours (Figure 5B) and was marked after 24 and 48 hours (Figure 6A). Cytochrome *c* release was commonly associated with eosinophils exhibiting evidence of apoptotic nuclear condensation (Figure 5B). Stimulation with IL-5 for 6 hours maintained the association of cytochrome *c* with the mitochondria (Figure 5C), and inhibited cytochrome *c* release compared with medium control after 24 and 48 hours (Figure 6A). z-VAD.fmk had no inhibitory effect on cytochrome *c* release compared with medium control (Figure 6A). Cytochrome *c* release was also observed in eosinophils treated with STS for 2 hours (Figure 5D), and after 6 hours of STS treatment almost 100% of eosinophils exhibited cytochrome *c* release, which was not inhibited by z-VAD.fmk (Figure 6B). Due to downstream proteolytic degradation of the cell, accurate determination of cytochrome *c* release beyond 6 hours of STS treatment was not possible. Therefore, spontaneous and STS-induced eosinophil apoptosis involved caspase-independent cytochrome *c* release. IL-5 inhibited spontaneous eosinophil apoptosis at or prior to release of cytochrome *c*. The mechanism by which cytochrome *c* is released from the mitochondria during apoptosis is unclear. We investigated the possible role of Bax in eosinophil apoptosis because its translocation to the mitochondrial membrane facilitates the release of cytochrome *c* in many models of apoptosis.[20,21]

Cytosol-to-mitochondria translocation of Bax during eosinophil apoptosis

Spontaneous eosinophil apoptosis was associated with a caspase-independent redistribution of Bax from the cytosol to the mitochondria, which was inhibited by IL-5. Anti-Bax 6A7 detected diffuse cytosolic and nuclear Bax distribution in the majority of freshly isolated eosinophils (Figure 7A), indicating constitutive expression of the conformationally altered form of Bax. Interestingly, Bax expression was observed to localize predominantly to the nucleus compared with the cytosol in freshly isolated eosinophils (Figure 7A). Isotype-matched mouse negative control showed essentially no evidence of nonspecific staining (Figure 7B). Punctate Bax distribution that colocalized with the mitochondria was observed in eosinophils deprived of IL-5 for 6 hours (Figure 7C). This punctate distribution was commonly perinuclear and consistently associated with cells exhibiting characteristic apoptotic morphology of condensed nucleus and cytoplasm. Stimulation of eosinophils with IL-5 for 6 hours maintained the cytosolic and nuclear distribution observed in the freshly isolated eosinophils (Figure 7D), and prevented Bax translocation to the mitochondria observed during

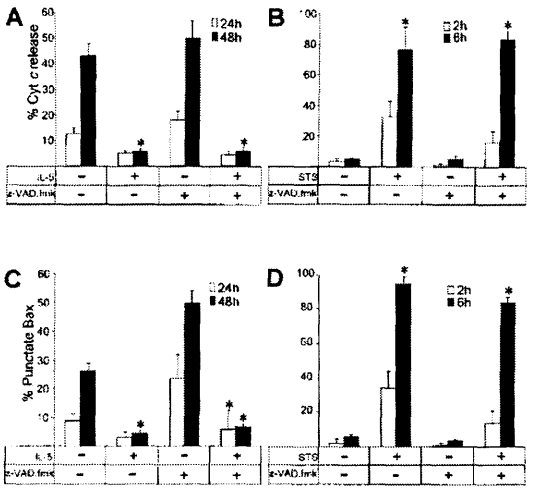

Figure 6. IL-5 prevents both cytochrome *c* release and Bax translocation to the mitochondria during spontaneous eosinophil apoptosis. Eosinophils were co-incubated in the presence or absence of either STS (10^{-5} M) or IL-5 (10^{-10} M) and z-VAD.fmk (100 μM) for the indicated times and the cells assessed for evidence of cytochrome *c* release (A,B) or Bax translocation to the mitochondria (C,D). Cytochrome *c* and Bax were detected by immunohistochemistry as described in "Materials and methods." The percentage of eosinophils exhibiting diffuse cytochrome *c* and aggregated Bax distribution were quantified by fluorescence microscopy. IL-5 inhibited cytochrome *c* release and Bax translocation observed during spontaneous eosinophil apoptosis after 24 and 48 hours. Quantification of subcellular localization in eosinophils exposed to STS for longer than 6 hours was not possible. STS rapidly induced cytochrome *c* release and Bax translocation after 2 hours, which was not inhibited by z-VAD.fmk. Results are expressed as the mean ± SE of 3 separate experiments. Asterisk indicates IL-5– or STS-treated eosinophils compared with the relevant untreated or z-VAD.fmk–treated control ($P < .05$).

spontaneous apoptosis after 24 and 48 hours (Figure 6C). In contrast, although z-VAD.fmk inhibited spontaneous apoptosis assessed by PS externalization, it increased the percentage of eosinophils showing Bax translocation to the mitochondria (Figure 6C). Treatment of eosinophils with STS for 2 hours also resulted in the marked appearance of punctate Bax distribution (Figure 7E), and similarly to cytochrome *c* release, nearly 100% of eosinophils exhibited translocated Bax after 6 hours (Figure 6D). STS-induced Bax redistribution was not inhibited by z-VAD.fmk (Figure 6D). To determine whether translocation of other proapoptotic Bcl-2 homologues occurred during apoptosis, the expression of Bik, Bak, and Bim was also investigated. Expression of Bik and Bak was undetectable by either immunoblotting or immunocytochemistry in peripheral blood eosinophils (data not shown). Bim expression was detected both by immunoblotting and immunocytochemistry, where it was localized to the cytoplasm. However, Bim did not redistribute during spontaneous or STS-induced apoptosis (data not shown).

Discussion

We have shown for the first time that IL-5 exhibits its potent antiapoptotic effect in eosinophils by preventing Bax translocation to the mitochondria, cytochrome *c* release, increased mitochondrial membrane permeability, and subsequent caspase activation. In nonapoptotic cells, Bax is a soluble, cytosolic monomer, with an N-terminus proposed to mask its hydrophobic C-terminus.[22] During the apoptosis of murine thymocytes, COS-7, and murine fibrosarcoma cell lines, it has been shown that a conformational change exposes the BH3 domain and C-terminus, facilitating oligomerization of Bax and translocation from the cytosol to the

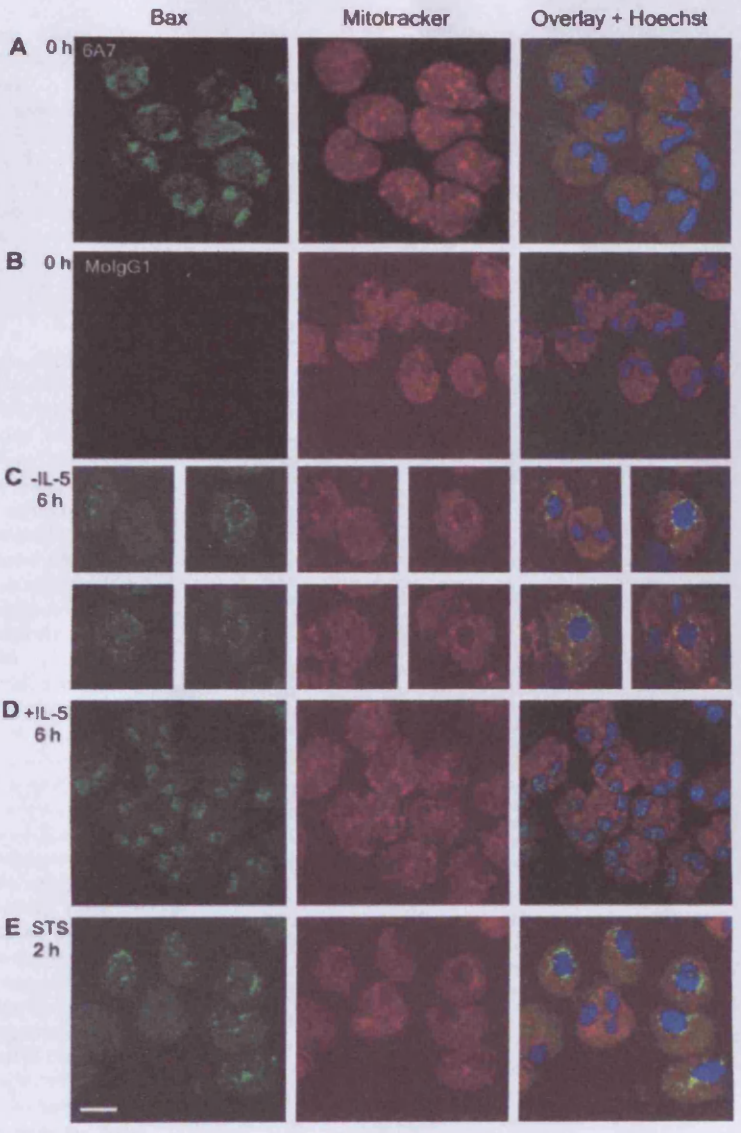

Figure 7. Bax translocates to the mitochondria during spontaneous and STS-induced eosinophil apoptosis. Immunocytochemistry was performed, as described in "Materials and methods," on fixed cytospins of freshly isolated eosinophils (A,B) or eosinophils cultured in the absence (C) or presence of IL-5 (10^{-10} M) for 6 hours (D) or STS (10^{-5} M) for 2 hours (E). Images were captured by confocal microscopy at × 100 magnification under oil immersion. Eosinophils were stained with monoclonal anti–Bax 6A7 (A,C-E) or isotype-matched control (B), and the mitochondria and nuclei stained with Mitotracker CMXRos and Hoechst 33258, respectively (A-E). Colocalization (yellow) of Bax (green) and mitochondria (red) was observed in single sections. Culturing in the absence of IL-5 for 6 hours and treatment with STS for 2 hours induced Bax translocation to the mitochondria. Bar represents 10 μm. Results are representative of experiments performed on 4 separate donors.

mitochondrial membrane, respectively.[19,22,48] Bax oligomers have channel-forming activity and trigger the release of cytochrome *c* from human melanoma cells[21] and isolated mitochondria.[20,49,50] We, and others, have previously shown that human peripheral blood eosinophils express high levels of Bax.[12,13] We now provide evidence that freshly isolated eosinophils constitutively express the conformationally altered form of Bax, detected by the monoclonal antibody 6A7 that specifically recognizes an N-terminal epitope inaccessible in soluble, cytosolic Bax.[22] Interestingly, despite diffuse Bax expression being detectable throughout the entire cell, expression was predominantly observed in the nucleus (Figure 7A). Lack of nuclear and cytosolic staining in the isotype-matched negative control would suggest that the staining was not nonspecific (Figure 7B). An association of Bax with the nuclear membrane and matrix in nonapoptotic cells has been previously reported in MCF-7 cells[51,52] and a number of human lung cancer cell lines.[53] However, the relevance of nuclear Bax expression is currently unclear. During both spontaneous and STS-induced apoptosis, Bax underwent a caspase-independent translocation to the mitochondria in human eosinophils. This colocalization was consistently observed in eosinophils exhibiting the condensed nuclear morphology characteristic of apoptosis and was inhibited by IL-5. Interestingly, Bax redistribution to the mitochondria seemingly increased with z-VAD.fmk (Figure 6C), probably due to the delayed onset of the caspase-mediated execution phase of apoptosis and the consequent proteolytic degradation of cells cultured for up to 48 hours, thereby prolonging the association of Bax with the mitochondria. This is the first report of Bax translocation to the mitochondria during spontaneous apoptosis of human granulocytes due to cytokine deprivation. A recent report has demonstrated that Bax inserts into the mitochondrial membrane and cytochrome *c* is released during apoptosis of neutrophils, induced by a 15°C to 37°C temperature

transition.[54] Similarly, in this temperature arrest system, Bax translocation to the mitochondria was shown to be of crucial importance in the execution of granulocyte apoptosis.

Caspase-independent release of cytochrome *c* from mitochondria was observed in eosinophils undergoing STS-induced and spontaneous apoptosis, with the latter inhibited by IL-5. The mechanism by which cytochrome *c* is released from the mitochondria remains unclear. It has been proposed that Bax induces the opening of the permeability transition pore complex via interaction with the adenine nucleotide translocator, resulting in mitochondrial membrane depolarization and cytochrome *c* release.[55] Spontaneous eosinophil apoptosis clearly involved caspase-independent loss of $\Delta\Psi m$, which was inhibited by IL-5.

Receptor-mediated and chemical/stress-induced caspase activation are associated with apical processing of caspase-8 and -9, respectively. Although recent studies have reported activation of caspases in eosinophils in response to Fas, growth factor withdrawal, and dexamethasone,[17,56,57] caspase processing has not been investigated in detail. We have demonstrated processing of caspase-3, -7, -8, and -9, and possible caspase-6 activation during spontaneous eosinophil apoptosis. Immunoblot analysis seemingly indicated caspase-3 processing proximal to caspase-8 processing. In receptor-mediated apoptosis, perturbations of the mitochondrial membrane are dependent on the activation of caspase-8. The time scale of activation of caspase-3 and -8, coupled with the observation that mitochondrial perturbations were not blocked by z-VAD.fmk, suggested that spontaneous eosinophil apoptosis is mediated by caspase-independent mitochondrial events and the consequent activation of the caspase-9/-3/-7 pathway. The processing and activation of caspase-8 observed after 24 hours may form an amplification loop mediated by caspase-3 and -6 as previously described.[35] The processing of caspase-9 was not as marked as the other caspases tested. This may be due to the relative efficacy of the antibodies used, although it must be noted that caspase-9 can be activated without proteolytic cleavage.[58] We have demonstrated that caspases are major effectors of the execution phase of spontaneous eosinophil apoptosis, and IL-5 induces eosinophil survival by inhibiting apoptosis before caspase activation.

Ligation of the IL-5 receptor induces rapid tyrosine phosphorylation and activation of juxtamembranous tyrosine kinases. The signal is propagated via the Janus kinase2 (Jak2)/signal transducers and activators of transcription (STAT) and Ras-Raf-1–mitogen-activated protein kinase (MAPK) cascades (Figure 8).[59,60] Activation of Jak2, Lyn, and Syk tyrosine kinases, and Src homology 2 phosphatase 2 tyrosine phosphatase (SHPTP-2) is crucial for IL-5–induced eosinophil survival.[60,61] SHPTP-2 activation and association with the adaptor protein Grb2 is proposed to couple the IL-5 receptor to the Ras signaling pathway,[61] supported by the requirement for Raf-1 serine/threonine kinase activation in IL-5–mediated suppression of eosinophil apoptosis.[60] The mechanism by which these signaling cascades integrate into the apoptotic pathway,

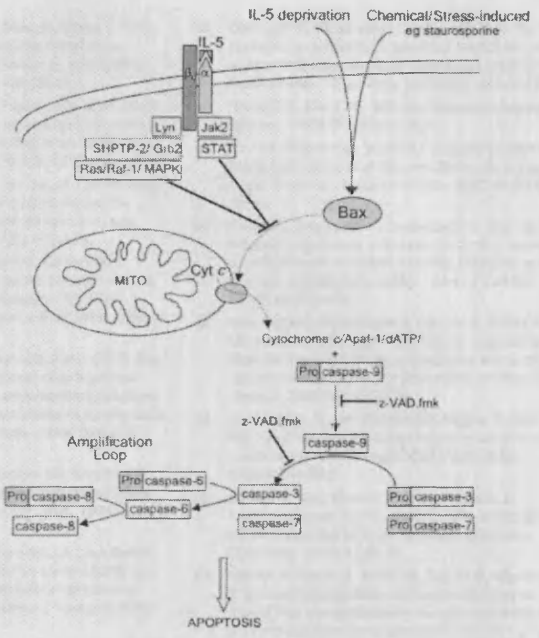

Figure 8. Proposed scheme for spontaneous apoptotic pathway in eosinophils and the inhibitory target of IL-5. Spontaneous eosinophil apoptosis involves caspase-independent Bax translocation to the mitochondria, cytochrome *c* release, and perturbation of the mitochondrial membrane followed by activation of caspase-9, -3, and -7, similar to the pathway of chemical-induced apoptosis. Caspase-3 activation of caspase-6 followed by activation of caspase-8 is proposed to form an amplification loop. IL-5 inhibits eosinophil apoptosis at an as yet undetermined site upstream of Bax translocation, cytochrome *c* release, and caspase activation. The IL-5 antiapoptotic signal is transduced by recruitment and activation of Jak2 and Lyn tyrosine kinases, and SHPTP-2 to the receptor, resulting in activation of Jak/STAT and Ras-Raf-MAPK pathways (see text for details). Dashed arrows represent protein translocation.

thereby regulating Bax translocation, is unknown and the subject of future investigation. Interactions with other proteins, such as the apoptosis suppressing Bcl-x_L, the expression of which is down-regulated during spontaneous apoptosis, but maintained or up-regulated on stimulation with GM-CSF and IL-5, may be involved.[18]

Elucidating the point in the signaling cascade that IL-5 inhibits apoptosis would enable specific inhibitors of growth factor–mediated eosinophil survival to be developed. This could have important therapeutic implications for asthma and related eosinophilic disorders.

Acknowledgment

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