The Role of Hypoxia and its Interaction with the PI3K/Akt Pathway in the Pathogenesis of Malignant Pleural Mesothelioma

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by

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ABSTRACT

The Role of Hypoxia and its Interaction with the PI3-K/pAkt Pathway in the Pathogenesis of Malignant Pleural Mesothelioma

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Malignant pleural mesothelioma (MPM) is a cancer of increasing incidence, with an invariably fatal outcome. Conventional oncological treatments for the majority of patients with MPM, allowing only palliation of the disease have driven the imperative to advance understanding of the disease pathobiology. Carbonic Anhydrase (CA) IX, a surrogate marker of hypoxia, is upregulated downstream of the stabilisation of hypoxia-inducible factor (HIF)-1α and is over-expressed in solid tumours. Protein Kinase B, or Akt, known to have important intracellular roles including resistance to apoptosis, is activated in human malignancies and upregulated in hypoxic conditions.

This body of work examined the expression of CA IX and phosphorylated Akt (pAkt) in tumour samples from patients with MPM, correlating expression with established prognostic factors. The role of pAkt and HIF-1α in the survival of an MPM cell line exposed to hypoxic conditions was also examined. Tumour samples from 200 patients with MPM were stained using pAkt and CA IX specific antibodies. The effect of hypoxia on apoptosis was evaluated in 4 mesothelioma cell lines and 1 benign mesothelial cell line, in the presence or absence of the phosphatidylinositol-3-kinase inhibitor, LY294002.

There was a positive association between the level of CA IX and pAkt staining, implying that intra-tumoural hypoxia may be a stimulus for Akt phosphorylation. On multivariate analysis increased expression of nuclear pAkt was found to be associated with a poor survival. In-vitro cell culture work showed that, although pAkt is expressed in normoxic conditions in the cell lines studied, in the JU77 cell line the rates of apoptosis were significantly increased in hypoxic conditions when the phosphorylation of Akt was blocked by LY294002.

This work provides evidence for the anti-apoptotic role of pAkt in hypoxic conditions in solid human malignancies. Phospho-Akt may represent a novel therapeutic target in MPM.

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LIST OF CONTENTS

ABBREVIATIONS

ABC	Horseradish-peroxidase labelled Streptavidin-biotin complex
AGC	cAMP-dependant protein kinase A/protein kinase G/protein kinase C
Akt	Protein kinase B
AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
AO	Antisense olignucleotides
Apaf-1	Apoptotic protease-activating factor-1
ARNT	Aryl hydrocarbon nuclear translocator
ATP	Adenosine triphosphate
BCL	B-Cell lymphoma gene
bFGF	basic Fibroblast growth factor
bHLH	basic Helix-loop-helix
BTS	British Thoracic Society
CA-IX	Carbonic Anhydrase-IX
CALGB	Cancer and Leukaemia Group B
cAMP	cyclic adenosine monophosphate
CDK	Cyclin-dependant kinase
CEA	Carcinoembryonic antigen
CO_2	Carbon dioxide
CRE	CREB-response element
CREB	cAMP-response element binding protein
CTMP	Carboxyl-terminal modulator protein
CTV	Clinical target volume
Da	Dalton (molecular weight)
DISC	Death-inducing signalling complex
DMLC	Dynamic multileaf collimation
DMSO	Dimethylsulphoxide

DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependant protein kinase
ECOG	Eastern Co-operative Oncology Group
EGF	Epidermal growth factor
EORTC	European Organisation for the Research and Treatment of Cancer
EPP	Extra-pleural pneumonectomy
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FCS	Foetal calf serum
FIH-1	Factor inhibiting HIF-1
FKHR	Forkhead in human rhabdomyosarcoma
FSC	Forward scatter
GARFT	Glycinamide ribonucleotide transformylase
GSK3	Glycogen synthase kinase 3
HBSS	Hanks balanced salt solution
HIF-1	Hypoxia inducible factor-1
HIF-1α	Hypoxia inducible factor-1α
HIF-1β	Hypoxia inducible factor-1β
HM	Hydrophobic motif
H_2O_2	Hydrogen peroxide
HRE	Hypoxia response element
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis protein
IC	Inhibitory concentration
ID	Inhibitory domain
IFN	Interferon
IGF	Insulin-like growth factor
IκB	Inhibitor of NF-κB
IKK	IκB kinase

IL	Interleukin
ILK	Integrin-linked kinase
IMIG	International Mesothelioma Interest Group
IMS	Industrial methylated spirits
k	Kilogram
KD	Kinase domain
LDH	Lactate dehydrogenase
MAPKAP-K2	Mitogen-activated protein kinase-activated protein kinase 2
Mdm2	Murine double minute 2
mTOR	Mammalian target of rapamycin
NF2	Neurofibromatosis type-2
NF-κB	Nuclear factor-κB
NHS	Normal human serum
NICE	National Institute for Clinical Excellence
Nip3	Nineteen kDa-interacting protein 3
NRS	Normal rabbit serum
NSCLC	Non-small cell lung cancer
Pa	Pascal (SI unit of pressure)
pAkt	phosphorylated Akt
PAS	Per-ARNT-AHR-Sim
PD	Pleurectomy/decortication
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependant kinase 1
PDT	Photodynamic therapy
Per	Drosophilia protein period
PET	Positron emission tomography
PH	Pleckstrin homology
PI3-K	Phosphatidylinositol 3-Kinase
PI(3,4)P2	Phosphatidylinositol-3,4-bisphosphate

PI(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B (Akt)
pO_2	Partial pressure of Oxygen
PP2A	Protein phosphatase 2A
PMT	Photomultiplier tube
pRB	Retinoblastoma protein
PS	Performance status
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and Tensin homolog deleted on chromosome 10
RAC-PK	Related to A and C protein kinase
Rb	Retinoblastoma
RBX-1	Ring-box protein 1
RHD	Rel homology domain
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
SHIP	Src homology 2 domain containing inositol polyphosphate phosphatase
Sim	Drosophilia protein single-minded
Smac/DIABLO	Second mitochondrial-derived activator of caspases
SMR	Soluble mesothelin-related proteins
SSC	Side scatter
SV 40	Simian Virus 40
Tag	Large-T antigen
tag	Small-t antigen
TNF	Tumour necrosis factor
TNM	Tumour-Node-Metastasis
TRADD	Tumour necrosis factor-1-associated death domain
TS	Thymidylate synthase

TSG	Tumour suppressor gene
UK	United Kingdom
UV	Ultra-violet
VATS	Video-assisted thoracoscopic surgery
VHL	von Hippel-Lindau tumour suppressor protein
WT-1	Wilm's tumour suppressor gene-1

LIST OF FIGURES

Figure 4.13 Expression of pAkt and HIF-1α in the JU77 cell line, with or without treatment with 50μM LY294002, with or without exposure to conditions of reduced environmental O_2 concentration

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

1.1 Background

Malignant pleural mesothelioma is an exceptionally aggressive, almost universally fatal neoplasm originating from mesothelial cells that form the serosal lining of the pleural, peritoneal and pericardial cavities (Carbone, Kratzke, & Testa 2002), in decreasing order of frequency (Bielefeldt-Ohmann, Jarnicki, & Fitzpatrick 1996). Despite a number of treatment modalities employed both previously and currently, the prognosis remains poor. Most published patient series have reported median survival ranging between 4 and 18 months (Calavrezos et al. 1988;Edwards et al. 2000;Metintas et al. 2001;Steele 2002).

1.1.1 Epidemiology

Since the first epidemiological evidence linking occupational asbestos exposure to the development of mesothelioma was published more than 40 years ago (Wagner, Sleggs, & Marchand 1960), a large body of work has established not only occupational, but geographical high risk areas (Britton 2002). Currently, 2000-3000 new cases are seen per year in the United States of America (Price & Ware 2004), reflected in the 5-10% increase in annual mortality in most industrialised countries (Britton 2002). In his original work, Peto *et al* predicted that mesothelioma will be responsible for about 250,000 deaths across Western Europe over the next 35 years before declining in incidence (Peto et al. 1999). However, since that time, the data have been refined to include new trends and statistical models now predict a peak of approximately 1950-2450 deaths per year between 2011 and 2015 in Great Britain. Additionally, it is estimated that between 1960 and 2050, MPM will account for 90,000 deaths in Great Britain, of which 65,000 will occur after 2001 (Hodgson et al. 2005). Despite this apparent reduction in the predicted peak in this country, a more concerning fact is that although great strides have been made in attempting to discontinue the use of asbestos, it is still being extensively mined and used in many parts of the world, especially in parts of Asia (Nicholson 2001).

1.2 Diagnosis and Management of Malignant Pleural Mesothelioma

1.2.1 Clinical Features

The patient with diffuse pleural mesothelioma is three times more likely to be male and usually presents in the fifth to seventh decade with worsening dyspnoea over the preceding weeks or months. This may be accompanied by a cough. Dull, non-specific chest pain is commonly seen and may occasionally be pleuritic (2001). In the early stages, dyspnoea is often due to the presence of a large, bloodstained pleural effusion, but as the disease progresses, a thick rind of tumour that prevents lung expansion replaces the parietal and visceral pleural layers (figure 1.1). The affected hemi-thorax, right more often than the left (Sugarbaker et al. 1996), displays the physical signs appropriate to thickened pleura, effusion and poor lung expansion, which include reduced breath sounds and dullness to percussion. Chest wall and abdominal masses, ascites and finger clubbing may all be seen. Systemically, the patient may complain of weight loss and intermittent profound sweating. The disease is locally aggressive and invades into intra-thoracic structures, although the frequency of finding distant spread is increasing (2001).

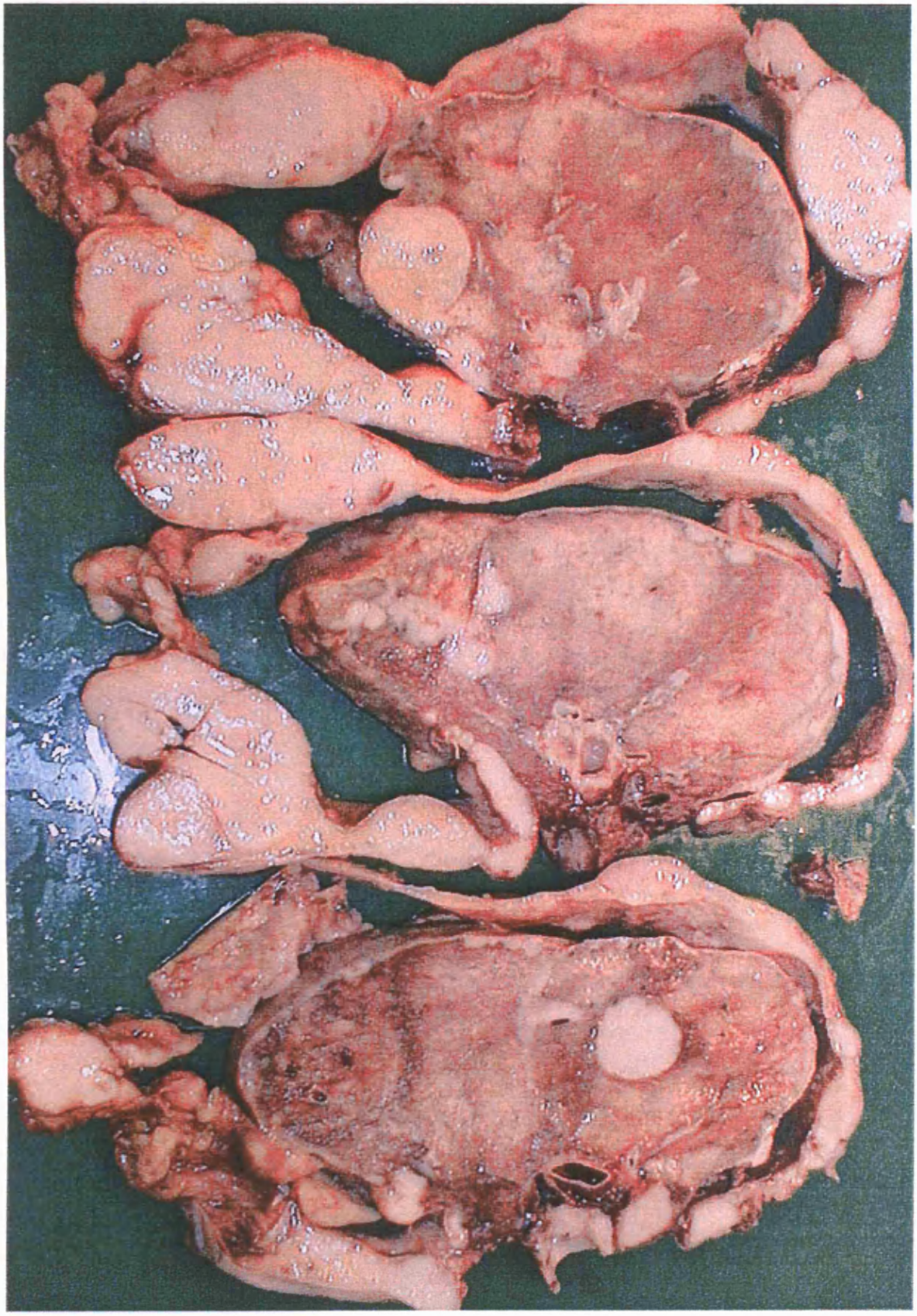
1.2.2 Histology

Histological diagnosis has in the past proven a challenge, often because the rarity of this disease prevented adequate exposure for most pathologists to tumour specimens. Now however, much work has been completed, throwing light over this diagnostic dark spot, especially in terms of immunohistochemical techniques.

There are three cellular types: epithelioid, making up about 50% of cases; sarcomatoid, containing connective tissue elements; and mixed, or biphasic, composed of a varying amount of both. Using light microscopy, sarcomatoid disease is usually recognisable to the experienced eye. The real diagnostic difficulty is most commonly encountered in the differentiation of epithelioid mesothelioma and metastatic adenocarcinoma. In a previous review, Ordonez concluded that calretinin, cytokeratin 5/6 and Wilm's tumour suppressor gene-1 (WT-1) are the best positive markers of epithelioid MPM aiding differentiation from adenocarcinomas of unknown origin. However, cytokeratin 5/6 and WT-1 are frequently expressed in squamous carcinomas. Carcinoembryonic antigen (CEA) and the

monoclonal antibodies B72.3 and MOC-31 are the most sensitive and specific negative markers for differentiating mesothelioma from pulmonary adenocarcinomas, although CEA is uncommonly expressed in ovarian malignancies (Ordonez 2002). In addition, the negative markers E-Cadherin and thyroid transcription factor-1, along with the positive markers N-Cadherin, thrombomodulin, BerEP4 and LeuM1(CD15) have also been advocated (Abutaily, Addis, & Roche 2002).

Figure 1.1 Pathological sectioning of a lung surrounded by a rind of MPM, including intra-parenchyma invasion.



1.2.3 Staging

Staging is most frequently achieved by radiological techniques in the first instance, followed if necessary by surgical intervention. After the initial chest radiograph, computed tomography (CT) of the thorax (figure 1.2a) and upper abdomen is used to identify the extent of the disease. Magnetic resonance imaging (MRI) (figure 1.2b) is useful with multiplanar capabilities and increased sensitivity for the detection of tumour extension into the diaphragm, interlobar fissures and the chest wall (Boraschi et al. 1999;Heelan et al. 1999;Knuuttila et al. 2001;Marom et al. 2002). It has recognised value in the delineation of T3 from T4 disease, in the context of selection for radical surgery (Stewart et al. 2003). Positron emission tomography (PET), utilising radiolabelled 18-fluorodeoxyglucose (FDG) as a marker is already being used both as a staging tool and in assessment of treatment response (Benard et al. 1999;Carretta et al. 2000;Gerbaudo et al. 2002;Schneider et al. 2000;Zubeldia, Abou-Zied, & Nabi 2000).

Thoracoscopy plays an important role in the diagnosis and staging of pleural mesothelioma. It is often difficult to achieve adequate tumour samples for diagnosis from blind, percutaneous biopsy or with CT guidance (Adams et al. 2001). In this situation video-assisted thoracoscopy can be carried out, allowing complete drainage of any effusion, assessment of the entire hemi-thorax, biopsy of tumour and hilar or mediastinal lymph nodes and pleurodesis if required, in most cases.

1.2.3.1 Clinical Staging Systems

Several staging systems have been proposed, the earliest being that by Butchart et al in 1976 (Butchart et al. 1976) (table 1.1). Since then four further systems have been developed, only one of which managed to correctly stratify survival incorporating the assessment of resection margins and nodal status (Sugarbaker et al. 1999) (table 1.2). However, the TNM system proposed by the International Mesothelioma Interest Group in 1995 (Rusch 1995), developed using new data regarding the impact of T and N status on survival, is being increasingly used in clinical trials to aid comparability of results (table 1.3).

Figure 1.2a CT of the thorax showing left-sided pleural disease (arrow).

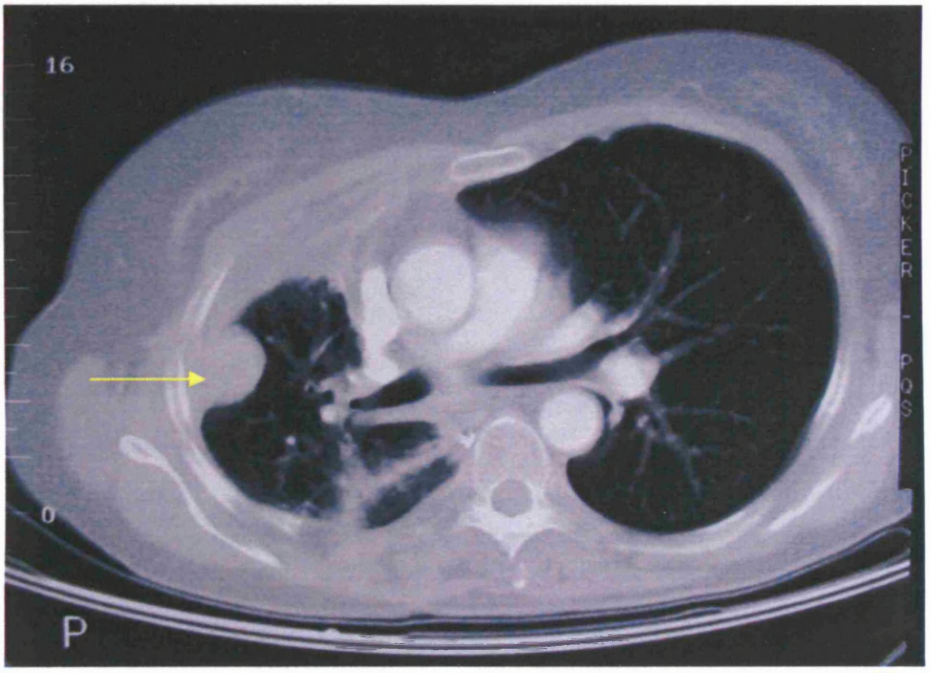


Figure 1.2b MRI of the thorax showing enhancement of the left pleura with bulky tumour over the diaphragm (thick arrow), extending into the oblique fissure (thin arrow).

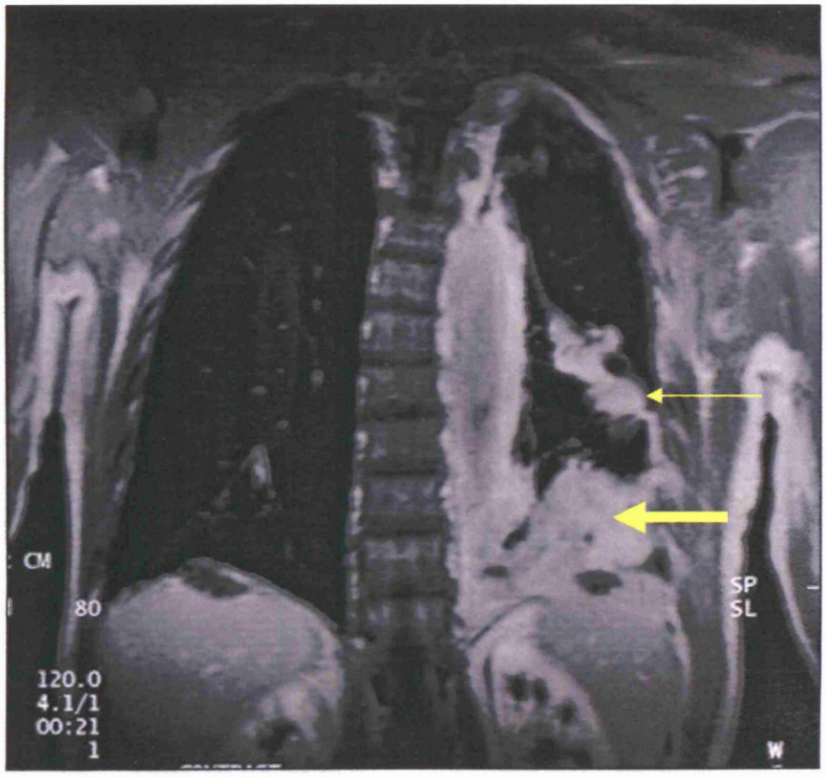


Table 1.1 The Butchart clinicopathological staging system for diffuse malignant mesothelioma (Butchart et al. 1976)

Stage	Clinicopathological Staging
I	Tumour confined to the ipsilateral pleura, lung and pericardium
II	Tumour invading chest wall or involving mediastinal structures, eg. Oesophagus, heart, opposing pleura Lymph node involvement within the chest
III	Tumour penetrating diaphragm to involve peritoneum directly Lymph node involvement outside chest
IV	Distant blood-borne metastases

Table 1.2 The Brigham staging system for malignant pleural mesothelioma (Sugarbaker et al. 1999)

Stage	Definition
I	Disease completely resected within the capsule of the parietal pleura without adenopathy: ipsilateral pleura, lung, pericardium, diaphragm, or chest wall disease limited to previous biopsy sites
II	All of stage I, with positive resection margins and/or intrapleural adenopathy
III	Local extension into chest wall or mediastinum; into the heart or through the diaphragm or peritoneum; or with extrapleural lymph node involvement
IV	Distant metastatic disease

Table 1.3 The International Mesothelioma Interest Group staging system for malignant pleural mesothelioma (Rusch 1995)

Stage		**Description**
Tumour (T) Stage	**T1a**	Tumour limited to ipsilateral pleura, including mediastinal and diaphragmatic pleural, without involvement of visceral pleura
	T1b	T1a + scattered foci of tumour involving the visceral pleura
	T2	Tumour involving each of ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: • Involvement of diaphragmatic muscle • Confluent visceral pleural tumour (including fissures), or extension of tumour from visceral pleura into underlying pulmonary parenchyma
	T3	Locally advanced, but potentially resectable tumour. Tumour involves all of the ipsilateral pleural surfaces with at least one of the following features: • Involvement of endothoracic fascia • Extension into mediastinal fat • Solitary, completely respectable focus invading into the soft tissues of the chest wall • Non-transmural involvement of the pericardium
	T4	Locally advanced, technically unresectable tumour. Tumour involves all of the ipsilateral pleural surfaces with at least of the following features: • Diffuse extension or metastatic spread into the chest wall with or without rib destruction • Direct trans-diaphragmatic spread extension to the peritoneum • Direct extension to the contralateral pleura, any mediastinal organ or the spine
Nodal (N) Stage	**Nx**	Regional lymph nodes (LNs) cannot be assessed
	N0	No regional lymph node metastases
	N1	Involvement of the ipsilateral bronchopulmonary or hilar LNs
	N2	Involvement of subcarinal or ipsilateral mediastinal LNs (including the internal thoracic LNs)
	N3	Involvement of contralateral mediastinal or internal thoracic LNs, or any supraclavicular LNs
Metastases (M) Stage	**Mx**	Presence or absence of distant metastases cannot be assessed
	M0	No distant metastases
	M1	Distant metastases present
Overall Staging		**Corresponding TNM Stage**
Stage Ia		T1a N0 M0
Stage Ib		T1b N0 M0
Stage II		T2 N0 M0
Stage III		Any T3 M0 Any N1 M0 Any N2 M0
Stage IV		Any T4 Any N3 Any M1

1.2.3.2 Prognostic Scoring Systems

Several published patient series (Calavrezos et al. 1988;Edwards et al. 2000;Metintas et al. 2001;Steele 2002) have provided details of various clinicopathological variables found to be of prognostic significance including histological subtype, tumour stage, performance status, gender, chest pain, weight loss, haemoglobin concentration, white cell count, platelet count and lactate dehydrogenase level. The Cancer and Leukaemia Group B (CALGB) and the European Organisation for the Research and Treatment of Cancer (EORTC) have produced scoring systems using combinations of these variables to allow the categorisation of patients into different prognostic groups (Curran et al. 1998;Herndon et al. 1998) (figure 1.3 and table 1.4, respectively). Recently, several novel biological markers of prognosis have been proposed, based on immunohistochemical examination of tumour samples. Amongst others, both angiogenesis (using microvessel density as a marker) and cyclooxygenase-2 expression have been shown to correlate with a poor outcome (Edwards et al. 2001;Edwards et al. 2002).

1.2.4 Chemotherapy

Over the last 30 years or more, virtually every class of chemotherapeutic agent has been used in the treatment of mesothelioma (Baas 2002;Ong & Vogelzang 1996;Ryan, Herndon, & Vogelzang 1998), and in general terms the reported successes have been limited (Singhal & Kaiser 2002). There are likely to be at least several reasons for this. Firstly, because mesothelioma is relatively rare, many studies have enrolled only small numbers. Secondly, different groups have used varied staging systems and methods of stratification of patients, making meaningful comparisons difficult. Thirdly, in terms of response and outcome, some studies have attempted to look at objective measurements such as a reduction in tumour bulk, which is notoriously difficult, and others have addressed symptom control and quality of life issues. Also, some have measured survival from diagnosis date compared with the date of treatment commencement, which has led to statistical anomalies.

Figure 1.3 Flow chart showing the derivation of the Cancer and Leukaemia Group B prognostic scoring system for patients with mesothelioma. Times in months represent median survival figures assigned to the respective terminal groupings. (Herndon et al. 1998).

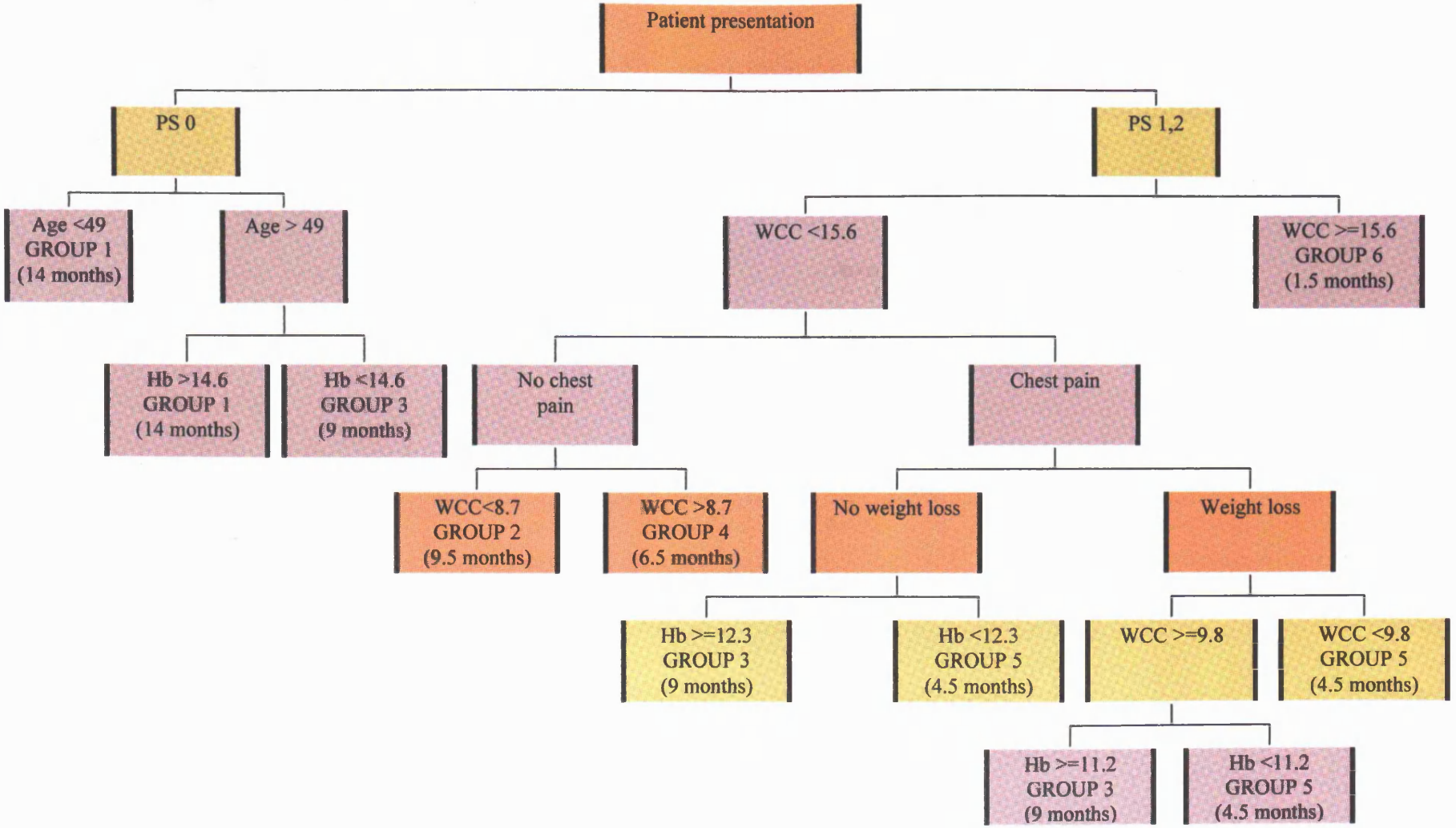


Table 1.5 The European Organisation for Research and Treatment of Cancer prognostic scoring system for patients with pleural mesothelioma (Curran et al. 1998).

Factor	**Category**	**Risk Score**
White Cell count	$>8.3x10^9/L$	+0.55
Performance Status	1 or 2	+0.60
Histology	"probable" or "possible" sarcomatoid	+0.52 +0.67
Gender	Male	+0.60

Patients are assigned to a "good" prognosis group (40% 1 year survival) if the sum of the risk scores </=1.27 (ie. 2 factors or less). Those with a risk score sum of >1.27 fall into a "poor" prognosis group 12% 1 year survival).

A meta-analysis by Berghmans et al, published in May 2002, stated that combination therapy with the anthracycline, Doxorubicin, and the platinum agent, Cisplatin had the highest consistent response rate of 28% at that time (Berghmans et al. 2002). They also commented that alone, Cisplatin appeared to be the most active agent. Other single agents evaluated include the vinca alkaloid, Vinorelbine, which was shown to produce a partial response in 24% of 29 patients treated (Steele et al. 2000) and the pyrimidine antimetabolite, Gemcitibine, which in three separate studies produced response rates varying from 0% to 31% (Kindler et al. 2001;Kindler & van Meerbeeck 2002). Interestingly, in combination with a platinum agent, Gemcitabine has promising activity, giving response rates from 16% to 48% in the phase II setting (Byrne et al. 1999;Nowak et al. 2002a;van Haarst et al. 2002). Other agents used either alone or in combination have included antimetabolites (Raltitrexed, Methotrexate and 5-Fluorouracil), alkylating agents (Ifosfamide and Cyclophosphamide) and camptothecins (Topotecan and Irinotecan).

Quality of life assessments have provided important evidence for the benefits of chemotherapy in this disease. Using single agent Vinorelbine, 47% of patients demonstrated an improvement in respiratory symptoms and 74% in psychological feeling (Steele et al. 2000). Byrne et al demonstrated an increase in the vital capacity of responding patients leading to symptomatic benefit in a single centre phase II trial of Cisplatin and Gemcitabine (Byrne et al. 1999). Using the same combination of drugs in a multicentre trial, it was even more encouraging that vital capacity and global quality of life remained stable in all patients and improved significantly in responding patients (Nowak et al. 2002). Thirty-nine patients receiving the combination of Mitomycin C, Vinblastine and Cisplatin (MVC) graded their own symptoms, with 62% reporting improvements, especially in cough and pain (Middleton et al. 1998).

1.2.4.1 Pemetrexed

Perhaps the most promising recent development has been the anti-tumour activity associated with the antimetabolite, Pemetrexed. Pemetrexed is a multitargeted antifolate that inhibits thymidylate synthase (TS), glycinamide ribonucleotide transformylase (GARFT) and dihydrofolate reductase (DHFR) (Calvert & Bunn, Jr. 2002;Curtin & Hughes 2001). A phase III study comparing pemetrexed

and cisplatin with cisplatin alone has shown an improvement in not only median survival, but also time to disease progression (Vogelzang et al. 2003). It is not certain at present why an antifolate should be so active in this disease, but recent work has shown consistent over expression of the alpha folate receptor in mesothelioma, which may be responsible, at least in part. This does not appear to vary according to the histological subtype (Bueno et al. 2001).

1.2.4.2 Intra-pleural and Hyperthermic Chemotherapy

With the lack of convincing benefits of conventional delivery methods, investigators have looked to other possible options. Intrapleural chemotherapy has been shown to be technically feasible, producing an increased concentration of the drug in close proximity to tumour cells and a prolonged systemic drug exposure, with reduced toxicity complications when compared to intravenous delivery (Bogliolo et al. 1991). An increase in median survival was seen in a series of 28 patients who underwent radical debulking surgery and intrapleural chemotherapy, followed by systemic chemotherapy, but the local treatment had no effect in reducing locoregional recurrence (Rusch et al. 1994). For those with unresectable or inoperable disease and troublesome, recurrent intra-pleural collections, there is some experience with the use of implantable pleural catheters allowing repeated delivery of chemotherapy, which has been shown to provide benefit in symptom improvement (Omasa et al. 2001;Shoji et al. 2002). In an attempt to harness the improved anti-tumour effects with hyperthermia (Matsuzaki et al. 1995), instillation of agents at temperatures above that of body temperature have shown promising results in terms of increasing local tumour control (de Bree et al. 2002;Yellin et al. 2001) and higher local tissue concentrations are achievable than with normothermic chemotherapy (Ratto et al. 1999). In a further variation of this theme, Bakhshandeh et al have recently reported experience employing the conventional systemic delivery of ifosfamide, carboplatin and etoposide chemotherapy, but using whole body hyperthermia to 41.8 °C (Bakhshandeh et al. 2003). Most recently however, the results of a phase I study of intraoperative hyperthermic cisplatin immediately following radical surgery were presented at the 2007 annual meeting of the American Society of Clinical Oncologists (ASCO), with patients in conventional better prognostic groups appearing to derive the most benefit (Zellos et al. 2007)

1.2.4.3 Neoadjuvant Chemotherapy

The considerable morbidity associated with radical surgical procedures undertaken in this disease has led to the concept of treatment with chemotherapy prior to any definitive operative procedure. Initially this was felt likely to increase intra-operative technical difficulties because of the obliteration of the usual tissue planes and an association with increased post-operative complications (Sugarbaker et al. 1999). However, favourable results have been seen in a phase II study of initially 19 patients given 3 cycles of Cisplatin and Gemcitabine, followed by extra-pleural pneumonectomy (EPP) (Weder et al. 2004). An expansion of this series has shown manageable complications at an acceptable rate (Opitz et al. 2006).

1.2.5 Surgery

The role of surgery in this disease can be divided into three broad categories. Surgery, often required for the purposes of diagnosis and staging, also has a role in the palliation of symptoms, including debulking procedures, and finally, its most controversial guise, in the radical resection of tumour (Waller 2003).

1.2.5.1 Video-Assisted Thoracoscopic Surgery (VATS)

VATS has proven to be a useful surgical technique in this disease. It is frequently employed to obtain adequate tissue to allow histological diagnosis, but at the same sitting may be used for symptom control of malignant effusion by pleurodesis (Benko, Molnar, & Horvath 1999;Furedi et al. 1999) and by parietal pleurectomy (Grossebner et al. 1999;Waller, Morritt, & Forty 1995). It has now been shown that VATS techniques can be used reliably and safely for visceral decortication for those patients in whom more radical procedures are not possible (Martin-Ucar et al. 2001) (figure 1.4a).

1.2.5.2 Extra-pleural pneumonectomy

Without doubt, EPP is the most radical surgical procedure carried out for this disease. It provides maximal cytoreduction of tumour by *en bloc* removal of parietal and visceral pleurae, with the ipsilateral lung, pericardium and hemidiaphragm (figure 1.4b). Mediastinal lymphadenectomy is performed and the pericardium and hemidiaphragm are then reconstructed (Rusch, Piantadosi, & Holmes 1991;Sugarbaker, Mentzer, & Strauss 1992). As may be anticipated there is considerable

morbidity associated with this procedure including cardiac dysrhythmias, most commonly supraventricular tachycardias, rapid pneumonectomy space filling with symptomatic mediastinal shift, bronchopleural fistula, chylothorax, patch disruption and empyema (Stewart et al. 2005). In addition to this, previously this procedure has been associated with a high operative mortality. One early series reported a mortality rate of 31% (Butchart et al. 1976), but as experience has grown, this has been reduced to approximately 5% (Pass et al. 1997;Rusch 1999;Sugarbaker et al. 1999).

1.2.5.3 Pleurectomy/Decortication

An alternative to EPP is pleurectomy/decortication (PD). This involves a parietal pleurectomy and visceral tumour decortication, with resection and reconstruction of the ipsilateral pericardium and hemidiaphragm, but avoids any significant lung resection (Rusch 1993). This procedure is not as technically demanding as EPP (Zellos & Sugarbaker 2002) and has been shown to have a lower operative mortality (less than 5%) (Rusch 1997). It is acknowledged that local recurrence is a problem with this technique (Pass et al. 1997b;Rusch 1994), and this may be explained by difficulties in achieving clearance of tumour from the interlobar fissures (Zellos & Sugarbaker 2002), although evidence may be emerging that, for early stage disease, improved survival can be achieved with PD (Flores et al. 2007).

Figure 1.4a Parietal pleural tumour over the mediastinal and diaphragmatic surfaces seen at thoracoscopy

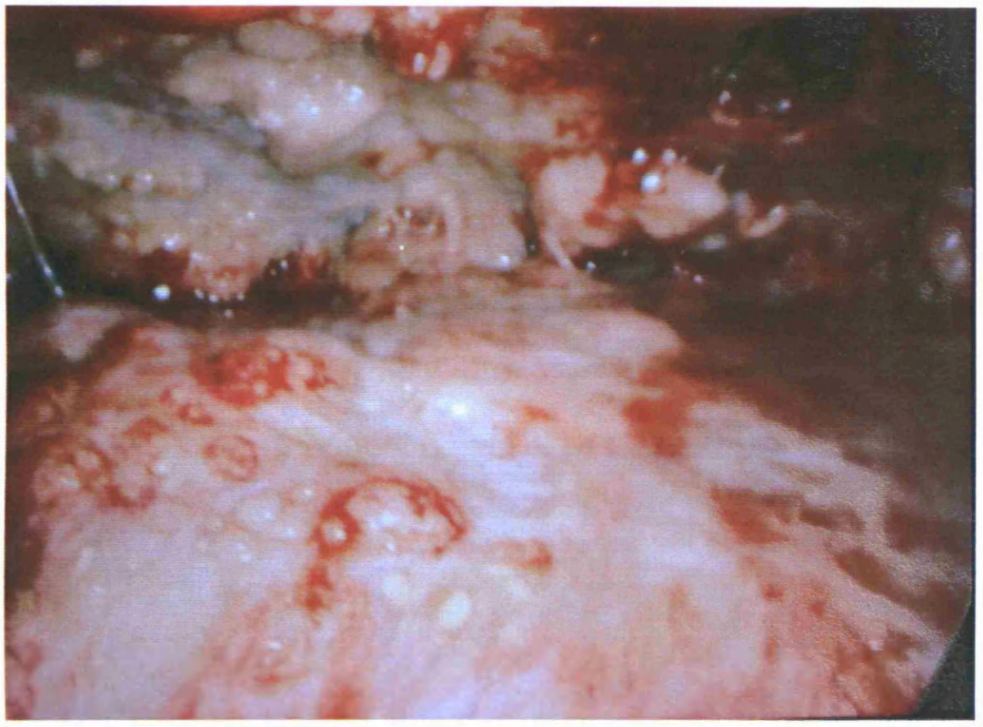
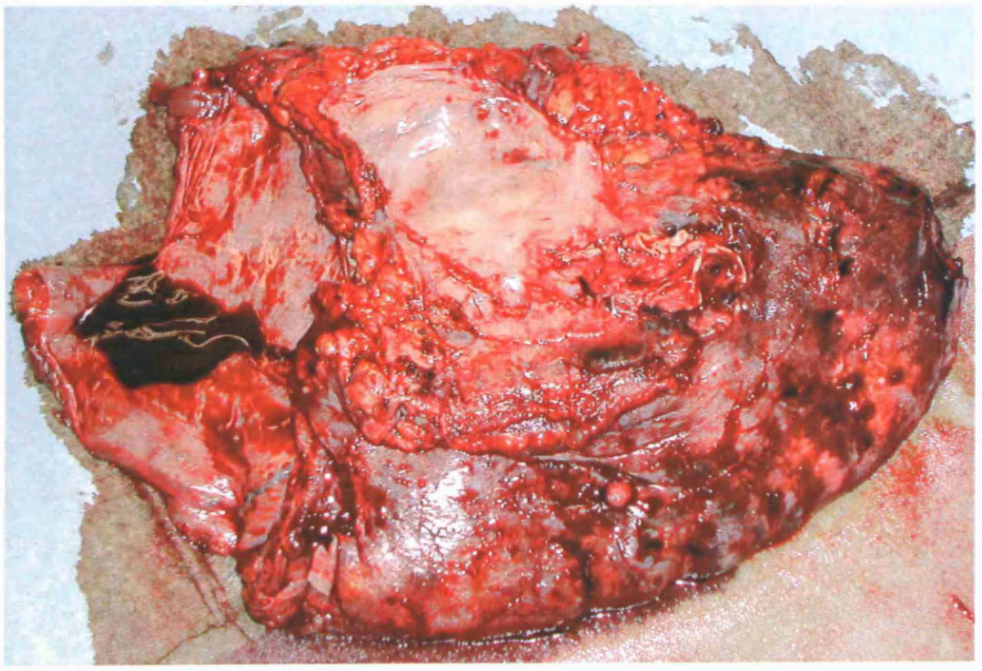


Figure 1.4b *En-bloc* specimen removed at EPP



1.2.6 Radiotherapy

In-vitro work has established that mesothelioma cell lines are unexpectedly radio-sensitive (Carmichael et al. 1989), but clinical application of this is limited by intra-thoracic structures. Because of the massive field sizes required, organs such as the heart, lung, oesophagus, liver and even the ipsilateral kidney can be significantly irradiated during treatment (Singhal & Kaiser 2002). Radiation therapy alone in this disease has failed to increase survival (Steele & Rudd 2000) because of the dose-limiting problems, with response rates reported to be as low as 3% (Zellos & Sugarbaker 2002). In an attempt to provide greater protection to underlying structures, especially the lung, combined photon and electron beam techniques have been described, using central lung blocking to reduce doses to non-diseased areas (Kutcher et al. 1987). Despite weaknesses in its use as a single mode of treatment, radiotherapy is effective in the prevention or reduction of tumour seeding within the chest wall at wound sites (Boutin, Rey, & Viallat 1995;Senan 2003) and appears to have a role in combination with other therapies.

1.2.6.1 Radiotherapy, Including Brachytherapy, as an Adjunct to Surgery

Surgical treatment alone for mesothelioma has been shown to lead to high rates of local recurrence (Baldini et al. 1997), of up to 80% in some reports (Rusch et al. 2001). The use of radiotherapy has been suggested as an additional treatment in an attempt to increase disease control. Some success has been achieved with high-dose hemithoracic radiotherapy in the post-operative period following either EPP or PD (Rusch et al. 2001).

Another technique involves the use of intra-operative radiotherapy to areas of high risk for residual disease (fissures, pericardium and diaphragm), with conformal external beam radiotherapy post-operatively (Lee et al. 2002).

The theoretical benefits of delivering high doses of localised radiation treatment with maximal sparing of surrounding areas has provided the impetus to evaluate the use of brachytherapy in this disease (Raben & Mychalczak 1997). Unfortunately, only limited success has been achieved with the instillation, either permanently with seeds or implants, or temporarily with phosphate colloids, of

radioactive materials into the pleural cavity was combined with surgery and post-operative conformal radiotherapy in a series of patients at the Memorial Sloane-Kettering Cancer Center (Hilaris et al. 1984;Rusch 1997).

1.2.6.2 Intensity-Modulated Radiotherapy (IMRT)

IMRT is a relatively new technique developed previously to enable a reduction in the field sizes of conformal radiotherapy in the treatment of centrally located lung cancers (Brugmans et al. 1999;Derycke et al. 1998). In mesothelioma, the aim is again to improve local control through delivery of the maximum radiotherapeutic dose possible whilst obtaining the greatest sparing of surrounding normal tissues. In a report by Meeks et al, IMRT was found to be advantageous for large, irregular targets with critical structures in close proximity (Meeks et al. 1998), precisely the conditions that are seen in mesothelioma.

Briefly, the process involves careful immobilisation of the patient in the treatment position. Critical structures and the clinical target volume (CTV) are then delineated, taking into account potential uncertainties due to movement. With the desired doses and dose tolerances, inverse planning is used to optimise the spatial distribution of radiation intensity within each field. Each radiation beam is mathematically described as a collection of small (typically 1cm x 1cm) beamlets. The intensity of each of these beamlets can be varied to achieve the desired dose distribution. Once an optimal plan is achieved, a template plan can be established for future treatments, thereby reducing the total planning time needed. The delivery of these beamlets, or IMRT fields, is achieved by dynamic multileaf collimation (DMLC), a technique in which the leaf pairs of a multileaf collimator are swept across the field at different rates to vary the dose rate from the accelerator. Some early results from the MD Anderson Cancer Center in Texas, USA, have shown promising early control of the disease following EPP (Ahamad et al. 2003). This technique is now being explored and refined in many centres all over the world.

1.2.7 Trimodality Therapy

Combination therapies for mesothelioma have developed out of necessity due to the poor outcome of patients treated with a single modality. Some of the options for incorporating two treatment modalities have already been examined, but some centres have taken this one step further with the addition of a third technique. Initially, trimodality therapy consisted of surgery, usually EPP or PD, followed by chemo-radiotherapy. However, with the increasing popularity of chemotherapy prior to surgery, it is likely that this neoadjuvant approach will become the mainstay.

1.3 Novel Therapies for Malignant Pleural Mesothelioma

The lack of randomised-controlled trial evidence of effective treatment for mesothelioma has provided clinicians with an almost unique opportunity to explore alternative avenues in the management of this disease. New ideas, involving immunotherapy and its combination with chemotherapy, gene therapy, angiogenesis inhibitors and other novel inhibitors are being investigated. Much of this work has been developed as a result of our currently inadequate, but increasing, understanding of the biology of this disease (Schwarzenberger, Byrne, & Kolls 1999).

1.3.1 Photodynamic Therapy

Photodynamic therapy (PDT) describes the local or systemic administration of a chemical compound that accumulates within tumour cells and, when activated by light of a particular wavelength, produces toxic oxygen species that lead to cell death (Singhal & Kaiser 2002;Sterman, Kaiser, & Albelda 1999), possibly through induction of apoptotic pathways. As well as direct tumouricidal effects, PDT also has indirect effects on tumour vasculature, causing haemorrhagic necrosis of tissue (Pass & Pogrebniak 1992). Despite several trials, no definitive benefits have been shown (Baas et al. 1997;Moskal et al. 1998;Pass et al. 1997) and the treatment itself has led to development of significant complications (Luketich et al. 1996;Temeck & Pass 1995). The development of new photosensitising drugs with greater tissue penetration, increased production of oxygen species and fewer systemic side effects may enhance further investigation of this adjuvant therapy (Hahn, Smith, & Friedberg 2001;Krueger et al. 2003).

1.3.2 Immunotherapy

Several cytokines have been investigated for their activity in this disease, after displaying the ability to inhibit growth of human or murine mesothelioma cell lines. They include interleukin (IL)-2, IL-12, TNF-α and interferon (IFN)-α, -β and -γ.

IL-12 has been shown to have very definite anti-tumour activity in a murine MPM model (Caminschi et al. 1998). Systemic administration at the time of tumour inoculation prevented tumour growth in 70% of mice. Intra-lesional administration in the same study led to temporary regression of tumour growth associated with increased CD4 and CD8 lymphocyte counts. Using an IL-12 gene and successfully transfecting it into a murine model, the same group have shown that paracrine secretion of IL-12 can induce immunity against mesothelioma, without the toxicity associated with systemic administration (Caminschi et al. 1999).

There is some evidence illustrating the toxic effects of TNF-α against human mesothelioma cell lines implanted in nude mice (Ohnuma et al. 1993), but this cytokine has provoked only limited tumour response in the clinical situation, despite being well tolerated by patients (Stam et al. 2000). This may be explained considering the identification of a TNF-α inhibitor in pleural fluid that may be produced by tumours to evade the cytotoxic action of TNF-α itself (Martinet et al. 1992).

The interferons have all been shown to inhibit growth of mesothelioma cell lines to varying degrees (Nowak et al. 2002). They are known to increase the expression of human leucocyte antigen (HLA) antigens on malignant cell membranes (Christmas 1991), which may in turn produce better targets for the immune system. Despite this, the overall clinical experience has been disappointing.

As well as the use of various cytokines as single therapies, numerous studies have evaluated anti-tumour activity using combinations of different cytokines. For instance, TNF-α has been combined with IL-2 (Bowman et al. 1991) and IFN-γ (Nowak et al. 2002) and IFN-γ with activated macrophages (Monnet et al. 2002), but unfortunately the results have been less than impressive or the treatments too toxic.

1.3.2.1 Combined Immunochemotherapy

Theoretical synergistic effects between standard chemotherapy agents and cytokine treatments have prompted researchers to evaluate their roles when used in combination in this disease. Most studies have used the most active chemotherapeutic agents in combination with either interferons or interleukins. Examples include the anti-metabolite methotrexate combined with IFN-α and –γ (Halme et al. 1999), cisplatin, mitomycin and IFN-α-2a compared with best supportive care (Metintas et al. 1999), and carboplatin has been combined with IFN-α-2a (O'Reilly et al. 1999). Although some studies have reported successes in terms of tumour response, the situation has been clouded by the appearance of significant side effects, including myelosuppression with anaemia, thrombocytopaenia, neutropaenia and renal toxicity (Parra et al. 2001;Purohit et al. 1998).

1.3.3 Gene Therapy

Continuing the search for effective treatments in mesothelioma has led inexorably to the rapidly evolving and developing field of gene therapy. The acknowledgement of genetic influences on diseases in general and indeed the findings that certain conditions can be attributed to specific genetic abnormalities have helped to create the supposition that manipulation of an individuals' genome in various different, but very specific ways, may alter the outcome of the underlying disease process. For several reasons, pleural mesothelioma is an excellent candidate for this work. As previously established, there exist no effective standard therapies, providing sound ethical ground. The tumour remains localised until late in its course and the pleural cavity itself is very accessible for tumour biopsy, vector delivery and subsequent assessment of response to treatment. Also, the local delivery of treatment should theoretically reduce any systemic side effects (Nowak et al. 2002;Sterman, Kaiser, & Albelda 1999).

The possibility of re-expression or reactivation of tumour suppressor genes within active tumour has been considered as one potential avenue of application of gene therapy. The $p16^{INK4a}$ gene product has produced the most interest so far. Frizelle et al have shown, following transduction of a $p16^{INK4a}$ expressing adenovirus, in-vitro cell-cycle arrest, reduction in cell growth and eventual cell death. In mesothelioma xenografts, inhibition of tumour formation, tumour growth arrest and reduced

tumour size and spread were all seen (Frizelle et al. 1998). Prolonged survival was also seen in the transduced xenografts (Frizelle et al. 2000). Another group has reported similar success, with the overexpression of transfected $p14^{ARF}$ leading to cell cycle arrest in phase G1, and apoptotic cell death (Yang et al. 2000).

Despite problems with toxicity, the potential efficacy of immunotherapy with different cytokines in mesothelioma, delivered either locally or systemically, has led to the investigation of gene therapy to augment the immune response. Researchers at the University of Western Australia have used a replication-restricted Vaccinia virus (VV) as a vector for intra-tumoural IL-2 transfection in six patients with treatment-resistant mesothelioma. A T-cell infiltrate was seen in 50% of tumour biopsies and VV-IL-2 mRNA was detectable up to 3 weeks following administration. Encouragingly there were no significant toxicities reported but there was also no evidence of tumour regression (Mukherjee et al. 2000). The same group have also reported findings combining gene therapy with surgical debulking in a murine model. After surgical debulking, vaccinations with tumour transfectants encoding genes for IL-4, IL-2, GM-CSF and B7-1 were given at sites away from tumour masses. Transfectants that expressed B7-1 or produced high levels of GM-CSF, in combination with debulking, effectively delayed tumour growth (Mukherjee et al. 2001). More recently, in a study using a replication-defective adenovirus and IFN-γ, in 10 human mesothelioma cell lines in nude mice, Gattacceca et al have shown prolonged production of the cytokine in the transfected cell lines, with a delay in tumour development (Gattacceca et al. 2002). Although the mechanisms are still to be fully elucidated, there is some evidence that tumour-specific CD4+ and CD8+ T cells are key effectors in tumour eradication in this situation.

A further idea has been to attempt to deliver a toxic or "suicide" gene into a tumour cell that leads to destruction of that cell. This is also referred to as molecular chemotherapy. The most promising avenue, and the most extensively studied, involves the herpes simplex virus (HSV)-thymidine kinase (tk) gene. Although non-toxic on its own, it increases cell sensitivity to nucleoside analogues, for example, Ganciclovir (GCV). It achieves this through monophosphorylation of this drug, which is then rapidly converted to a triphosphate form, which is a potent inhibitor of DNA

polymerase, thereby reducing DNA replication. A phenomenon known as the "bystander effect" has been observed with this treatment. This involves the destruction of neighbouring, non-transduced cells either by toxic GCV metabolites passing between cells via gap junctions or apoptotic vesicles, or by generation of an anti-tumour immune response (Sterman, Kaiser, & Albelda 1999). It has been shown by several groups that, using a replication-deficient adenovirus encoding HSVtk (Ad.HSVtk), efficient transduction of mesothelioma cells can be achieved and, in combination with systemic ganciclovir administration, this vector is effective in the treatment of human mesothelioma tumours in murine models (Esandi et al. 1997;Hwang et al. 1995;Smythe et al. 1995). Progressing from this, Sterman et al reported a phase I study, carried out between 1995 and 1997. Twenty-six patients underwent intra-pleural administration of Ad.HSVtk at doses ranging from 10^9 plaque-forming units (PFU) to 10^{12} PFU, followed by 2 weeks of intravenous ganciclovir. Gene transfer was detectable in 17 out of 25 patients evaluated in a dose-dependent fashion. Strong anti-adenoviral humoral and cellular immune responses were noted and tk protein was found on immunohistochemical evaluation to have penetrated 40-50 cell layers below the mesothelial surface (Sterman et al. 1998;Sterman, Kaiser, & Albelda 1998;Sterman, Kaiser, & Albelda 1999). Although dose-limiting toxicities were never reached, systemic side effects were observed and work is continuing to refine these techniques, with second-generation adenoviral vectors (Lanuti et al. 1999) and the addition of systemic steroids (Sterman et al. 2000).

Antisense oligonucleotides (AO) are RNA sequences designed to alter expression of certain genes in a very specific manner. Currently in mesothelioma, this involves in-vitro work only, concentrating on two particular areas. Survivin, a member of the inhibitors of apoptosis gene family, has been found to be overexpressed in freshly resected mesothelioma specimens by Xia et al. They have transfected anti-survivin oligonucleotides into survivin-positive and survivin-negative cell lines, finding significantly increased rates of apoptosis in the survivin-positive cell lines (Xia et al. 2002). In addition to this, and progressing along a similar route, Smythe et al have used bcl-xl AO. The bcl-xl gene is part of the bcl-2 family of genes, and again, is important in apoptotic homeostasis. After treating mesothelioma cell lines with the bcl-xl AOs, significant apoptosis was seen when compared with bcl-xl sense oligonucleotides (Smythe et al. 2002).

1.3.4 Vaccines

To further manipulate the immune response to mesothelioma, an avenue of research has focused on the production of vaccines for this disease. One approach has been the use of an allogeneic, irradiated ovarian carcinoma cell line, retrovirally transfected with the HSVtk gene (PA-1-STK cells) and then administered intra-pleurally, combined with systemic GCV treatment (Schwarzenberger et al. 1998). This treatment was found to be effective in killing both mouse and human mesothelioma cells in a dose-dependent manner. A phase I study in 6 patients has shown that this is a well-tolerated treatment, with an increase in CD8 T-cells in pleural fluid and that the PA-1-STK cells home to tumour sites very well in vivo (Harrison, Jr. et al. 2000;Schwarzenberger et al. 1998).

1.3.5 Anti-Angiogenesis Therapy

1.3.5.1 Bevacizumab

Bevacizumab is a recombinant anti-VEGF monoclonal antibody that blocks VEGF binding to its receptors. Pre-clinical and early clinical evaluation has been very encouraging (Ferrara 2002), with decreased vascular permeability, vessel regression in murine models (Jain 2002) and good synergistic effects with conventional chemotherapy (Kindler 2004).

1.3.5.2 Tyrosine Kinase Inhibition

In addition to VEGF and COX-2, the role of epidermal growth factor receptor (EGFR) as an autocrine growth factor has been suggested, and high levels of expression have been seen in up to 70% of mesothelioma samples (Janne et al. 2002).

As with VEGF inhibition, the current strategies for EGFR involve manipulation of the activity of its tyrosine kinase. ZD1839 (Iressa) is an orally administered, highly selective EGFR tyrosine kinase inhibitor that has been shown to be active in mesothelioma cell-lines. Treatment with ZD1839 has produced significant dose-dependent reductions in colony formation ranging from 41% to 89% using sarcomatoid and epithelioid cell-lines respectively (Janne et al. 2002). In the same study ZD1839 was found to be at least as effective against mesothelioma cell-lines as against a non-small

cell lung cancer line. More recently, translating this *in-vitro* work to the clinical situation, the EGFR tyrosin kinase inhibitor, Erlotinib, was trialled in a Phase II study in patients with previously untreated MPM. Despite high expression of the EGFR in 75% of patients, disease stabilisation as the best response occurred in only 14 (42%) patients (Garland et al. 2007).

The inhibition of the tyrosine kinase activity of the VEGF receptors flk-1 and flt-1 is the mode of action of the drug SU5416 (Haspel et al. 2002). Favourable results from phase I and phase II studies in haematological malignancies and other solid tumours (Rosen 2001), have led to national trials in mesothelioma in the United States. Because of a mode of action complimentary to classical cytotoxic chemotherapy it was postulated that there might be synergistic effects in combination. Unfortunately however, the combination of SU5416 with different chemotherapeutic drugs has led to significant bleeding and thromboembolic events (Kuenen et al. 2002;Manley et al. 2002;Marx et al. 2002) that are likely to preclude further combination trials.

1.3.5.3 Thalidomide

Another novel agent with anti-angiogenic potential is thalidomide. This is thought to act by inhibiting the actions of VEGF, tumour necrosis factor (TNF)-α and basic fibroblast growth factor (bFGF) (Nowak, Lake, Kindler, & Robinson 2002b). Bass et al have published results of a phase II clinical trial with once daily Thalidomide in 40 patients with mesothelioma. Eleven (27.5%) patients had greater than 6 months of disease stabilisation, with a median survival of 7.5 months. Almost half of the subjects developed troublesome constipation and 2 patients suffered grade II neurotoxicity (Baas et al. 2005).

1.3.5.4 Platelet-Derived Growth Factor inhibition

The polyprotein PDGF was originally identified in serum as a mitogen for fibroblasts, smooth muscle cells and glial cells (Betsholtz, Karlsson, & Lindahl 2001). It acts via a cell surface tyrosine kinase receptor (George 2001;Heldin & Westermark 1999) regulating cell cycle transition from phase G0 to S (Stice, Vaziri, & Faller 1999). It is thought to act as an autocrine growth factor (Pogrebniak, Lubensky, & Pass 1993). STI-571 (Imatinib, Gleevec) is a potent 2-phenylaminopyrimidine tyrosine

kinase inhibitor affecting PDGF-α and –β receptors which has recently been evaluated in a Phase II trial. Unfortunately, in a similar fashion to EGFR tyrosine kinase inhibition, a very poor response was seen in the clinical situation, with none of the 25 patients treated showing any demonstrable response (Mathy et al. 2005).

1.3.6 Current United Kingdom-Based Clinical Trials

To address the lack of a randomised controlled trial evidence-base for the treatment of MPM, there are currently four national trials actively recruiting patients in the UK. Firstly, the Mesothelioma And Lung Cancer Study (MALCS) is an epidemiological study examining the occupational history of those patients diagnosed with either MPM or lung cancer, in an attempt to identify the at risk occupations, especially in younger groups of patients. Secondly, the MesoVATS trial is a study designed to compare the role of VATS cyto-reductive pleurectomy with talc pleurodesis in patients with suspected or proven MPM, to assess the potential benefits offered by the more invasive pleurectomy. Thirdly, the Mesothelioma And Radical Surgery (MARS) trial, which has been designed to assess the impact of some of the most aggressive treatments for this disease. Patients in this trial will all receive one of three chemotherapy regimens and then go on to be randomised to either EPP or not. The option of radiotherapy is also available after surgical randomisation. This trial is still at an early stage. Finally, the British Thoracic Society MSO1 trial which was designed to examine active supportive care and compare this with either of two different chemotherapy regimens plus active supportive care. This trial has completed recruitment of more than 400 patients and the first results were revealved at the 2007 ASCO Annual meeting, indicating that active supportive care was at least as good as the chemotherapy regimens studied in this cohort of patients (Muers et al. 2007). At least in part, this information is presented with thanks to the National Macmillan Mesothelioma Resource Centre February 2007 bulletin.

1.4 The Pathobiology of Malignant Pleural Mesothelioma

1.4.1 Introduction

It is accepted that around 80% of people who develop mesothelioma have occupational or environmental exposure to asbestos at a level higher than background. Fortunately, approximately

only 5% of people exposed develop the disease. As demonstrated by this aetiological discrepancy, one can be certain that there are other factors involved in its development. Likely candidates include Simian Virus 40, radiation, other minerals such as erionite and previous pleural scarring (Manning, Vallyathan, & Mossman 2002) (Rizzo et al. 2001) (Carbone, Kratzke, & Testa 2002).

It has been suggested that the pathogenesis of mesothelioma relies on three processes, which may act to reinforce each other. Genetic alterations, a chronic inflammatory response and a process of immuno-evasion may all contribute to an environment where inappropriate responses to autocrine and paracrine growth factors are seen (Bielefeldt-Ohmann, Jarnicki, & Fitzpatrick 1996) (O'Byrne & Dalgleish 2001). The development of this disease is associated with a latent period of 30 years or more, a longer period being seen with lower levels of exposure to asbestos (Manning, Vallyathan, & Mossman 2002).

1.4.1.1 Simian Virus 40

Simian Virus 40 (SV 40) is a DNA tumour virus that has been implicated in the causation of MPM. It has been found to induce MPM in hamsters (Carbone et al. 1997) and has been identified in 60-80% of MPM tumour samples in the USA (Carbone et al. 1999;Carbone, Rizzo, & Pass 2000). It is likely that this was due to manufacture of polio vaccine in the USA in the 1950s and 1960s using cell cultures of rhesus monkey kidney cells. SV 40 is endogenous to this particular species of monkey (Carbone, Kratzke, & Testa 2002).

The virus itself encodes 2 principal proteins thought to be responsible for its malignant potential. These are the large T-antigen (Tag) and the small t-antigen (tag). The large Tag is capable of contributing to the transformation of a number of cell types via interaction with the retinoblastoma (Rb) and p53 tumour suppressor proteins (Ali & DeCaprio 2001). The small tag works to enhance the function of the large Tag, largely through its binding to and inhibition of protein phosphatase 2A (PP2A) (Rundell & Parakati 2001) which has an important role in the regulation of cell growth and proliferation. It has been found that human mesothelial cells are infected far more easily than other types of human cell and that these cells are resistant to the usual process of cell lysis that follows large

Tag-mediated SV 40 replication. In addition, the accumulation of the large Tag within the cell leads to a much accelerated rate of cellular transformation (Bocchetta et al. 2000).

Despite all of the laboratory and theoretical evidence indicating a role for SV 40 in the pathogenesis of MPM contradicting laboratory-based studies have shown the complete absence of detectable SV 40 in 100% of tumour samples examined from several different world regions, including the USA (Manfredi et al. 2005). It is likely that the debate on the role of SV 40 in MPM will continue for some time to come.

1.4.1.2 Genetic Abnormalities

Although there is good evidence that exposure to asbestos can be linked to the future development of MPM, there are certain areas of high geographical risk where there is a low occupational exposure to asbestos. Several villages in the Cappadocian region of central Turkey were found to have incredibly high rates of MPM with up to 50% of deaths attributable to the condition. Initial work suggested the causative factor to be a naturally occurring fibrous zeolite, erionite, which was found in the stone material used to build houses in the region (Carbone, Kratzke, & Testa 2002). In addition, the variation of MPM incidence between different families in the same region prompted the suggestion of a genetic component to the disease. Epidemiological maps constructed of families in the region revealed evidence for a genetic component to the disease, possibly autosomal dominant in nature (Roushdy-Hammady et al. 2001). It is likely that exposure to erionite, combined with a genetic susceptibility to MPM, are responsible for the high rates of the disease seen in this region (Carbone, Kratzke, & Testa 2002).

A high frequency of genomic losses has consistently been observed in MPM, with many cell lines exhibiting homozygous deletion of the 9p21 region (Carbone, Kratzke, & Testa 2002), or more specifically, co-deletion of p15 and p16 genes within the chromosome band 9p21 (Xio et al. 1995). Loss of this region includes the CDKN2A locus which encodes the tumour suppressor genes (TSG) $p14^{ARF}$ and $p16^{INK4a}$. In those cases where $p16^{INK4a}$ is not lost through deletion, it has been shown to be inactivated by promoter hypermethylation (Wong et al. 2002), often with maintenance of the wild-type

retinoblastoma gene (pRB) (Frizelle et al. 1998), leading to loss of cell cycle regulation. Another TSG frequently mutated in MPM is the neurofibromatosis type 2 (NF2) TSG (Xiao et al. 2002). Of particular note, many human cancers are known to have p53 mutations, which have never been demonstrated in MPM (Metcalf et al. 1992;Mor et al. 1997). Indeed, it would appear that the presence of wild-type p53, a central regulator of the cell cycle and apoptosis, is much more common (Fennell & Rudd 2004;Leard & Broaddus 2004). Finally, it has been suggested that there may be an increased susceptibility to the disease following asbestos exposure in those people with Ehlers-Danlos syndrome or Marfan's syndrome (Bisconti, Bisetti, & Bidoli 2000).

1.4.1.3 Immune response in MPM

There is increasing evidence that an environment of altered immunity exists in malignant disease and plays a central role in the development of that process (O'Byrne et al. 2000). In mesothelioma, observations have been made regarding tumour cytokine and prostaglandin production leading not only to mechanisms of immune evasion (Fitzpatrick, Peroni, & Bielefeldt-Ohmann 1995) but also to suppression of the individuals' immune system itself, with abnormal humoral and cell-mediated systems, abnormal cell-mediated antibody-dependent cellular toxicity, and defective macrophage and natural killer cell function (Lew et al. 1986;Sterman, Kaiser, & Albelda 1999).

1.4.1.4 Abnormal angiogenesis

As our understanding of the molecular pathobiology of mesothelioma has increased, it has become apparent that certain mechanisms are vitally important in the process of tumour development. One such process is angiogenesis. Hypoxia, produced by the tumour out-growing its blood supply, causes the stimulation of angiogenesis by the activation of different, but inter-related cellular pathways. Vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) are amongst those upregulated or increasingly expressed, either directly or indirectly, to increase angiogenesis and so reduce the hypoxia. The involvement of these factors in the pathogenesis of mesothelioma is still to be fully elucidated but much evidence exists of their potential roles. VEGF has been shown not only to be an autocrine growth factor in mesothelioma (Konig et al. 2000), but also to be prognostically linked with a high microvessel density (Nowak et al. 2002). The contribution to tumourigenesis made

by COX-2 is well established, especially in colorectal cancer (Dempke et al. 2001), and its over-expression has been found to correlate with a poor prognosis in mesothelioma (Edwards et al. 2002).

1.4.2 Apoptosis

Apoptosis is an energy-dependent multi-step process of programmed cell death, distinct from necrosis, which is seen in both neoplastic and non-neoplastic cell populations. The term itself is derived from the Greek word meaning, "falling off", as in leaves from a tree. It was originally described by Kerr *et al* in 1972 (Kerr, Wyllie, & Currie 1972), as a series of morphological changes noticed through ultra structural studies, to differentiate this mode of cell death from coagulative necrosis in non-neoplastic tissues. It was very quickly realised that apoptosis was also taking place within populations of tumour cells (Danilevicius 1973) and that apoptosis may occur in cell populations exposed to adverse conditions such as hypoxia or toxins, where the environment is not "so inclement" as to lead to coagulative necrosis (Wyllie 1974). Since that time extensive work has been completed and apoptosis has come to be recognised as an essential process in the deletion of damaged cells, in development and normal physiological cell turnover (Leard & Broaddus 2004) as well as a disrupted process in many pathological processes including cancers.

In the normal mammalian cell there are two principal pathways by which apoptosis takes place, the extrinsic and intrinsic pathways, however they both lead to the same final common pathway involving activation of an evolutionarily conserved family of cysteine proteases, known as caspases, that characteristically cleave their specific substrates after aspartate residues (Baliga & Kumar 2002).

The extrinsic pathway is activated through the tumour necrosis factor (TNF) superfamily of cell surface death receptors and their activation by specific ligands including TNF-α and FasL. Activation of the death receptors leads to the recruitment of adaptor proteins, such as TNF receptor-1-associated death domain (TRADD) and Fas-associated death domain (FADD), which, with the respective receptor, form the death-inducing signalling complex (DISC). The DISC then recruits and activates the initiator caspase, caspase-8 (Fennell & Rudd 2004), which in turn leads to the activation of the effector caspases (-3, -6 and -7) and eventual apoptosis.

The intrinsic pathway is stimulated by DNA damage and cellular stress, including hypoxia and appears to be inexorably linked with intracellular mitochondria. Pro-apoptotic members of the B-cell lymphoma gene (BCL)-2 family lead to the release of Cytochrome c, second mitochondria-derived activator of caspases (Smac/DIABLO) and apoptotic protease-activating factor-1 (Apaf-1) from mitochondria. Cytochrome c binds with Apaf-1 and procaspase 9 in the presence of adenosine triphosphate (ATP) to form the apoptosome. Within the apoptosome, activated caspase-9 then cleaves and activates the effector caspases -3,-6 and -7. Despite the apparent difference in the starting points of these 2 pathways, there appear to be an increasing number of ways for "crosstalk" to occur between the pathways, allowing both to be activated producing a synergistic amplification of the resulting apoptotic cascade (Leard & Broaddus 2004).

There exist critical regulators of apoptosis that act in either pro- or anti-apoptotic fashions. The largest family is the Bcl-2 family of proteins with over 20 members (Baliga & Kumar 2002). Each member of the Bcl-2 family have been shown to contain at least one of four domains known as Bcl-2 homology (BH) domains, numbered 1-4 (Schultz & Harrington, Jr. 2003). In addition, they can be functionally divided into 2 principal groups, those that act in a pro-apoptotic fashion (including Bax, Bak, Bid, Bad, Bim and Bok) and those that are anti-apoptotic (including Bcl-2, Bcl-X_L, Bcl-W and MCl-1). These proteins act largely in and around the mitochondrial membrane. They are able to hetero- and homodimerise, thereby providing fine control of the apoptotic mechanism (Leard & Broaddus 2004), with the fate of the individual cell being reliant, at least in part, on the relative concentrations of the individual family members present at any one time(Schultz & Harrington, Jr. 2003). In addition to the Bcl-2 family, the inhibitor of apoptosis proteins (IAPs), which include XIAP and survivin are able to inhibit the activation of caspase-9 and downstream caspases, providing a further level of control (Zangemeister-Wittke & Hopkins-Donaldson 2005).

1.4.3 Akt/Protein Kinase B

A novel serine/threonine protein kinase of the cAMP-dependent protein kinase A/protein kinase G/protein kinase C (AGC) super family was discovered by 3 independent groups in 1991. The first group, using the process of homology cloning, identified a protein with a kinase domain similar to

that within the protein kinases A and C, therefore naming the protein *R*elated to *A* and *C* *P*rotein *K*inase (RAC-PK) (Jones et al. 1991). Shortly after this, a second group, cloning the same cDNA, identified the protein, naming it protein kinase B (PKB) (Coffer & Woodgett 1991). Finally, a third group identified the product of the murine oncogene, v-akt, as a cellular homologue of PKB and named it c-akt (Bellacosa et al. 1991). Since that time, 3 distinct isoforms have been identified and are now referred to as PKBα (or Akt1), PKBβ (Akt2) and PKBγ (Akt3), which although products of different genes, share an homology greater than 80% at the amino acid level (Nicholson & Anderson 2002).

The structural features of the Akt isoforms can be seen in figure 1.5. Each isoform, with a molecular weight of approximately 56 kilo-Daltons (kDa), consists principally of 3 domains. The amino (N)-terminal pleckstrin homology (PH) domain has been shown to bind 3-phosphoinositides (Nicholson & Anderson 2002), allowing interaction with membrane phospholipids. The sequence of approximately 100 amino acids was originally found in pleckstrin, the major phosphorylation substrate for PKC in platelets (Hanada, Feng, & Hemmings 2004). The kinase catalytic domain in the central region of the molecule shares a high degree of similarity with PKA, PKC and other members of the AGC kinase family. It contains an activation loop which, when active, regulates access to the catalytic site of Akt (Nicholson & Anderson 2002). Within the activation loop is a threonine residue (see figure 5 for the isoforms variations), the phosphorylation of which is required for partial activation of the molecule. Following on from this, is the carboxy (C)-terminal extension containing a sequence of approximately 40 amino acids (Song, Ouyang, & Bao 2005). This region possesses the F-X-X-F/Y-S/T-Y/F hydrophobic motif (HM) (where X is any amino acid) which is characteristic of the AGC kinase family (Hanada, Feng, & Hemmings 2004). Phosphorylation of the all important serine residue within the HM is necessary for full activation of the Akt molecule.

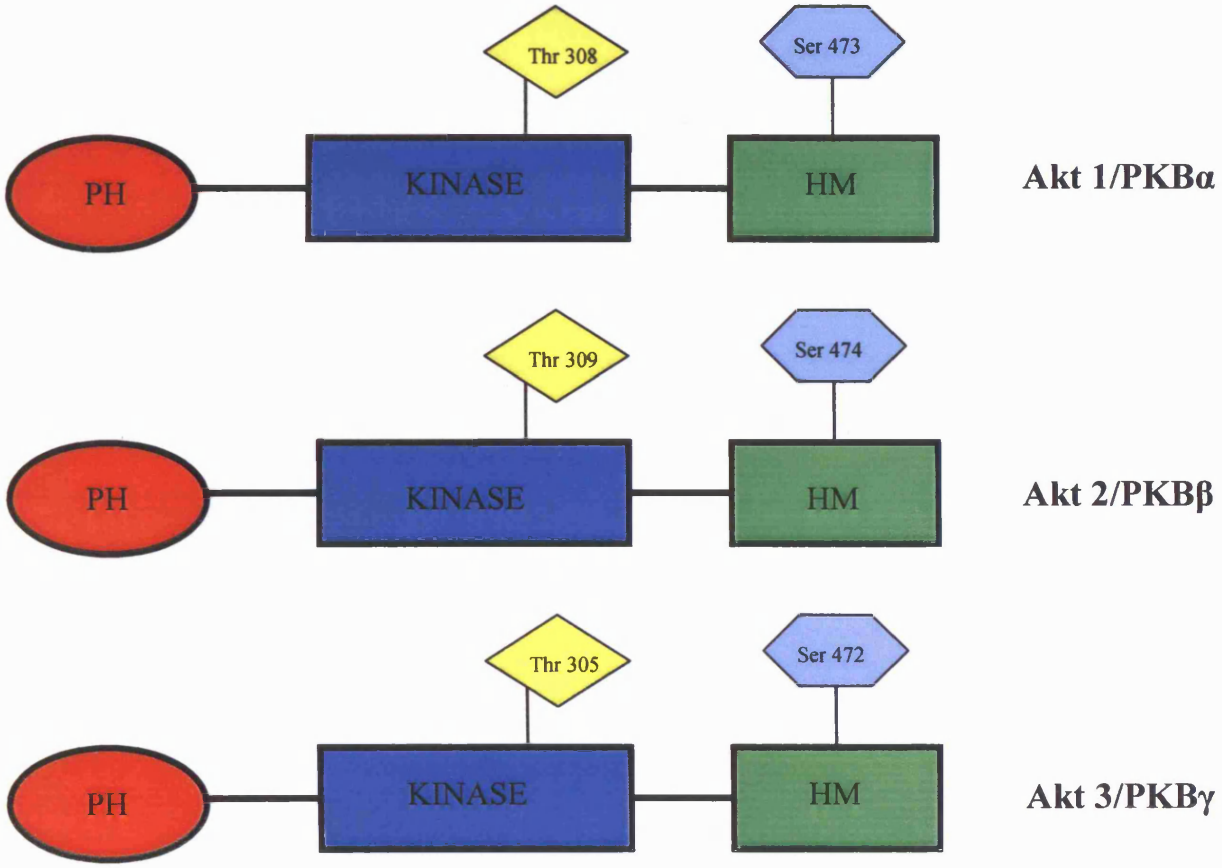


Figure 1.5 Schematic representations of the structural isoforms of Akt. Each isoforms consists of an N-terminal pleckstrin homology (PH) domain, a kinase domain and a C-terminal regulatory domain, or hydrophobic motif (HM). Sites of phosphorylation for activation are indicated.

1.4.4 Phosphatidylinositol 3-Kinase and Akt/PKB Signalling

1.4.4.1 Activation of Akt

With recognition of its potential importance, the activation of Akt has been extensively investigated. Although in no way a comprehensive list, Akt has been shown to be activated by platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin and epidermal growth factor (EGF) (Burgering & Coffer 1995). Following membrane receptor activation, the intracellular mechanism most intensively investigated is the phosphatidylinositol 3-kinase (PI3-K) -dependent

activation of Akt (Hanada, Feng, & Hemmings 2004). The PI3-Ks, are a family of lipid kinases, characterised by their ability to phosphorylate the 3'-OH position of inositol phospholipids (Fresno Vara et al. 2004) of which there 3 principal classes (I*a* and *b*, II and III) (Wymann, Zvelebil, & Laffargue 2003). Class I PI3-Ks (physiologically the most important) will be discussed further.

Class I PI3-Ks are heterodimers, activated by tyrosine kinase (1*a*) and G-protein-coupled receptors (1*b*) (Song, Ouyang, & Bao 2005), and consisting principally of a regulatory and a catalytic subunit. The regulatory subunit (p85 in figure 1.6) contains two Src-homology 2 (SH2) domains which have high affinities for phosphorylated tyrosine residues in activated cell membrane receptors (Wymann, Zvelebil, & Laffargue 2003). Through association with cell membrane receptors, the p85 subunit brings the catalytic subunit (p110 in figure 1.6) into approximation with the plasma membrane, in turn leading to the phosphorylation of phosphatidylinositol (PtdIns)-4,5-bisphosphonate (PI(4,5)P2), producing the second messenger PtdIns-3,4,5-triphosphate (PIP3). The major importance of phospholipids like PIP3 is their ability to recruit, with high affinity, proteins with a PH domain, like Akt, to the plasma membrane. In addition to this role, PIP3 is thought to have a role in the modulation and facilitation of the activation of Akt (Frech et al. 1997).

The multi-site phosphorylation of Akt that takes place following its translocation to the plasma membrane is a process of increasing complexity (figure 1.6). A 63 kDa phosphoinositide-dependent kinase, PDK1, is responsible for the phosphorylation of Akt within its activation loop at threonine 308 in the case of Akt1 (Alessi et al. 1997). PDK1 contains a PH domain allowing its binding to the plasma membrane. Comparatively, the phosphorylation of the serine residue (S473 in Akt1) is a process still incompletely understood. Numerous putative kinases have been suggested to have a role in the process, but none have been shown to convincingly fulfil the role. Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2), integrin-linked kinase (ILK) 1, protein kinase C βII (PKCβII), DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutant (ATM), rapamycin-insensitive mTOR complex (TORC2) have all been postulated, with *in-vitro* evidence for and against their roles (Fayard et al. 2005). Further work has identified two tyrosine

residues within the activation loop, the phosphorylation of which has been shown to be independently important in the activation of Akt (Hanada, Feng, & Hemmings 2004).

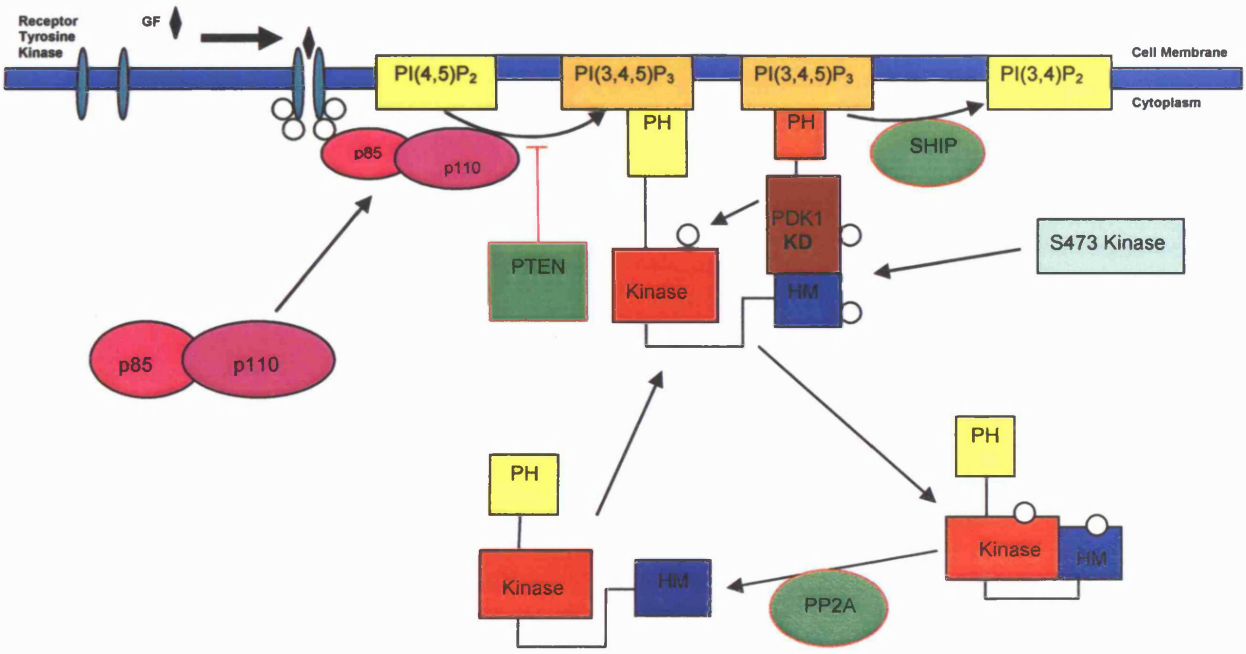


Figure 1.6 A schematic representation of the PI3-K-dependent activation of Akt1 by the phosphorylation of residues threonine 308 and serine 473. Although the mechanism has been broadly established, certain specifics remain elusive, for example the precise nature of the serine^{473}-kinase is yet to be determined. In terms of negative regulation, PTEN prevents the activation of Akt through the inhibition of the actions of PI3-K, and PP2A is responsible for the de-activation of pAkt by dephosphorylation.

Finally, Scheid *et al* suggested that, based on available evidence, the phosphorylation of serine 473 occurs initially on arrival at the plasma membrane; although they remain unsure as to how exactly this happens. The then phosphorylated HM domain interacts with PDK1, increasing its activity, leading to threonine 308 phosphorylation within the activation loop. The HM domain then preferentially associates with its own kinase domain, stabilising the pAkt molecule in a fully active state (Scheid & Woodgett 2003). The fully active pAkt then detaches from the plasma membrane, translocating to the cytosol and nucleus to phosphorylate its downstream targets (Alessi et al. 2002).

Akt can be activated via PI3-K-independent mechanisms. *In vitro*, Ca^{2+}/Calmodulin-dependent kinase has been shown to directly activate Akt. Also, periods of cellular stress, possibly through association with the heat shock protein (Hsp) 27, lead to the activation of Akt (Song, Ouyang, & Bao 2005). However, the details and significance of these routes of Akt activation remain to be fully elucidated.

In common with many other cellular mechanisms, the activation of Akt is, under normal circumstances tightly regulated by numerous controls. Two of these, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Src homology 2 domain containing inositol polyphosphate phosphatase (SHIP) are phosphatases that dephosphorylate PIP3 to PI(4,5)P2 and PI(3,4)P2 respectively (Nicholson & Anderson 2002).

Great interest has been expressed in PTEN, a dual-specificity phosphatase with both lipid and protein substrates. Deletions or mutations in the *PTEN* gene have been demonstrated in a number of malignancies including gliomas, endometrial cancers, breast, ovary, thyroid, bladder and small-cell lung cancers (Mohiuddin et al. 2002) which may well, at least in part, be due to increased levels of pAkt. Although the overall effect of the loss of functioning lipid phosphatase activity of PTEN in tumours is not in question, the details of its precise functions are still to be fully elucidated. For example, it is likely that PTEN and Akt are closely linked with the actions of the *p53* tumour suppressor gene and the interaction of its protein product with Murine double minute (Mdm) 2, which facilitates the ubiquitin ligation and subsequent degradation of p53 by the proteasome. Briefly,

Mdm2, a downstream target of pAkt, when phosphorylated translocates to the nucleus and associates with p53, leading to its ubiquitin ligation and subsequent degradation, thereby allowing the continued survival of a potentially mutated cell. PTEN, by blocking the activation of Akt, prevents Mdm2 phosphorylation, which in turn inhibits its movement into the nucleus, so preserving the apoptosis-inducing functions of p53 (Mayo & Donner 2002). In addition to this, there has been renewed interest in the protein tyrosine phosphatase action of PTEN. It has been shown to be capable of down-regulating the actions of the cell-cycle control protein, cyclin D1 in the breast cancer cell line MCF-7 (Leslie & Downes 2002). Further more, it may have a role in the suppression of metastasis (Leslie & Downes 2004). Finally, demonstrating its potential role in MPM, forced over-expression of the *PTEN* gene leads to increased rates of apoptosis and a decrease in the kinase activity of pAkt in two mesothelioma cell lines (Mohiuddin et al. 2002).

Quite differently to PTEN, it is thought that SHIP does not play quite such a vital role in the control of PI3-K and Akt. SHIP converts PIP3 to PI(3,4)P2, rather than PI(4,5)P2. This may well represent a mechanism to control the duration and the magnitude of the effect of increasing the concentration of PIP3 (Leslie & Downes 2002). In addition, it is possible that the production of PI(3,4)P2 is the active conversion of one second message to another signal, for a purpose as yet undetermined (Leslie & Downes 2002). In addition to PTEN and SHIP, a third phosphatase, protein phosphatase 2A (PP2A) dephosphorylates pAkt, possibly within the nucleus, returning it to its inactive state (Brazil, Yang, & Hemmings 2004). Several other proteins have been shown to bind and negatively regulate Akt itself. Over-expression of carboxyl-terminal modulator protein (CTMP) leads to a reduction of threonine 308 and serine 473 phosphorylation (in Akt1), resulting Akt inactivation. Keratin K10, a component of cytoskeletal structure, binds Akt, inhibiting its intracellular translocation, so preventing its activation. Finally, the Akt binding protein Trb3, has been shown to bind to the central region of the kinase domain of Akt, and when over-expressed reduces phosphorylation and inhibits Akt activity (Hanada, Feng, & Hemmings 2004).

1.4.4.2 Functions and downstream targets of Akt

The number of downstream targets of Akt and their respective functions is daunting at first glance. Principally, they can be classified as having roles in cell survival and apoptosis, or the regulation of cell cycle progression. The amino acid sequence, Arg-Xaa-Arg-Xaa-Xaa-[Ser/Thr]-Hyd (where Xaa is any amino acid and Hyd is a bulky hydrophobic amino acid) defines the minimal consensus peptide sequence required for recognition for phosphorylation of a protein by Akt (Alessi et al. 1996). Although this sequence has been identified in over 500 known intra-cellular proteins (Nicholson & Anderson 2002), there are thought to be additional levels of structural requirements to determine actual substrates. Currently, over 50 putative substrates for Akt have been identified (Hanada, Feng, & Hemmings 2004).

Within tissues, the processes of cell proliferation and cell death are vital to the overall balance of the system. Akt has been recognised as having a critical role in the regulation of cell survival (Song, Ouyang, & Bao 2005). Akt is responsible for the phosphorylation of a number of transcription factors positively influencing anti-apoptotic factors and negatively affecting pro-apoptotic processes. The forkhead (FKHR) family of transcription factors usually reside in the nucleus and lead to the transcription of pro-apoptotic genes such as *Fas-L* and *Bim* (Nicholson & Anderson 2002). The phosphorylation of FKHR by pAkt leads to its exclusion from the nucleus and its association and sequestration with 14-3-3 binding proteins preventing its actions. The pAkt pathway also has a positive transcriptional effect on anti-apoptotic gene expression. Firstly, through the phosphorylation and activation of cyclic-AMP (cAMP)-response element binding protein (CREB), the anti-apoptotic genes *Bcl-2* and *mcl-1* are upregulated. Secondly, pAkt is known to phosphorylate and activate the IκB-kinase (IKK) complex, which mediates the degradation of IκB, preventing its inhibitory action on the transcription factor nuclear factor-κB (NF-κB). This will be discussed in further detail later. Without inhibition, NF-κB translocates to the nucleus, stimulating the transcription of inhibitor of apoptosis genes, *cIAP1* and *cIAP2*. In addition to its involvement with transcription factors, pAkt directly influences key regulators of the apoptosis cascade. For example, BAD, a pro-apoptotic member of the Bcl-2 family is prevented from interacting with Bcl-2 and Bcl-X_L localised on the mitochondrial membrane by pAkt phosphorylation. Phosphorylated BAD forms a complex with 14-3-

3 binding protein and is sequestered in the cytoplasm. Also, phosphorylation of procaspase-9 by pAkt prevents the Cytochrome c-induced cleavage of procaspase-9, so blocking its intrinsic apoptotic protease activity (Hanada, Feng, & Hemmings 2004). The anti-apoptotic effect of Akt on p53, via phosphorylation of Mdm2 has already been discussed (see section *1.3.4.1*). Finally, pAkt affects cell survival through its interaction with cellular metabolic pathways. Phospho-Akt has a stimulatory effect on glucose transporters (GLUT), increasing the uptake of glucose into cells in response to insulin stimulation (Franke et al. 2003)and at the same time, inhibit the action of glycogen synthase kinase 3 (GSK3), preventing the inhibition of glycogen synthase (Hanada, Feng, & Hemmings 2004). In addition, pAkt may have a direct effect on mitochondrial hexokinase activity which acts to preserve mitochondrial integrity by maintaining the coupling between glucose metabolism and ATP synthesis (Nicholson & Anderson 2002).

As well as the role of pAkt in cell survival, it also affects a multitude of process involved with cell cycle progression. In addition to its role in glucose metabolism, GSK3 is thought to phosphorylate cyclin D1 leading to its degradation by the proteasome. By phosphorylation and inactivation of GSK3 by pAkt, stable cyclin D1 leads to shortened cell cycle times. Furthermore, not only does GSK3 inactivate cyclin D1, it is also capable of inhibiting the β-catenin-induced transcription of the *cyclin D* gene through phosphorylation of β-catenin. Once again, this action is blocked by GSK3 phosphorylation by pAkt. The mammalian target of rapamycin (mTOR) has been postulated as a direct target for pAkt. Activation of mTOR leads not only to the upregulation of ribosomal cyclin D mRNA translation, but may also regulate cyclin D at a transcriptional level through the phosphorylation of CREB and its interaction with the CREB-response element (CRE) in the promoter region of *cyclin D*. The retinoblastoma protein (pRB) prevents the transition of cells from G1 to the S phase of the cell cycle. It is inactivated by cyclin-dependent kinase (CDK) which, predictably, requires cyclin for efficient phosphorylation of pRB. A family of CDK inhibitors complicate matters further. The production of two members, $p21^{CIP1}$ and $p27^{KIP1}$ is mediated at least in part by the transcriptional effect of a member of the FKHR family of proteins, AFX. Therefore, through phosphorylative inhibition of FKHR, there will be a reduction in the production of these two cell cycle regulators. In addition to the effect of pAkt on FKHR, both $p21^{CIP1}$ and $p27^{KIP1}$ are direct

targets for pAkt, phosphorylation leading to their nuclear exclusion. Cyclin D1 has been found to be upregulated in many human cancers and it can be seen from the above description that pAkt can influence its impact at many levels (Nicholson & Anderson 2002).

1.4.5 Nuclear Factor-κB

NF-κB is a ubiquitously expressed dimeric transcription factor composed of homo- or heterdimers of the 5 principal Rel family members: p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100 (Gilmore et al. 2002). The most common heterodimer, p50-p65, is known specifically as NF-κB. All members of the family contain an N-terminal domain of approximately 300 amino acids, known as the Rel homology domain (RHD) which is responsible for dimerisation, nuclear localisation and DNA binding. In unstimulated cells, inactive NF-κB is sequestered in the cytoplasm through interaction with members of the inhibitor of NF-κB (IκB) family which bind the RHD, masking the nuclear localisation and DNA binding sequences. In response to a multitude of signals, IκB is phosphorylated by the IκB kinase (IKK) complex which causes the ubiquitin-dependent 26S proteosomal degradation of IκB. Consequently, NF-κB translocates to the nucleus leading to target gene expression. The IKK complex consists of 1 regulatory subunit (IKKγ) and 2 kinase subunits (IKKα and IKKβ) which are responsible for IκB phosphorylation (Lin & Karin 2003). A huge number of NF-κB target genes have been identified and principally, their functions involve immune and stress responses, with anti-apoptotic and cell proliferative effects particularly pertinent in the process of malignant disease. It is not surprising that NF-κB has been found to be upregulated in many forms of human cancer, including breast, ovary, prostate, kidney, liver, colon, pancreas and thyroid (Gilmore et al. 2002).

In addition to the variety of upstream signals that are capable of NF-κB activation, pAkt is capable of NF-κB activation, via the actions of IκB and IKK (Kane et al. 1999). For example, the interruption of Akt activation in a melanoma cell line has been shown to decrease NF-κB promoter activity (Dhawan et al. 2002). Furthermore, there is thought to be a role for Akt in Ras- and TNF-α-induced NF-κB activation (Li & Stark 2002). However, in apparent contradiction, more recent evidence has pointed to the position of NF-κB upstream of Akt activation, required for the tumour necrosis factor (TNF)-mediated activation of Akt in the NIH3T3 cell line (Meng et al. 2002). This is

likely to be a reflection of the complexity of the signalling pathways involved and may be cell type specific.

1.4.6 The Role of Hypoxia

Hypoxia, most easily defined as a reduction in the normal levels of tissue oxygenation, occurs in many different clinical situations including acute and chronic cardiovascular and respiratory conditions, but especially so within developing solid tumours. Once a tumour reaches more than 1-2mm in diameter, oxygen supply by diffusion is unable to meet its metabolic demands and it must stimulate angiogenesis to provide oxygenated blood. Unfortunately, the aberrant nature of tumoural neovascularisation often leads to the failure of these demands being met. The result is a hostile, hypoxic intra-tumoural environment, with an environmental oxygen concentration far below that of the surrounding normal tissue. The adaptation of malignant cells to this environment may provide a natural selection process for cells capable of surviving in these conditions. This is reflected in the findings that high levels of intra-tumoural hypoxia have been reported to lead to poorer outcomes in carcinomas of the cervix and head and neck, and also soft tissue sarcomas (Swinson et al. 2004).

1.4.6.1 Hypoxia Inducible Factor-1

Hypoxia inducible factor (HIF)-1 is a heterodimeric transcription factor, initially reported by Semenza *et al* in 1992 (Semenza & Wang 1992). It consists of a 120 kDa hypoxia responsive element, HIF-1α and a 91-94 kDa constitutively expressed element, HIF-1β (otherwise known as aryl hydrocarbon receptor nuclear translocator, ARNT) (Wang et al. 1995). Both contain basic helix-loop-helix (bHLH) and Per-ARNT-AHR-Sim (PAS) domains (Wang, Jiang, Rue, & Semenza 1995) which are required for dimerisation and DNA binding. In addition, HIF-1α also contains N-terminal (N-TAD) and C-terminal (C-TAD) transactivation domains that are central to transcriptional activities and interaction with co-activators (Bardos & Ashcroft 2004) (figure 1.7). Structurally similar to HIF-1α, two further proteins, HIF-2α and HIF-3α, have been identified. Hypoxia inducible factor-2α has the ability to heterodimerise with HIF-1β, with target genes varying from HIF-1α. The role of HIF-3α has so far been poorly defined (Semenza 2004).

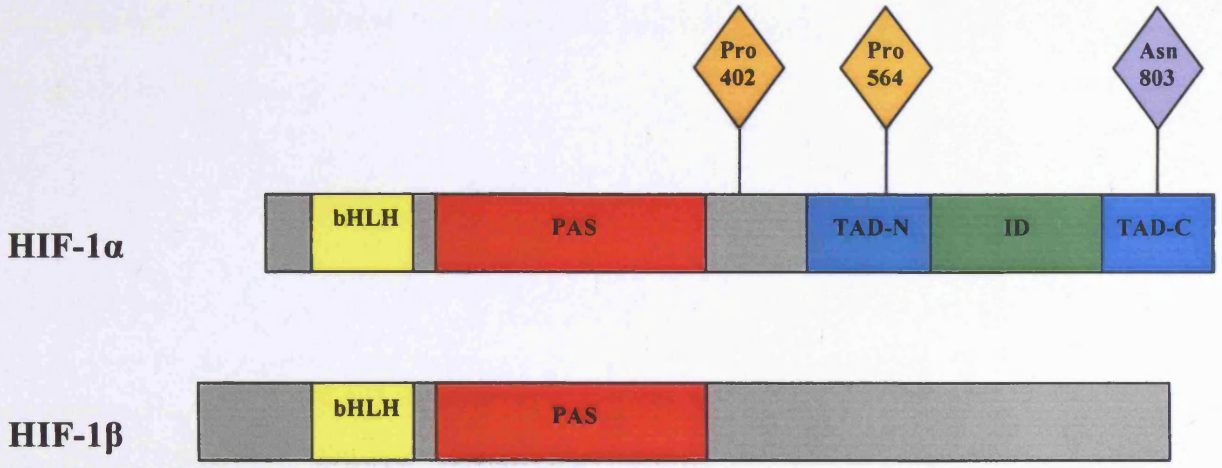


Figure 1.7 Schematic representations of HIF-1α and HIF-1β. Prolyl hydroxylation of residues 402 and 564 leads to ubiquitination and degradation by the proteasome, whereas hydroxylation of Asn 803 prevents interaction with the co-activator p300. These control mechanisms are both oxygen-dependent.

The activity of HIF-1 is regulated by the availability of HIF-1α, which itself is dependent on the presence or absence of oxygen and certain other activators of cellular signalling including growth factors. In the presence of oxygen, HIF-1α is modified by the prolyl hydroxylation of residues 402 and 564. This prolyl hydroxylase requires not only oxygen, but also ferrous iron and 2-oxoglutarate for activity (Harris 2002). Hydroxylation then allows the binding of the von Hippel-Lindau tumour suppressor protein (VHL), which forms part of the multiprotein ubiquitin E3 ligase complex with elongin B, elongin C, cullin 2 and ring box protein (RBX)-1 that targets HIF-1α for degradation by the 26S proteasome (figure 1.8). *In vitro* work with hypoxia-reperfusion models has shown the half-life of HIF-1α to be less than 5 minutes, and may in fact be less than 60 seconds (Semenza 2004). Exerting further control on transcriptional activity is factor inhibiting HIF-1 (FIH-1) which forms a ternary complex with HIF-1α and VHL, which leads to histone deacetylase recruitment repressing transactivation domain function. In addition to this, FIH-1 catalyses hydroxylation of the Asn 803 residue in the TAD-C domain preventing its interaction with p300, a key co-activator required for

transcriptional activation. In conditions of hypoxia, prolyl hydroxylation is inhibited, VHL is unable to bind and HIF-1α remains stabilised.

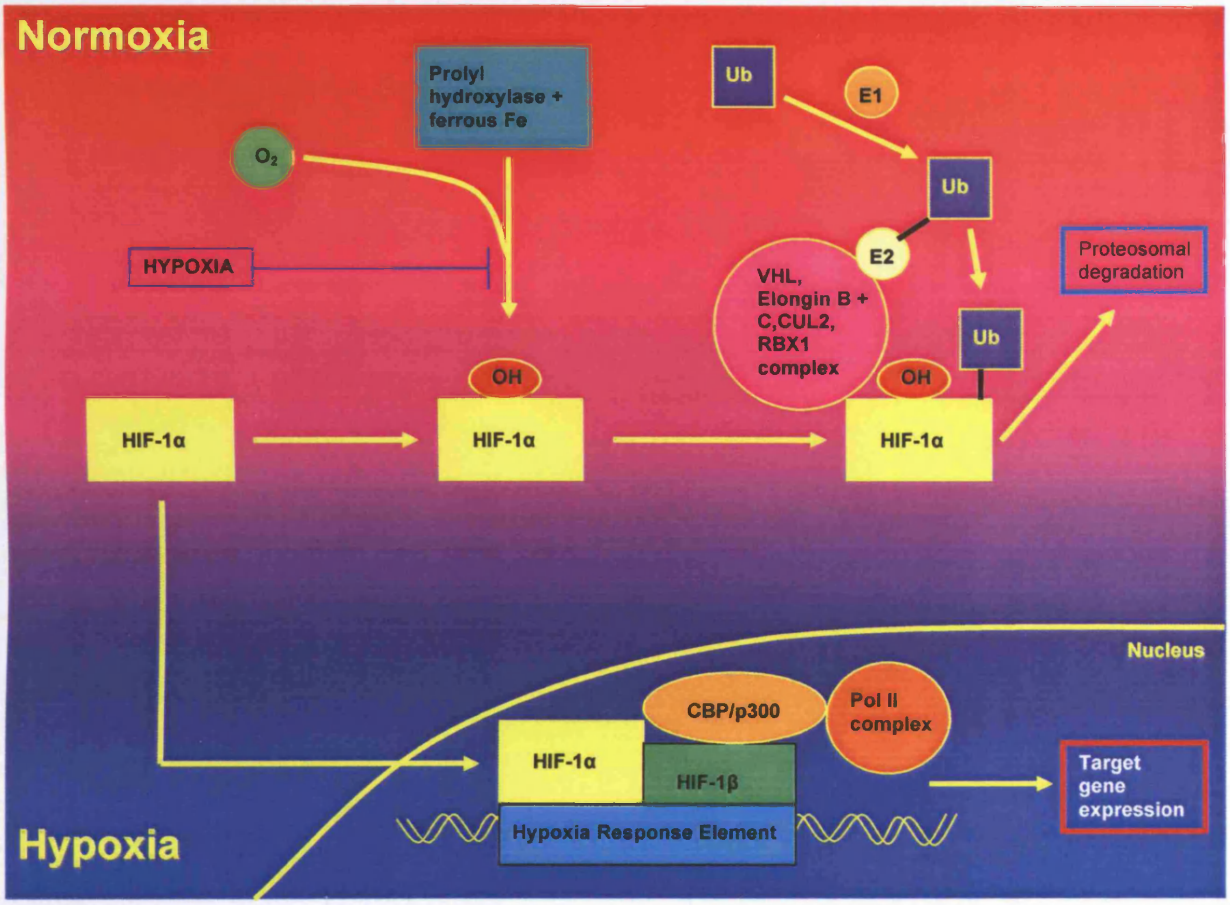


Figure 1.8 A schematic representation of the regulation of HIF-1α in normoxic conditions and the activation of HIF-1 in conditions of hypoxia

The stabilised HIF-1α translocates to the nucleus, binds with HIF-1β (Chilov et al. 1999) and its co-activators p300 and DNA polymerase complex, Pol II and leads to target gene expression (figure 1.8). The binding of target genes is facilitated by a conserved sequence (5'-CGTG-3') in the promoter region of target genes, known as the hypoxia response element (HRE). A multitude of target genes

have been identified for HIF-1, with such diverse roles as angiogenesis (eg. VEGF), cell growth (eg. IGF-2) and metabolism (eg. GLUT-1) (Semenza 2002). For example in a study by Akakura *et al*, pancreatic cancer cell lines found to exhibit constitutive expression of HIF-1α were found to be more resistant to apoptosis due to hypoxia and nutrient deprivation when compared with cell lines without constitutive expression (Akakura et al. 2001). Considering this, it is little wonder that immunohistochemical studies have shown frequent and dramatic over expression of HIF-1α in numerous human cancers (Talks et al. 2000) likely resulting from both intra-tumoural hypoxia and genetic mutations (Semenza 2002).

There is increasing evidence for the involvement of the PI3-K/Akt pathway in the "web" of signalling pathways associated with HIF-1. It is likely that this relationship is conserved despite varying degrees of normoxia, or hypoxia or in malignant disease. Stimulation of cells with a variety of growth factors binding to their cognate tyrosine kinase receptors has been shown to induce the expression of HIF-1α, HIF-1 DNA binding and target gene expression in non-hypoxic conditions (Semenza 2002a;Stiehl et al. 2002). This can be blocked by the inhibition of the PI3K pathway. Further more, over expression of *PTEN* leads to a significant reduction in HIF-1α expression (Jiang et al. 2001;Zhong et al. 2000). Whether Akt is activated upstream or downstream of HIF-1α stabilisation and HIF-1 transactivation remains unclear. For example, *in vitro* work studying the neuronal cell line RN46A showed that Akt activation was critical to HIF-1 transcriptional activity (Zhang et al. 2003), implying an upstream position for Akt. However, other reports have shown that Akt is not required for the hypoxic induction of HIF-1 (Arsham et al. 2002) and that HIF-1and Akt have independent mechanisms to increase tumour growth and angiogenesis (Arsham et al. 2004). The likelihood is however, that the interaction of pAkt and HIF-1 will be variable and cell-type specific. This has been convincingly demonstrated by Alvarez-Tejado *et al* using three different neoplastic cell lines, showing that although exposure to hypoxia always led to the induction of HIF-1α, this was not always the case for pAkt (Alvarez-Tejado et al. 2002).

Although the vast majority of signalling pathways associated with HIF-1 elicit a survival response, there is evidence that HIF-1 may act in a pro-apoptotic fashion under certain circumstances.

For example, it has been shown that HIF-1 leads to the upregulation of a pro-apoptotic member of the Bcl-2 family, nineteen kDa-interacting protein (Nip) 3 (Piret et al. 2002). More interestingly however, is the association of HIF-1α with p53 in hypoxic conditions, resulting in the stabilisation and transcriptional activation of p53, with inhibition of HIF-1-dependent transactivation. This has been shown to be controlled by the phosphorylation status of HIF-1α, with dephosphorylated HIF-1α preferentially binding to p53 (in more severe, prolonged hypoxia), in comparison with the phosphorylated form, binding to HIF-1β (in conditions of lesser hypoxia) (Suzuki, Tomida, & Tsuruo 2001). To further emphasize the effect of the degree of hypoxia, it may be that Akt signalling has a dual effect on HIF-1α stabilisation. Mottet *et* al have demonstrated in a hepatoma cell line that during a five hour incubation in hypoxic conditions, the pAkt-induced deactivation of GSK3 was necessary for HIF-1α stabilisation. However, during longer periods of hypoxia (16 hours), Akt is inactivated, leading to the upregulation of GSK3 which then down-regulates HIF-1α (Mottet et al. 2003). A further paradox is illustrated by the effect of carbon monoxide (CO) and nitric oxide (NO) on HIF-1 activation. In conditions of hypoxia, HIF-1 leads to upregulation of nitric oxide synthase and haemoxygenase-1 which synthesise NO and CO respectively. However, CO and NO both inhibit hypoxia-induced HIF-1α expression and HIF-1 DNA binding and transcriptional activity. Conversely, in conditions of normoxia, NO has exactly the opposite effects. It is possible that, in combination, CO and NO form stable compounds unable to activate HIF-1, rather than reactive oxygen species when they are present alone (Semenza 2001).

In addition to its undoubted involvement with pAkt, HIF-1 is very probably entwined in NF-κB-related signalling. For example, Figueroa *et al* have demonstrated that in a hepatocellular carcinoma cell line exposed to hypoxic conditions, selective inhibition of NF-κB significantly blocks HIF-1 protein expression (Figueroa et al. 2002). Furthermore, NF-κB has been shown to lead to the accumulation of HIF-1α, following TNF-α stimulation in normoxic condtions, despite HIF-1α maintaining its interaction with VHL and subsequent ubiquitination (Zhou, Schmid, & Brune 2003).

1.4.6.2 Carbonic Anhydrase IX

Carbonic anhydrase (CA) IX has become a focus of attention in many different solid tumours because of its potentially critical role in several cellular processes conducive to the malignant phenotype. CA IX is a transmembrane zinc metalloenzyme (Potter & Harris 2003), found predominantly in cancer cells (Ivanov et al. 2001) that catalyses the reversible hydration of carbon dioxide (CO_2) to form bicarbonate (HCO_3^-) and protons (H^+) (Breton 2001). It is upregulated in a hypoxic environment, through increased transcription of the *CA9* gene which contains a hypoxia response element (HRE) in its promoter region, downstream of the transcriptional complex HIF-1 (Wykoff et al. 2000). As an alternative to the role of hypoxia in the transcription of CA IX, *in vitro* work may suggest a mechanism less reliant on hypoxic conditions. High density cultures of HeLa cells, shown to have increased levels of PI3-K activity, demonstrate induction of CA IX despite only minimal levels of HIF-1 (Kaluz et al. 2002). Given the nature of the PI3-K/Akt signalling pathway, it is entirely conceivable that deregulated Akt activation, for example in malignancy, has a role in the induction of CA IX with or without significant hypoxia.

Its role in the malignant process has not been fully elucidated, but several possibilities exist, either alone or in combination. It has been hypothesised that it may have a role in proliferation and oncogenesis (Nogradi 1998), acting as a proto-oncogene (Potter & Harris 2003). Accounting for this, CA IX has a remarkable DNA-binding capacity and its expression in otherwise cytologically normal cervical smears has been identified to be a warning sign for dysplasia or in-situ carcinoma (Nogradi 1998). Also, transfection of in-vivo fibroblasts with the CA IX gene resulted in an, albeit transient, alteration in behaviour, with uncontrolled proliferation and morphological changes (Pastorek et al. 1994). Another potential function may be related to control of the tumour microenvironment, in particular the pH. The extra-cellular acidic tumour environment has long been thought to be due to lactate production from hypoxic anaerobic metabolism (Heacock & Sutherland 1990) (Griffiths et al. 2001), but this may not be the case. The acidic environment produced by the growth of tumour cells unable to synthesise lactate has shown that other processes must be involved (Wykoff, Beasley, Watson, Turner, Pastorek, Sibtain, Wilson, Turley, Talks, Maxwell, Pugh, Ratcliffe, & Harris 2000) and CA IX would be an ideal candidate. CA IX mediated diffusion of metabolic CO_2 out of the cell,

conversion to HCO_3^- and H^+ and exchange of HCO_3^- back into the cell would provide the appropriate environment. The tumoural benefits of the correct acidic microenvironment include an adverse effect on cell-mediated immunity (Potter & Harris 2003), conditions that would be favoured by tumour cells compared with normal tissues, enhanced selection pressure for apoptosis-resistant clones (Potter & Harris 2003), and an increased breakdown of extra-cellular matrix with a corresponding increase in the potential for invasion and metastasis (Gillies et al. 2002).

1.5 Study Hypotheses

1. Intra-tumoural hypoxia within malignant pleural mesothelioma tumour samples leads to the increased phosphorylation of Akt and the upregulation of CA IX, which leads to a poorer outcome in this disease.
2. Inhibition of Akt activation, through inhibition of the PI3-K pathway, increases the rates of apoptosis of malignant pleural mesothelioma cell lines in environmental conditions of reduced O_2 concentrations.

1.6 Aims of the Study

The aims of this study were:

1. To evaluate, by immunohistochemistry, the expression of pAkt and CA IX in MPM and to correlate that expression with patient survival and known clinicopathological risk factors in this disease.
2. To evaluate the effects of hypoxia on MPM cell lines, through the expression of pAkt and HIF-1α and also its effect on apoptosis with inhibition of Akt phosphorylation using the PI3-K inhibitor, LY294002.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 General Materials and Equipment

All the general chemicals and reagents were purchased from Sigma-Aldrich Company Ltd (Poole, UK), unless otherwise specified.

Vectabond reagent, from Vector Laboratories Inc. (Burlingame, CA).

DPX, xylene and IMS, from Merck Chemicals (Poole, UK).

Microscope, from Olympus (London, UK).

Amersham Hyperfilm, from Amersham Biosciences (Amersham, UK).

Citric acid monohydrate, 12% ammonia solution, sodium chloride, haematoxylin, sodium hydroxide pellets and hydrochloric acid, from Fisher Scientific UK Ltd (Leicester, UK).

Immunohistochemistry and Western Blotting isotype control antibodies, from Dako (Cambridgeshire, UK).

2.1.2 Primary Antibodies

2.1.2.1 Immunohistochemistry

The original concentrations and the working dilutions of the primary antibodies utilised in the immunohistochemistry and western blotting protocols are detailed in table 2.1.

1. *Phospho-Akt:* Anti-phospho-Akt (Ser^{473}) IHC specific polyclonal rabbit IgG (9277), from New England Biolabs (Beverly, MA).
2. *CA IX:* Anti-CA IX monoclonal mouse IgG2b (M75) antibody, received as a gift from Professor J Pastorek, (Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic), recognising the N-terminal extra-cellular proteoglycan domain of human CA IX (Pastorekova et al. 1992). The specificity of this antibody for CA IX has been confirmed by Western blots and immunostaining of COS-7 cells transfected with CA IX cDNA (Saarnio et al. 1998).

2.1.2.2 Western Blotting

1. *Phospho-Akt:* Anti-phospho-Akt (Ser^{473}) monoclonal mouse IgG2b antibody (587F11), from Cell Signalling Technology Inc. (Beverly, MA), recognising only Akt phosphorylated at serine 473.
2. *Akt1:* Anti-Akt1 (B-1) monoclonal mouse IgG1 antibody (sc-5298), from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), recognising amino acids 345-480 of human Akt1.
3. *HIF-1α:* Anti-HIF-1α monoclonal mouse IgG1 antibody (610959), from BD Transduction Laboratories (BD Biosciences, Oxford, UK), recognising the amino acid sequence 610-727 of human HIF-1α.
4. *α-Tubulin:* Anti-α-tubulin (B-7) monoclonal mouse IgG2a antibody (sc-5286), from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), recognising amino acids 149-448 of human α-tubulin.

2.1.3 Secondary Antibodies

2.1.3.1 Immunohistochemistry

1. Biotinylated polyclonal goat anti-rabbit immunoglobulins (E0432), 1:300 (Dako, UK).
2. Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins from the Envision+ System-HRP (K4007) (Dako, UK).
3. Peroxidase-conjugated rabbit anti-mouse immunoglobulins (P0161), 1:200 (Dako, UK).

2.1.3.2 Western Blotting

1. Peroxidase-conjugated goat anti-mouse immunoglobulins (P0447), 1:2000 (Dako, UK).

Table 2.1 Original concentrations and working dilutions for primary antibodies utilised in the immunohistochemistry and western blotting protocols

Antibody	Original concentration	Working dilution
Immunohistochemistry		
pAkt	100 µg/mL	1:100
CA IX	5 µg/mL	1:50
Western Blotting		
pAkt	100 µg/mL	1:1000
Akt1	200 µg/mL	1:1000
HIF-1α	250 µg/mL	1:500
α-Tubulin	200 µg/mL	1:1000

2.14 Cell Lines

1. *MeT-5A:* A benign, immortalised human mesothelial cell line, was obtained as a kind gift from Dr WA Swain (Thoracic Oncology Research Group, University of Leicester, UK). This cell line was derived from normal human mesothelial cells, transfected with an SV40 early region DNA plasmid. The cells express the SV40 large T antigen, but continue to exhibit features of mesothelial cells and remain non-tumourigenic after 1 year of subcutaneous inoculation into athymic nude mice (Ke et al. 1989).
2. *JU77:* An MPM cell line established from the pleural effusion of a patient with epithelial MPM (Manning et al. 1991).
3. *ONE58:* An MPM cell line established from the pleural effusion of a patient with epithelial MPM (Manning et al. 1991).
4. *CRL-5915:* An MPM cell line established from a pleural effusion in 1988, donated to and stored in the American Type Culture Collection (ATCC®).
5. *CRL-5820:* An MPM cell line established from a pleural effusion in 1976, donated to and stored in the ATCC®.

All 4 MPM cell lines were obtained as kind gifts from Dr YC Gary-Lee (Visiting Research Fellow, Wellcome Centre for Human Genetics, Oxford, UK).

2.1.4 Cell Culture Media

The MeT-5A cell line was grown in DMEM/F-12 medium (GIBCO), containing 10% FCS, 100ng/mL hydrocortisone, 2.5mg/mL insulin, 2.5mg/mL transferrin, 2.5mg/mL selenium, 50 units/mL penicillin V, 50 μg/mL streptomycin and 250 ng/mL amphotericin B. The JU77, ONE58 and CRL-5915 cell lines were grown in RPMI 1640 medium (Invitrogen), containing 10% FCS, 50 units/mL penicillin V, 50 μg/mL streptomycin and 250 ng/mL amphotericin B. In addition to this, 4.5g/L glucose was added for the CRL-5820 cell line.

2.1.6 Kits

Streptavidin-biotin complex (ABC) HRP (K0337) and the Envision+ System HRP (K4007) kits were purchased from Dako (Cambridgeshire, UK). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences (Amersham, UK).

2.1.7 Buffers and Reagents

Buffers and stock solutions were made from analytical grade chemicals and reagents, and prepared using 18Ω ultra pure water. Where necessary, the pH was altered using 12M HCl or 5M NaOH.

2.1.7.1 General

10 x Tris Buffered Saline (TBS)	*Final Concentration*
24.2g Tris base	200 mM
80g NaCl	137 mM

This was made up to 1000mL with water and the pH adjusted to 7.6.

10 x Phosphate Buffered Saline (PBS)	*Final Concentration*
80g NaCl	137mM
2g KCl	2.67 mM
22g Na_2HPO_4	8 mM
2g KH_2PO_4	1.5 mM

This was made up to 1000mL with water and the pH adjusted to 7.6. This was stored at 4°C and diluted to 1x for the final working solution.

1 x High-salt TBS-Tween 20 (HS-TBS-T)	*Final Concentration*
50mL 1 M Tris-HCL pH 8.0	50 mM
30mL 5 M NaOH	150 mM
1 mL Tween 20	0.1% v/v

This was made up to 1000mL with water.

20 x Citric Acid Buffer	*Final Concentration*
42g Citric acid Monohydrate	200 mM

This was made up to 1000mL with water and the pH adjusted to 6.0. This was stored at 4°C and diluted 1:20, to give a final working solution concentration of 10 mM.

2.1.7.2. Immunohistochemistry

0.5% Hydrogen Peroxide (for CA IX IHC)	*Final Concentration*
12.5mL 12% H_2O_2 sol.	0.5%
287.5mL H_2O	

3% Hydrogen Peroxide (for NF-κB IHC)	*Final Concentration*
75mL 12% H_2O_2 sol.	3%
225mL Methanol	

3% Hydrogen Peroxide (for pAkt IHC)	*Final Concentration*
75mL 12% H_2O_2 sol.	3%
225mL HS-TBS-T	37.5 mM Tris-HCL
	0.11 M NaCl

Immunohistochemistry background blocking solutions	*Final Concentration*
10% normal human serum (NHS) in 1 x TBS *(CA IX)*	10% v/v
5% goat serum in HS-TBS-T *(pAKT)*	5% v/v
20% normal rabbit serum (NRS) in PBS *(NF-κB)*	20% v/v

Mayers Haematoxylin Solution	*Final Concentration*
2g Haematoxylin	3.31 mM
100g Aluminium Potassium Sulphate ($AlK(SO_4)_2.12H_2O$)	110 mM
100g Chloral Hydrate ($C_2H_3Cl_3O_2$)	300 mM

This was dissolved in 2000mL of water whilst warming. Once dissolved, the following were added:

2g Citric acid Monohydrate ($C_6H_3Cl_3O_2$)	5 mM

400mg Sodium iodate ($NaIO_3$)	1 mM

2.1.7.3. Western Blotting

10% sodium dodecyl sulphate (SDS)	*Final Concentration*
10g SDS powder	10% w/v

This was dissolved in 100mL water and stored at room temperature until required.

10% Ammonium Persulphate (APS)	*Final Concentration*
10g APS powder	10% w/v

This was dissolved in 100mL water and stored at 4°C until required.

RIPA Lysis Buffer	*Final Concentration*
50mL 1 x PBS	1.37 mM NaCl
	270 µM KCl
	800 µM Na_2HPO_4
	150 µM KH_2PO_4
0.5mL Igepal	5% v/v
0.5mL 10% SDS	5% v/v
250mg deoxycholic acid	12.7 mM

This solution was prepared and stored at 4°C until required.

920mg sodium orthovanadate	1 mM
210mg sodium fluoride	1 mM
108mg β-glycerophosphate	1 mM

These were made up individually in 50mL water and stored at 4°C until required.

Complete Mini (PIC mix) (1 tablet in 1mL water)

70mg phenylmethylsulphonylfluoride (PMSF) saturated in 1mL 70% Ethanol

To make the working lysis buffer, 900µL RIPA buffer, 100µL PIC mix, 10µL sodium orthovanadate, 10µL sodium fluoride, 10µL β-glycerophosphate and 5µL PMSF were mixed immediately prior to use.

Laemmli 2 x concentrate Sample Buffer

Used at a ratio of 1:1 with samples for immunoblotting.

10% Resolving Gel	*Final Concentration*
5.84mL 30% Acrylamide/Bis-acrylamide	32% v/v
6.72mL 1 M Tris (pH 8.8)	370 mM
4.8mL H_2O	
180µL 10% SDS	1% v/v
400µL 10% APS	2.2% v/v
20µL N,N,N',N'-tetramethylenediamine (TEMED)	0.1% v/v

Stacking Gel	*Final Concentration*
1.33mL 30% Acrylamide/Bis-acrylamide	13% v/v
1.25mL 1 M Tris (pH 6.8)	122 mM
7.36mL H_2O	
100µL 10% SDS	1% v/v
200µL 10% APS	2% v/v
16µL TEMED	0.16% v/v

10 x SDS Running Buffer	*Final Concentration*
30.3g Tris base	250 mM
144g Glycine	2 M
10g SDS	0.1% w/v

This was made up to 1000mL with water and the pH adjusted to 8.3. It was stored at 4°C until required and diluted 10x for the working solution.

Transfer Buffer	*Final Concentration*
1.52g Tris base	12.5 mM
14.44g Glycine	200 mM
100mL Methanol	10% v/v

This was made up to 1000mL with water.

1 x TBS-Tween 20 (TBS-T)	*Final Concentration*
100mL 10 x TBS	
200 mM Tris base	
80 g NaCl	137 mM
1mL Tween 20	0.1% v/v

This was made up to 1000mL with water.

Blocking Buffer (5%)	*Final Concentration*
2.5g Marvel milk powder	5% w/v
50mL TBS-T	

Stripping Buffer	*Final Concentration*
350µL 2-mercaptoethanol	100 mM
10mL 10% SDS	20% v/v
3.12mL 1 M Tris (pH 6.8)	62.5 mM

This was made up to 50mL with water. It was prepared fresh for each use.

2.1.7.4 Assessment of Cell Viability and Apoptosis

10 x Annexin V Binding Buffer(pH 7.4)	*Final Concentration*
0.1M Hepes	10mM
1.4M NaCl	140mM
25mM $CaCl_2$	2.5mM

This was stored at 4°C and diluted to 1x with water prior to use.

Fluorescein Isothiocyanate-conjugated Annexin V (Annexin V-FITC)

Propidium Iodide	*Final Concentration*
Diluted with an equal volume of water prior to use	25μg/mL

2.2 Patients

2.2.1 Local Regional Ethics Committee Application and Approval

Formal application to the Leicestershire and Rutland Regional Health Authority local regional ethics committee (LREC) was made via the Research and Development Department based at Leicester General Hospital. A dual application was submitted, allowing the establishment of a mesothelioma tissue bank and database in addition to approval for this work. In addition to the prospective collection of tissue samples, this LREC approval included retrospective approval for specimens previously collected by a former researcher. Examples of both the patient information sheet and the consent form can be found in appendix 2.

2.2.2 Patient Population and Data Collection

The patient population on which the immunohistochemistry and correlative analysis of survival and clinicopathological variables is based was formed by past and present patients of the Department of Thoracic Surgery at Glenfield Hospital, or formerly the Department of Cardiothoracic Surgery at Groby Road Hospital, Leicester.

Patients with a diagnosis of malignant pleural mesothelioma were either identified retrospectively through the Department of Pathology or, from 29 January 2003, invited prospectively to participate in the study. All medical notes and detailed pathology reports were reviewed and clinicopathological data were entered into a secure database (included in the LREC approval detailed previously) which had previously been established by Mr J G Edwards, PhD (Edwards 2002). The database was adapted and expanded for the current study and to allow ongoing collection of data regarding future patients. A total of 200 patients with corresponding pathological samples available for analysis were included in the immunohistochemistry part of this study, although at the end of this

researchers' period of study the database itself contained details on 393 patients, almost certainly making this the largest database on patients with a diagnosis of malignant pleural mesothelioma in the United Kingdom. A representation of the Access form used for data collection is shown in Appendix I.

2.3 Methods

2.3.1 Immunohistochemistry

2.3.1.1 Paraffin-embedded tissue sample selection and preparation

Following routine pathological processing within the Department of Pathology at GGH, haematoxylin and eosin stained histology slides were reviewed to identify those with the greatest proportion of tumour. Two hundred cases were chosen, the corresponding tissue blocks were obtained and individual sections of 4 μm thickness were cut to onto glass slides pre-treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37°C to assist with section-to-slide adhesion.

2.3.1.2 Immunohistochemical methods

Consecutive formalin-fixed, paraffin-embedded tissue sections from the same 200 tissue blocks described in *2.3.1.1* were used. Similar techniques were used for both primary antibodies, but with notable differences (tables 2.1 and 2.2). The techniques described had been previously employed by Dr D Swinson (CA IX) and Mr A Shah (pAkt). Both methods were re-optimised by this researcher. As controls, positives were identified from work carried out by the previously named researchers. Benign pleural samples, isotype controls (where monoclonal primary antibodies were used) and negative controls with omission of the primary antibody were also employed.

2.3.1.3 Interpretation of Immunostaining

All interpretation was carried out on an Olympus light microscope (Olympus, London, UK). All slides were assessed by this researcher. Independent assessment was carried out by Dr D Richardson (CA IX and pAkt). Slides were assessed under conditions blinded to patient data and outcome. Slides stained for pAkt were assessed for the degree of nuclear, cytoplasmic and

membranous staining. Those stained for CA IX were assessed for cytoplasmic and membranous staining only, with nuclear staining being counted as either present or absent, with no gradation. The following semi-quantitative scale was used: 0% (no staining), 0; less than 5%, 1; ≥ 5% to less than 25%, 2; ≥ 25% to less than 50%, 3; ≥ 50% to less than 75%, 4; ≥ 75%, 5. In all cases the percentage figure applies only to the volume of tumour staining. To aid in this distinction, the corresponding sections stained conventionally with haematoxylin and eosin were available to aid identification of tumour islands. Any discrepancies in interpretation were resolved by consensus using a conference microscope. A Consultant pathologist (Dr J L Jones) adjudicated on persisting unresolved differences. In the assessment of cytoplasmic (c) and nuclear (n) Akt, and nuclear CA IX, all slides were assessed by this researcher, with a random selection of 40 slides from each series assessed by a second observer (Mr J G Edwards). In validation of this, good agreement was achieved according to Cohens' Kappa (cpAkt, kappa score 0.6, $p<0.0001$; npAkt, kappa score 0.7, $p<0.0001$; nCA IX, kappa score 0.7, $p<0.0001$).

Table 2.2 Method for pAkt immunostaining

Incubate paraffin-embedded slides for 15 minutes at 60°C
Dewax and rehydrate through graded alcohols (xylene → 99% IMS x 2 → 95% IMS x 2) 2 mins each
Wash in distilled H_2O for 5 mins
Antigen retrieval. Boil for 2 mins in 10M Citric acid in pressure cooker.
Rinse in distilled H_2O
Wash in running tap water for 10 mins
Wash in HS-TBS-T for 5 mins
Block endogenous peroxidase with 3% H_2O_2 (in HS-TBS-T) for 10 mins at RT
Wash in HS-TBS-T for 5 mins x 3
Block background staining using 5% goat serum in HS-TBS-T (100µL per slide), for 60 mins at RT
Wash in HS-TBS-T for 5 mins x 3
Incubate with primary antibody, diluted 1:100 in 5% goat serum, 100µL per slide, overnight at 4°C
Wash in HS-TBS-T for 5 mins x 3
Incubate with secondary antibody, diluted 1:300 in 5% goat serum, 100µL per slide, for 30 mins at RT
Immediately, make up ABC, with HS-TBS-T, leave to equilibrate for 30 mins at RT
Wash in HS-TBS-T for 5 mins x 3
Incubate slides with ABC (100µL per slide) for 15 mins at RT
Wash in HS-TBS-T for 5 mins x 3
Mix DAB (1 drop of DAB concentrate, for every 1mL of buffer solution)
Incubate with DAB solution (100µL per slide) for 2 mins
Rinse in distilled H_2O
Counterstain with Mayer's Haematoxylin for 10 secs
Rinse in running tap water for 5 mins
Dehydrate through graded alcohols (reverse of step 2)
Mount using DPX mountant and coverslips

Table 2.3 Method for CA IX Immunostaining

Incubate paraffin-embedded slides for 15 minutes at 60°C
Dewax and rehydrate through graded alcohols (xylene → 99% IMS x 2 → 95% IMS x 2) 2 mins each
Wash in TBS for 5 mins
Block endogenous peroxidase with 0.5% H_2O_2 (in distilled H_2O) for 30 mins at RT
Wash in TBS for 5 mins x 3
Block background staining using 10% NHS diluted in TBS (100µL per slide) for 15 mins at RT
Wash in TBS for 5 mins x 3
Incubate with primary antibody (M75), diluted 1:50 in distilled water, 100µL per slide, for 30 mins
Wash in TBS for 5 mins x 3
Incubate with secondary polymer from Envision+ kit, 2 drops per slide, for 30 mins at RT
Wash in TBS for 5 mins x 3
Mix DAB (1 drop of DAB concentrate, for every 1mL of buffer solution)
Incubate with DAB solution (100µL per slide) for 5 mins
Rinse in running tap water for 5 mins
Counterstain with Mayer's Haematoxylin for 10 secs
Rinse in running tap water for 5 mins
Dehydrate through graded alcohols (reverse of step 2)
Mount using DPX mountant and coverslips

2.3.2 Cell Culture

2.3.2.1 Establishing cultures from frozen

Cell vials retrieved from storage in a liquid nitrogen dewer were placed in an incubator at 37°C/5% CO_2 until they were completely thawed. They were then added to 5mL of warmed medium and additives appropriate to the cell line in a 25cm^2 flask. The flask was then placed in the incubator at 37°C/5% CO_2 for 8 hours or overnight. After this time the medium was changed to reduce the effect of dimethyl sulphoxide (DMSO) added to the original cryomix at the time of cell freezing. Once cells reached, or approached a confluent stage of growth in 25cm^2 flasks, they were transferred to 75cm^2 and then 150cm^2 flasks using the technique described below. All cell culture work was performed under aseptic conditions within a laminar air flow fume hood using pre-warmed reagent solutions.

2.3.2.2 Routine subculture of cell lines

Once cells reached confluency within a flask they required either transfer into a bigger flask or passaging. After pre-warming all the necessary reagents, a solution of 1x trypsin in 1xPBS was made up from stock. The medium within the culture flask was poured off and the adherent cells were washed with Hanks Balanced Salt solution (HBSS). Depending on the size of flask 3-5mL of the trypsin/PBS solution was added and the flask was placed in the incubator at 37°C until the cells had detached, usually less than 5 minutes. Once cell detachment had occurred, an equal volume of warmed culture medium was added to neutralise the effects of the trypsin and an appropriate volume of cell suspension was added to a new flask.

After a cell line had been established from frozen, they were routinely cultured in 150cm^2 flasks to reduce the number of passages. However, for most of the cell line experiments the 25cm^2 flasks provided an ample supply of viable cells, whilst preserving culture medium and other reagents. For the production of lysates for western blotting, 75cm^2 flasks were used to increase the protein yield.

2.3.2.3 Preparing cells for storage

Cells were harvested as per the above protocol. Following cell detachment using the trypsin/PBS solution, the cell suspension (trypsin neutralised with medium) was pipetted into a sterile universal container and centrifuged for 5 minutes at 1500rpm. The supernatant was then discarded and the remaining cell pellet was re-suspended in a solution of 90% foetal calf serum (FCS) and 10% DMSO. The cryomix suspension was then divided into eppendorf ampoules in 1mL aliquots, which were then frozen to -80°C overnight and placed in liquid nitrogen for longer term storage.

2.3.3 Treatment of cells in culture

2.3.3.1 Exposure to hypoxic environmental conditions

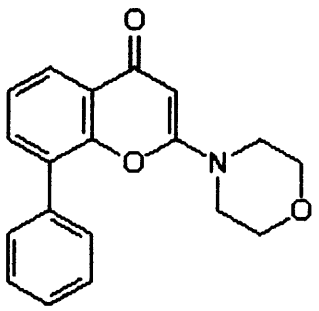
Routinely, cells were cultured in standard incubators at 37°C, in an atmosphere of 21% O_2 and 5% CO_2 with the balance made up of N_2. To alter and control the environmental O_2 concentration an RS Biotech Galaxy R incubator (RS Biotech, UK) was used. Oxygen levels were confirmed using an Analox oxygen meter placed in the incubator. For all hypoxia experiments cells were grown until flasks were approximately 75% confluent. The culture flasks were removed from a conventional incubator, the medium was discarded and the cells were washed once with HBSS. Fresh medium was then added to the flasks and they were placed into the Galaxy R incubator under the appropriate environmental conditions.

2.3.3.2 LY294002

The naturally occurring bioflavinoid Quercetin has been shown to inhibit multiple protein kinases, including PI3-K. In the search for increased specificity, other flavinoid derivatives were evaluated with LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (figure 2.2) demonstrating complete and specific PI3-K activity inhibition at an inhibitory concentration $(IC)_{50}$ of 1.4μM (Vlahos et al. 1994). The mechanism of action is through competitive inhibition of the ATP-binding site (Davies et al. 2000). LY294002 was obtained from Calbiochem (UK) as a 10mM solution in DMSO. To obtain appropriate working concentrations, varying volumes of LY294002 were added to culture flasks containing cells and medium immediately prior to flasks being placed in incubators

set to produce experimental environmental conditions (section 4.4.1, chapter four). Duplicate experiments were conducted with DMSO alone added as control flasks.

Figure 2.1 The molecular structure of LY294002



2.3.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This process of protein separation uses an anionic detergent (in this case SDS) to give the proteins a negative charge, in combination with a reducing agent and heat to denature cellular polypeptides, allowing their separation according to molecular weight.

2.3.4.1 Preparation of lysates

To allow for the semi-quantitative probing of cellular proteins following exposure of cells to varying environmental conditions, lysates were produced. Flasks of cells were removed from the incubator. The medium was discarded and the cells were washed once with cold HBSS. Then, 500μL of the RIPA lysis buffer was added to lyse the remaining adherent, viable cells. This process would usually take 2-3 minutes. The floor of the flask was then scraped with a sterile scraper to remove any remaining cells and the lysis suspension was pipetted into a 2mL eppendorf, passed through a 14 gauge needle 5 times and left to clarify on ice for 30 minutes. Following this, the suspension was centrifuged for 20 minutes at 14,000 rpm at 4°C. Finally, the supernatant was pipetted into a sterile 1mL eppendorf for storage prior to protein measurement and further use.

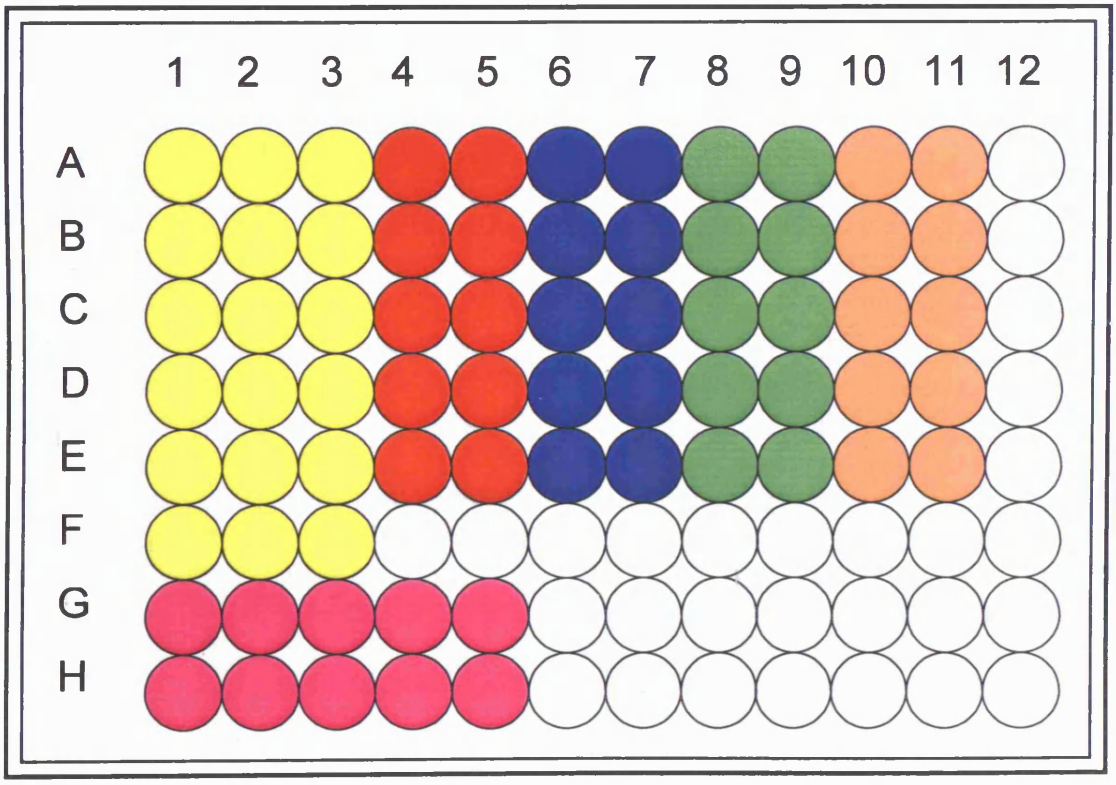
2.3.4.2 Bio-Rad DC Microplate Protein Assay

To allow the accurate comparison of cell lysates produced as above, a colorimetric assay for determining protein concentration was used. The Bio-Rad DC protein assay, which works along

similar lines to the test first developed by Lowry in 1951 (Lowry et al. 1951) was chosen as it is compatible with anionic detergents, and can be adapted for use with microtitre plates and a microplate reader to allow the processing of reasonably large numbers of samples.

With each run, a standard concentration curve was established, using six known concentrations, ranging from 0.0 – 2.0mg/mL of bovine serum albumin (BSA) in RIPA lysis buffer. For each standard, three wells on the microtitre plate were used to allow for minor pipetting errors. For the samples to be tested, two wells were used. Figure 2.2 shows an example plate layout.

Figure 2.2 An example of possible microtitre plate layout of standards and samples for the Bio-Rad DC microplate protein assay. The standards, in yellow, show the 6 different concentrations, with 3 repeats for each. For the samples, consider five different time periods in an hypoxic environment represented by the rows, for five different cell lines, represented by each colour in the columns (with the exception of the "pink", which is the opposite due to space limitations on the 12x8 plate).



Into each well, 5µL of either standard or sample was pipetted as appropriate. A working Bio-Rad reagent was then prepared by mixing 20µL of Reagent S with every 1mL of Reagent A required. Twenty-five microlitres of this mixture was then pipetted into every well containing standard or sample. Then, 200µL of Bio-Rad Reagent B was also added to each well. The contents of each well were then mixed by careful pipetting, using a clean tip for each well and dispersing any bubbles that may have formed. The microtitre plate was then covered and allowed to sit for 15 minutes at RT. Finally, the absorbance of the standards and samples were read using an automated spectrophotometric microplate reader set at 690 nm. A standard curve was derived from the averaged standard readings and the protein concentrations of the samples were calculated using this curve.

2.3.4.3 Sample preparation

Stored lysates of known protein concentration were mixed with an equal volume of 2x Laemmli sample buffer (Sigma-Aldrich, UK) in a 1mL eppendorf tube. This sample buffer consists of 2-mercaptoethanol which reduces the intra and inter-molecular disulfide bonds. It also contains SDS and glycerol, and bromophenol blue which provides a marker that runs ahead of the proteins and also makes it easier to see the sample during loading. Each lysate/sample buffer mix was then heated to 99°C for 10 minutes.

2.3.4.4 Gel preparation

The Bio-Rad mini Protean III kit was used to house the resolving and stacking gels. This was assembled according to the manufacturers' instructions and was tested with water first to ensure there were no leaks. The resolving gel was made up according to the table below (table 2.4). This was carefully pipetted into the apparatus, avoiding air bubbles, to a level approximately 2cm from the top. A layer of Isobutanol was then added above the gel to prevent diffusion of atmospheric O_2 into the gel which would hinder the polymerisation process. The layer of Isobutanol was poured off after the gel had set, usually approximately 10 minutes, and then the stacking gel was added above the resolving gel (for recipe, see table 2.4). A 10-toothed comb was added into the top of the stacking gel to form the wells for the samples to be placed into. The stacking gel was then allowed a further period of 10

minutes to set before the comb could be removed. For the resolving gel, a 10% acrylamide gel was used to allow the adequate separation of proteins in the 15-100 KDa range.

2.3.4.5 Electrophoresis

The gels within their glass housing were then submerged in 1x SDS running buffer and the samples were loaded into the wells using a micro-pipette, loading the correct volumes so that each well contained 50µg of protein. A molecular weight marker, SeeBlue® Plus2 (Invitrogen, UK) and the appropriate controls were also added to each gel run. The gels were run at 100mA (amp constant, ~120V) until the marker line reached the bottom of the gel.

Table 2.4 The component reagents and their volumes required for the resolving and stacking gels in SDS-PAGE.

Reagent	**10% Resolving Gel (µL)**	**Stacking Gel (µL)**
30% w/v Acrylamide and bis-acrylamide	2970	670
1M Tris (pH 8.8)	3360	625
distilled H_2O	2400	3680
10% SDS	90	50
10% ammonium persulphate (APS)	200	100
N,N,N',N'-tetramethylenediamine (TEMED)	10	8

2.3.5 Western Blotting

2.3.5.1 Electroblotting

A Polyvinylidene Difluoride (PVDF) membrane (Immobilon P™, Sigma-Aldrich, UK) was cut to the size of the gel. It was equilibrated by submersion in methanol for 30 seconds. All the components required for protein transfer to the PVDF membrane were then soaked in cold transfer buffer for 15 minutes. Using the Bio-Rad wet blotting apparatus the components were assembled in the following order: negative electrode; fibre pad; blotting paper; gel; PVDF membrane; blotting paper; fibre pad; positive electrode, with transfer anticipated from negative to positive. Care was taken to minimise bubbles between the layers by rolling with a 10mL pipette at each stage of construction. The apparatus was then submerged in transfer buffer and run at 200mA (amp constant, ~130V) for 60 minutes. Protein transfer was confirmed by staining with Ponceau S (Sigma-Aldrich, UK) and the molecular weight marks were scored onto the edge of the PVDF membrane in indelible ink.

2.3.5.2 Protein detection

To reduce background staining the PVDF membrane was blocked with 5% milk in TBS-T for 60 minutes at RT. The membrane was then incubated with the primary antibody in 5% milk in TBS-T overnight (for 16 hours) at 4°C. All primary antibodies were used at a concentration of 1:1000 unless otherwise stated. The PVDF membrane was then washed 3 times for 5 minutes in TBS-T. It was then incubated with an HRP-conjugated secondary antibody, diluted 1:2000 in 5% milk in TBS-T for 2 hours at RT. After 3 further 5 minute washes in TBS-T the PVDF membrane was exposed to a 1mL mix of the two enhanced chemiluminescence (ECL) solutions (Amersham Life Sciences, UK) for 1 minute. The membrane was then carefully wrapped in Saran wrap and exposed to Amersham Hyperfilm (Amersham Life Sciences, UK) for a period of time that, once optimised, was used for all further experiments. Film exposure to the PVDF membrane and development were carried out under safe light conditions.

2.3.5.3 Membrane stripping and re-probing

To allow probing for different proteins, the membranes were stripped of the primary and secondary antibodies attached during the western blot process. This was achieved by placing the PVDF membrane in a 50mL plastic vial containing stripping buffer and placing the sealed vial in a shaking water bath at 50°C for 30 minutes. The membrane was then removed and washed 3 times for 5 minutes in TBS-T. The membrane was then re-treated with the ECL developer kit to ensure adequacy of the stripping. The membrane was then re-blocked with 5% milk in TBS-T and re-probed with the appropriate primary antibody.

2.3.6 Assessment of cell viability

2.3.6.1 Trypan Blue Staining

Trypan blue is a vital dye that is completely excluded from living cells. It can be used in a method to determine the proportion of viable cells within a population.

A cell culture flask was removed from the incubator. The medium was poured off into a sterile universal and kept aside. The adherent cells in the flask were then washed with pre-warmed HBSS. This was also collected into the universal. Five millilitres of 1xtrypsin/1xPBS solution was added to the flask to detach adherent cells. Once the cells had detached, 5mL of culture medium was added to neutralise the effect of the trypsin. The resulting suspension was then pipetted into the universal container which was then centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in 1mL of Hepes buffer solution (pH 7.4). Equal volumes (10 μL) of Hepes/cell suspension and trypan blue were then mixed and the numbers of stained (non-viable) and non-stained (viable) cells were counted using a haemocytometer slide.

2.3.6.2 Cell counting using a haemocytometer slide

The haemocytometer is a specialised microscope slide on which two grids are engraved, in the central region of the slide, leaving it 0.1 mm lower than the rest of the slide. Each grid is made up of twenty five large squares, each containing 16 smaller squares of area 1/400 mm^2. Once a cover-slip is applied over the central grids, this creates an area of known volume of 0.1 mm^3. By applying 10μL of

the cell suspension and trypan blue mix under each end of the cover slip, capillary action will ensure coverage of the mixture over the grids. By counting the total number of cells (stained and non-stained) in the 25 large square grid, counting cells in the squares and those touching the top and left borders only, the calculation to determine the concentration of cells in a suspension is given by:

$$\text{Concentration (cells/mL)} = \text{Average cell count of both grids} \times 10^4$$

This allows aliquots of known numbers of cells to be produced for further experimentation.

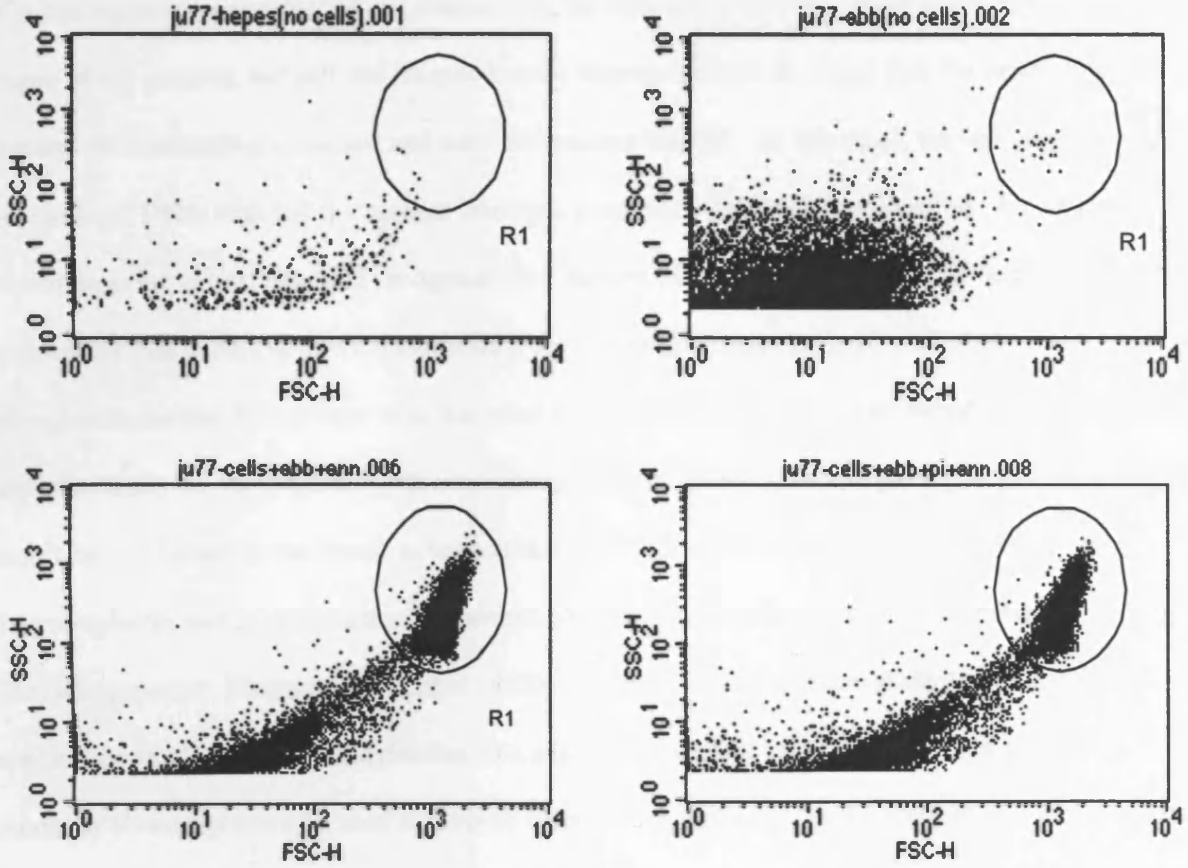
2.3.6.3 Flow cytometry and assessment of apoptosis

Flow cytometry is a general term for the process of analysing cells or other biological material by detection of the differing light absorbing or fluorescing properties of those cells or material. The piece of equipment that performs this process is known as a flow cytometer. The system used in this study was the *FACSCalibur*™ flow cytometer, using *CellQuest PRO*™ analysis software (Becton Dickinson, Cowley, Oxford, UK).

2.3.6.3.1 Identification of cell populations and optimisation

Prior to undertaking any meaningful experimental evaluation, all cell lines must be visually "identified" and optimised for use with a particular flow cytometer. Identification of the JU77 cell line is demonstrated in figure 2.3. Electrical compensation for spectral overlap was achieved for each cell line, completing the process of optimisation.

Figure 2.3 Identifying and gating for the Ju77 cell line. The analysis of experimental components first without the cells of interest and then with, allow identification of the cell population. In future analyses, only those "events" occurring within "R1" are counted.



2.3.6.3.2 Assessment of apoptosis

As previously noted, apoptosis is an energy-dependent process involving the co-ordinated "self-destruction" of individual cells without the triggering of an inflammatory response characteristic of more indiscrete processes of cell destruction, for example, necrosis. Despite this, towards the final stages of the process, the cell and its membranes degrade to such an extent that the morphological features differentiating apoptosis and necrosis become blurred. At this stage, the staining of intracellular DNA with the fluorescent vital dye, propidium iodide (PI) is possible. A process occurring prior to this, one well recognised as a feature of apoptosis, is the loss of membrane asymmetry that occurs with the translocation (or flipping) of membrane phospholipid phosphatidylserine (PS) groups from the inner to the outer aspect of the cell membrane. Annexin V is one of a family of polypeptides with a molecular weight of ~32 KDa. Originally isolated from human umbilical cord arteries and found to have anticoagulant properties, the Annexins inhibit thromboplastin and clotting factor Xa through phospholipid-clotting factor interactions (Reutelingsperger, Hornstra, & Hemker 1985). Annexin V binds preferentially to phospholipid species, particularly phosphatidylserine, in a calcium dependent manner (Vermes et al. 1995). Annexin V can therefore be used to identify cells in early apoptosis at the point of loss of membrane asymmetry. These features can be utilised in the flow cytometric differentiation of cells that are viable, undergoing apoptosis (and indeed whether they are early or late in the process) or dead within a distinct population.

For the apoptosis assay, following assessment of cell viability and counting by trypan blue staining, 2 x 10^5 cells were suspended in 200µL 1x Annexin V binding buffer. One microlitre of Annexin V-FITC (BD Biosciences Pharmingen) was added and incubated at 4°C for 10 minutes in the dark. One microlitre of PI (BD Biosciences Pharmingen) was added to the cell suspension immediately prior to analysis. The varying environmental conditions of cell culture prior to analysis will be described in the individual results sections.

2.3.7 Statistical methods

The software package SPSS v14 for Windows (SPSS, Chicago, IL) was used to perform the statistical analysis.

For the immunohistochemistry, groups were dichotomised into categorical variables above and below the median score (2, ≥25 to less than 50%) or, in the case of nuclear CA IX, the presence or absence of staining. Survival curve estimations were obtained using the Kaplan-Meier method (Kaplan & Meier 1958), with univariate prognostic factors identified using the log rank test. A Cox proportional hazards regression model was used to analyse variables identified on univariate analysis with $p<0.1$ in a multivariate model. A forward stepwise selection procedure was used, with variables added to the model according to a partial likelihood ratio test, using the entry criterion of $p<0.05$. The Chi-squared test was used to identify associations between the CA IX and pAkt staining. The degree of agreement between the individual immunohistochemistry scorers was assessed using Cohen's Kappa test.

Numerical data obtained by flow cytometric analysis are expressed as means +/- the standard error of the means. The presence or absence of significant differences between results was determined using the Mann Whitney U test to compare between environmental conditions, or one-way analysis of variance (ANOVA) when three or more means were compared. ANOVA was combined with Tukey's post-hoc test to assess which means differed.

CHAPTER THREE

PHOSPHO-AKT AND CA IX EXPRESSION IN MPM

3.1 Introduction

Activated, or phosphorylated-Akt has a potentially important role in both cell proliferation and survival through cell cycle regulation and anti-apoptotic mechanisms (Nicholson & Anderson 2002). The expression of pAkt in MPM has previously only been reported in small numbers of cases (Altomare et al. 2005) and the prognostic implications of its expression, or its correlation with other clinicopathological prognostic markers has never been investigated in MPM.

Carbonic Anhydrase IX, known to be up-regulated in hypoxic conditions, has been postulated as a surrogate marker of hypoxia in lung cancer (Swinson et al. 2003). Additionally, it may have a role in proliferation and oncogenesis (Nogradi 1998) although the mechanism through which this could occur is not clear. One possibility is its potential ability to accentuate the acidity of the peri-tumoural extra-cellular space, thereby providing neoplastic cells with an environment favouring malignant growth and metastasis over normal cells (Potter & Harris 2003).

As part of the overall study, the aims of this section were to examine the immunohistochemical expression of pAkt and CA IX in a large series of paraffin-embedded MPM tumour samples for each of which a detailed history is available and follow-up is complete. The correlation of expression with known clinicopathological parameters and the relationship between each other is explored. The prognostic significance of pAkt and CA IX expression was also assessed, in univariate and multivariate models.

3.1.1 Patient Demographics

There were 180 men and 20 women, with a median age at time of diagnosis of 61 years (range 41-86). There were 126 (63%) epithelioid tumours, 36 (18%) biphasic and 38 (19%) sarcomatoid. Seventy-three (36.5%) patients underwent palliative debulking surgical procedures. Sixty-four patients had more extensive surgical treatment, with 7 (3.5%) undergoing PD and 57 (28.5%) undergoing EPP. Fifty (25%) patients had open surgical biopsy alone. In 13 (6.5%) patients the method of obtaining histological diagnosis was either unknown or achieved through non-surgical biopsy, these patients were excluded from further statistical analyses. At the time of assessment, the

overall median survival from the time of surgical intervention was 8.2 (range 1-74) months. One-, 2- and 3-year survival rates were 36%, 16% and 8% respectively. Twenty patients died within 30 days of operation giving a 30 day mortality of 11%, and 10.5% in those patients undergoing extra pleural pneumonectomy, which is in line with other published series (Aziz, Jilaihawi, & Prakash 2002;Rusch & Venkatraman 1996). These patients were excluded from further survival analyses to avoid the bias of non-cancer related deaths.

3.2 pAkt Immunostaining

3.2.1 Results

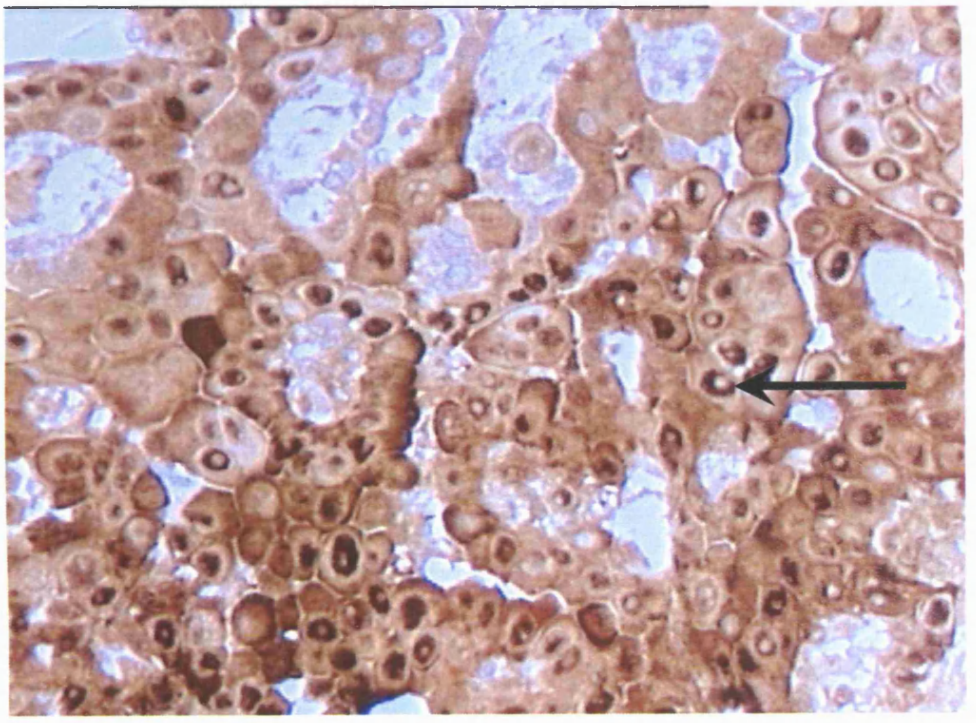
Phospho-Akt was frequently expressed in this cohort of patients, being present in 86% of tumour samples. It was localised principally to the cytoplasm and the nucleus, occasionally with marked nucleolar sparing (figure 3.1a). Although nuclear staining was never seen in the absence of cytoplasmic staining, a strong proportional correlation was noted between the two with regard to the extent of staining ($p<0.001$, χ^2). There was no significant difference in the distribution of staining between the histological subtypes, although the overall appearance was markedly different (figure 3.1b) and there was always greater expression of pAkt seen in the epithelial subtype. An isotype control, using normal rabbit IgG in place of the primary antibody can be seen in figure 3.3c.

3.2.2 Correlation with clinicopathological factors

Clinical prognostic factors in this disease, defined and validated previously (Curran, Sahmoud, Therasse, van Meerbeeck, Postmus, & Giaccone 1998);(Herndon, Green, Chahinian, Corson, Suzuki, & Vogelzang 1998);(Edwards, Abrams, Leverment, Spyt, Waller, & O'Byrne 2000), were correlated with cytoplasmic, nuclear and overall pAkt expression, using the χ^2 test (or Fishers exact test where total numbers in individual cells were <5). There were no associations noted between cytoplasmic and overall pAkt staining, however a haemoglobin of <14, considered a poor prognostic factor, was significantly associated with a greater proportion of samples with increased npAkt expression ($p=0.033$) (table 3.1).

Figure 3.1 Phospho-Akt expression in MPM, showing a) an epithelial tumour with intense cytoplasmic and nuclear staining, and also nucleolar sparing (arrow), b) a sarcomatoid tumour with less intense cytoplasmic and nuclear staining (x400) and c) an isotype control slide using normal rabbit IgG in place of the primary antibody

a)



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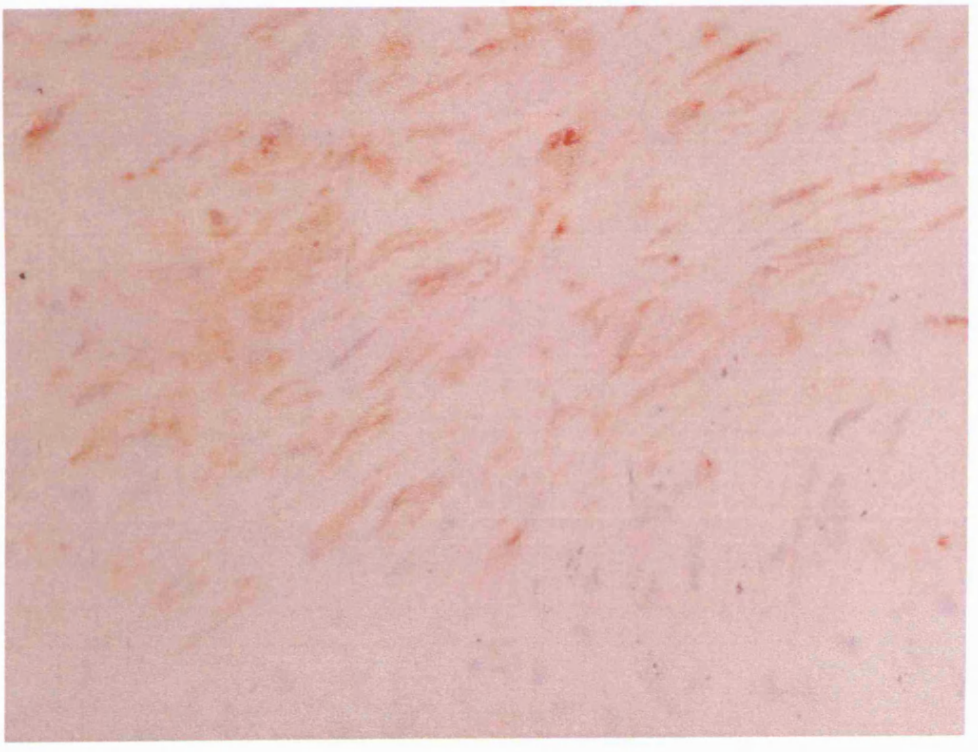

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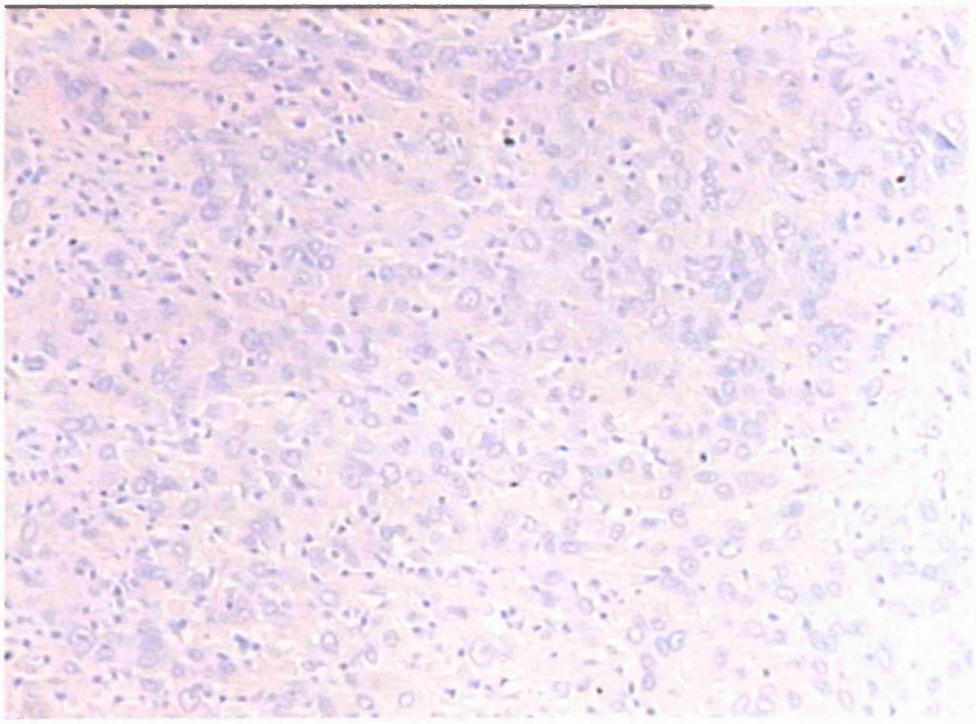


Table 3.1 Correlations between pAkt immunostaining and clinicopathological factors (χ^2 test).

Variable	Overall pAkt staining		p (χ^2)	Nuclear pAkt staining		p (χ^2)	Cytoplasmic pAkt staining		p (χ^2)
	<med	>med		<med	>med		<med	>med	
Gender									
Male	74	92	0.52	104	62	0.62	116	50	0.89
Female	7	12		13	6		13	6	
Chest Pain									
No	28	35	0.74	45	18	0.06	47	16	0.22
Yes	49	68		67	50		77	40	
>5% Weight loss									
No	38	59	0.23	62	35	0.76	64	33	0.24
Yes	38	42		50	31		60	21	
ECOG PS									
0	41	52	0.99	58	35	0.73	66	27	0.80
1 or 2	40	51		59	32		63	28	
Cell type									
Epithelial	46	71	0.13	73	44	0.89	78	39	0.28
Biphasic	14	18		20	12		26	11	
Sarcomatoid	21	15		24	12		25	6	
WBC									
=/< $8.3x10^4$	25	40	0.33	39	26	0.62	45	20	0.98
> $8.3x10^4$	52	61		72	41		78	35	
Platelets									
=/< $400x10^9$	47	66	0.55	69	44	0.64	76	37	0.48
> $400x10^9$	30	35		42	23		47	18	
Haemoglobin									
> 14 g/dL	41	61	0.29	57	45	**0.033**	69	33	0.59
=/< 14 g/dL	37	40		55	22		55	22	
CALGB									
1	19	20	0.42	24	15	0.94	25	14	0.33
2	1	8		6	3		4	5	
3	36	41		51	26		59	18	
4	4	4		5	3		6	2	
5	15	21		20	16		23	13	
6	4	7		7	4		8	3	
EORTC									
Low risk	33	48	0.48	48	33	0.35	54	27	0.44
High risk	46	54		66	34		72	28	

3.3 CA IX Immunostaining

3.3.1 Results

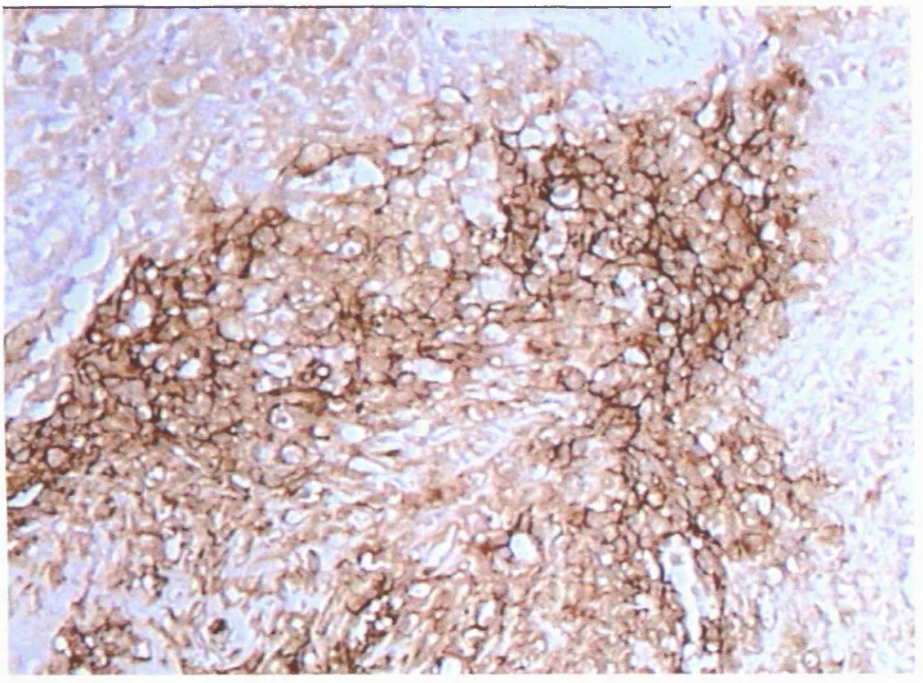
CA IX was expressed even more extensively that pAkt, being recorded in 97% of samples in this cohort. The distribution of CA IX was mainly cytoplasmic and membranous (figure 3.2a); with fewer samples (36%) displaying nuclear staining (figures 3.2b and c). Unique to the epithelial subtype, marked polarisation of membranous staining was occasionally seen (figure 3.2 d). There was a strong correlation between those samples with nuclear staining and increased cytoplasmic and membranous staining ($p<0.001$, χ^2). In addition, increased expression of CA IX was strongly associated with the epithelial histological subtype, with progressively less staining seen in biphasic and sarcomatoid tumours ($p<0.001$, χ^2) (figures 3.2e and f). To confirm the specificity of the CA IX antibody, IgG2b isotype controls were performed (figures 3.2 g and h). Furthemore, benign pleural samples were also examined to confirm the marked upregulation of CA IX in malignant disease (figure 3.2i).

3.3.2 Correlation with clinicopathological factors and pAkt immunostaining

Increased expression of CA IX was associated with several indicators of improved outcome; the absence of chest pain, no significant weight loss, an Eastern Co-operative Oncology Group (ECOG) a performance status (PS) of 0, epithelial histology and a low risk EORTC grouping. Although there was some variation when the subcellular localisation of CA IX was considered more specifically, all the above were found to be significantly associated with overall CA IX immuno-staining (table 3.2). In spite of the apparent differences in the distribution of the expression of CA IX and pAkt, a significant proportional correlation was observed between cytoplasmic CA IX and overall pAkt expression ($p=0.021$, χ^2) (table 3.3).

Figure 3.2 CA IX expression in MPM, showing a) epithelial MPM (x100); epithelial MPM with b) nuclear staining (x400) (arrow), c) perinuclear staining (x400) (arrow) and d) with polarised membranous staining (x100) (arrow); e) sarcomatoid MPM (x100) with f) notable nuclear staining (x400) (arrow); isotype controls for g) epitheial and h) sarcomatoid subtypes; i) very limited staining in benign pleural disease

a)



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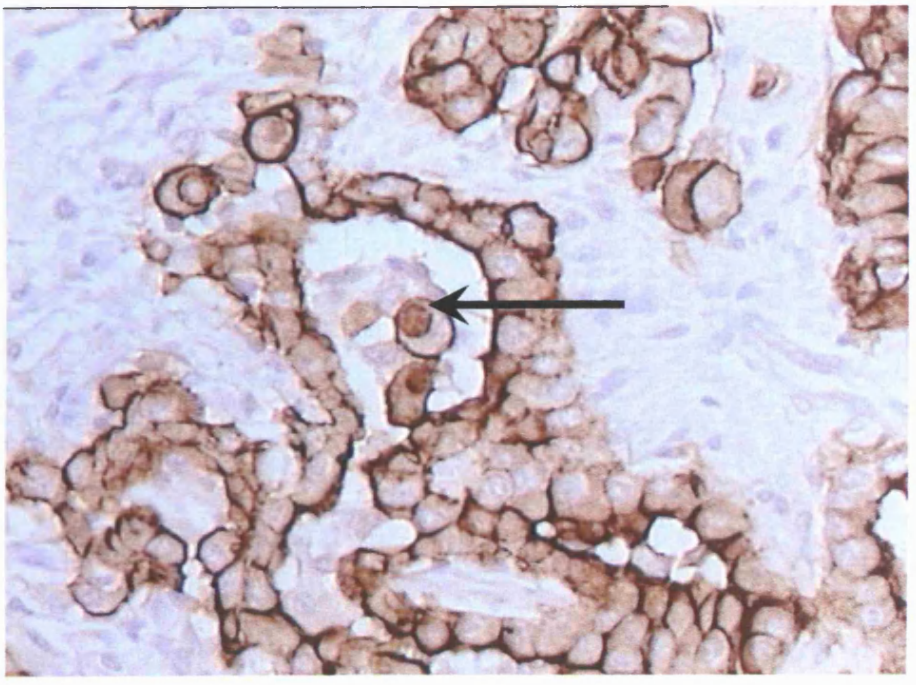

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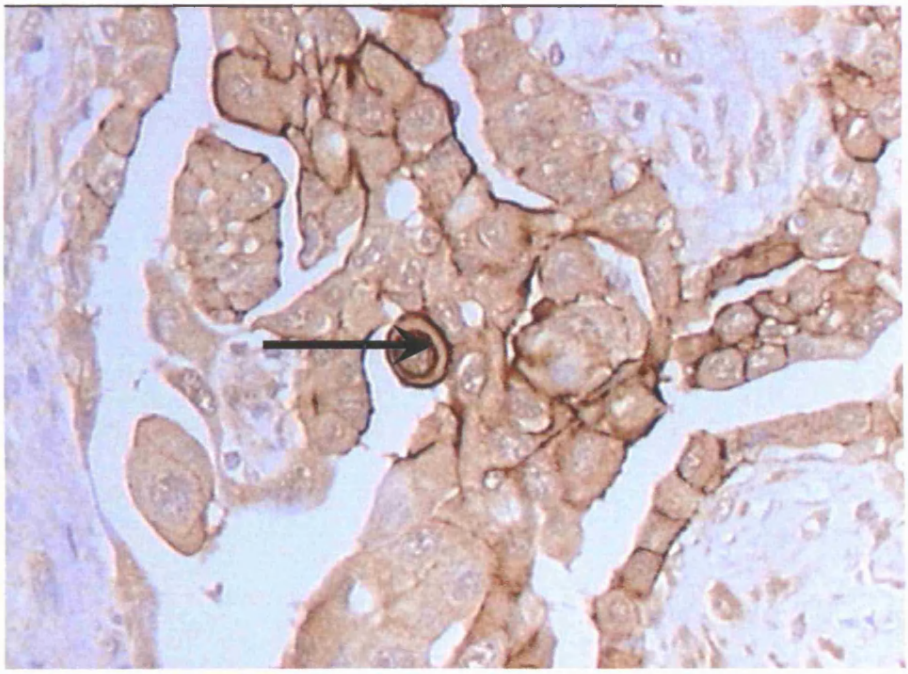

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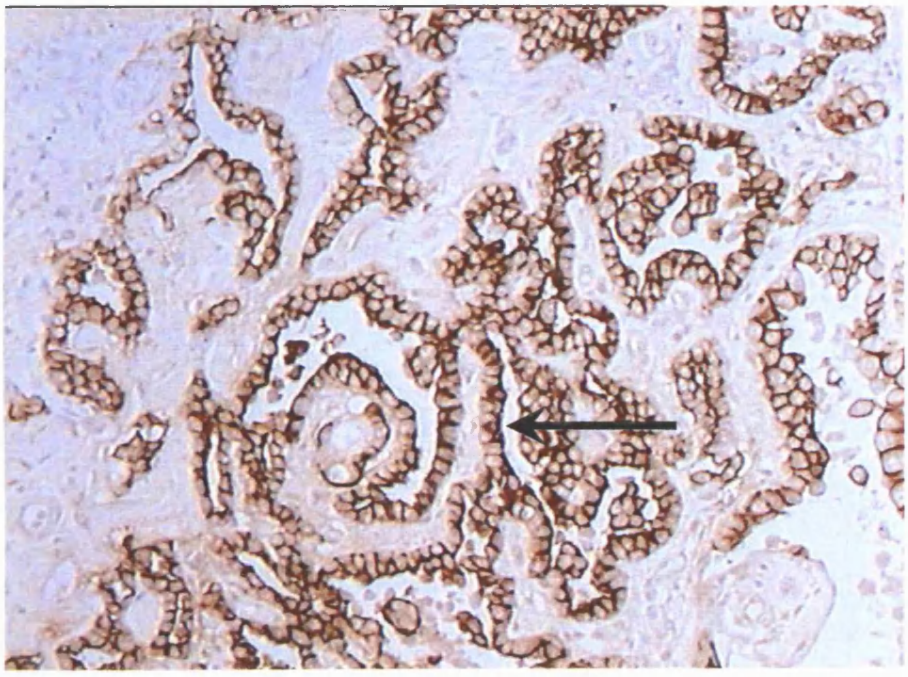

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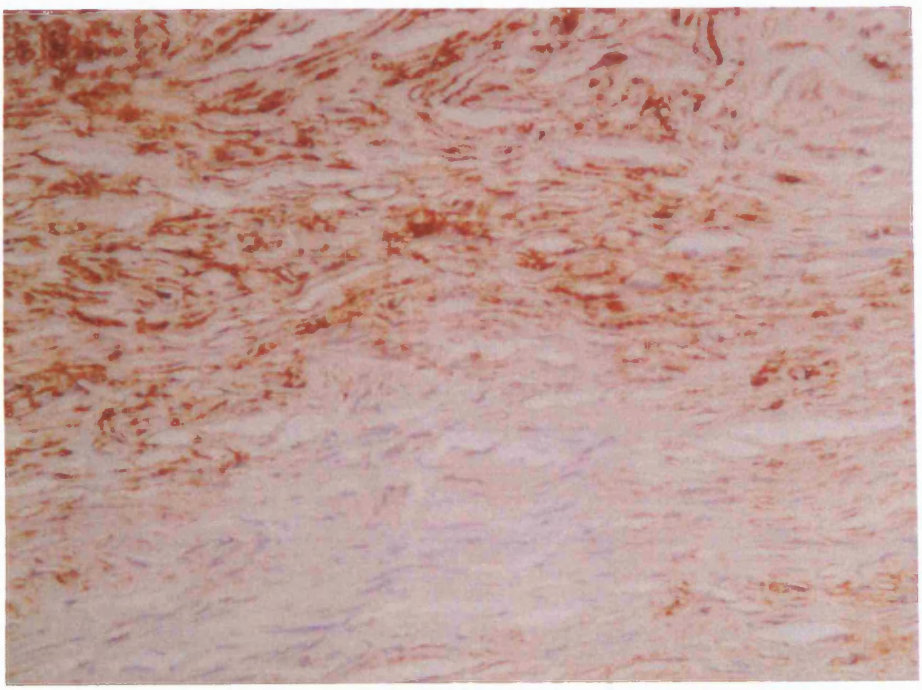

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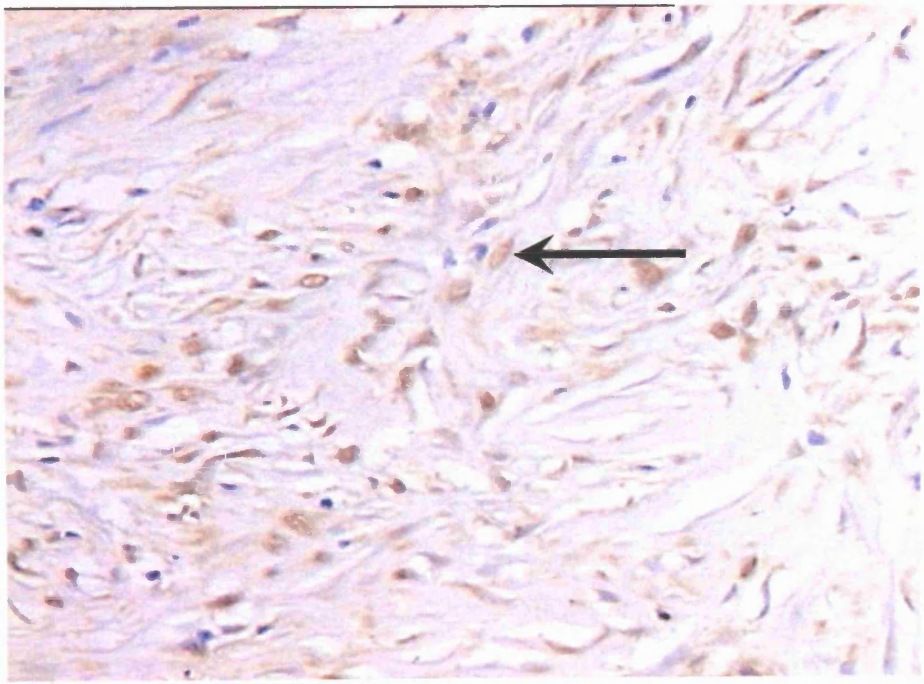
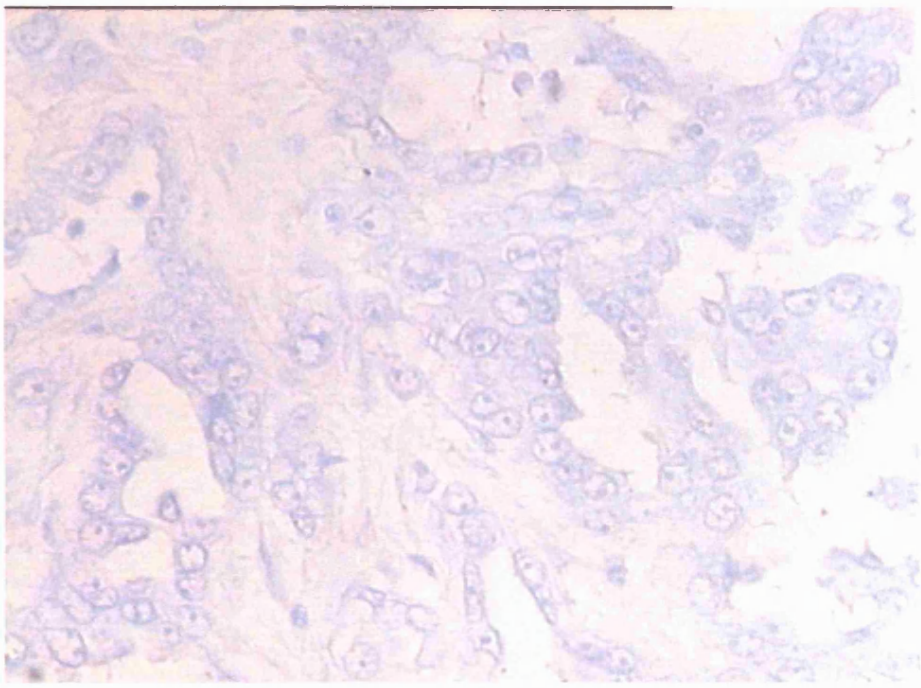


g)



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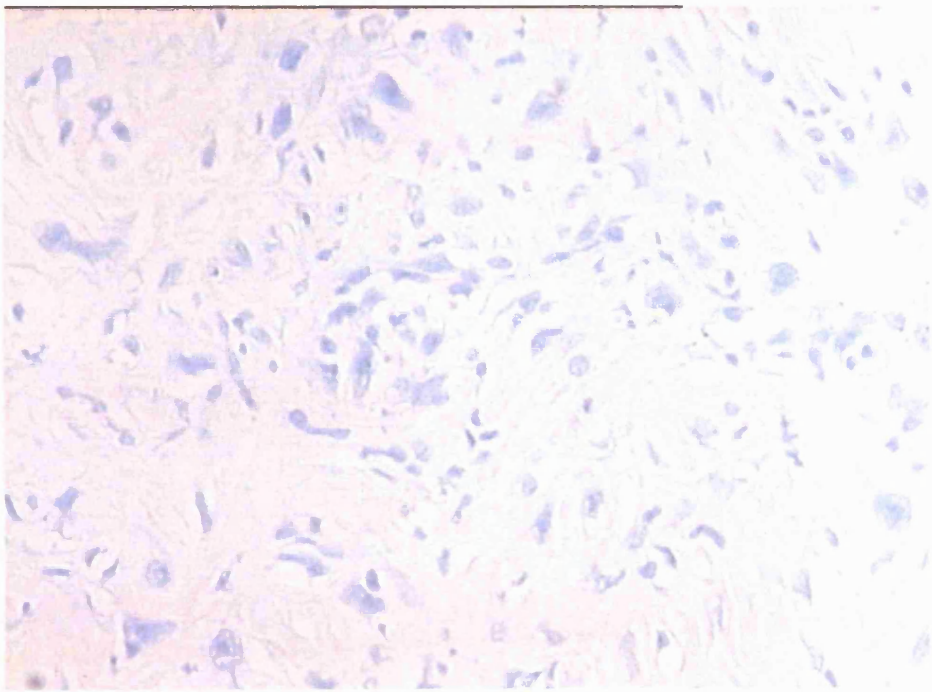


i)

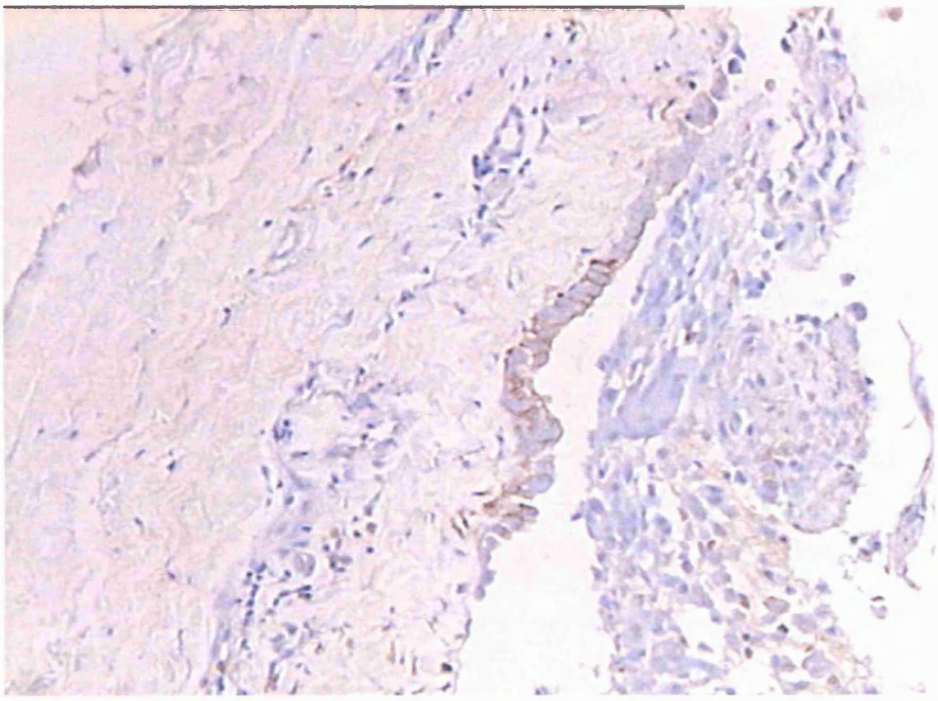


Table 3.2 Correlations between CA IX immunostaining and clinicopathological factors (χ^2 test)

Variable	Overall CA IX		p (χ^2)	Membranous CA IX		p (χ^2)	Cytoplasmic CA IX		p (χ^2)	Nuclear CA IX		p (χ^2)
	<med	>med		<med	>med		<med	>med		<med	>med	
Gender												
Male	75	93	0.28	87	81	0.22	93	75	0.51	108	59	0.56
Female	6	13		7	12		9	10		11	8	
Chest Pain												
No	16	47	**<0.001**	21	42	**0.001**	30	33	0.18	34	28	**0.046**
Yes	63	56		71	48		69	50		83	36	
>5% Weight loss												
No	34	65	**0.01**	45	54	0.18	44	55	**0.005**	63	35	0.99
Yes	43	38		45	36		53	28		52	29	
ECOG PS												
0	32	63	**0.009**	39	56	**0.013**	46	49	0.10	54	40	**0.047**
1 or 2	48	43		54	37		55	36		65	26	
Cell type												
Epithelial	38	81	**<0.001**	51	68	**0.003**	55	64	**0.001**	68	50	**<0.001**
Biphasic	16	16		16	16		18	14		34	2	
Sarcomatoid	27	9		27	9		29	7		17	15	
WBC												
=/< $8.3x10^4$	25	41	0.18	32	34	0.52	32	34	0.15	35	31	**0.023**
> $8.3x10^4$	55	59		61	53		68	46		79	34	
Platelets												
=/< $400x10^9$	50	65	0.73	60	55	0.86	61	54	0.37	72	43	0.69
> $400x10^9$	30	35		33	32		39	56		42	22	
Haemoglobin												
> 14 g/dL	29	48	0.13	59	43	0.09	61	43	0.28	66	37	0.95
=/< 14 g/dL	51	53		34	45		39	38		49	28	
CALGB												
1	13	26	0.22	15	24	0.28	19	20	0.31	22	17	0.24
2	3	6		4	5		3	6		3	6	
3	35	44		41	38		45	34		52	26	
4	2	6		3	5		3	5		5	3	
5	20	16		23	13		23	13		27	9	
6	7	4		7	4		8	3		7	4	
EORTC												
Low risk	22	61	**<0.001**	28	55	**<0.001**	37	46	**0.013**	45	37	**0.016**
High risk	57	43		64	36		63	37		72	28	

Table 3.3 Correlations between CA IX and pAkt immunostaining (χ^2).

		Overall pAkt		Cytoplasmic pAkt		Nuclear pAkt	
		<med	>med	<med	>med	Absent	Present
Overall CA IX	<med	39	41	54	26	50	30
	>med	42	63	75	30	67	38
	p (χ^2)		0.23		0.56		0.85
Membranous CA IX	<med	43	50	61	32	60	33
	>med	38	54	68	24	57	35
	p (χ^2)		0.50		0.22		0.72
Cytoplasmic CA IX	<med	52	49	72	29	67	34
	>med	59	55	57	27	50	34
	p (χ^2)		**0.021**		0.61		0.34
Nuclear CA IX	Absent	54	63	83	34	76	41
	Present	26	44	45	22	40	27
	p (χ^2)		0.33		0.59		0.48

3.4 Survival Analyses

3.4.1 Univariate analysis

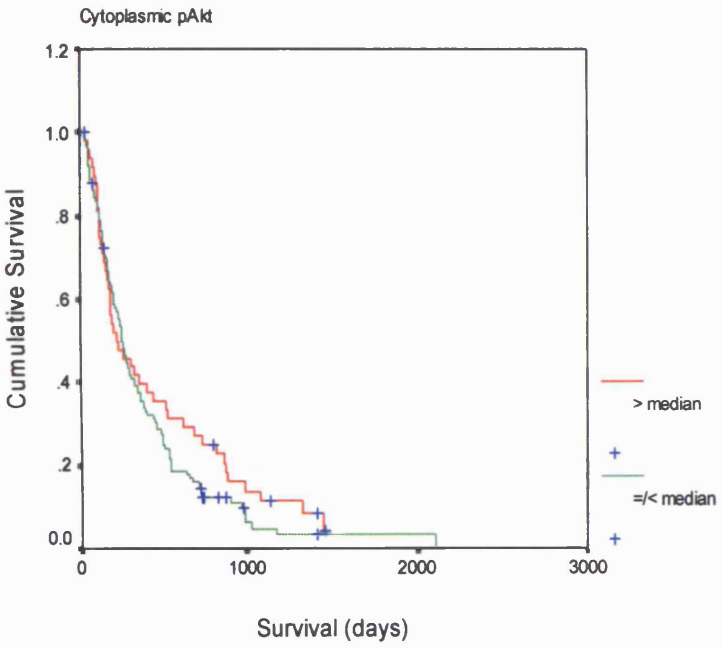
With regard to pAkt, the extent of cytoplasmic or nuclear expression was seen to have little effect on prognosis (p=0.27 and p=0.10, respectively), except when the data were analysed according to histological subtype. In this situation it was seen that an increased expression of nuclear pAkt was associated with a poorer outcome in the epithelial subtype (233 versus 491 days, p=0.047) (figure 3.3).

Univariate analysis revealed that increased overall expression of CA IX (either membranous or cytoplasmic > median) was associated with an improved survival (307 versus 175 days, p=0.01). Considered individually, cytoplasmic staining for CA IX was found to be predictive of an improved survival (p=0.04), but membranous or nuclear staining were not (p=0.08 and p=0.06, respectively) (figure 3.4). The extent of CA IX expression was not prognostic within a given histological subtype when these were considered separately.

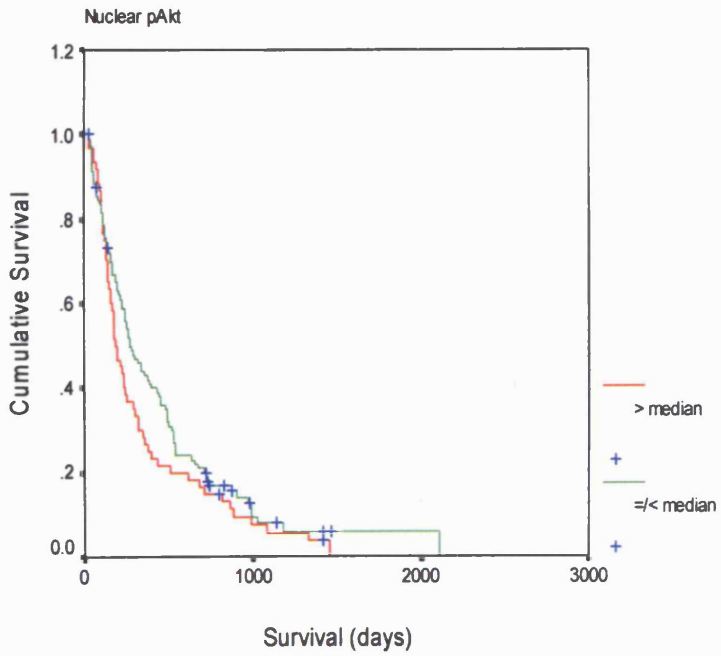
In addition, clinical variables were included in the univariate survival analysis, all of which continued to stratify survival according to previously published work on this cohort of patients (Edwards, Abrams, Leverment, Spyt, Waller, & O'Byrne 2000), including the CALGB and EORTC prognostic scoring systems. Individual indicators of poor prognosis are detailed in table 3.4.

Figure 3.3 Kaplan-Meier survival curves of pAkt expression, showing the effects of high and low a) cytoplasmic (p=0.27) and b) nuclear staining (p=0.10). Histological subtype analysis revealed no difference for c) cytoplasmic pAkt (p=0.43), but d) increased nuclear pAkt expression in the epithelial subtype was found to be indicative of a poorer prognosis (233 versus 491 days, p=0.047 log rank).

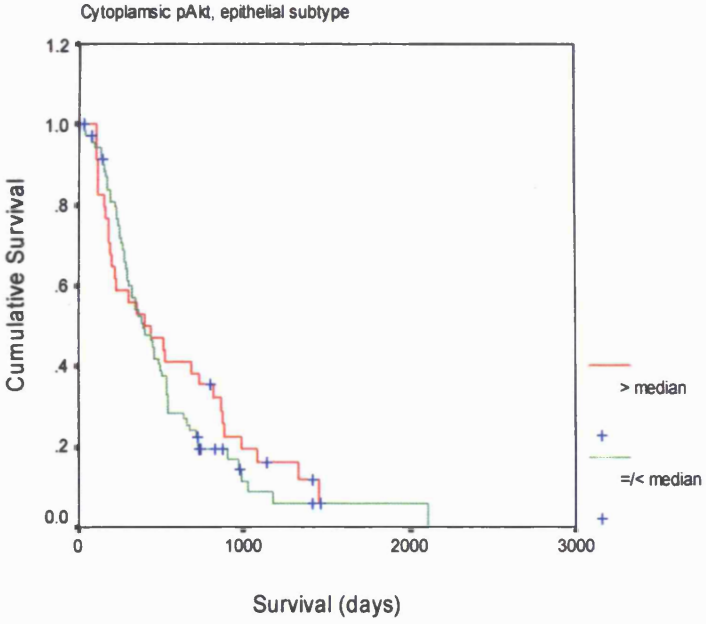
a)



b)



c)


Cytoplamsic pAkt, epithelial subtype
Cumulative Survival
1.2
1.0
.8
.6
.4
.2
0.0
0
1000
2000
3000
Survival (days)
> median
=/< median

d)

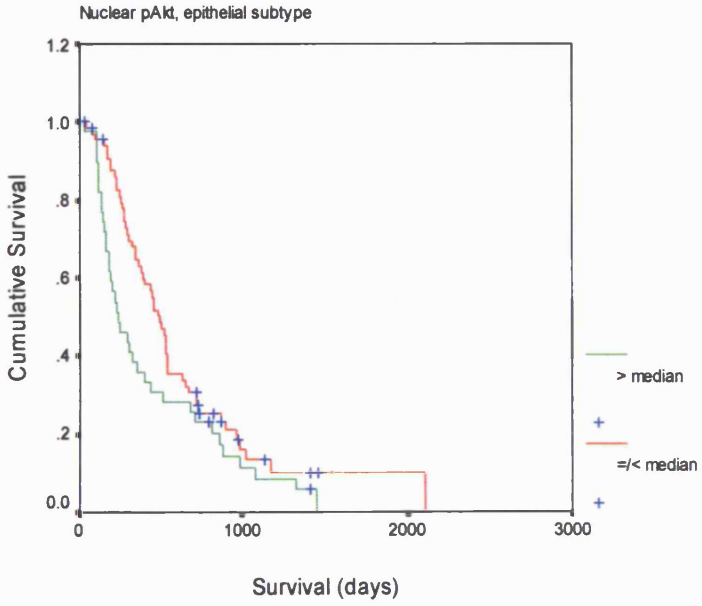
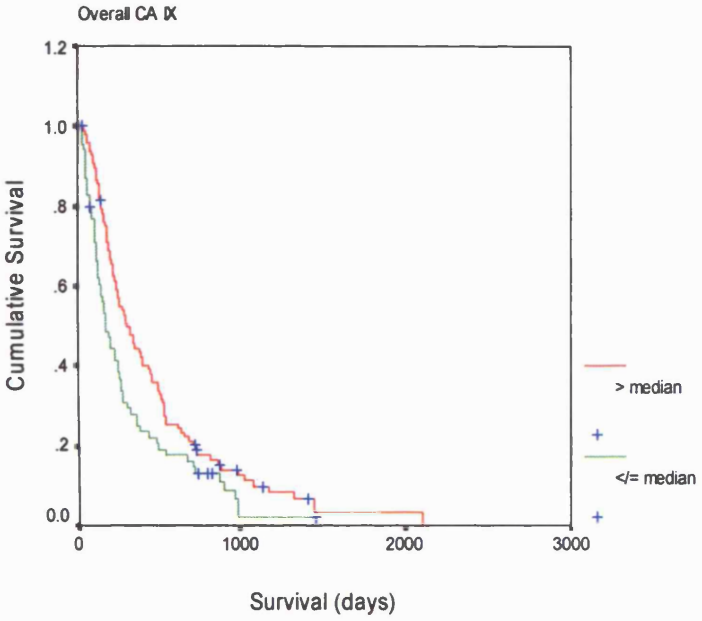
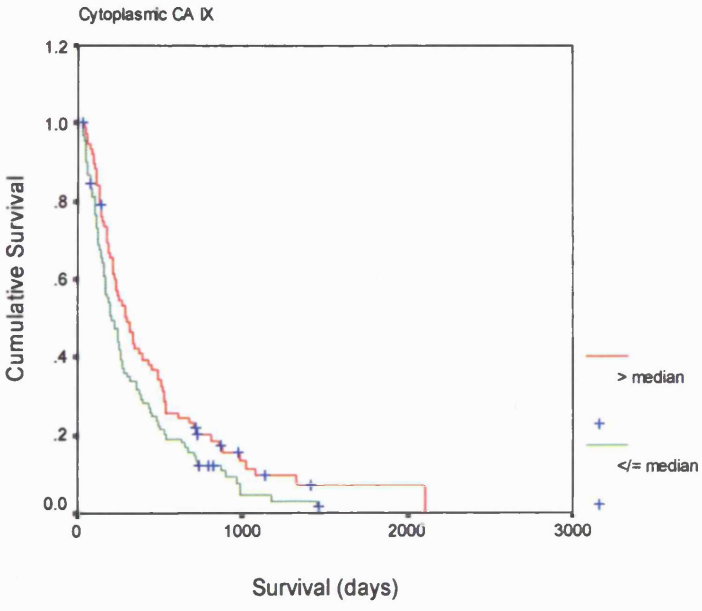

Nuclear pAkt, epithelial subtype
Cumulative Survival
1.2
1.0
.8
.6
.4
.2
0.0
0
1000
2000
3000
Survival (days)
> median
=/< median

Figure 3.4 Kaplan-Meier survival curves of CA IX expression showing the apparent positive effect of increased a) overall CA IX (p=0.01) and b) cytoplasmic CA IX (p=0.04). Although a trend was preserved, c) membranous CA IX and d) nuclear CA IX expression were not prognostic indicators (p=0.08 and p=0.06, respectively).

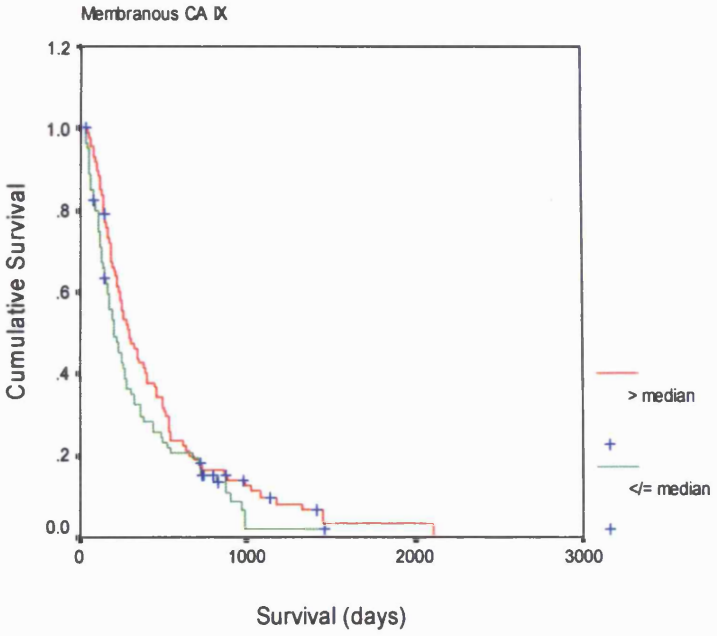
a)



b)



c)


Membranous CA IX
Cumulative Survival
1.2
1.0
.8
.6
.4
.2
0.0
0
1000
2000
3000
Survival (days)
> median
</= median

d)

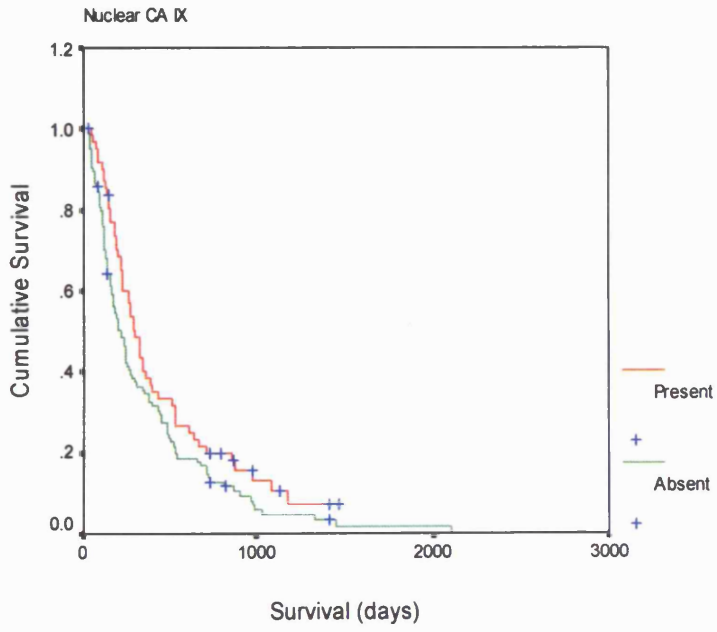

Nuclear CA IX
Cumulative Survival
1.2
1.0
.8
.6
.4
.2
0.0
0
1000
2000
3000
Survival (days)
Present
Absent

Table 3.4 Univariate survival analysis (Kaplan-Meier, log rank).

Variable		Median survival (days)	p value
Gender	Male	247	0.24
	Female	259	
Chest pain	No	324	**<0.001**
	Yes	186	
Weight loss >5%	No	307	**<0.001**
	Yes	172	
Histology	Epithelial	382	**<0.001**
	Biphasic	175	
	Sarcomatoid	108	
ECOG Performance status	0	345	**<0.001**
	1/2	157	
White blood cell count	</=8.3x10^4	296	0.06
	>8.3x10^4	200	
Platelets	</=400x10^9	296	**0.009**
	>400x10^9	175	
Haemoglobin	>14 g/dL	434	**<0.001**
	</=14 g/dL	192	
CALGB score	1	497	**<0.001**
	2	538	
	3	246	
	4	247	
	5	129	
	6	75	
EORTC score	Low risk	345	**<0.001**
	High risk	151	
Nuclear CA IX	Negative	213	0.06
	Positive	296	
Membranous CA IX	=/<median	203	0.08
	>median	292	
Cytoplasmic CA IX	=/<median	213	**0.04**
	>median	307	
Overall CA IX	=/<median	175	**0.01**
	>median	307	
Nuclear pAkt	=/<median	277	0.10
	>median	186	
n pAkt (Epithelial subtype only)	=/<median	491	**0.047**
	>median	233	
Cytoplasmic pAkt	=/<median	249	0.27
	>median	215	
Overall pAkt	=/<median	266	0.36
	>median	246	

3.4.2 Multivariate analysis

The poor prognostic factors identified previously, including the prognostic scoring systems, were entered into the multivariate analysis. On analysis, non-epithelial histological subtype, a high CALGB score, weight loss of greater than 5% and an increased expression of nuclear pAkt were identified as independent risk factors (table 3.5).

Table 3.5 Multivariate survival analysis of clinicopathological and biological prognostic variables (identified by the log rank test, entry criterion p=/<0.1) by Cox Proportional Hazards analysis. Only cases for which data were complete in all categories were included (n=153).

Variable		Number	HR	95 % CI		p Value
Nuclear pAkt	=/< Median	94	1			**0.040**
	> Median	59	1.44	1.01	2.05	
Histology	Epithelial	95	1			**<0.001**
	Biphasic	28	6.10	3.57	10.4	
	Sarcomatoid	30	2.16	1.38	3.39	
Weight loss >5%	No	86	1			**0.016**
	Yes	67	1.62	1.09	2.39	
CALGB score	1	31	1			**0.003**
	2	7	0.81	0.31	2.12	
	3	72	1.52	0.96	2.43	
	4	6	1.91	0.66	5.57	
	5	30	2.11	1.15	3.90	
	6	7	8.27	3.25	21.0	
Haemoglobin	>14 g/dl	63				0.16
	</=14 g/dl	90				
Chest Pain	No	56				0.82
	Yes	97				
ECOG Performance status	0	81				0.34
	1 or 2	72				
Platelets	</=400x10^9/l	99				0.30
	>400x10^9/l	54				
White Blood Cell Count	</= 8.3x10^4/l	58				0.62
	>8.3x10^4/l	95				
EORTC score	Low risk	72				0.73
	High risk	81				
Membranous CA IX	=/< median	74				0.28
	> median	79				
Cytoplasmic CA IX	=/< median	83				0.63
	> median	70				
Nuclear CA IX	No	96				0.32
	Yes	57				
Overall CA IX	=/< median	64				0.38
	> median	89				

3.5 Discussion

This work represents the first to examine the immunohistochemical expression of both CA IX and pAkt in a large cohort of patients with MPM. The expression of pAkt in MPM has not been previously widely reported, but comparisons can be made with studies in other solid tumours. Predominantly cytoplasmic staining, with occasional nuclear staining, was seen in approximately 35% of HNSCC samples in a study by Amornphimoltham *et al* (Amornphimoltham et al. 2004). In addition to this, Tanno *et al* identified intense nuclear and cytoplasmic expression of pAkt in 84% of human bile duct cancer samples analysed (Tanno et al. 2004). Despite the similarities seen between these studies and the present, isolated cytoplasmic expression has been noted in breast cancer (Vestey et al. 2005), with predominantly membranous expression reported in prostate cancer (Malik et al. 2002). It is worthy to remember that no membranous expression was seen in this study.

Variable sub-cellular localisation of pAkt may represent different levels of activation or the targeting of different intra-cellular substrates. Recent work detailing the expression of pAkt in NSCLC has shown that increased expression of pAkt was found to be associated with a favourable prognosis. However, when the data were considered according to sub-cellular localisation, npAkt expression correlated with nodal metastases, well known as a poor prognostic factor in NSCLC (Shah et al. 2005). In further support of this role for npAkt, a study by Vasko *et al* of pAkt expression in thyroid cancer showed nuclear localisation of pAkt to be associated with increased tumour aggressiveness and invasion (Vasko et al. 2004). Also, increased overall expression of pAkt is more commonly associated with a poorer outcome, for example, in terms of a reduced disease-free survival (breast cancer (Vestey, Sen, Calder, Perks, Pignatelli, & Winters 2005)), a shorter time to progression (HNSCC (Amornphimoltham, Sriuranpong, Patel, Benavides, Conti, Sauk, Sausville, Molinolo, & Gutkind 2004)) or increased resistance to radiotherapy (bile duct cancer (Tanno, Yanagawa, Habiro, Koizumi, Nakano, Osanai, Mizukami, Okumura, Testa, & Kohgo 2004)).

The isoform Akt1 possesses a functional leucine-rich nuclear export sequence which, when mutated leads to the nuclear accumulation of Akt1 (Saji et al. 2005). In addition, data regarding the intra-cellular interactions of Akt and particularly the importance of nuclear localisation are emerging.

In a study by Adini *et al*, pAkt was found to at least partially rely on co-localisation for nuclear accumulation with RhoB (Adini et al. 2003), the endothelial cell small GTPase implicated in cell survival (Wheeler & Ridley 2004). In the rat phaeochromocytoma cell line PC12, intra-nuclear Akt has been shown to associate particularly with nucleolin (Borgatti et al. 2003), the nucleolar protein with proposed roles including ribosome biogenesis, cell proliferation and growth (Tuteja & Tuteja 1998). Furthermore, growth factor treatment of the osteoblast-like clonal cell line MC3T3-E1 revealed a significant increase in the nuclear translocation of Akt, strongly implicating its involvement in signalling pathways that mediate cellular proliferation (Borgatti et al. 2000).

The expression of CA IX has been documented previously (Ivanov, Liao, Ivanova, Danilkovitch-Miagkova, Tarasova, Weirich, Merrill, Proescholdt, Oldfield, Lee, Zavada, Waheed, Sly, Lerman, & Stanbridge 2001), but included only 8 mesothelioma samples and said nothing of the distribution of staining or its potential role in prognostication. The pattern of staining seen in this series is validated by reports in different tumours. Membranous and cytoplasmic staining have been reported in pancreatic and renal cell carcinomas (Bui et al. 2003;Juhasz et al. 2003), with nuclear staining described in non-small cell lung cancer (NSCLC) (Swinson, Jones, Richardson, Wykoff, Turley, Pastorek, Taub, Harris, & O'Byrne 2003). In addition to this, marked upregulation of CA IX has been seen around areas of necrosis (Hedley et al. 2003) and also areas of increasing hypoxia, based on *in*-vivo eppendorf readings of intra-tumoural oxygen levels in carcinoma of the cervix (Loncaster et al. 2001) and more recently in NSCLC and head and neck cancer (Le et al. 2006). Increased expression of CA IX has recently been shown to correlate strongly with increased levels of the hypoxia-regulated protein Galectin-1 in head and neck squamous cell carcinomas (HNSCC) (Le et al. 2005). Precisely what this says about the role of CA IX in the malignant process is still to be clarified, but the polarisation of membranous staining may give an indication. Saarnio *et al* reported prominent polarisation of staining on the basolateral surface of benign enterocytes, potentially placing CA IX at sites of active cell proliferation (Saarnio, Parkkila, Parkkila, Waheed, Casey, Zhou, Pastorekova, Pastorek, Karttunen, Haukipuro, Kairaluoma, & Sly 1998b), with similar work in colorectal tumours supporting this role in malignant disease (Saarnio et al. 1998a).

Previously published work in different epithelial malignancies has identified CA IX as an indicator of poor prognosis (Swinson et al. 2003) and increased tumour aggressiveness (Kim et al. 2005b). Despite this apparently contradicting evidence, in this analysis CA IX expression was associated with a more favourable prognosis. In mesothelioma, this is conversely perhaps unsurprising given its increased expression in epithelial tumours which represent a histological variant well known to be associated with a better outlook (Steele 2002). The extent of CA IX provided no discriminatory prognostic value when expression was analysed within the epithelial subtype alone. Why there should be less expression of CA IX in the biphasic and sarcomatoid subtypes is not clear, but a difference in embryological origin of the cell types from which the tumours develop and their degree of differentiation are potential reasons. Further work is required to more clearly delineate the situation.

3.6 Summary

To conclude this chapter, this work has demonstrated for the first time the marked expression of pAkt and CA IX in paraffin-embedded sections of human malignant pleural mesothelioma. Markedly variable expression is seen, between both histological subtypes and the differing sub-cellular compartments. Perhaps most interesting, is the potential role of increased nuclear pAkt expression in terms of both treatment possibilities through selective inhibition and also a future role in biological prognostication.

CHAPTER FOUR

THE EFFECTS OF HYPOXIA ON MALIGNANT PLEURAL MESOTHELIOMA AND MESOTHELIAL CELL LINES

4.1 Introduction

Hypoxia of any tissue results from an inadequate supply of oxygen leading to compromise in the metabolic biological function of that tissue. Macroscopically, intra-tumoural hypoxia has been shown to have a significant impact on survival and response to treatments in numerous different solid tumours including cervical, head and neck and breast cancers and also soft tissue sarcomas (Hockel & Vaupel 2001). An apparent paradox is encountered on the microscopic level, as tumour progression has also been associated with an increased intra-tumoural microvessel density (Semenza 2002b). This is explained by structural and functional imperfections frequently seen in the hastily formed tumour microvasculature, thereby leading to conditions of hypoxia (Harris 2002;Vaupel, Kallinowski, & Okunieff 1989).

Conditions of intra-tumoural hypoxia have been recreated *in vitro* through the exposure of a variety of malignant cell lines to reduced environmental O_2 concentrations, attempting to unravel the adaptive anti-apoptotic and proliferative mechanisms. For example, Lee *et al* have recently shown that exposure of lung cancer cell lines to 1% O_2 leads to up-regulation of pAkt, conferring increased resistance to ultra-violet (UV)- and etoposide-mediated apoptosis (Lee et al. 2006). In addition, hypoxia has been demonstrated to produce prolonged Akt phosphorylation in RN46A neuronal cells, required for increased resistance to apoptosis (Zhang, Gozal, Sachleben, Jr., Rane, Klein, & Gozal 2003). However, complicating the simplistic view of an individual role for pAkt, it has been proposed that activation of the PI3-K/pAkt pathway is required for the hypoxic stabilisation of HIF-1α in HepG2 (Mottet, Dumont, Deccache, Demazy, Ninane, Raes, & Michiels 2003), PC-3 and DU145 (Jiang, Jiang, Zheng, Lu, Hunter, & Vogt 2001) cell lines, although there is also contradicting work demonstrating that HIF-1α induction can be achieved independent of pAkt (Alvarez-Tejado, Alfranca, Aragones, Vara, Landazuri, & del Peso 2002). However, as may be predicted, work using the HIF-1-deficient murine hepatoma cell line, BpRcl, has revealed that activated Akt has potent oncogenic and angiogenic abilities itself (Arsham, Plas, Thompson, & Simon 2004), independent of HIF-1.

The aims of this section were to assess the impact of reduced environmental O_2 concentration on the viability of a benign mesothelial cell line and 4 MPM cell lines. In conjunction with this, the

expression of pAkt and HIF-1α through SDS-PAGE and immunoblotting were to be explored. Furthermore, addition of the PI3-K inhibitor, LY294002, to cell cultures, was designed to examine the presence or absence of a role for pAkt in cell survival under hypoxic conditions.

4.2 The effect of a reduced environmental O_2 concentration on cell viability over a varying time-course

4.2.1 Experiment 1

4.2.1.1 Methods

Separate flasks containing cultures of the 5 cell lines were placed into an atmosphere of 0.5% O_2 for a period of 16 hours. The period of 16 hours was chosen because previous work had shown the up-regulation of pAkt in a lung cancer cell line incubated for varying periods up to 24 hours (Lee, Lee, Kim, Han, Shim, & Yoo 2006). In addition to this, work with human monocytes had demonstrated the up-regulation of HIF-1α through hypoxic exposure over a 16-hour time period (Burke et al. 2002). Corresponding flasks were also kept in normoxic (20.9% O_2) conditions.
All flasks were processed according to the previously described method (chapter two). This was repeated on 2 separate occasions.

The trypan blue positive counts and the cell concentration were obtained immediately following removal of cells from the cultures flasks. The percentage of cells found to be annexin V positive or "viable" was determined by flow cytometry. These techniques, performed consecutively in the laboratory, are more fully described in chapter 2 (section 2.3.6) and were identical for all cell line experiments.

4.2.1.2 Results

Exposure of both MPM and mesothelial cell lines to reduced environmental O_2 concentration of 0.5% for a period of 16 hours appeared to have little effect on the cell populations in terms of the measured parameters. No significant differences were seen between those populations kept in conditions of normoxia when compared with those kept in hypoxic conditions. Four different outcome measures, obtained from two different but chronologically consecutive techniques, are shown

in table 4.1. As the exposure to 0.5% O_2 for 16 hours appeared to have little effect on the cells, it was decided to progress with longer time-points.

Table 4.1 Experiment 1 - Comparison of the effect of a lowered environmental O_2 concentration of 0.5% on a variety of measures of cell viability, compared with normoxic conditions. Means were compared with the Mann-Whitney U test (n=2, for each environmental condition for each cell line).

Cell line		Experimental Conditions		p value
		0.5% O_2 for 16 hours (Mean +/- SEM)	20.9% O_2 for 16 hours (Mean +/- SEM)	
MeT5A	Trypan blue positive (%)	2.9 +/- 2.1	1.4 +/- 0.3	1.0
	Cell concentration (10^6cells/μL)	1.4 +/- 0.1	1.5 +/- 0.3	0.7
	Annexin V positive (%)	5.7 +/- 1.9	5.4 +/- 0.8	1.0
	Viable cells (%)	84.1 +/- 1.7	82.3 +/- 1.8	0.4
JU77	Trypan blue positive (%)	1.9 +/- 0.4	1.5 +/- 0.3	0.4
	Cell concentration (10^6cells/μL)	3.2 +/- 0.7	3.0 +/- 0.5	0.7
	Annexin V positive (%)	2.8 +/- 0.7	2.9 +/- 0.2	1.0
	Viable cells (%)	86.1 +/- 1.8	82.8 +/- 2.0	0.4
ONE58	Trypan blue positive (%)	8.6 +/- 5.2	0.4 +/- 0.4	0.1
	Cell concentration (10^6cells/μL)	1.2 +/- 0.1	1.6 +/- 0.1	0.1
	Annexin V positive (%)	4.5 +/- 2.0	4.8 +/- 0.1	1.0
	Viable cells (%)	75.5 +/- 1.5	80.4 +/- 3.0	0.1
CRL-5915	Trypan blue positive (%)	2.4 +/- 0.0	2.8 +/- 0.5	1.0
	Cell concentration (10^6cells/μL)	1.6 +/- 0.3	1.6 +/- 0.2	1.0
	Annexin V positive (%)	0.5 +/- 0.1	0.3 +/- 0.1	0.2
	Viable cells (%)	75.2 +/- 0.4	82.3 +/- 2.3	0.1
CRL-5820	Trypan blue positive (%)	3.9 +/- 2.7	4.4 +/- 1.5	1.0
	Cell concentration (10^6cells/μL)	0.6 +/- 0.1	0.8 +/- 0.2	0.4
	Annexin V positive (%)	9.0 +/- 2.8	7.9 +/- 0.2	1.0
	Viable cells (%)	58.3 +/- 5.3	69.7 +/- 5.5	0.1

4.2.2 Experiment 2

4.2.2.1 Methods

Separate flasks containing cultures of the benign mesothelial cell line, MeT5A and the MPM cell line, JU77, were incubated in an environmental O_2 concentration of 0.1% for a total of 96 hours. At 24 hour intervals a flask of each cell line was processed for analysis. Corresponding flasks were also cultured in normoxic conditions and repeated on 3 separate occasions. The O_2 concentration was reduced and the 24 hour time-points were chosen in an attempt to extract the most extensive and comprehensive data possible, given the apparent lack of effect seen in experiment 1. Furthermore, the 3 additional MPM cell lines were incubated for 96 hours at 0.1% O_2, with corresponding normoxic flasks and repeated on 2 separate occasions.

A potential mechanism of survival in hostile conditions is a reduction in cellular metabolic activity. As an indirect measure of this, in the MeT5A and JU77 cell lines the culture medium pH was recorded at each time point as well as at the beginning of the experiment.

4.2.2.2 Results

As in the previous experiment at 0.5% O_2 over a 24 hour period, there were no significant differences between the measured parameters at any of the time-points (tables 4.2, 4.3, 4.4 and 4.5; figure 4.1). A marked drop in pH in both cell lines measured was observed throughout the course of the experiment, but was always greater in the flask medium from cells cultured in a hypoxic environment. This was most marked over the first 24 hour period (figures 4.2a and 4.2b).

Table 4.2 Experiment 2 - Comparison of the effect of environmental O_2 concentration of 0.1% over a 24 hour period on a variety of measures of cell viability, compared with normoxic conditions. Means were ranked and compared with the Mann-Whitney U test (n=3, for each environmental condition for each cell line).

Cell line		Experimental Conditions		p value
		0.1% O_2 for 24 hours (Mean +/- SEM)	20.9% O_2 for 24 hours (Mean +/- SEM)	
MeT5A	Trypan blue positive (%)	7.5 +/- 1.0	7.8 +/- 0.5	0.8
	Cell concentration (10^6cells/μL)	1.0 +/- 0.8	0.7 +/- 0.4	0.8
	Annexin V positive (%)	7.6 +/- 2.2	11.6 +/- 3.7	0.4
	Viable cells (%)	70.1 +/- 4.7	62.0 +/- 3.0	0.3
JU77	Trypan blue positive (%)	4.2 +/- 2.3	2.1 +/- 2.1	0.5
	Cell concentration (10^6cells/μL)	1.7 +/- 1.4	1.4 +/- 1.3	0.4
	Annexin V positive (%)	13.3 +/- 5.0	10.9 +/- 0.3	0.5
	Viable cells (%)	71.6 +/- 4.5	74.2 +/- 3.8	0.3

Table 4.3 Experiment 2 - Comparison of the effect of environmental O_2 concentration of 0.1% over a 48 hour period on a variety of measures of cell viability, compared with normoxic conditions. Means were ranked and compared with the Mann-Whitney U test (n=3, for each environmental condition for each cell line).

Cell line		Experimental Conditions		p value
		0.1% O_2 for 48 hours (Mean +/- SEM)	20.9% O_2 for 48 hours (Mean +/- SEM)	
MeT5A	Trypan blue positive (%)	5.9 +/- 2.5	5.0 +/- 3.6	0.8
	Cell concentration (10^6cells/μL)	1.2 +/- 0.8	1.3 +/- 0.6	0.8
	Annexin V positive (%)	8.2 +/- 1.6	8.9 +/- 2.0	0.8
	Viable cells (%)	73.6 +/- 3.1	68.8 +/- 4.7	0.3
JU77	Trypan blue positive (%)	4.7 +/- 0.4	3.8 +/- 2.3	0.5
	Cell concentration (10^6cells/μL)	2.6 +/- 2.2	1.7 +/- 1.5	0.3
	Annexin V positive (%)	8.0 +/- 1.5	11.6 +/- 2.9	0.8
	Viable cells (%)	73.1 +/- 3.5	73.6 +/- 6.0	0.8

Table 4.4 Experiment 2 - Comparison of the effect of environmental O_2 concentration of 0.1% over a 72 hour period on a variety of measures of cell viability, compared with normoxic conditions. Means were ranked and compared with the Mann-Whitney U test (n=3, for each environmental condition for each cell line).

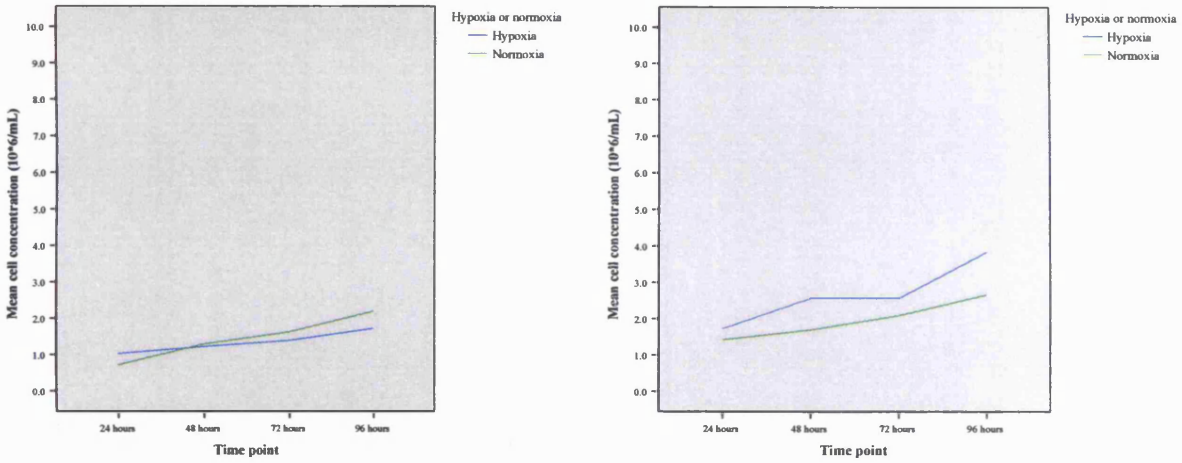
Cell line		Experimental Conditions		p value
		0.1% O_2 for 72 hours (Mean +/- SEM)	20.9% O_2 for 72 hours (Mean +/- SEM)	
MeT5A	Trypan blue positive (%)	8.0 +/- 1.2	6.1 +/- 2.1	0.3
	Cell concentration (10^6cells/μL)	1.4 +/- 0.8	1.6 +/- 0.7	0.5
	Annexin V positive (%)	4.9 +/- 1.0	6.5 +/- 1.3	0.3
	Viable cells (%)	75.3 +/- 9.2	78.7 +/- 1.1	0.5
JU77	Trypan blue positive (%)	4.7 +/- 0.9	4.2 +/- 0.9	0.8
	Cell concentration (10^6cells/μL)	2.6 +/- 1.8	2.1 +/- 1.4	0.5
	Annexin V positive (%)	8.0 +/- 4.2	6.5 +/- 2.8	0.8
	Viable cells (%)	73.0 +/- 2.9	78.9 +/- 1.3	0.1

Table 4.5 Experiment 2 - Comparison of the effect of environmental O_2 concentration of 0.1% over a 96 hour period on a variety of measures of cell viability, compared with normoxic conditions. Means were ranked and compared with the Mann-Whitney U test (n=3 for MeT5A and JU77 cell lines and n=2 for ONE58, CRL-5915 and CRL-5820 cell lines, for each environmental condition for each cell line).

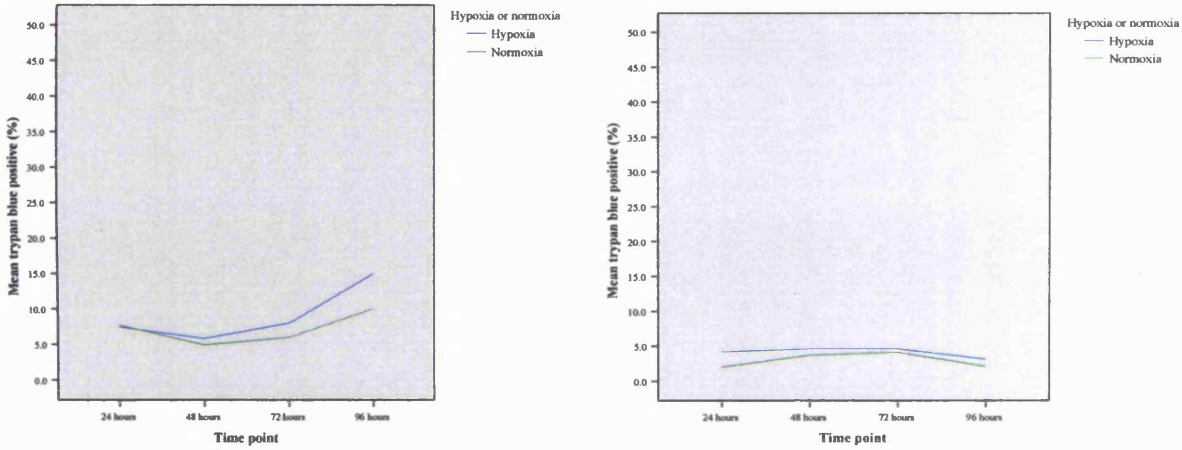
Cell line		Experimental Conditions		p value
		0.1% O_2 for 96 hours (Mean +/- SEM)	20.9% O_2 for 96 hours (Mean +/- SEM)	
MeT5A	Trypan blue positive (%)	15.0 +/- 3.0	10.1 +/- 1.7	0.3
	Cell concentration (10^6cells/μL)	1.7 +/- 0.8	2.2 +/- 1.0	0.5
	Annexin V positive (%)	6.0 +/- 3.2	7.8 +/- 4.8	0.8
	Viable cells (%)	67.6 +/- 4.2	74.0 +/- 5.6	0.5
JU77	Trypan blue positive (%)	3.2 +/- 1.2	2.2 +/- 0.7	0.3
	Cell concentration (10^6cells/μL)	3.8 +/- 1.8	2.7 +/- 1.0	0.6
	Annexin V positive (%)	8.0 +/- 4.9	6.7 +/- 7.6	0.5
	Viable cells (%)	74.0 +/- 6.2	79.7 +/- 3.9	0.8
ONE58	Trypan blue positive (%)	6.7 +/- 2.3	7.9 +/- 4.1	1.0
	Cell concentration (10^6cells/μL)	0.9 +/- 0.0	1.4 +/- 0.4	0.1
	Annexin V positive (%)	5.1 +/- 0.6	7.0 +/- 1.3	0.2
	Viable cells (%)	65.5 +/- 0.7	66.0 +/- 9.8	1.0
CRL-5915	Trypan blue positive (%)	3.6 +/- 1.4	3.9 +/- 1.1	0.7
	Cell concentration (10^6cells/μL)	0.7 +/- 0.2	0.9 +/- 0.5	1.0
	Annexin V positive (%)	1.6 +/- 0.7	1.3 +/- 0.4	0.7
	Viable cells (%)	72.2 +/- 3.4	78.1 +/- 3.6	0.4
CRL-5820	Trypan blue positive (%)	4.2 +/- 1.2	4.8 +/- 0.7	0.4
	Cell concentration (10^6cells/μL)	0.9 +/- 0.3	0.8 +/- 0.3	0.4
	Annexin V positive (%)	11.7 +/- 5.0	15.5 +/- 8.0	0.4
	Viable cells (%)	49.6 +/- 4.2	54.8 +/- 10.8	1.0

Figure 4.1 Trends of the effects of up to 96 hours of 0.1% O_2 compared with normoxic conditions in a benign mesothelial (MeT5A, left column) and MPM (JU77, right column) cell line. A – mean cell concentration (x10^6), B – mean trypan blue positive (%), C – mean annexin V positive (%), D – mean viable cells (%).

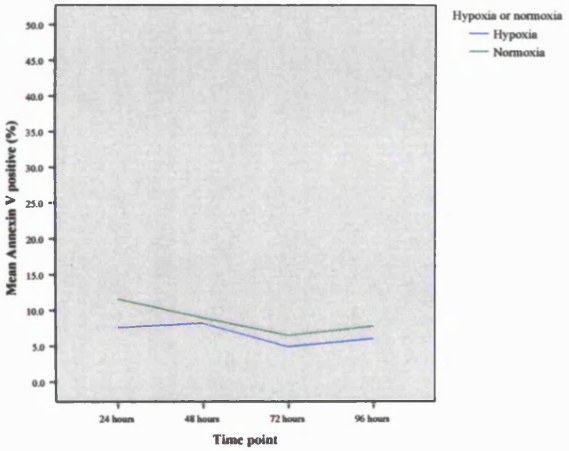
A

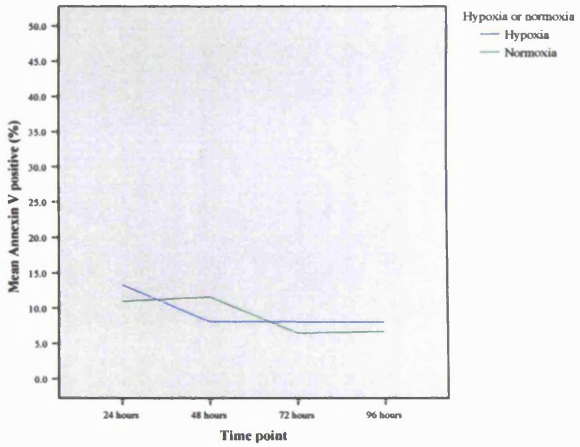


B

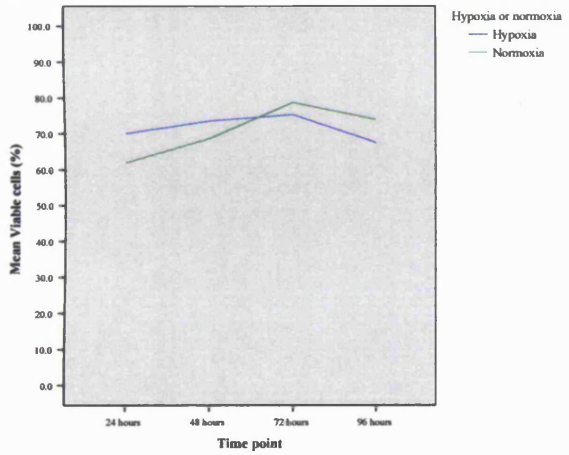


C


Hypoxia or normoxia
Hypoxia
Normoxia
Mean Annexin V positive (%)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
24 hours
48 hours
72 hours
96 hours
Time point


Hypoxia or normoxia
Hypoxia
Normoxia
Mean Annexin V positive (%)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
24 hours
48 hours
72 hours
96 hours
Time point

D


Hypoxia or normoxia
Hypoxia
Normoxia
Mean Viable cells (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
24 hours
48 hours
72 hours
96 hours
Time point

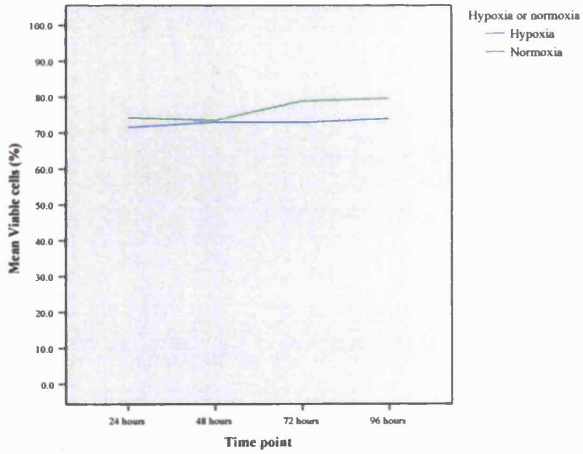

Hypoxia or normoxia
Hypoxia
Normoxia
Mean Viable cells (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
24 hours
48 hours
72 hours
96 hours
Time point

Figure 4.2a Plot demonstrating the change seen in culture medium pH for the MeT5A cell line measured at each 24 hour time-point throughout the experiment and also at the beginning (values expressed are means of 2 experiments).

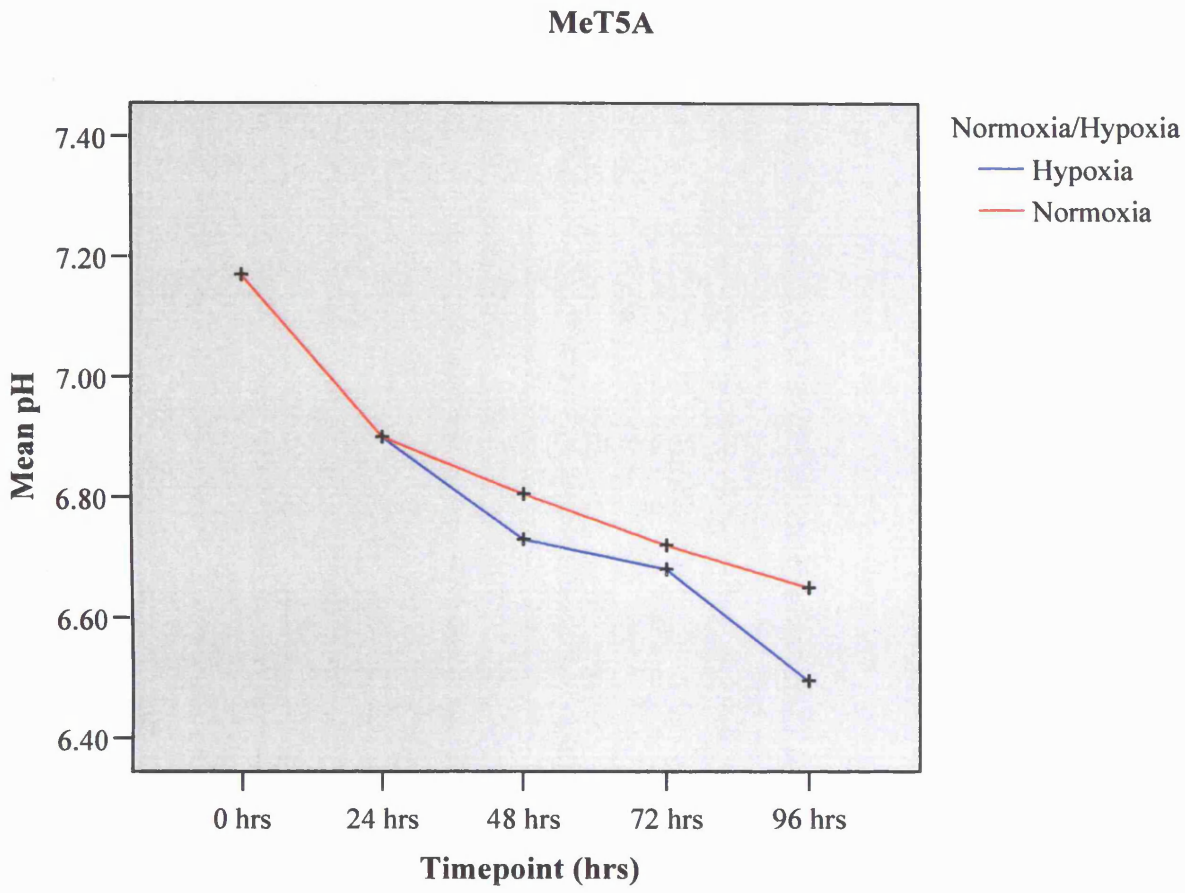
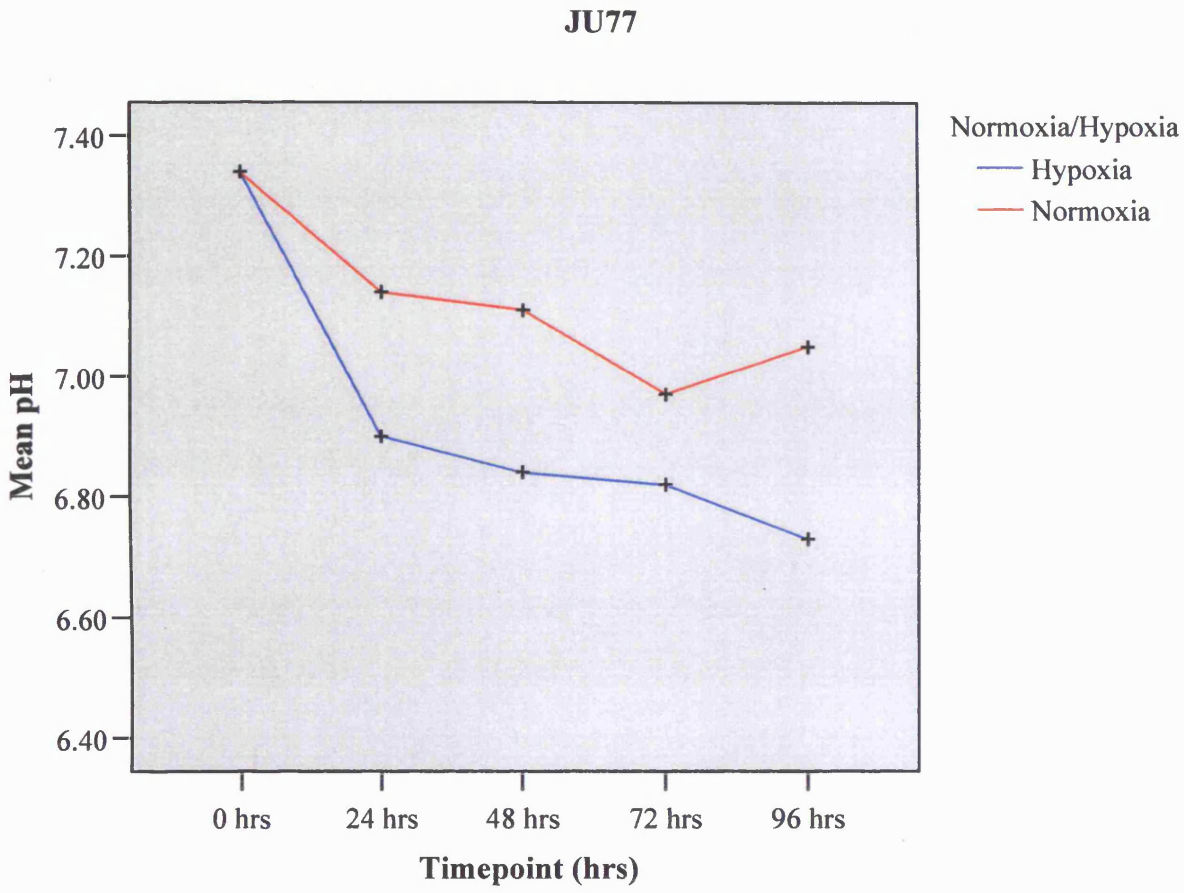


Figure 4.2b Plot demonstrating the change seen in culture medium pH for the JU77 cell line measured at each 24 hour time-point throughout the experiment and also at the beginning (values expressed are means of 2 experiments).



4.2.3 Western Blotting

4.2.3.1 Methods

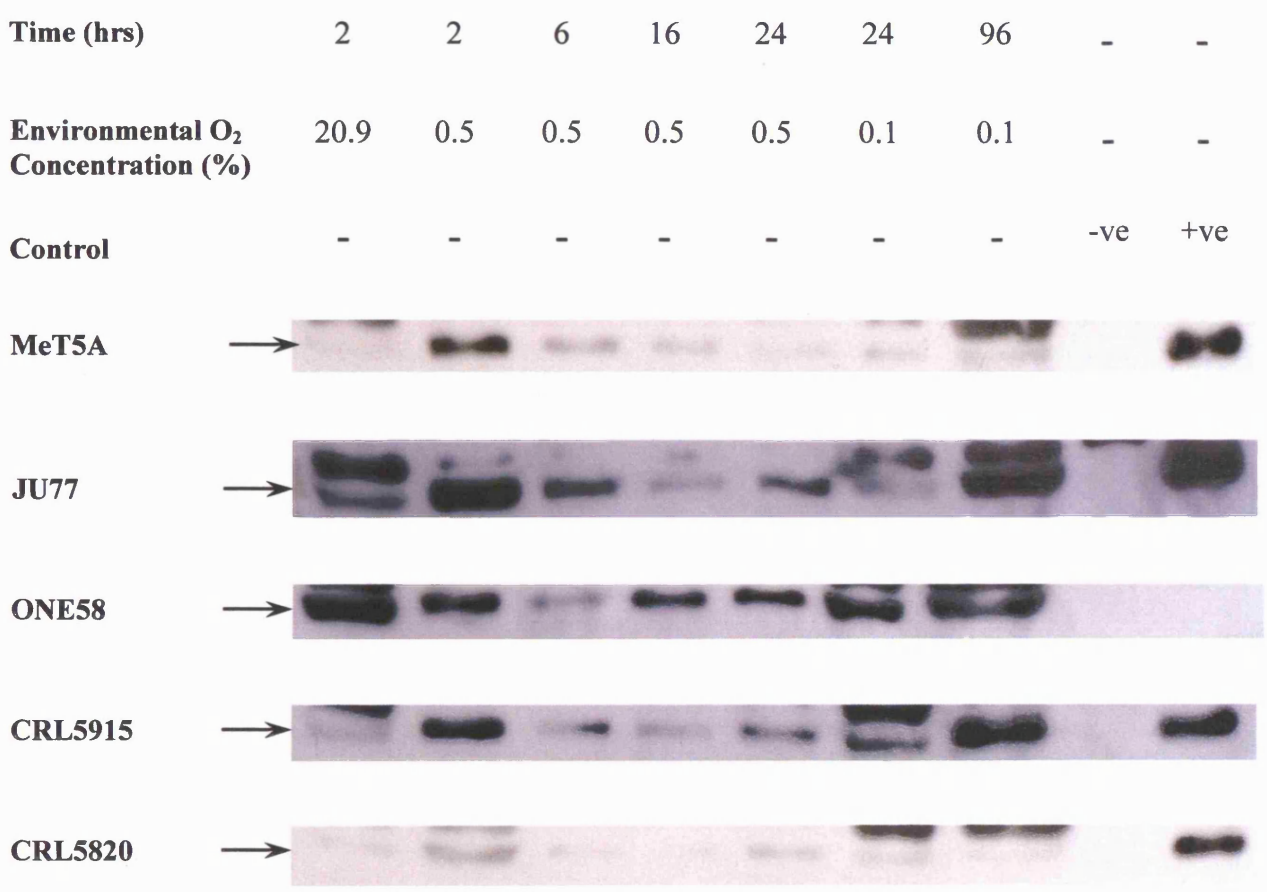
Although there were minimal differences seen between cell cultures exposed to hypoxic conditions and analysed by flow cytometry, it appeared reasonable that the up-regulation of pAkt or HIF-1α was possible over shorter time periods of exposure. For this reason, and to produce a time-course, cell cultures were exposed to either 0.5% O_2 for time-points at 2, 6, 16 and 24 hours. Additional cultures were exposed to 0.1% O_2 for 24 and 96 hours. Cells were processed and lysates created using the previously described methods, but without the addition of phosphatase inhibitors.

In addition to cell line lysates, control samples were added to gels for SDS-PAGE. For pAkt immunoblotting a whole cell lysate produced from a mouse embryo fibroblast cell line (NIH/3T3) stimulated with PDGF (sc-3803), was employed. As a negative control, the un-stimulated version (sc-2210) was used. Both were purchased from Santa Cruz Biotechnology, Inc. (USA). For HIF-1α immunoblotting, the human ductal breast carcinoma cell line T47D was used (kindly donated by Dr Bernard Burke, University of Leicester, Leicester, UK). This has been shown previously to express HIF-1α following exposure to hypoxic conditions (Ameri et al. 2002). Positive control lysates were created following exposure to 0.1% O_2 for 16 hours, with corresponding negative controls kept at 20.9% O_2. These controls were used for all further experiments.

4.2.3.2 The effect of varying the environmental O_2 concentration over a variable time-period on the expression of pAkt

Variable expression of pAkt was seen throughout the different cell lines (figure 4.3). In the benign mesothelial cell line, MeT5A, minimal phosphorylation of Akt was observed at 20.9% O_2. This was in stark contrast to that seen in the ONE58 cell line, and to a lesser degree in the JU77 and CRL5915 cell lines. All of the cell lines investigated then showed up-regulation of pAkt expression following exposure to 0.5% O_2 for 2 hours. From this time-point onwards, the MeT5A and CRL5820 cell lines showed poor expression of pAkt, regardless of the length of exposure (6-96 hours) or the degree of hypoxia (0.5-0.1% O_2). In the JU77, ONE58 and CRL5915 cell lines a consistent finding was the up-regulation of pAkt expression at the 96 hour time-point following exposure to 0.1% O_2.

Figure 4.3 The variable expression of phosphorylated-Akt in a benign mesothelial and malignant pleural mesothelioma cell lines. Cells were exposed to conditions of varying environmental O_2 concentration over varying periods of time, lysed and proteins separated by SDS-PAGE. After transfer of proteins, the PVDF membranes were immunoblotted with anti-pAkt (Ser^{473}) antibody. Bands were visualised by chemiluminescence. Representative blots are shown of experiments repeated in duplicate. The arrow indicates the lower bands on each of the blots are those of interest.



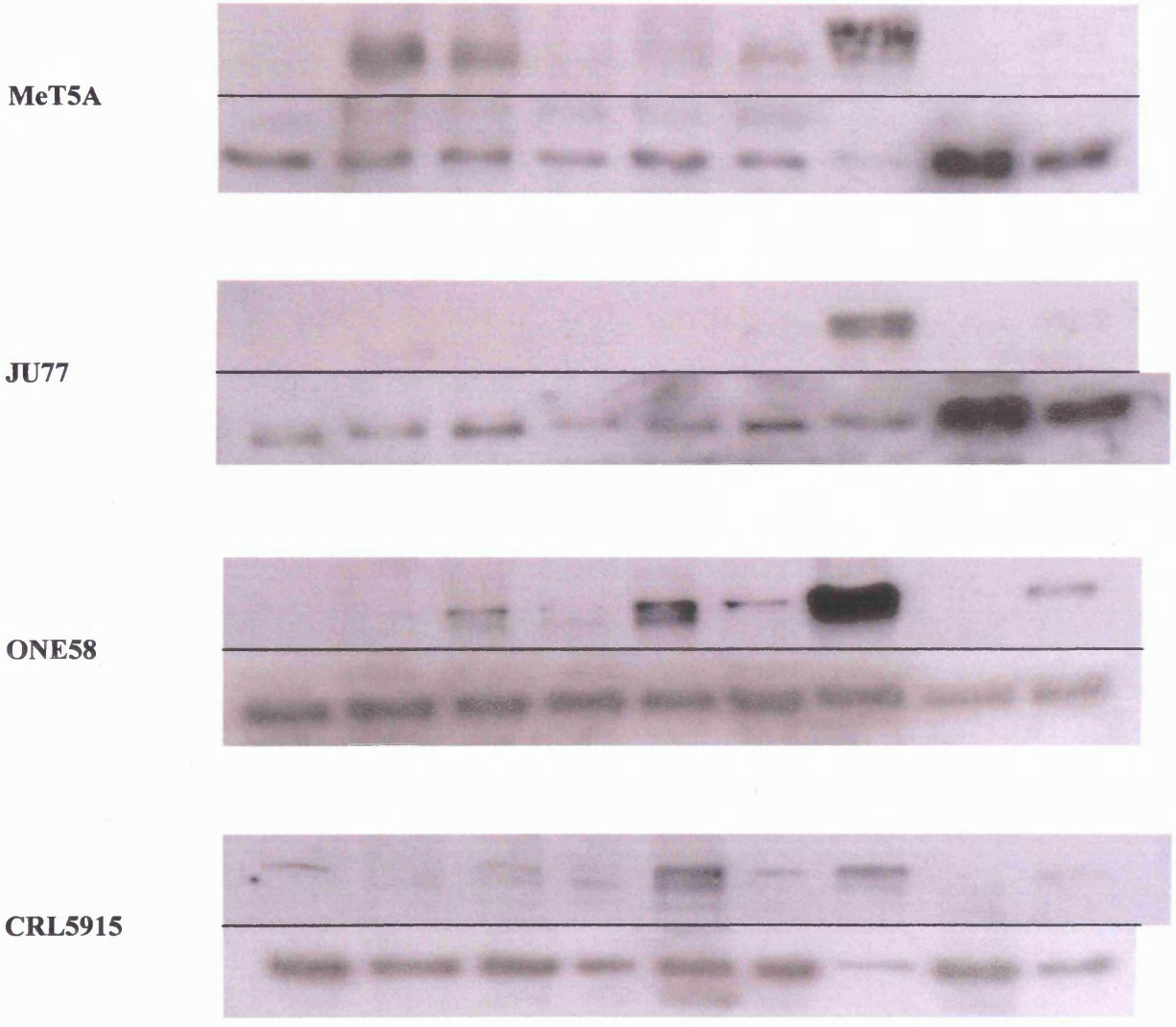
4.2.3.3 The effect of varying the environmental O_2 concentration over a variable time-period on the expression of HIF-1α

HIF-1α expression in the 4 cell lines examined is shown in figure 4.4. Aside from that seen in the malignant cell line, CRL5915, there was no expression noted in any of the remaining benign or malignant cell lines when cultured at 20.9% O_2. Expression was then seen in all cell lines with most notable up-regulation once again at 96 hours exposure to 0.1% O_2. This can most likely be regarded as a true increase in expression when the corresponding α-Tubulin probed blots are considered, showing either equivalent or reduced total protein levels loaded onto those lanes.

Closer inspection of the blots shown in figure 4.4 revealed two very closely associated bands at around the 120 kDa level, visible very easily in the ONE58 and CRL5915 cell line blots. Furthermore, variable expression of these two bands is clearly visible which are likely to represent either the phosphorylated (higher band) or the de-phosphorylated (lower band) forms of HIF-1α (Suzuki, Tomida, & Tsuruo 2001). The phosphorylated form appears to be the most regularly expressed in the ONE58 and CRL5915 cell lines until exposure to 0.1% O_2 for 96 hours, when the de-phosphorylated form becomes equally prominent. At least, this is the case for the MeT5A, JU77 and ONE58 cell lines.

Figure 4.4 The variable expression of HIF-1α in a benign mesothelial and malignant pleural mesothelioma cell lines. Cells were exposed to conditions of varying environmental O_2 concentration over varying periods of time, lysed and proteins separated by SDS-PAGE. After transfer of proteins, the PVDF membranes were immunoblotted with anti-HIF-1α antibody (shown in the upper of each pair of blots). Bands were visualised by chemiluminescence. Blots were then stripped and re-probed for α-Tubulin (shown in the lower of each pair of blots). Representative blots are shown of experiments repeated in duplicate.

Time (hrs)	2	2	6	16	24	24	96	-	-
Environmental O_2 Concentration	20.9	0.5	0.5	0.5	0.5	0.1	0.1	-	-
Control	-	-	-	-	-	-	-	-ve	+ve



4.3 The effect of hydrogen peroxide as a stimulus to the induction of apoptosis

From initial experiments described above, there was an apparent lack of impact of using a reduction in environmental O_2 concentration as the primary stimulus to apoptosis, independent of the time-period of exposure. Therefore, hydrogen peroxide (H_2O_2) was investigated as an additional stimulus. The following experiments were conceived to obtain information on not only the concentration of H_2O_2 required to produce apoptosis in benign and malignant cell lines, but also to examine the effects of varying the exposure time-period.

4.3.1 Experiment 3

4.3.1.1 Methods

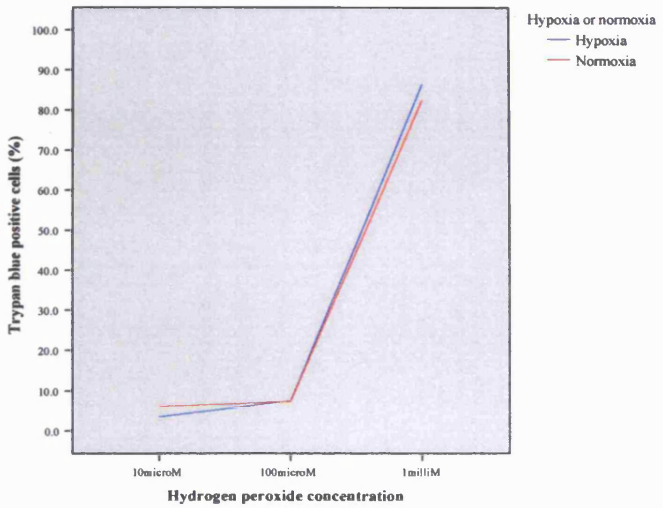
Flasks containing approximately 75% confluent cultures of the MeT5A cell line were prepared as previously described. Following the addition of fresh medium, the appropriate volumes of 1M H_2O_2 were added to achieve working concentrations of 10μM, 100μM and 1mM. Flasks were then exposed to environmental O_2 concentration of 0.5% for 24 hours, with reciprocal flasks kept at 20.9% O_2 for comparison.

4.3.1.2 Results

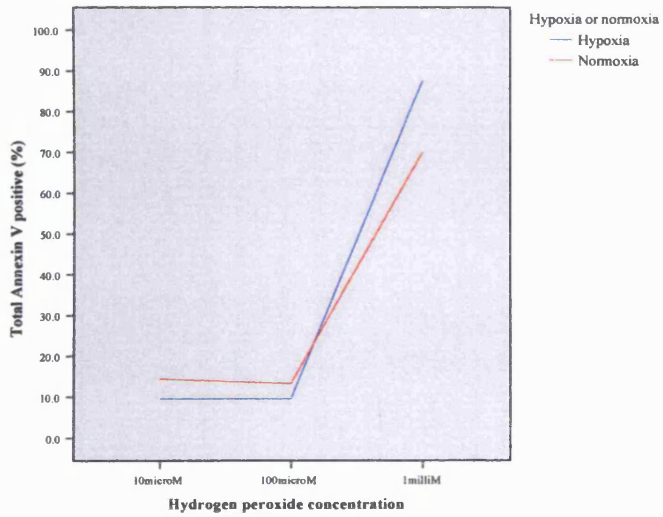
A notable decrease in cell viability was seen with the addition of 1mM H_2O_2, but there remained very little difference at 10μM and 100μM. In addition, there appeared to be only minimal differences between hypoxic and normoxic environmental conditions (figure 4.5).

Figure 4.5 Exposure of MeT5A cell cultures to varying concentrations of H_2O_2, in conditions of reduced environmental O_2 concentration and normoxia over a 24 hour period. A – Trypan blue positive cells (%), B – total annexin V positive cells (%), C – viable cells (%).

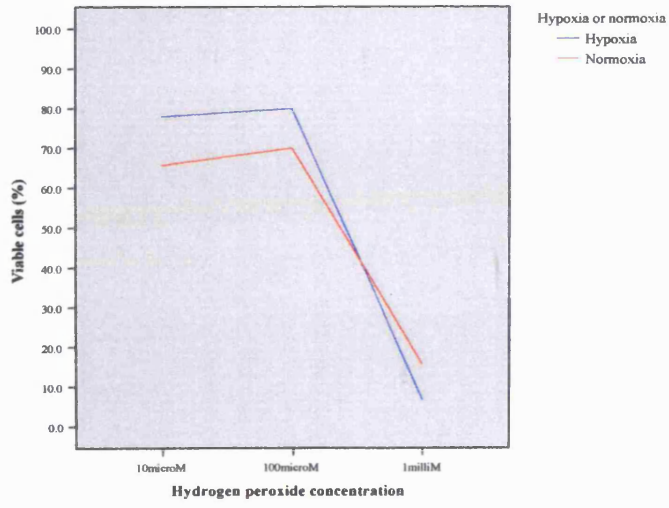
A



B



C



4.3.2 Experiment 4

4.3.2.1 Methods

Flasks containing approximately 75% confluent cultures of the JU77 cell line were prepared as previously described. Following the addition of fresh medium, the appropriate volumes of 1M H_2O_2 were added to achieve working concentrations of 250μM, 500μM and 750μM. Flasks were then exposed to environmental O_2 concentration of 0.5% for 24 hours, with reciprocal flasks kept at 20.9% O_2 for comparison.

4.3.2.2 Results

As with the benign mesothelial MeT5A cell line, an increase in the numbers of apoptotic cells with a corresponding decrease in numbers of viable cells were seen with increasing concentrations of H_2O_2 in an approximately linear relationship, in the JU77 cell line (figure 4.6). With higher numbers of Annexin V positive cells and fewer viable cells, there is a suggestion that the JU77 cultures may be more prone to the apoptotic stimulus in conditions of reduced environmental O_2 concentration.

4.3.3 Experiment 5

4.3.3.1 Methods

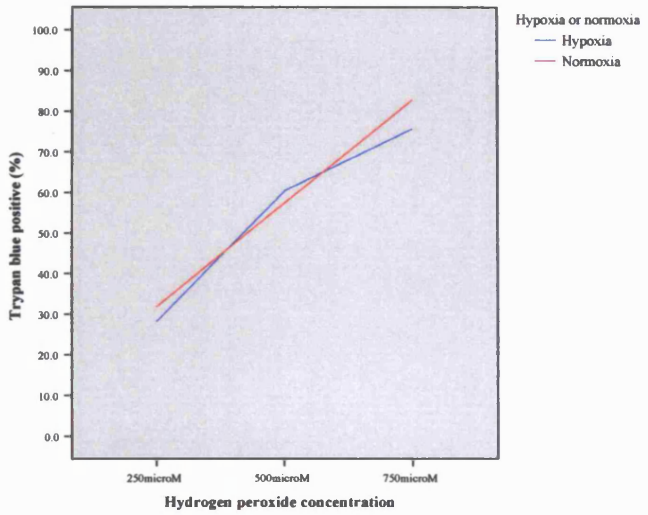
Flasks containing approximately 75% confluent cultures of the JU77 cell line were prepared as previously described. Following the addition of fresh medium, the appropriate volume of 1M H_2O_2 were added to achieve a working concentration of 250μM. Flasks were then exposed to environmental O_2 concentration of 0.5% for 2, 4 and 6 hours, with reciprocal flasks kept at 20.9% O_2 for comparison.

4.3.3.2 Results

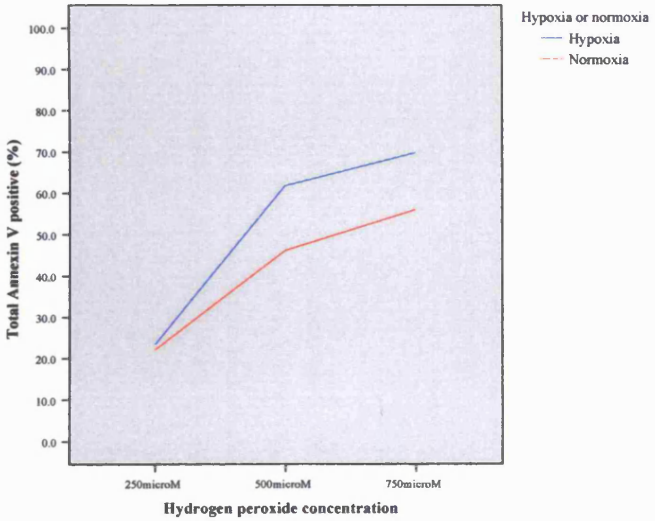
Exposure of cultures of the malignant JU77 cell line had minimal effects on the rates of apoptosis as measured by trypan blue dye or Annexin V uptake (figure 4.7). Even with 6 hours exposure the values did not reach those seen after 24 hours of exposure. No apparent differences were observed between conditions of hypoxia and normoxia.

Figure 4.6 Exposure of JU77 cell cultures to varying concentrations of H_2O_2, in conditions of reduced environmental O_2 concentration and normoxia over a 24 hour period. A – Trypan blue positive cells (%), B – total annexin V positive cells (%), C – viable cells (%).

A



B



C

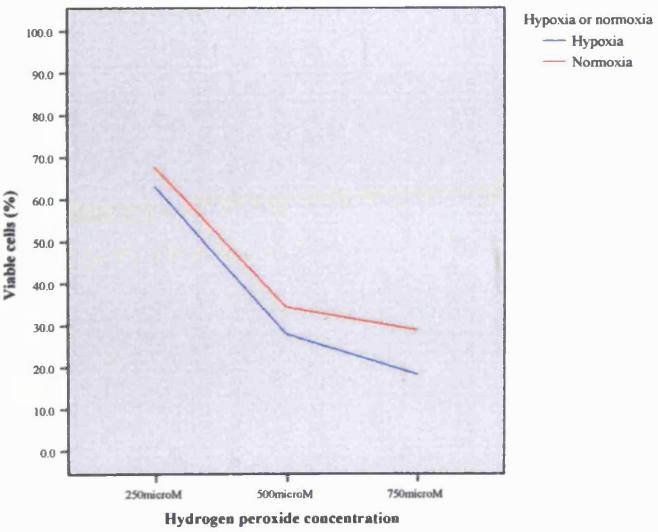
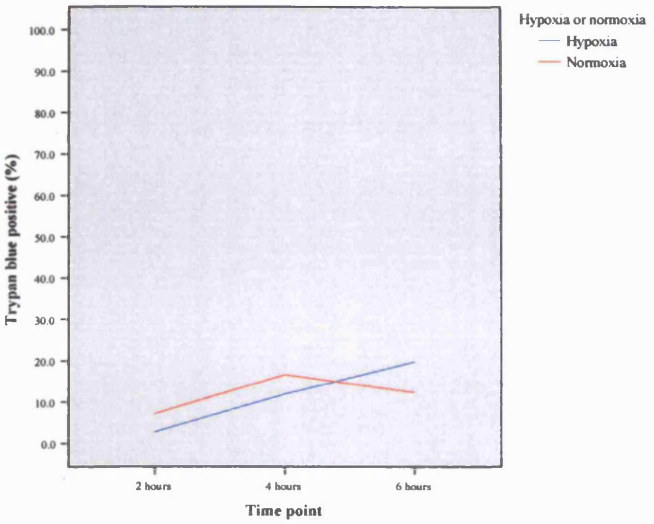
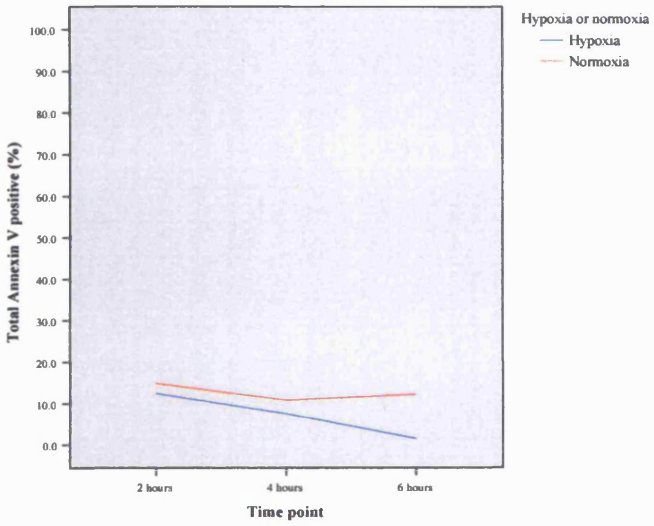


Figure 4.7 Exposure of JU77 cell cultures to 250μM H_2O_2, in conditions of reduced environmental O_2 concentration and normoxia over 2, 4 and 6 hour time-periods. A – Trypan blue positive cells (%), B – total annexin V positive cells (%), C – viable cells (%).

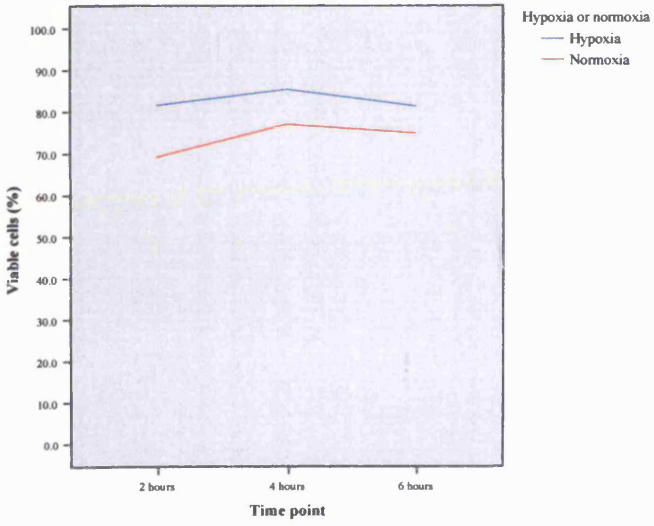
A



B



C



4.3.4 Experiment 6

4.3.4.1 Methods

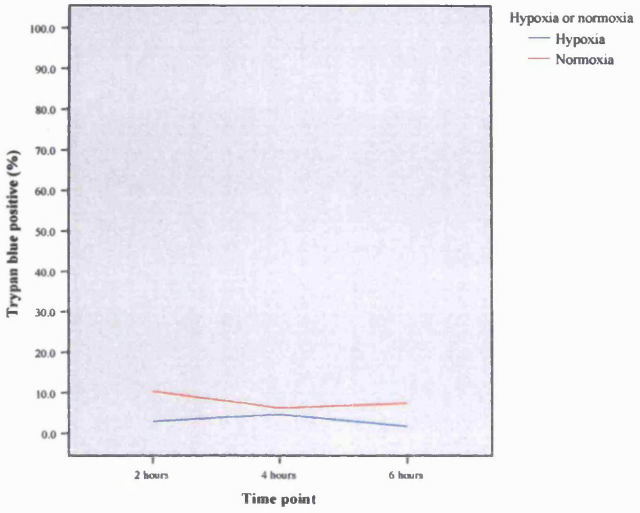
Flasks containing approximately 75% confluent cultures of the JU77 cell line were prepared as previously described. Following the addition of fresh medium, the appropriate volumes of 1M H_2O_2 were added to achieve working concentrations of 500μM. Flasks were then exposed to environmental O_2 concentration of 0.5% for 2, 4 and 6 hours, with reciprocal flasks kept at 20.9% O_2 for comparison.

4.3.4.2 Results

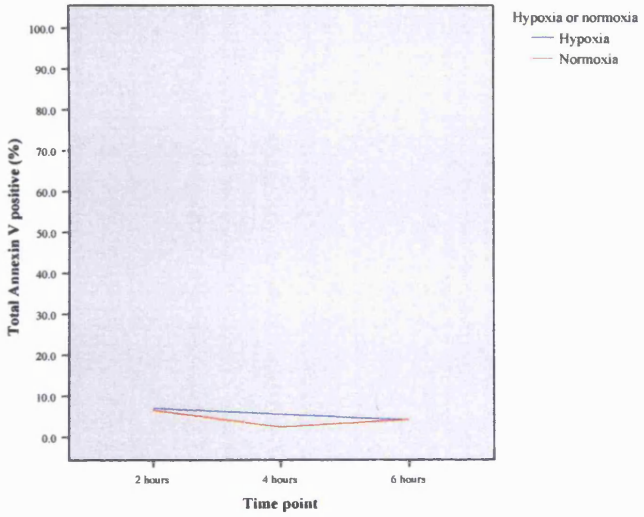
In a similar fashion as to the previous experiment, exposure of cultures of the JU77 cell line to 500μM H_2O_2 for 2, 4 or 6 hours had very minimal effects on cell survival in terms of a stimulus to the induction of apoptosis (figure 4.8). Once again, no differences were seen between cultures placed in conditions of reduced environmental O_2 concentration, with those kept at atmospheric levels.

Figure 4.8 Exposure of JU77 cell cultures to 500μM H_2O_2, in conditions of reduced environmental O_2 concentration and normoxia over 2, 4 and 6 hour time-periods. A – Trypan blue positive cells (%), B – total annexin V positive cells (%), C – viable cells (%).

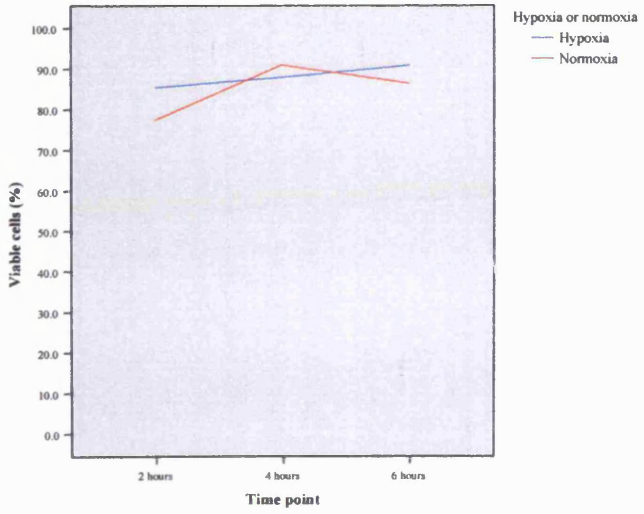
A



B



C



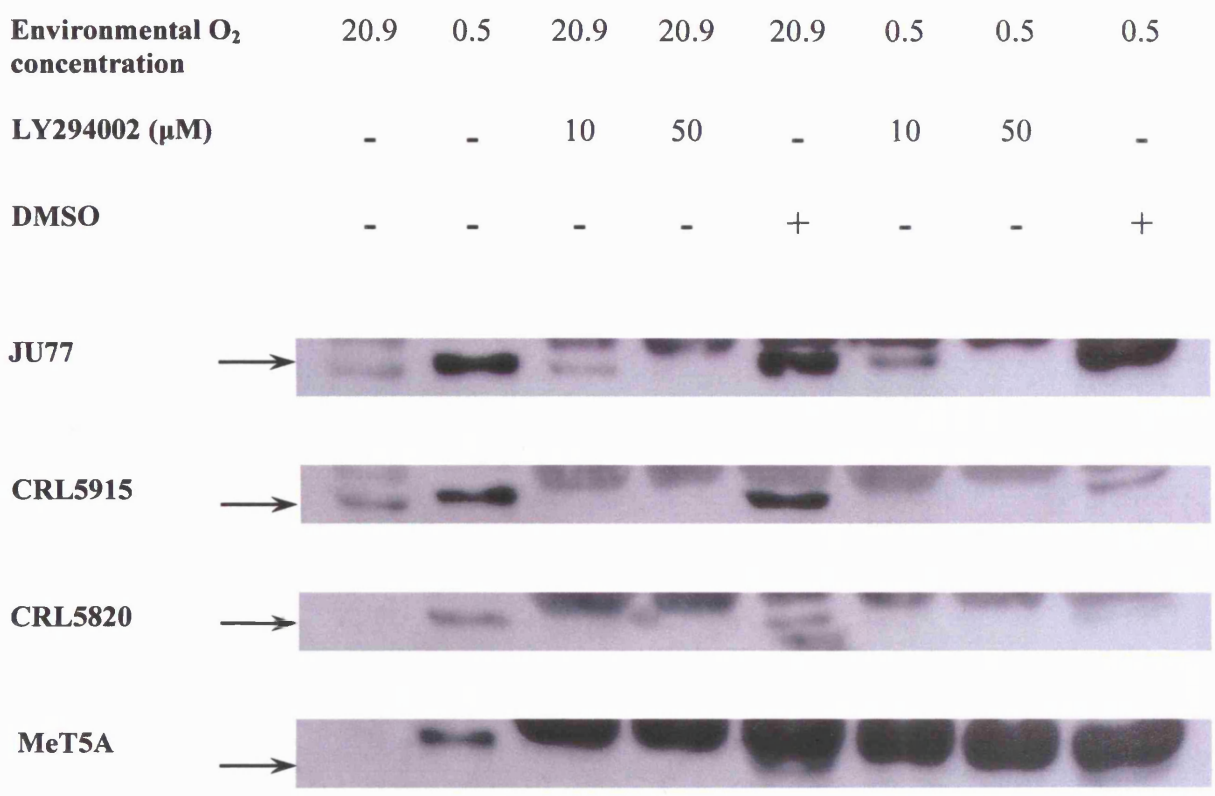
4.4 The effect of the PI3K/pAkt inhibitor LY294002 on cell viability over a varying time-course

4.4.1 Concentration course

The aim of the addition of LY294002 to cell cultures was to inhibit the phosphorylation of Akt as completely as possible, allowing the effect of this on cell survival to be assessed. LY294002, the flavinoid-derived small molecule inhibitor of PI3-K has been widely used in cell signalling research at a variety of working concentrations. Davies *et al* showed that LY294002 at a concentration of 10μM was required to achieve 50% inhibition (the IC_{50}) of PI3K, with exposure over a time-period of 10-40 minutes (Davies et al. 2000). In keeping with this, *in vitro* cell line work detailing the effects of hypoxia on the human pancreatic cancer cell line L3.6pl, demonstrated the presumed partial inhibition of PI3-K activity with 2.5μM and complete inhibition of activity with 25μM, through the partial and complete inhibition of expression of pAkt in cell lysates on western immunoblotting (Yokoi & Fidler 2004). However, concentrations of LY294002 up to 50μM have been used by different groups of researchers (Blancher et al. 2001;Kaluz et al. 2002;Zhong et al.2000).

A concentration course was undertaken with cells exposed to 10μM and 50μM LY294002 for a period of 2 hours with the environmental O_2 concentration at either 0.5% or 20.9%. Although 10μM LY294002 did lead to the apparent complete inhibition of the phosphorylation of Akt in 3 of the four cell lines examined, it took 50μM in the JU77 cell line (figure 4.9). This was therefore used as the final concentration.

Figure 4.9 Concentration course of the inhibitory action of LY294002 on the phosphorylation of Akt. Flasks of cell cultures were treated with either 10μM or 50μm of LY294002 for 2 hours in conditions 0.5% or 20.9% O_2. Lysates were then produced in the standard way, with protein separation by SDS-PAGE and, following protein transfer to PVDF membranes, band visualisation by chemiluminescence. The arrow indicates the lower bands on each of the blots are those of interest.



4.4.2 Experiment 7

4.4.2.1 Methods

Separate flasks containing cultures of all cell lines were prepared according to described methods. Following the addition of fresh culture medium, the appropriate volume of 10mM LY294002 was added to obtain a final concentration of 50μM. Flasks were then incubated at an environmental O_2 concentration of 0.5%, with corresponding flasks kept at 20.9% O_2, for a period of 2 hours, as these conditions had previously been shown to lead to a complete inhibition of Akt phosphorylation. This was repeated on 2 occasions for the MeT5A, ONE58, CRL5915 and CRL5820 cell lines because of time constraints. For the JU77 cell line, in addition to those flasks containing the LY294002, appropriate controls were also run on at least 3 separate occasions.

To allow a more thorough analysis, data collected from the JU77 cell line has been considered and analysed independently from that obtained from the remaining 4 cell lines.

4.4.2.2 Results

For the benign mesothelial cell line (MeT5A) and 3 of the MPM cell lines (ONE58, CRL5915 and CRL5820), the addition of LY294002 had no significant impact on the measured parameters of cell viability regardless of exposure to reduced environmental O_2 concentration or not, although there was a trend to reduced cell viability measured by flow cytometry (table 4.6).

In apparent contradiction to the previous results, the addition of 50μM LY294002 did appear to make cultures of the J77 cell line less robust and more susceptible to the apoptosis-inducing effects of hypoxia (table 4.7, figures 4.10, 4.11 and 4.12). Although there were no differences seen with the use of trypan blue dye or when considering cell concentration itself, significantly more cells stained positively for Annexin V when exposed to LY294002, than when cultured in 0.5% O_2 alone (22.2% versus 8.7%, p=0.002, Tukey's post-hoc test). In addition, when the proportion of viable cells were considered between the same two groups there were significantly fewer viable cells seen in the LY294002-exposed group (69.1% versus 87.5%, p=0.003, Tukey's post-hoc test). It is worthy of note that although a greater mean percentage of Annexin V positive cells were seen in the LY294002-

treated group kept in 20.9% O_2, there was no significant difference between this and those flasks cultured in 20.9% O_2 without the addition of LY294002 (16.4% versus 9.5%, p=0.16). Similarly, although the addition of DMSO alone did result in a greater percentage of Annexin V positive cells and fewer viable cells when compared with flasks in normoxic and hypoxic conditions alone, the differences did not reach statistical significance (11.4% versus 9.5%, p=0.98 and 86.5% versus 85.7%, p=1.00 respectively for normoxia; 16.6% versus 8.7%, p=0.19 and 73.5% versus 87.5%, p=0.07 respectively for hypoxia).

Table 4.6 Effect of 50μM LY294002 on cell viability in one benign mesothelial (MeT5A) and 3 MPM (ONE58, CRL5915 and CRL5820) cell lines. Culture flasks were exposed to conditions of reduced environmental O_2 concentration for 2 hours, with reciprocal flasks in normoxic conditions. Means were ranked and compared with the Mann-Whitney U test (n=2, for each environmental condition for each cell line).

Cell line		Experimental Conditions		p value
		0.5% O_2 for 2 hours + 50 μM LY294002 (Mean +/- SEM)	20.9% O_2 for 2 hours + 50 μM LY294002 (Mean +/- SEM)	
MeT5A	Trypan blue positive (%)	3.6 +/- 1.0	2.3 +/- 1.1	0.4
	Cell concentration (10^6cells/μL)	1.3 +/- 0.0	1.2 +/- 0.1	0.3
	Annexin V positive (%)	39.0 +/- 18.3	31.5 +/- 1.6	1.0
	Viable cells (%)	53.9 +/- 13.2	65.1 +/- 0.8	0.1
ONE58	Trypan blue positive (%)	3.1 +/- 0.8	0.6 +/- 0.6	0.1
	Cell concentration (10^6cells/μL)	1.3 +/- 0.2	1.5 +/- 0.1	0.2
	Annexin V positive (%)	28.6 +/- 4.7	28.8 +/- 11.1	1.0
	Viable cells (%)	54.3 +/- 3.9	67.4 +/- 11.9	0.1
CRL-5915	Trypan blue positive (%)	3.9 +/- 2.8	0	0.1
	Cell concentration (10^6cells/μL)	0.9 +/- 0.1	0.8 +/- 0.1	0.7
	Annexin V positive (%)	2.9 +/- 0.3	6.6 +/- 3.9	0.1
	Viable cells (%)	77.6 +/- 3.0	88.3 +/- 4.3	0.1
CRL-5820	Trypan blue positive (%)	7.6 +/- 3.3	3.5 +/- 0.1	0.1
	Cell concentration (10^6cells/μL)	0.9 +/- 0.3	0.9 +/- 0.1	1.0
	Annexin V positive (%)	16.4 +/- 0.6	7.9 +/- 4.4	0.1
	Viable cells (%)	61.9 +/- 23.2	81.1 +/- 6.8	0.4

Table 4.7 Effect of 50μM LY294002 on various measures of cell viability in the JU77 cell line, in conditions of reduced environmental O_2 concentration or atmospheric concentration (20.9% O_2). Flasks containing solvent (DMSO) only acted as controls. Values are given as means +/- SEMs of at least 3 separate experiments. Larger F statistic values and $p<0.05$ indicate significant differences between mean values obtained. Emboldened figures were identified as significantly different within groups on Tukey's post-hoc test ($p=0.002$ for Annexin V positive; $p=0.003$ for viable cells) (see figures 4.10 and 4.11).

	Experimental Conditions						ANOVA		Post-Hoc tests
	Normoxia	Hypoxia	Normoxia + 50μM LY294002	Hypoxia + 50μM LY294002	Normoxia + DMSO	Hypoxia + DMSO	F statistic	p value	
Trypan blue positive (%)	1.0 +/- 0.4	1.0 +/- 0.1	0.7 +/- 0.3	0.6 +/- 0.2	0.4 +/- 0.1	0.9 +/- 0.2	0.5	0.7	-
Cell concentration (10^6cells/μL)	3.6 +/- 0.6	4.1 +/- 0.4	3.2 +/- 0.3	3.5 +/- 0.7	3.3 +/- 0.4	2.6 +/- 0.1	0.6	0.7	-
Annexin V positive (%)	9.5 +/- 1.4	**8.7 +/- 1.5**	16.4 +/- 1.1	**22.2 +/- 5.5**	11.4 +/- 0.6	16.6 +/- 2.1	7.7	0.002	0.002
Viable cells (%)	85.7 +/- 2.7	**87.5 +/- 1.8**	79.0 +/- 1.1	**69.1 +/- 4.0**	86.3 +/- 0.9	73.5 +/- 2.6	7.6	0.002	0.003

Figure 4.10 Effect of LY294002 on rates of apoptosis in cultures of JU77 under conditions of hypoxia or normoxia, measured by Annexin V staining (*post-hoc tests performed identify a significant difference between highlighted groups, p=0.002). Values are expressed as means +/- SEMs of at least 3 separate experiments.

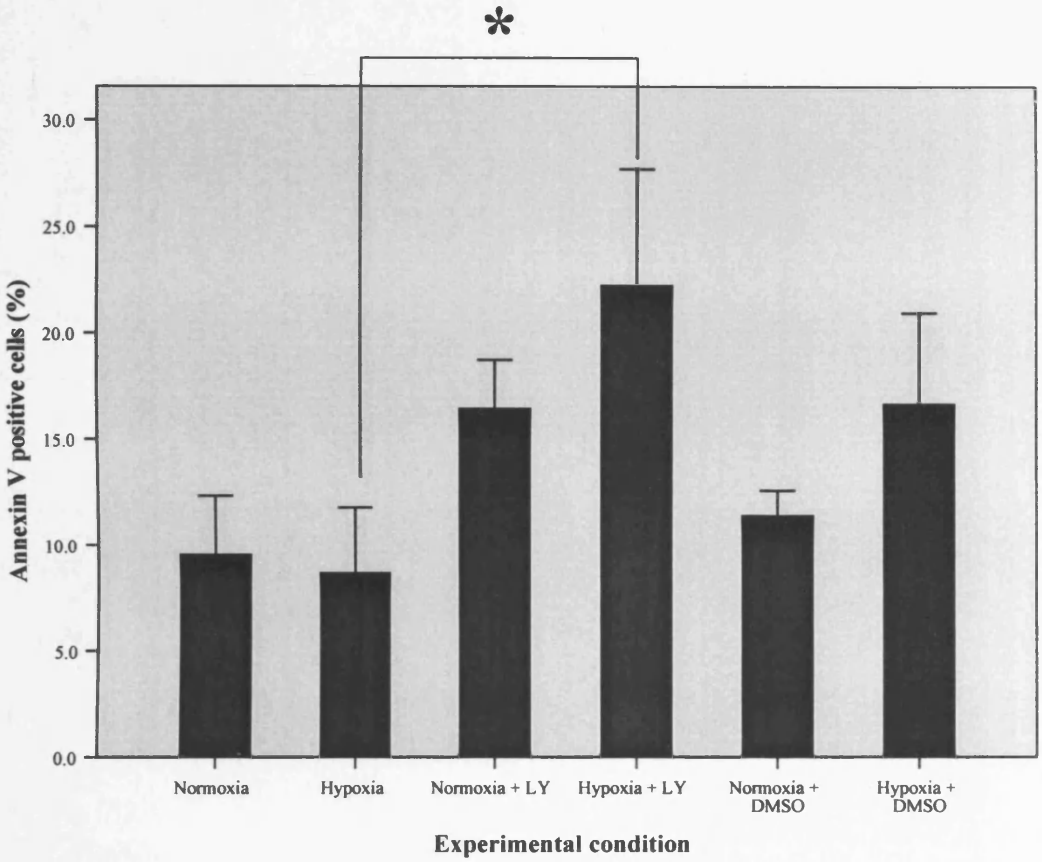


Figure 4.11 Effect of LY294002 on cell viability in cultures of JU77 under conditions of hypoxia or normoxia, measured by exclusion of Annexin V (*post-hoc tests performed identify a significant difference between highlighted groups, p=0.003). Values are expressed as means +/- SEMs of at least 3 separate experiments.

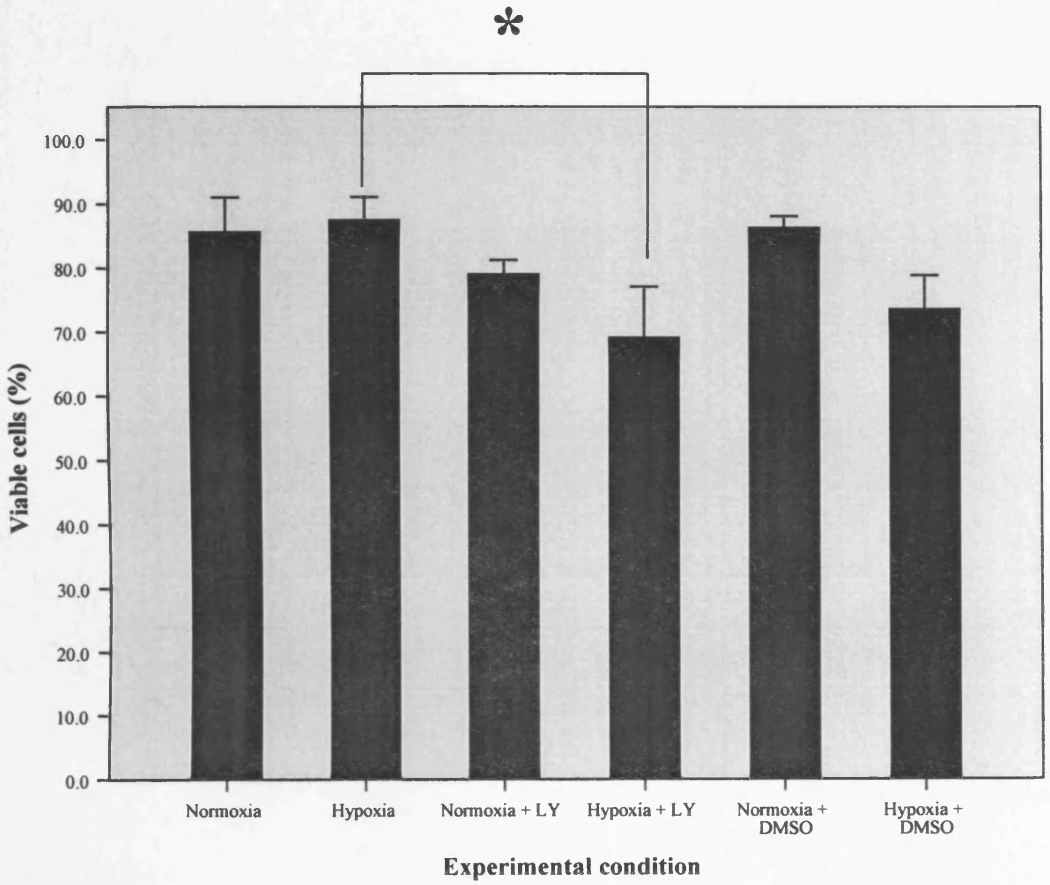
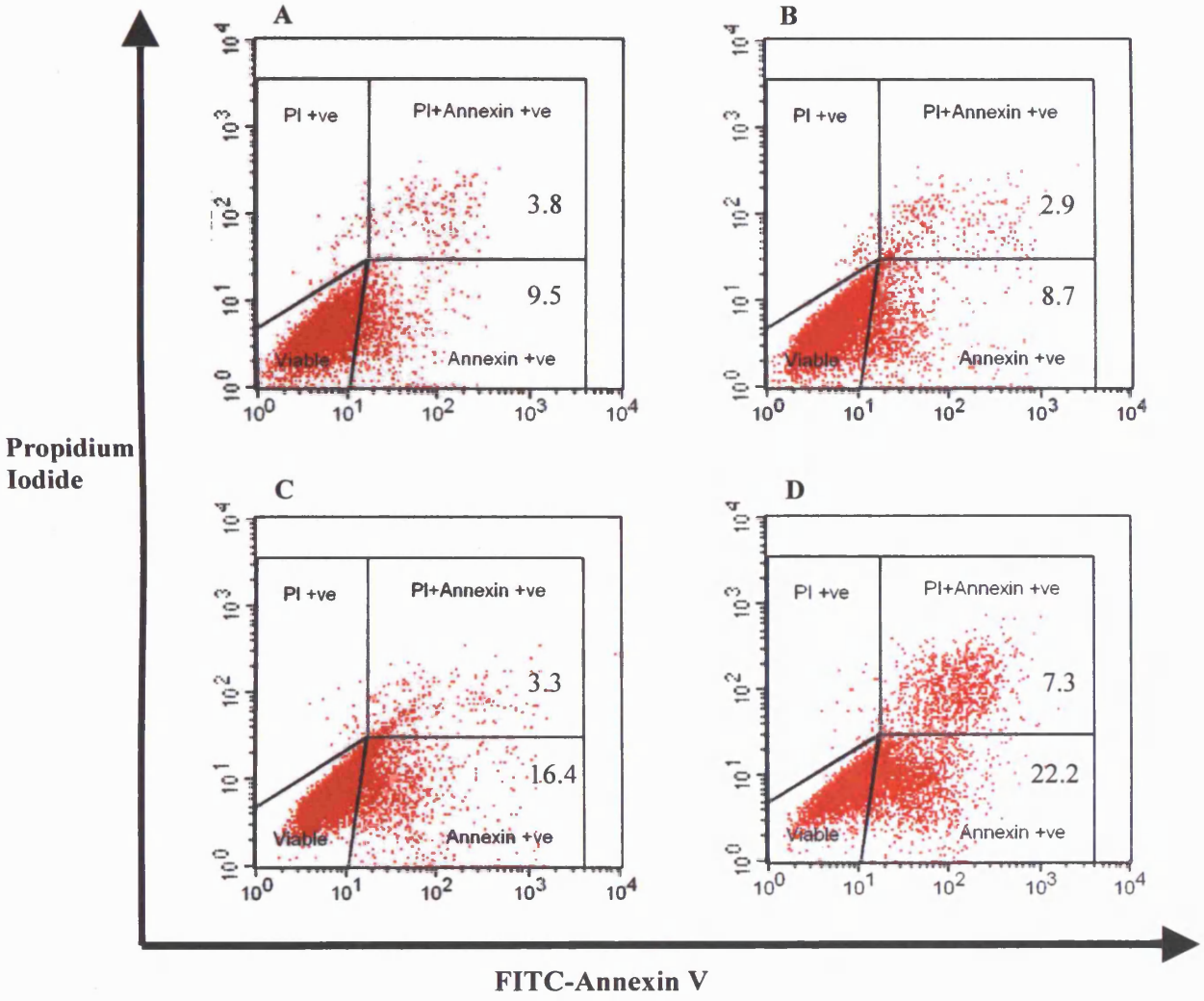


Figure 4.12 FACS plots of the effects of LY294002 on rates of apoptosis in cultures of JU77. Figures given within quadrants represent mean percentages, illustrating the significant increase in apoptotic (Annexin V positive) cells in cultures of cells exposed to LY294002 and 0.5% O_2 for a time-period of 2 hours. Plots shown are representative of at least 3 separate experiments. A – Normoxia; B – Hypoxia; C – Normoxia + LY294002; D – Hypoxia + LY294002



4.4.3 Western Blotting

4.4.3.1 Methods

Flasks containing cultures of the JU77 cell line were prepared, treated with LY294002 and placed into the appropriate environmental conditions as described previously. The flasks were then processed for lysate production according to previously described methods but with the addition of phosphatase inhibitors to the RIPA buffer.

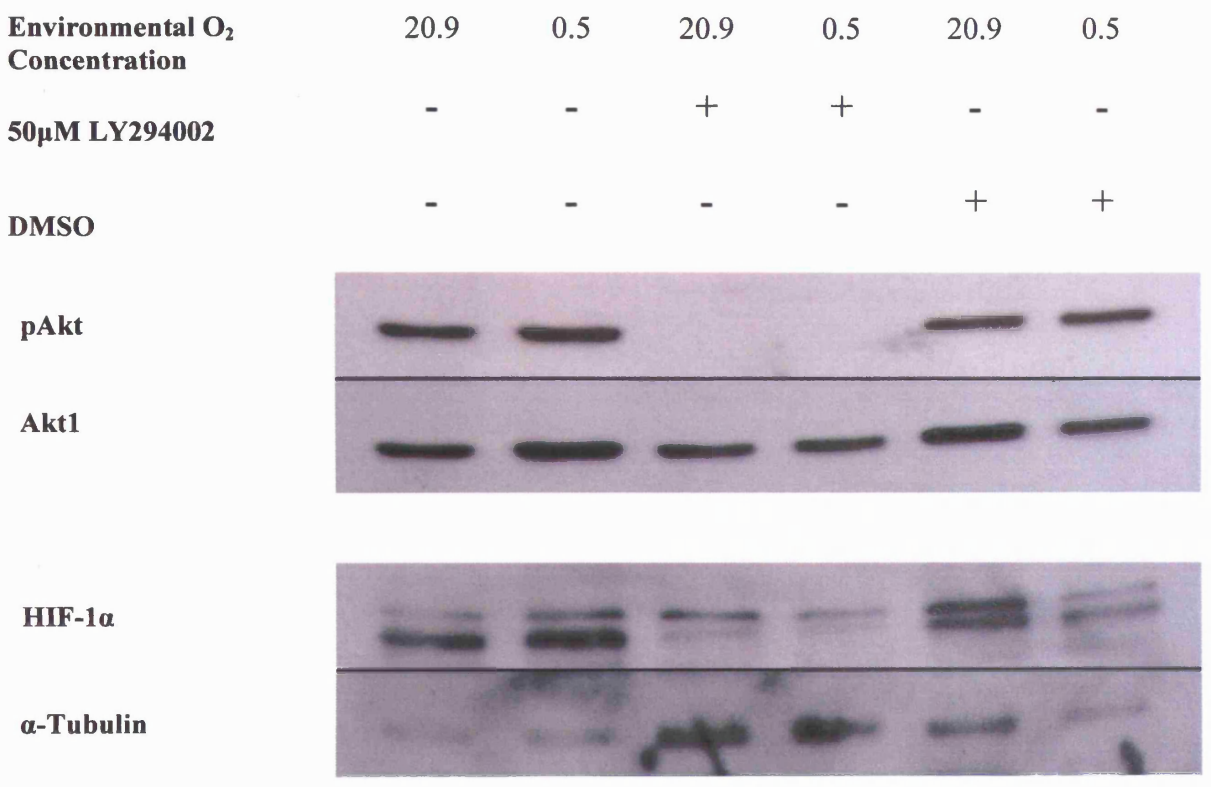
4.4.3.2 The effect of LY294002 on the expression of pAkt in conditions of reduced environmental O_2 concentrations

The addition of 50μM LY294002 led to the complete inhibition of Akt phosphorylation in lysates produced from cultures of the JU77 cell line (figure 4.10). This occurred regardless of exposure to reduced environmental O_2 concentrations. The presence of a strong band under normoxic conditions (20.9% O_2) would suggest the constitutive up-regulation of pAkt in this cell line.

4.4.3.3 The effect of LY294002 on the expression of HIF-1α in conditions of reduced environmental O_2 concentrations

The addition of LY294002 had a less profound effect on the up-regulation and expression of HIF-1α than on pAkt (figure 4.10). However, it did appear to produce a marked down-regulation of the de-phosphorylated form (lower band) of the protein, with the phosphorylated form remaining more prominent.

Figure 4.13 Expression of pAkt and HIF-1α in the JU77 cell line, with or without treatment with 50μM LY294002, with or without exposure to conditions of reduced environmental O_2 concentration for a 2 hour time-period. Uppermost blots show pAkt expression, with blots stripped and re-probed for Akt1. Lowermost blots show HIF-1α expression, with blots stripped and re-probed for α-Tubulin. Blots shown are representative of experiments repeated in duplicate.



4.5 Discussion

The aim of this series of experiments was to examine the effect of hypoxia on a benign mesothelial cell line and 4 MPM cell lines. This was accomplished through the use of a variety of techniques used to assess cells through various stages of programmed cell death (apoptosis) or as viable cells. Results from the different techniques were included together (tables 4.1-4.7), listed according to cell types. It may be expected that the percentage of trypan blue positive cells and the percentage of viable cells would total 100%, but this was not seen and is likely due to the differing techniques used and also the time delay involved in carrying them out with the cells necessarily out of culture medium. Should the hypoxic insult have a notable impact on any of these cell populations, a significant difference in at least one of these measures may have been expected. In addition, it is worth noting that the lack of difference between the cell concentrations could perhaps imply that the cell lines grew at least as well in the hypoxic conditions as they did in conditions of atmospheric oxygen concentrations. Furthermore, in the initial experiments over 96 hours, three of the five cell lines (ONE58, CRL5915 and CRL5820) had consistently greater numbers of viable cells in the populations kept at atmospheric Oxygen concentrations when compared with those exposed to hypoxic conditions, although it is very unlikely that there were identical numbers of cells inoculated into each flask at the beginning of each experiment.

The production of an acidic extra-cellular environment has been described as a fundamental property of the malignant phenotype (Ivanov et al. 2001) and is thought to benefit tumour growth, metastasis and resistance to treatment (Gillies et al. 2002;Ivanov et al. 2001;Potter & Harris 2003;Rofstad et al. 2006). In this work, a marked reduction in the measured pH of culture medium of both benign and malignant cell cultures was seen over the course of the 96 hour experiments. Although there may well be some buffering effect of the culture medium itself, it is reasonable to assume that cultures remained metabolically active, with glycolysis producing lactic acid, leading to the reduction in pH. This would appear consistent with the small amount of cell death seen even after 96 hours at an environmental O_2 concentration of 0.5%, further reinforced by the greater drop in pH seen in those cultures kept in conditions of reduced environmental O_2 concentrations, where an increase in anaerobic metabolism may be expected. Lactic acid levels

specifically were never measured, but may have given a more accurate assessment of metabolic activity.

The production of lactate may not be the only cause for acidification of the culture medium. By using a lactate dehydrogenase (LDH)-deficient ras-transfected Chinese hamster ovarian cell line that produces negligible quantities of lactic acid, Yamagata *et al* have shown that the production of lactic acid by tumour cells may not be the major mechanism of extra-cellular fluid acidification (Yamagata et al. 1998). As alluded to in the introduction of chapter 3, it is possible that CA IX may well have a role in this process through the hydration of carbon dioxide (CO_2) to produce carbonic acid (Griffiths et al. 2001). Given that 97% of MPM tumour samples investigated in this work were positive for CA IX on immunohistochemistry, it would seem plausible that there is a role for CA IX in the observed reduction in culture medium pH. Supporting this hypothesis, Svastova *et al* have shown that the acidification of culture medium under hypoxic conditions is CA IX-dependent, by the prevention of acidification through CA IX inhibition (Svastova et al. 2004). Indeed, further work has shown that selective inhibition of CA IX activity with sulphonamides has shown that acidification can be reversed, which may present novel anti-cancer treatment possibilities (Cecchi et al. 2005).

The addition of H_2O_2 as a stimulus to apoptosis has been demonstrated through *in vitro* cell line work (Taylor et al. 2004). It has been shown to induce mitochondrial cytochrome c release, DNA fragmentation and caspase activation (Cardoso et al. 2004). The experiments carried out by this researcher were replicated too few times to draw any firm conclusions, but it would appear plausible that the cell lines investigated contain robust functioning anti-apoptotic mechanisms, requiring prolonged exposure to high concentrations of H_2O_2 to trigger cell death. For example, in the MeT5A cell line, it required exposure to 1mM H_2O_2 for 24 hours to produce a noticeable increase in the levels of apoptosis. Similarly, in the JU77 cell line, concentrations of H_2O_2 equal to or greater than 500μM for 24 hours were required. Indeed, this effect was largely abolished by reducing the exposure time to anything less than 6 hours.

Despite the acceptance of the significance of tumoural hypoxia to the overall malignant process, the definitions of degrees and absolute levels of hypoxia remain more ethereal and are poorly specified in many studies. In a review by Hockel *et al*, the point of the reduction of the partial pressure of O_2 (pO_2) below a critical threshold for "hypoxia" to develop appears to depend not only on definition, but also on the component measured (ranging from the partial pressure in end-capillary blood to the oxidation status of cytochrome c) and the method of that measurement (Hockel & Vaupel 2001). The pO_2 of normal tissue depends principally on its function and blood flow. The critical intra-tumoural pO_2 below which detrimental changes have been observed is ~1.3kPa (Hockel & Vaupel 2001), in comparison with the pO_2 of arterial blood which is usually 10-fold greater than this.

Addressing the environmental O_2 concentrations chosen for this study, Yokoi *et al* have previously shown the up-regulation of Akt phosphorylation in the human pancreatic cancer cell line L3.6pl when exposed to hypoxic conditions of 1% O_2 compared with normoxia (Yokoi & Fidler 2004). Similar findings have been demonstrated in the rat phaeochromocytoma cell line, PC12 (Beitner-Johnson et al. 2001). However, despite the minimal hypoxic stimulus apparently required for the phosphorylation of Akt in certain cell lines, previous work with human monocyte-derived macrophages has demonstrated that hypoxic induction of HIF-1α occurs at 0.5% and 0.1% O_2, but not at 1% or 20.9% (Burke et al. 2002). Furthermore, using Clarke electrodes, Allen *et al* have demonstrated that, with the inflow of 100% Nitrogen at 5L/min, it can take more than 3 hours for the partial pressure of O_2 to fall to ~20mmHg (~2.5kPa) and equilibrate within culture medium in flasks. However, in the presence of a viable cell line (in this case the non-small cell lung cancer (NSCLC) cell line, A549) the partial pressure of O_2 was observed to fall to a lower level (~6mmHg, ~0.8kPa) and equilibrate more rapidly over a period of around 90 minutes (Allen, Schneider, & White 2001). The period of 2 hours was therefore considered a practical minimum time-point throughout all cell line experiments to reasonably allow culture medium equilibration with environmental O_2 concentration and it was considered appropriate to use 0.5% and 0.1% as experimental O_2 concentrations to ensure a sufficient hypoxic stimulus.

An atmospheric O_2 concentration of 20.9% was used for the "normoxia" controls, as this is provides the environmental conditions in which the cell lines have been optimised for growth. Because of cellular metabolism it is likely that the culture medium pO_2 is actually lower than 20.9%. No effort was made to increase the pO_2 for these cultures as this is would be considered hyperoxic conditions, providing an additional stressor for the cell cultures (Frank et al. 1989). For the purposes of this work, the ability to regulate the environmental Oxygen concentration through the use of a specialised incubator provided the most consistent, controllable experimental conditions. In addition, a small volume of medium (5mL in a T25 flask) was used to minimise the barrier to diffusion and to reduce the time to gaseous equilibration.

Although up-regulated at varying time-points in conditions of hypoxia, western blotting of lysates created from cultures kept in normoxic conditions would imply that pAkt may well be constitutively up-regulated in several of the cell lines investigated. This would not be without precedent. Previous work has indicated the constitutive expression of pAkt in the MPM cell lines I-45 and REN (Rascoe et al. 2005). The functional implications of this finding have also been investigated. A study by Box *et al* has demonstrated in the Hep3B hepatocellular carcinoma cell line, that constitutive activation of Akt is associated with a reduction in hypoxia-induced cell cycle arrest (Box & Demetrick 2004). Furthermore, using another hepatocellular carcinoma cell line, HepG2, constitutively active nuclear pAkt may have a role in cell transformation and immortality (Syed et al. 2002). Contrary to this, the constitutive activation of HIF-1α was less well demonstrated in this study although it has been shown previously, with particular reference to a study examining a number of pancreatic cancer cell lines which has demonstrated an anti-apoptotic advantage conferred on those cell lines constitutively expressing HIF-1α (Akakura et al. 2001).

The author is not aware of any previous studies that have examined the expression of pAkt and HIF-1α over such a prolonged time-period as 96 hours. It is perhaps unsurprising that, given the likely environmental conditions within the culture medium at 96 hours, there is marked up-regulation of both pAkt and HIF-1α. However, perhaps more interestingly is the double-band expression of HIF-1α that is clearly visible on numerous occasions. HIF-1α has been proposed to

have both pro- and anti-apoptotic actions, with exposure to severe hypoxia and even anoxia leading to the induction of apoptosis (Piret et al. 2002). This apparent switch of the usual role assigned to HIF-1α has been shown to depend not only on cell type (Piret et al. 2002), but more importantly on the phosphorylation state of HIF-1α. Suzuki *et al* have demonstrated in the breast carcinoma cell line, MCF-7, that a slower migrating band on western blotting is the phosphorylated form of HIF-1α and associates with HIF-1β, whereas the more rapidly migrating band is the de-phosphorylated form of HIF-1α and has a tendency to associate with p53 (Suzuki, Tomida, & Tsuruo 2001). In this study, it was found that the more slowly migrating band was predominant in the earlier experiments, implying an anti-apoptotic role for HIF-1α. In contrary to this, the final experiment over a 2 hour period revealed a stronger expression of the de-phosphorylated form, although this was noticeably reduced in the cultures treated with LY204002. Although the down-regulation of the de-phosphorylated form of HIF-1α may imply a lack of apoptotic stimulation, it does not necessarily follow that there should be an increase in anti-apoptotic processes, which may explain to some extent the increased rates of apoptosis seen in these cultures. Furthermore, the activation of Akt leads to Mdm2 activation and translocation to the nucleus, which in turn leads to the proteosomal degradation of p53 (Mayo & Donner 2002). Clearly, the complete inhibition of Akt phosphorylation by LY294002 would inhibit this anti-apoptotic mechanism. It could be postulated that the effect of interference with the PI3-K/pAkt pathway is of greater importance to cell survival than the effect of HIF-1α in this cell line. Further work is needed to clarify this situation.

This work has shown that the PI3-K/pAkt pathway plays an anti-apoptotic role in MPM cells exposed to hypoxic conditions. Complete inhibition of the phosphorylation of Akt has been clearly demonstrated by treatment with 50μM LY294002, which corresponds to an increase in the rates of cellular apoptosis, compared with cultures not treated. LY294002 prevents the phosphorylation of PI3-K through competitive ATP-inhibition (Vlahos et al. 1994), which in turn prevents the activation of Akt. However, it has become apparent that LY294002 is not a specific inhibitor of PI3-K and also inhibits the actions of CK2, which has led to some researchers recommending its use in combination with another PI3-K inhibitor, such as Wortmannin, which does not inhibit CK2 (Davies et al. 2000). Indeed, although not the focus of this work, it is worthy

to note that CK2 itself is capable of an anti-apoptotic role through the inhibition of PTEN (Miller et al. 2002) and the prevention of caspase-mediated degradation (Litchfield 2003). Although the incidental inhibition of CK2 may complicate the picture presented in the current study, recent work has shown that the specific inhibition of CK2 in Jurkat cells leads to the induction of apoptosis, which appears to occur via the inhibition of Akt phosphorylation (Di Maira et al. 2005). Therefore, although the inhibition of Akt phosphorylation may well be occurring via two distinct intra-cellular pathways with LY294002 treatment, for the purposes of this study that may not be as important as initially thought. Akt-specific small molecule inhibitors have recently been developed (Castillo et al. 2004;Gills & Dennis 2004) which will help enormously with further work required to clarify the situation.

4.6 Summary

This study has shown that MPM cell lines are resistant to hypoxia as a stimulus to apoptosis. A possible explanation for this finding may well be the up-regulation of pAkt and HIF-1α, although this expression is variable between different cell lines, and in the case of HIF-1α may well require conditions of prolonged, severe hypoxia. The cell lines are remarkably resistant to acidic culture medium, but it is suggested that this may represent a fundamental part of the malignant phenotype. Finally, it would appear that the addition of 50μM LY294002 leads not only to the complete inhibition of the phosphorylation of Akt but that this is associated with increased levels of apoptosis under hypoxic conditions. This may indicate that pAkt plays a role in the survival of MPM cells under hypoxic conditions, promoting the role of Akt inhibition as a novel treatment option in this disease.

CHAPTER FIVE

CONCLUDING DISCUSSION

This study is the first to report results of the immunohistochemical expression of pAkt and CA IX in large numbers of paraffin-embedded samples of MPM. The finding of 86% of tumour samples staining positive for pAkt would appear to correlate with the finding of normoxic expression of pAkt in all cell lines, a finding that was particularly prominent in the ONE58 and JU77 lines. Recent work from the University of Texas has reported the constitutive activation of Akt in two different mesothelioma cell lines, I-45 and REN (Rascoe et al. 2005), which would be consistent with the findings. More specifically, the nuclear subcellular localisation of pAkt, found to be an independent predictor of a poorer outcome, presents an interesting topic of further study given the evidence for its prognostic relevance presented in this study and also its association with a poorer outcome in other malignancies (Shah et al. 2005;Vasko et al. 2004). Furthermore, the nuclear localisation of pAkt may well have a role in benign disease processes given work by Shiraishi *et al* identifying the nuclear targeting of Akt as responsible for mediating the inhibition of apoptosis in ischaemia-reperfusion of cardiomyocytes (Shiraishi et al. 2004).

Numerous recent studies have identified the activation and up-regulation of Akt as playing an important role in MPM. Altomare *et al* have demonstrated that through the inhibition of Akt phosphorylation an MPM cell line was rendered increasingly prone to apoptosis, although in this study the stimulus was cisplatin rather than hypoxia (Altomare et al. 2005). In the same study, 17 of 26 (65%) human MPM specimens examined showed pAkt staining, which although lower than the 86% positive rate in this study does add to the validity of this work. Perhaps the most interesting finding in this study is the increased rates of apoptosis seen in conjunction with the inhibition of Akt phosphorylation. In agreement with this, apoptotic resistance was significantly reduced through LY294002 treatment in a novel spheroid model of MPM tumour cells created by Kim *et al* (Kim et al. 2005). Furthermore, a pro-metastatic phenotype, described by Cole *et al* in MPM cells, has been down-regulated by the addition of LY294002 (Cole, Jr. et al. 2006), an effect which may well occur via the PI3-K/pAkt pathway. Finally, several studies have examined potential routes of activation of Akt in MPM, for example, via the receptor tyrosine kinase, EphB4 (Xia et al. 2005), or via the hepatocyte growth factor-stimulated met receptor (Jagadeeswaran et al. 2006;Mukohara et al. 2005).

Considering in combination the work presented in this study and additional data from various laboratory-based studies, there can be little genuine doubt that the phosphorylation of Akt is, in some way, involved in anti-apoptotic mechanisms in MPM. This study however, is, as far as the author is aware, the first to consider hypoxia as the primary stimulus to apoptosis. The link between pAkt and HIF-1α has been explored, but never before in MPM and the picture remains less than clear. There is recent *in vitro* evidence in invasive breast cancer cell lines that pAkt is required for the up-regulation of HIF-1α in conditions of hypoxia (Gort et al. 2006). However Arsham *et al* have shown that pAkt is neither required for the stabilisation of HIF-1α in hypoxic conditions, nor is capable of stimulating HIF-1 target gene expression (Arsham et al. 2002). Additionally, the same group have shown that both pAkt and HIF-1α independently induce tumour growth and angiogenesis in a hepatoma cell line (Arsham et al. 2004). To further complicate the picture, there is evidence that pAkt is capable of up-regulating the expression of HIF-1α, independent of hypoxic conditions in a HER2 over-expressing cell line (Li et al. 2005), and to contradict again, Sasabe *et al* have shown that the forced expression of HIF-1α in an oral squamous cell carcinoma cell line leads to the phosphorylation and activation of Akt (Sasabe et al. 2005). It is the supposition of this author that the precise interaction is likely to be cell-type specific and small molecule, specific inhibitors of HIF-1α (Chau et al. 2005;Tan et al. 2005) and pAkt (Castillo et al. 2004;Gills & Dennis 2004) will enable more focused, disease- or cell-type specific work in the future.

During the period over which this study was completed, developments in the research and treatment of MPM have continued unabated. Many have focused on the clinical management of all aspects of the disease, but largely comprise retrospective case series or at best, small number randomised trials. Phase I and II trials continue to examine novel treatments, particularly small molecule inhibitors, both alone and in combination with other chemo-therapeutic agents. An EORTC-registered phase II trial (protocol number 08052) using the proteosome inhibitor Bortezomib in combination with cisplatin is a good example of this and is currently recruiting patients. Gene therapy is still its early stages (Sterman 2005), although there are indicators of effective anti-tumour immune responses (Sterman et al. 2005). Traditional treatments of surgery,

chemotherapy and radiotherapy continue to be improved. A recent Cochrane review of the role of Pemetrexed in combination with Cisplatin in MPM has revealed that, in comparison with other chemotherapy or best supportive care, there may be a survival benefit associated with its use in patients of good performance status (Green et al. 2007). In addition, following an appeal, the National Institute for Clinical Excellence has recently published a final appraisal determination that is likely to lead to the adoption of Pemetrexed and Cisplatin as the recognised standard chemotherapy for MPM. Although encouraging results with certain, focused types of radiotherapy have been described (Stevens et al. 2005) once again there is a lack of randomised controlled trial evidence of the benefits of radiotherapy in general in this disease (Ung et al. 2006). New surgical approaches and techniques are being developed to reduce the morbidity associated with radical surgery for this condition (Martin-Ucar et al. 2004) and our understanding of the significance of lymph node status in MPM continues to evolve (Edwards et al. 2006).

Despite its resistance to treatment, patients diagnosed at an early stage have consistently derived survival benefits (Rusch 1995;Stewart et al. 2004), prompting the search for markers of early disease, especially given the commonly seen latent periods of 30 years or more from time of exposure to development of the disease (Britton 2002). Recent work has focused on two such markers. Osteopontin is a phosphorylated glycoprotein, over-expressed in several malignancies including MPM. Pass *et al* have shown that serum Osteopontin levels may be used to differentiate between individuals exposed to asbestos without MPM and those exposed to asbestos, with MPM (Pass et al. 2005). Additionally, the finding that 84% of a cohort of patients with MPM had raised serum concentrations of soluble mesothelin-related proteins (SMP), compared with only 2% of people with other non-MPM lung and pleural diseases (Robinson et al. 2003), has raised exciting possibilities for screening (Creaney & Robinson 2005). This data, validated in a separate cohort of patients (Scherpereel et al. 2006), has driven the development of a commercially available, robust test for use in the diagnosis of and monitoring of patients with MPM (Beyer et al. 2007).

In conclusion, this study has reported the poor prognosis associated with increase expression of nuclear pAkt in MPM tumour samples, and has suggested that hypoxia may play a

role in the expression of pAkt given the association with CA IX, the surrogate marker of hypoxia. It has also shown that both pAkt and HIF-1α are up-regulated in conditions of hypoxia in MPM cell lines and that the PI3-K inhibitor, LY294002, inhibits the phosphorylation of Akt and possibly leads to a down-regulation in the de-phosphorylated form of HIF-1α in conditions of hypoxia and atmospheric O_2 concentrations. Phospho-Akt appears to have a role in the prevention of hypoxia-induced apoptosis in an MPM cell line, with mounting evidence in various solid tumours implicating the importance of the nuclear sub-cellular localisation of activated Akt. There are currently approximately 2000 new cases of MPM a year in this country (Peto et al. 1995) and there are predicted to be 250,000 deaths throughout Western Europe over the next 30 years or so (Peto et al. 1999). The British Thoracic Society (BTS) is currently updating its original guidelines on the management of patients with MPM published in 2001 (2001) and a national framework consultation document on the standards of care expected for patients with MPM is currently in circulation. This work provides further evidence for the importance of the Akt pathway in the malignant process in this disease and its potential role as a novel target for treatment of this disease and it is hoped that it may stimulate future work and the formulation of novel treatment strategies for patients suffering from this at present, incurable disease.

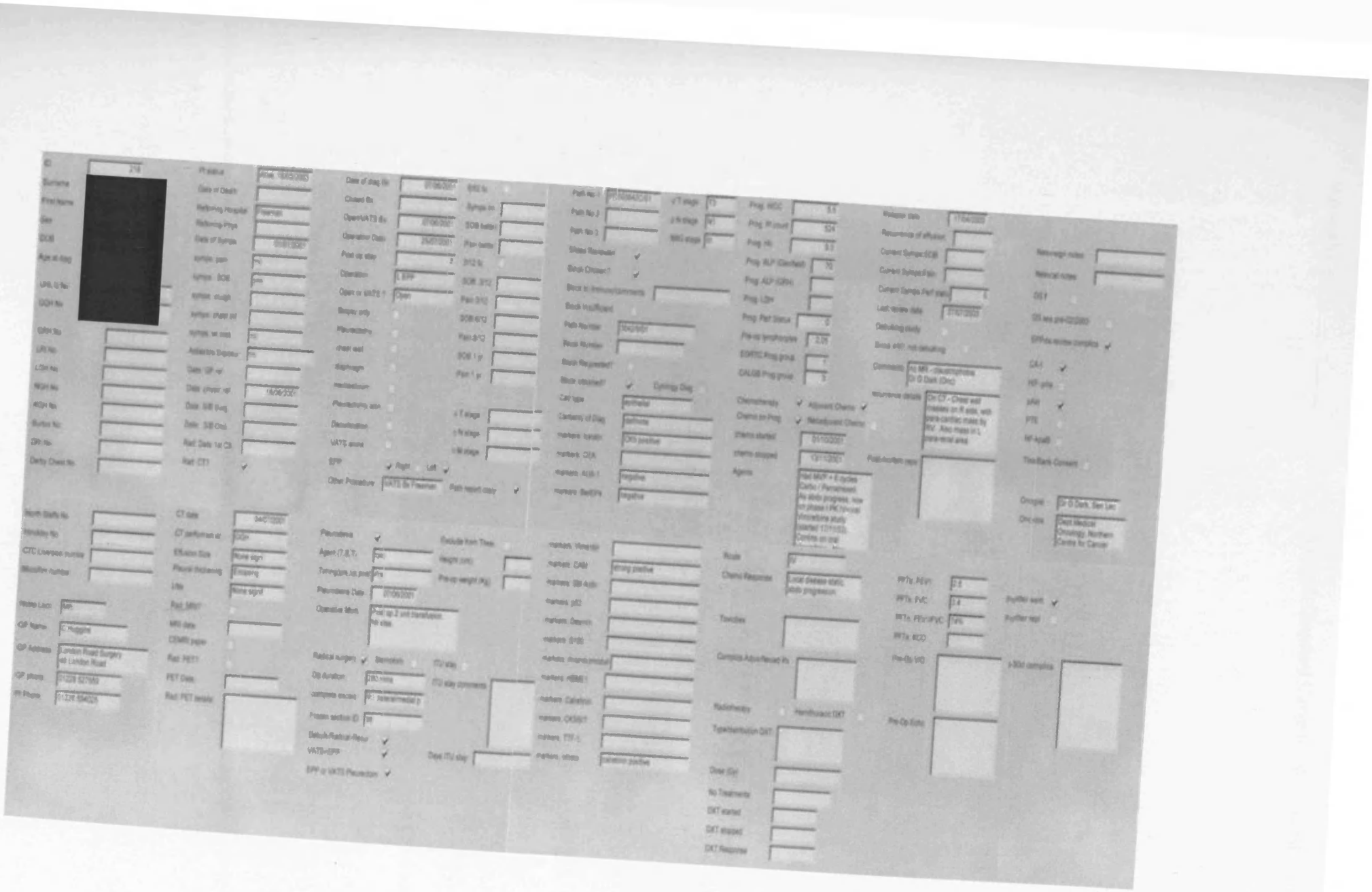
APPENDIX I

EXAMPLE DATABASE DATA CAPTURE FORM

SPECIAL NOTE

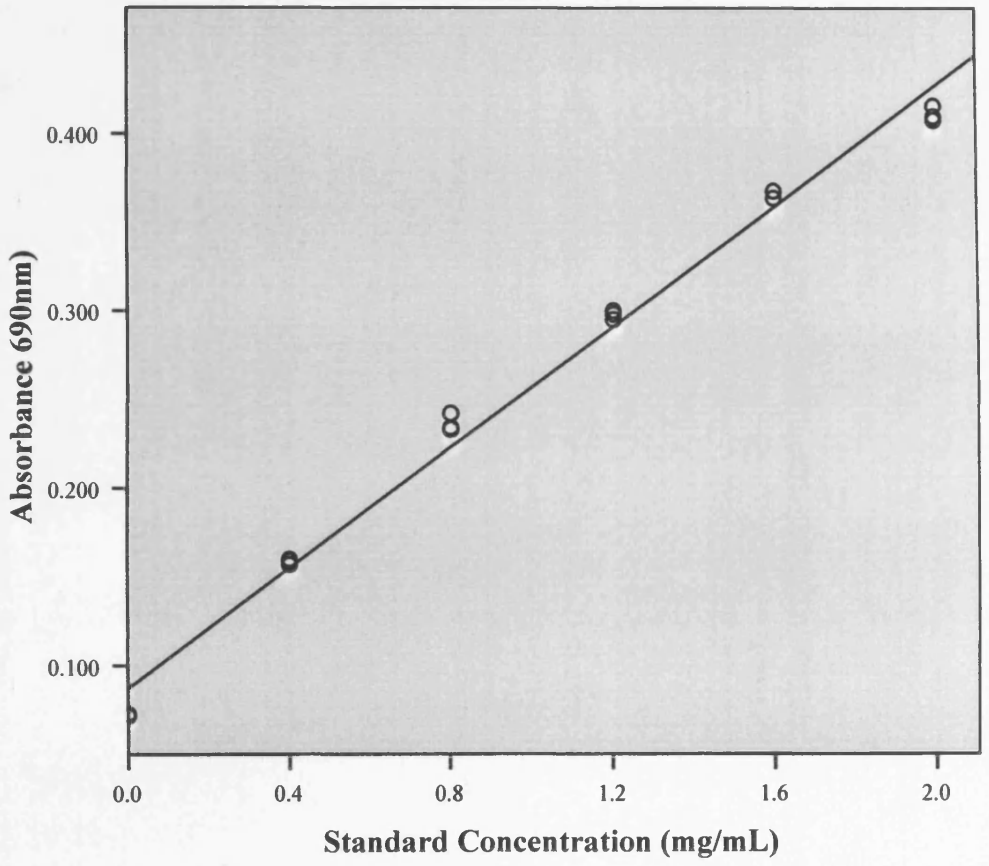
THE FOLLOWING IMAGE IS OF POOR QUALITY DUE TO THE ORIGINAL DOCUMENT.

THE BEST AVAILABLE IMAGE HAS BEEN ACHIEVED.



APPENDIX II

EXAMPLE STANDARD CURVE FOR WESTERN BLOTTING LYSATES



For each set of lysates created throughout this work, the protein concentration was derived using a standard graph similar to that shown above ($R^2 = 0.991$, $p<0.0001$).

APPENDIX III

CONSENT FORMS AND PATIENT INFORMATION SHEETS

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PATIENT CONSENT FORM

Title of Study: The Role of Hypoxia and its Interaction with the PI3K/pAkt/PTEN Pathway in the Pathogenesis of, and the Immunodiagnosis of Malignant Pleural Mesothelioma

Principal Investigator: Mr David A Waller
Contact Details: Telephone 0116 2563539
Facsimile 0116 2563139

This form should be read in conjunction with the Patient Information Leaflet, Version 3, 28.11.2003.

I agree to take part in the above study as described in the Patient Information Leaflet.

I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet.

I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.

I understand that tissue samples and associated clinical data may be transferred to non-commercial research partners of the University Hospitals of Leicester NHS Trust and Leicester University, but that the information will be anonymised prior to transfer.

I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment.

I understand that if research using my tissues produces information, which has immediate clinical relevance to me, I will be informed by my hospital consultant or GP and be given an opportunity to discuss the results.

I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue.

The samples which I hereby consent to donate are:

.....
.....
.....

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with .....and ask any questions. The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.

Signature of patient .....Date.....

(Name in BLOCK LETTERS)

.....

I confirm I have explained the nature of the Trial, as detailed in the Patient Information Leaflet, in terms which in my judgement are suited to the understanding of the patient.

Signature of Investigator ..... Date.....

(Name in BLOCK LETTERS)

.....

Printed on headed notepaper

PATIENT INFORMATION SHEET

Title of Study: The Role of Hypoxia and its Interaction with the PI3K/pAKT/PTEN Pathway in the Pathogenesis of, and the Immunodiagnosis of Malignant Pleural Mesothelioma

Principal Investigator and Delegated Tissue Bank Official: Mr David A Waller, Consultant Thoracic Surgeon, GGH, UHL NHS Trust

You may contact Mr Waller on: 0116 2563959
Facsimile: 0116 2563139

Name of Tissue Bank: The Leicestershire Mesothelioma Tissue Bank, Clinical Sciences Building, Glenfield Hospital.

Tissue Bank Custodian: University Hospitals of Leicester NHS Trust

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

1. What is the purpose of the study?

The study is designed to look at certain aspects of how mesothelioma grows, the factors that may be involved with growth and the impact of these factors on prognosis. We will also be looking at ways to test the potential dangers of new materials to prevent the problems that we now have following the use of asbestos previously.

When a patient has an operation for mesothelioma, pieces of the tumour (tissues) are kept afterwards. This is so that a pathologist can look at these tissues to check that adequate tumour has been removed successfully. As a normal part of the

operation, more tissue is removed than is actually needed by the pathologist in their assessment. We would like to use this extra tissue to carry out the study.

2. What will be involved if I take part in the study?

This study is a laboratory-based study and as such, will not directly involve any patients. The tissue samples will be collected during the time of your operation and there will be no further input required on your part. You will not be required to take any medication or have any further tests as part of this research study.

3. Who will have access to my tissue and will information obtained in the study be confidential?

Access to your tissues will only be available through the Leicestershire Mesothelioma Tissue Bank, which is controlled by the University Hospitals of Leicester. All your information will be treated with the usual degree of confidentiality under the Data Protection Act (1988). You will not be identified in any documents or publications relating to this research. Sometimes tissue from the tissue bank may be transferred to other researchers who are working in partnership with the University Hospitals of Leicester NHS Trust. If this is the case all samples will be anonymised before transfer.

We will notify your General practitioner should you agree to take part in the study.

4. What if I am harmed by this study?

As this research does not directly involve you, it is extremely unlikely that you will be harmed by this study. However, medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS, i.e. compensation is only available if negligence occurs.

6. What happens if I do not wish to participate in this study or wish to withdraw from it later?

If you do not wish to participate in this research or you wish to withdraw from it you may do so without justifying your decision and your future treatment will not be affected.

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PATIENT CONSENT FORM

The Leicestershire Mesothelioma Tissue Bank

Tissue Bank: Leicestershire Mesothelioma Tissue Bank
Location: Clinical Sciences Building, Glenfield Hospital, Groby Road, Leicester, LE3 9QP, UK

Tissue Bank Custodian: University Hospitals of Leicester NHS Trust

Principal Investigator and Delegated Tissue Bank Official: Mr David A Waller, Consultant Thoracic Surgeon, GGH, UHL NHS Trust

Patient Details [or affix address label]
Hospital Number
Name
Address
Date of Birth

This form should be read in conjunction with the patient information sheet, version number 6, 28/11/2003.

I understand that samples from the tissue bank and associated clinical data may be transferred to non-commercial partners of the University Hospitals of Leicester NHS Trust and Leicester University, but that the information will be anonymised prior to transfer.

I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS, i.e. compensation is only available if negligence occurs.

I understand that samples from the tissue bank will not be used to undertake any genetic tests whose results may have adverse consequences on mine or my families insurance or employment.

I understand that if research using my tissues produces information that has immediate clinical relevance to me, I will be informed by my hospital Consultant or General Practitioner and be given an opportunity to discuss the results.

I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue.

I would be willing to be contacted again regarding future use of this tissue for purposes not foreseen at the present time.

Please initial box

1. I confirm that I have read and understand the information sheet V6, 28/11/2003, for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from UHL NHS Trust or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

5. I agree to donate samples of my pleural tissue. ☐

Signature of Patient:.....Date.....

Name (in BLOCK LETTERS).....

I confirm that I have explained the purpose of the tissue bank, as detailed in the patient information sheet, in terms, which in my judgement are suited to the understanding of the patient.

Signature of individual taking consent: .....

Date:.....

Name (in BLOCK LETTERS).....

Printed on headed notepaper

PATIENT INFORMATION SHEET

Tissue Bank: Leicestershire Mesothelioma Tissue Bank
Location: Clinical Sciences Building, Glenfield Hospital, Groby Road, Leicester, LE3 9QP, UK

Tissue Bank Custodian: University Hospitals of Leicester NHS Trust

Principal Investigator and Delegated Tissue Bank Official: Mr David A Waller, Consultant Thoracic Surgeon, GGH, UHL NHS Trust

You may contact Mr Waller on: **Tel.** 0116 2563959
Fax. 0116 2563139

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

1. What is a tissue bank?

A tissue bank is a collection of tissue samples being stored long term that will be used to assist in future research into a specific disease or group of diseases, or investigate disease processes and their treatment. Tissue banks are a valuable research resource and are being increasingly established at local, regional and national level.

This tissue bank will contain samples of tissue donated by patients that have been removed at the time of surgery for mesothelioma.

2. What will the tissues in the tissue bank be used for?

The tissues will be used in experiments to help our understanding of mesothelioma. We will be looking at how this tumour grows, why some tumours spread and trying to find new treatments for mesothelioma.

All research that involves NHS patients or staff, information from NHS medical records or uses NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to minimum and balance against possible benefits and that you have been given sufficient information on which to make an informed decision to take part or not.

3. How much of my tissue will be taken?

During your operation, pieces of tissue are removed and kept so that a specialist can look at them. As part of the operation more tissue is removed than the specialist needs to evaluate. We would propose to use small amounts of this tissue from inside your chest cavity. The amount will usually be about the size of a 50p piece, but may be equivalent to the size of a little finger.

4. Who will have access to my tissue and how will confidentiality be maintained?

Access to your tissues will only be available through the Leicestershire Mesothelioma Tissue Bank, which is controlled by the University Hospitals of Leicester. All your information will be treated with the usual degree of confidentiality under the Data Protection Act (1988). Sometimes tissue from the tissue bank may be transferred to other researchers who are working in partnership with the University Hospitals of Leicester NHS Trust. If this is the case all samples will be made anonymised before transfer. You will not be identified in any way from your tissue sample.

5. Will I be contacted again in the future?

If, as a result of any research carried out on your tissue, new information becomes available which may have an impact on your care, this information will be discussed with you, either by your hospital Consultant or your General Practitioner.

6. Will I receive payment for the tissue that I donate to the tissue bank?

No, you will not receive any payment for the tissue you donate. It will be received as a gift. Neither yourself nor your relatives will benefit financially from any inventions that may result from the use of your tissues.

7. What happens if I do not wish to participate in this research, wish to withdraw from it later or wish to complain about the study?

If you do not wish to have your tissue held in the tissue bank you can withdrawal them without justifying your decision and your normal care and medical management will not be affected in any way.

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

In case of any problem, please contact Mr Waller (telephone number 0116 2563959).

Or: Research Office
Directorate of Research and Development
University Hospitals of Leicester NHS Trust
Leicester General Hospital
Gwendolen Road, Leicester LE5 4PW
Tel. 0116 258 8246

APPENDIX IV

SELECTED PUBLICATIONS

ELSEVIER

European Journal of Cardio-thoracic Surgery 24 (2003) 1019–1024

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

www.elsevier.com/locate/ejcts

Is there a role for pre-operative contrast-enhanced magnetic resonance imaging for radical surgery in malignant pleural mesothelioma?

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Abstract

Objective: To assess the use of contrast-enhanced magnetic resonance imaging (CEMRI) in addition to computed tomography in the pre-operative assessment of patients for radical surgery in malignant pleural mesothelioma. **Methods**: Over a 45-month period, 51 of 76 patients assessed (69 men and seven women), underwent extra-pleural pneumonectomy or radical pleurectomy/decortication. Post-operative pathological stage was correlated with radiological staging, with particular emphasis on tumour resectability. **Results**: Seventeen (22%) patients were found on CEMRI to have unresectable, but histologically unconfirmed disease, not previously seen on CT. Fifty-one (67%) patients proceeded to radical surgery, but pathological nodal data were incomplete in three, so excluding these patients from further analyses. The median pre-operative interval after CEMRI was 17 days. Two patients were found to have unexpectedly extensive disease at thoracotomy, thus the sensitivity of CEMRI for prediction of resectability was 97%. Using the International Mesothelioma Interest Group system, tumour stage was correctly predicted by CEMRI in 48% of patients, but understaged in 50% of cases, largely due to the underestimation of pericardial involvement, but this did not affect resectability and had no significant effect on prognosis. Nodal stage was correctly identified in 60% of patients. CEMRI was successful in predicting pathological tumour stage T3 or less (sensitivity of 85%; specificity of 100%), but less so in identifying tumour stage T2 or less (sensitivity of 23%; specificity of 96%) or N2 nodal disease (sensitivity 66%; specificity 73%). **Conclusions**: CEMRI is most useful in the differentiation of T3 and T4 disease and may be unnecessary at earlier stages. Its multiplanar tumour localisation abilities are of value in the assessment of resectability. It is unlikely to contribute significantly to nodal staging, but it remains a valuable adjunct in the selection of patients for radical surgery.

Keywords: Mesothelioma; Staging; Radical surgery; Magnetic resonance imaging

1. Introduction

The incidence of malignant pleural mesothelioma (MPM) is increasing and is expected to account for a quarter of a million deaths throughout Western Europe over the next 35 years [1]. Despite the traditionally nihilistic approach to treatment, there may be a role for radical surgery in early stage disease, in combination with other treatment modalities, and prolonged survival has been seen in selected patient groups [2,3].

Radiological evaluation of the extent of disease is therefore very important. Due to its availability, ease to perform, proficiency of interpretation and relative low cost, the traditional and most widely used method of assessment is computed tomography (CT). Magnetic resonance imaging (MRI) has been proposed as a useful imaging modality in MPM because of its superiority to CT in the assessment of mediastinal and great vessel involvement [4], and also diaphragmatic and chest wall invasion [5,6]. The addition of intravenous contrast enhancement to MRI has been shown to be of convincing diagnostic value aiding the prediction of malignancy in those with asbestos exposure and pleural lesions [6–8].

We report our single centre experience using contrast-enhanced MRI (CEMRI) in patients with mesothelioma under consideration for radical surgery. Using the results of surgical resection and intra-operative staging we have explored the correlation of radiological and pathological staging, with particular reference to its usefulness in the assessment of resectability.

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2. Materials and methods

Over a 45-month period, 76 patients with a diagnosis of non-sarcomatoid MPM, made by percutaneous or thoracoscopic pleural biopsy were assessed for radical surgery. Definitive histological evidence of sarcomatoid mesothelioma was a contraindication to further assessment, in accordance with local policy. There were 69 men and seven women, with a median age at diagnosis of 59 years (range 41–75 years).

All patients had previously undergone contrast-enhanced thoracic and upper abdominal CT to exclude those with unresectable disease due to local tumour invasion, contralateral pleural involvement or evidence of more distant metastatic disease. They then proceeded to contrast-enhanced MRI. A 1.5-Tesla Siemens Vision scanner was used, with T1 breath-hold 2D FLASH (fast low angle shot) images obtained in axial, sagittal and coronal planes, before and after intravenous administration of 0.1 mmol/kg gadolinium-diethylene-triamine pentaacetic acid (DTPA) contrast. Areas of interest identified on the CT scans were investigated further with T1 breath-hold gated and T1 2D FLASH cine images. Previous port, drain and biopsy sites were marked with oil capsules. MR images were reviewed independently by one of two specialist thoracic radiologists, and the tumour staged according to the International Mesothelioma Interest Group (IMIG) staging system [9]. Lymph nodes of short-axis diameter greater than 1.0 cm were considered pathologically enlarged, according to standard criteria for lung cancer [10]. In conjunction with radiological evaluation, the fitness of patients for surgery was assessed according to the British Thoracic Society guidelines for pneumonectomy [11].

Resectability was defined by the absence of mediastinal organ or full-thickness pericardial/myocardial involvement, absence of diffuse or multifocal chest wall disease, transdiaphragmatic extension or spread directly into the spine (any stage T4 disease). Contrast enhancement of sites of previous entry to the chest were not, in themselves, seen as any contraindication to radical treatment, as these were widely excised at surgery. Suitable patients then underwent extra-pleural pneumonectomy (EPP) or radical pleurectomy/decortication (PD) according to established techniques [12,13]. Systematic clearance of ipsilateral mediastinal and internal mammary lymph node groups was performed. The pre-operative CEMRI stage was compared to the final pathological stage obtained using the IMIG system mentioned previously.

2.1. Statistical analysis

The sensitivity, specificity, positive predictive value, negative predictive value, false positive rate and false negative rate were calculated to assess the ability of CEMRI to predict tumours of stage T3 or less. This was repeated for tumours of stage T2 or less and nodal disease of stage N2 or less. Time difference between CT and CEMRI, and patient survival, according to stage of pericardial involvement, was assessed using the Kaplan–Meier method [14].

3. Results

With CEMRI, contrast enhancement of tumour was seen in all patients (Fig. 1). CEMRI revealed the presence of 17 (22%) unresectable T4 or M1 tumours, which were not identified by CT. These were due to enhancement indicating mediastinal organ involvement in nine patients (Fig. 2), diffuse chest wall involvement in 2, infra-diaphragmatic spread in a further two and metastatic contralateral pleural disease in four patients. These findings were not confirmed histologically. In total, 51 patients proceeded to EPP or PD, and 25 patients had either no further surgical intervention or a lesser resection. Lesser resections included VATS debulking pleurectomy in five patients (T3 on CEMRI, but medically unsuitable for radical surgery) and chest wall resection for localised biphasic disease in two (T3 on CEMRI). One patient declined surgical intervention despite apparent resectability (T2N0 on CEMRI). The 17 patients with unresectable disease on CEMRI were included in this second group. For all patients, the median time interval

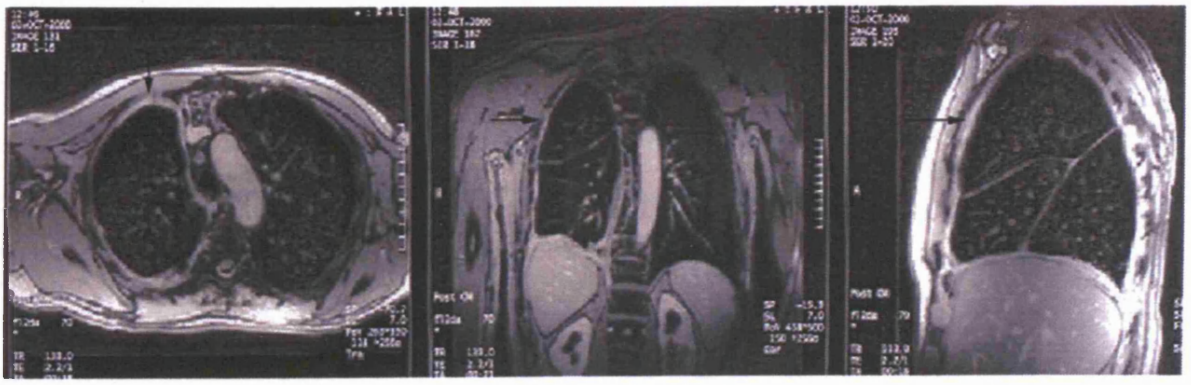

Fig. 1. Tumour enhancement of right-sided mesothelioma with CEMRI. This is seen surrounding the lung and involving the oblique and horizontal fissures.

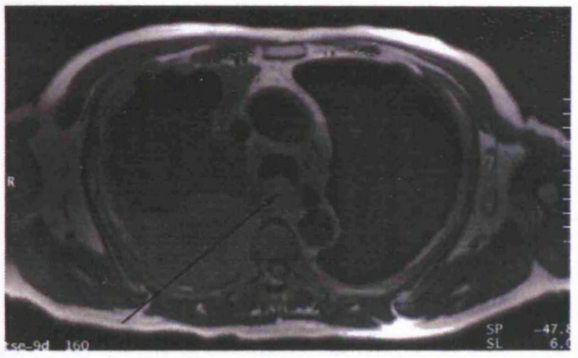

Fig. 2. T1 gated axial image showing unresectable right-sided mesothelioma, invading the trachea and oesophagus.

between CT and CEMRI was 43 days (range 0–609 days). In those who progressed to radical surgery the median time was 44 days (range 0–221 days) and in those with disease taken to be unresectable it was 25 days (range 2–609 days), $P = 0.82$.

Of the 51 patients in the radical surgery group, 44 had EPP and seven underwent PD. The median interval from CEMRI to radical surgery was 17 days (range 1–43 days). Two patients, in whom EPP was planned, were found to have more extensive mediastinal disease at thoracotomy than was predicted by CEMRI and so underwent PD. Thus, the sensitivity of the overall prediction of resectability was 97%. There was incomplete nodal staging in three of the patients who underwent PD and therefore they were excluded from all further analyses.

3.1. Pathological staging

Table 1 shows the breakdown of disease stages of the 48 patients with complete pathological information; 73% of patients were found to have stage III disease, and only five patients were within IMIG stages I or II.

3.2. Correlation of radiological and pathological staging

Concordance between MRI and pathological T stage was seen in 23 patients (48%). In 24 of the 48 cases (50%), the tumours were understaged. This was largely due to understaging of pericardial invasion (22 of 24 cases), but this was not found to compromise resectability. Overstaging of tumour was seen in one patient (2%). This occurred where apparent confluent visceral tumour (T2) seen on CEMRI was shown to be limited to scattered tumour foci on the visceral pleural surface (T1b) at thoracotomy. Considering nodal disease, 29 patients (60%) were correctly staged by CEMRI. This comprised 27 patients of status N0 and two patients with N2 disease. Eighteen patients were found to have more advanced nodal disease than predicted by MRI. Six (13%) of these had N1 disease and the remaining 12 (25%) had N2 disease where N0 status had been predicted. CEMRI overstaged nodal disease in one patient (2%), predicting N2 disease, where N0 was found.

Table 1
Comparison of IMIG stage, by MRI and surgical pathology in patients undergoing radical surgery ($n = 48$)

MRI stage	Pathological stage				
	I	II	III	IV	Total
I	0	0	0	0	0
II	1	4	16	1	22
III	0	0	19	5	24
IV	0	0	0	2	2
Total	1	4	35	8	48

Table 2
Stage T3 or less by MRI

		Pathological confirmation	
		T3 or less	Not T3 or less
Predicted on MRI	T3 or less	39	7
	Not T3 or less	0	2

In identifying those patients with stage T3 disease or less, CEMRI was found to have a sensitivity of 85% and a specificity of 100% (positive predictive value 100%; negative predictive value 22%; false positive rate 0%; false negative rate 15%) (Table 2). For T2 disease or less, the sensitivity fell to 23%, with a specificity of 96% (positive predictive value 83%; negative predictive value 59%; false positive rate 4%; false negative rate 77%) (Table 3). Using the same principle, but applying it to the prediction of N2 disease or less, for our series the sensitivity of CEMRI was 66%, and the specificity was 73% (positive predictive value 14%; negative predictive value 97%; false positive rate 26%; false negative rate 33%) (Table 4).

4. Discussion

This retrospective study represents a validation of contrast-enhanced MRI with the pathological results of radical surgery in malignant pleural mesothelioma, which has not previously been reported in detail.

Despite the finding of a number of patients with unresectable disease on CEMRI not previously identified on CT and although this was never confirmed histologically,

Table 3
Stage T2 or less by MRI

		Pathological confirmation	
		T2 or less	Not T2 or less
Predicted on MRI	T2 or less	5	17
	Not T2 or less	1	25

Table 4
Stage N2 or less by MRI

		Pathological confirmation	
		N2 disease present	No N2 disease present
Predicted on MRI	N2 disease present	2	1
	No N2 disease present	12	33

it is our observation that this discrepancy is due to the superior ability of CEMRI in the detection and assessment of malignant pleural disease, based on non-homogenous signal intensity or enhancement and certain specific morphological features. These features include circumferential pleural thickening, nodularity and irregularity. There is international agreement in this belief [6–8]. Based on this premise, and despite the successes achieved with limited debulking procedures [15], we felt ethically unable to subject patients to the considerable morbidity associated with, at the very least, exploratory thoracotomy. In some cases this would have required bilateral surgery. Although two patients did undergo thoracoscopic pleurectomy, the remaining patients chose continued treatment under the care of an oncologist. This explains the absence of pathological confirmation of the cases deemed unresectable on MRI.

In conflict with our opinion regarding CEMRI, a study by Heelen et al. comparing contrast-enhanced high-resolution CT reconstructs with unenhanced MRI found, at surgery, insignificant differences between the two techniques, leading the authors to find themselves unable to justify the increased cost of MRI [5]. It is apparent from the text that we have omitted any comparison with CT information in our cohort. The frequent supra-regional nature of patient referral to our centre and the widespread policy of obtaining CT scanning prior to biopsy means that, more often than not, CT imaging is obtained away from our centre leading to a lack of consistency, not only in the scan protocols, but using scanning equipment of different ages and from different manufacturers. For these reasons, a retrospective comparison of CT and CEMRI was considered impossible to justify. It is interesting to note that there was no significant difference in the time between CT and CEMRI in those patients undergoing radical surgery and those taken to be unresectable (radical, 44 days; unresectable, 25 days, $P = 0.82$), which would appear to discount time delay as a cause for unresectability.

It is important to appreciate that the process of the patient undergoing a CEMRI is not an insignificant event and there are contraindications. It has been shown that compared to CT, MRI leads to increased levels of anxiety in patients, due predominantly to the more enclosed environment [16]. To date, we have actually had only one patient who was unable to undergo CEMRI due to symptoms of claustrophobia. Within our local protocol for CEMRI, and relevant to the disease process itself, is the requirement for a 20-s breath-hold whilst obtaining images, which may lead to respiratory distress in those with significant intra-thoracic disease. Finally, those patients with metal implants such as pacemakers, are unable, or at least unsuitable, to undergo MR imaging.

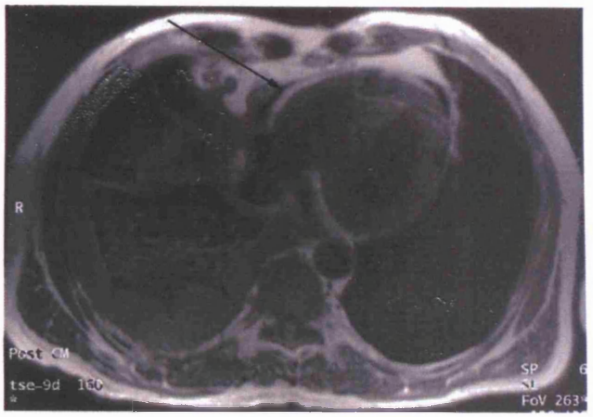

Fig. 3. T3 pericardium on CEMRI, T4 on pathology. Although a clear fat plane excludes involvement of the myocardium, the differentiation between partial and full-thickness pericardial disease is very difficult.

With a sensitivity of 85% and a specificity of 100%, CEMRI was very good at identifying those patients with stage T3 disease or less, therefore excluding unresectable stage T4 disease. However, over half of the cases were understaged due to more advanced pericardial involvement, although this was not found to compromise resectability. It is likely that no currently available imaging modality will prove sufficiently accurate to address the problem of staging pericardial involvement preoperatively (Fig. 3). The International Mesothelioma Interest Group staging system [9], developed in 1994, was based on the experiences of several pioneering investigators in the field of mesothelioma, and attempted to reconcile previously proposed staging systems using up to date information regarding the influence of T and N status on overall survival. Involvement of the pleura overlying the pericardium stages a tumour as either T1b or T2. With pericardial involvement the stage becomes T3. Should the tumour extend through to the internal surface of the pericardium, the stage advances to T4. All this occurs over the distance of a few millimetres, in close proximity to the constantly moving heart. Clearly, improving image definition will be difficult with currently available technology, despite the use of ECG cardiac gating. However, on examining this series of patients, we postulate that the worsening of prognosis with T4 disease may not apply to pericardial involvement. To illustrate this, the survival of patients found to have T3 and T4 pericardial involvement on pathological examination as either the worst, or equal to the worst area of disease was compared. There was no statistical difference in the median survival of the two cohorts (T3, 417 days; T4, 327 days, $P = 0.12$). Two possible explanations would appear plausible. The first concerns the small numbers in our series making definitive

statistical conclusions difficult and the second maybe that full-thickness pericardial involvement does not alter the prognostic significance and should not be considered as stage T4, unresectable disease. Greater numbers are needed to establish this with certainty. No patients with full-thickness pericardial tumour had evidence of malignant pericardial effusion or myocardial involvement.

CEMRI proved to be less than adequately accurate in assessing nodal disease (sensitivity 66%; specificity 73%, for N2 disease or less). Unfortunately 12 patients were predicted to have no nodal involvement and actually found to have N2 disease. In accounting for this, lymph nodes involved with metastatic tumour being of a size less than 1 cm in short-axis diameter may be implicated. This measurement is used as the radiological cut-off between uninvolved and involved lymph nodes in non-small cell lung cancer [10], but it is being increasingly realised to be a poor predictor of malignant nodal involvement in that disease [17]. There has been no comparable study in malignant pleural mesothelioma, but our data would imply a limited role, if any, for CEMRI in the assessment of nodal disease. Although not the focus of this work, given the data presented, there may, however, be an important role for cervical mediastinoscopy in the evaluation of patients under consideration for radical surgery.

This study was not designed to examine the ability of CEMRI to differentiate benign from malignant pleural disease, as pathological confirmation of MPM had already been obtained in all cases. It is, however, noteworthy that enhancement was seen in all cases. This property of tumour enhancement has previously been shown to be a useful indicator of malignant disease, although the consistently high negative predictive value of CEMRI may be of even greater value [7,8,18,19]. This raises the possibility of using CEMRI as a screening tool in this disease, or as an adjunct in the investigation of patients with pleural abnormalities identified on plain chest radiograph.

Within a regime of multimodality treatment, the idea of neoadjuvant chemotherapy is a concept of increasing interest in MPM. This idea has proven to be feasible in non-small cell lung cancer [20], with several trials active at present, including the LU22 trial in the United Kingdom [21]. The ability of CEMRI to differentiate between active and inactive pleural disease may be of great value in assessing patients treated with neoadjuvant chemotherapy prior to radical surgery for MPM. There are currently no published data on this subject.

The ability of PET scanning to evaluate actively metabolising cells, with extrapolation to the likelihood of malignancy, has led to a huge increase in the use of this technique. It has been shown to have a low false negative rate in the identification of malignant pleural disease [22] and be of value in the evaluation of the presence or absence of regional and distant metastases [23]. Its use is also likely to increase in the follow up of patients after radical surgery [24]. By measuring the intensity of isotope uptake, PET may have a role in prognostication in MPM [25]. Despite these advantages, it is unlikely to better the abilities of CEMRI in the assessment of local disease extent, although the use of fused PET and CT images is proving to be of benefit in patients with thoracic malignancies [26] and is likely to become more widely available in the near future.

It is acknowledged that CEMRI is inaccurate in the assessment of pericardial involvement, but this has made no difference in terms of resectability for our cohort of patients and may be less important in prognostic terms than previously thought. In the assessment of nodal status, CEMRI is unlikely to play a significant role. From a surgical viewpoint, its abilities lie in the differentiation of T3 and T4 disease, although it is perhaps less useful in the early stages of disease when it may confer no extra benefit compared to CT. It is very useful in the assessment of resectability and providing additional information through its multiplanar tumour localisation capabilities. It remains a valuable adjunct in the selection of patients for radical surgery.

Acknowledgements

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The Effect of Extent of Local Resection on Patterns of Disease Progression in Malignant Pleural Mesothelioma

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Background. We sought to determine whether or not there are differences in disease progression after radical or nonradical (debulking) surgical procedures for malignant pleural mesothelioma.

Methods. Over a 49-month period, 132 patients with malignant pleural mesothelioma underwent surgery. Fifty-three underwent extrapleural pneumonectomy and 79 underwent nonradical procedures. Time to evidence of clinical disease progression was recorded, as was the site(s) of that disease.

Results. One-hundred nineteen patients were evaluable, of which 59% (22 radical; 48 nonradical) had disease progression. Overall 30-day mortality was 8.5% (7.5% radical; 9% nonradical). The median time to overall disease progression was considerably longer after extrapleural pneumonectomy than debulking surgery (319 days vs 197 days, $p = 0.019$), as was the time to local disease progression (631 days vs 218 days, $p = 0.0018$). There was no preponderance of earlier stage disease in the radical surgery group. There was a trend toward prolonged survival in those undergoing radical surgery, but no significant difference between the groups (497 days vs 324 days, $p = 0.079$). In those who had extrapleural pneumonectomy, time-to-disease progression significantly decreased with N2 disease compared with N0/1 involvement (197 days vs 358 days, $p = 0.02$).

Conclusions. Extrapleural pneumonectomy may be preferable to debulking surgery in malignant pleural mesothelioma to delay disease progression and give greater control of local disease. Involvement of N2 nodes is associated with accelerated disease progression and is therefore a contraindication to extrapleural pneumonectomy.

(Ann Thorac Surg 2004;78:245–52)

The incidence of malignant pleural mesothelioma (MPM) continues to increase [1–3] and no single regimen has substantially and repeatedly altered the natural history of the disease for the majority of those affected [4, 5]. Unfortunately, a widespread nihilistic attitude towards MPM among many physicians has, in many cases, limited the role of surgery to that of obtaining histologic diagnosis or achieving symptomatic control of pleural effusions [6].

Lung parenchyma-sparing procedures have been proposed to have a role in patients with prohibitive comorbid conditions [7, 8] and they have led to notable symptomatic improvements [9]. They have been successfully combined with local adjuvant treatments [10, 11]. Despite this, the theoretical benefits of maximal tumor cytoreduction have led to the advocacy of radical surgery as a worthwhile modality in this disease [5, 12–15] and, in this context, multimodality treatments incorporating surgery and chemoradiotherapy have been shown to be feasible [16] and even demonstrated considerable survival benefits in select patients [17, 18]. We sought to evaluate our single-center experience of surgery for MPM in an attempt to assess the contribution of the choice of surgical procedure to differences in the patterns or onset of disease progression comparing radical with nonradical procedures.

Patients and Methods

Over a 49-month period, 132 patients underwent surgery for MPM. There were 121 men (92%) and 11 women (8%), with a median age at the time of diagnosis of 60 years (range 41–79 years). Fifty-three patients (40%) underwent radical surgery (median age 57 years) and 79 patients underwent nonradical procedures (median age 62.5 years). Within the nonradical group, 52 patients (39%) had open procedures (47 patients had tumor decortication and 5 patients had pleurectomy) and 24 (19%) had video-assisted thoracoscopic (VATS) interventions (6 patients had decortication and 18 patients had pleurectomy). Three patients (2%) had a localized chest wall tumor resection only and were further considered as part of the nonradical open-surgery group. Radical surgery, defined as extrapleural pneumonectomy (EPP), has been described in detail previously [19]. In variation to published techniques, the peritoneal cavity is opened with

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Abbreviations and Acronyms

CEMRI = contrast-enhanced magnetic resonance imaging
CT = computed tomography
EPP = extra-pleural pneumonectomy
IMIG = International Mesothelioma Interest Group
MPM = malignant pleural mesothelioma
N = nodal (stage)
PA = postero-anterior
PET = positron emission tomography
SUV = standardized uptake value
T = tumor (stage)
UICC = Union Internationale Contre le Cancer
VATS = video-assisted thoracoscopic surgery

removal of the hemidiaphragm, which is then reconstructed with a single Gortex patch (W. L. Gore & Associates, Flagstaff, AZ). The hemipericardium is reconstructed with Prolene mesh (Ethicon, Somerville, NJ) in all cases. All patients undergoing radical surgery have a mediastinal lymphadenectomy of stations 4, 7, and 10 at the time of surgery. Any station 5 nodes are removed in left-sided resections. All lesser lung parenchyma-sparing resections ranging from parietal pleurectomy to parietal and visceral surface tumor decortication with removal of the pericardium and hemidiaphragm [8] constituted nonradical procedures. Two patients due to undergo EPP were found to have unexpected local invasion at thoracotomy and so underwent tumor decortication. Patients undergoing surgical diagnostic biopsy or pleurodesis alone were excluded.

Radiologic assessment was initially performed with posteroanterior (PA) chest roentgenogram and then with computed tomography (CT). Contrast-enhanced magnetic resonance imaging (CEMRI) was used as indicated, most frequently in the radical surgery cohort [20]. Fitness for surgery was assessed according to British Thoracic Society guidelines [21]. Operation selection was dependent on the apparent stage of disease after appropriate clinical and radiologic investigation, the presence or absence of confounding comorbid conditions, and fully informed discussion with the patient. Patients in the radical surgery group had nonsarcomatoid tumors confirmed preoperatively in accordance with local policy.

Using information from preoperative imaging, operative findings, and pathologic detail, the tumor (T) status, nodal (N) status, and the overall stage was obtained using accepted tumor-node-metastasis (TNM) systems [22, 23]. In the nonradical group, where pathologic information regarding mediastinal lymphadenopathy was incomplete, nodal status was derived from cross-sectional imaging (Table 1).

All patients were reviewed at this center after their discharge from the hospital. Although there was no routine use of cross-sectional imaging during follow-up, all patients were subject to clinical examination and a PA chest roentgenogram. They did not undergo more extensive cross-sectional imaging unless there was clinical indication.

Patient demographic and operative details were collated using the departmental database and individual

Table 1. Patient Demographics (n = 132)

		Radical Surgery	Open Nonradical Surgery	Thoracoscopic Nonradical Surgery	*p* Value
Preop IMIG/UICC staging (pathologic stage)	I	1 (3)	2	10	
	II	18 (3)	18	9	
	III	34 (32)	27	3	<0.0001
	IV	0 (15)	8	2	
Histology	Epithelial	46	32	17	
	Biphasic	7	13	3	0.005
	Sarcomatoid	0	10	4	
ECOG performance status	0	39	19	8	
	1	14	31	14	0.0003
	2	0	5	2	
EORTC prognostic groups	1	35	25	4	
	2	18	30	20	0.002
CALGB prognostic groups	1	19	4	3	
	2	6	5	2	
	3	23	23	13	
	4	3	7	3	0.004
	5	2	13	2	
	6	0	3	1	

CALGB = US Cancer and Leukemia Group B; IMIG = International Mesothelioma Interest Group; ECOG = Eastern Cooperative Oncology Group; EORTC = European Organisation for Research and Treatment of Cancer; UICC = Union Internationale Contre le Cancer.

case notes were reviewed. Information regarding disease progression was categorized as either local, distant, or both. Where information was incomplete or absent, that patients' general practitioner was contacted in an attempt to collect as much complete information as possible.

Disease progression was defined as clinical or radiologic evidence of tumor with or without symptoms and with or without histologic or cytologic confirmation. In some cases, the presence or absence of tumor was confirmed or refuted at post-mortem examination. The boundaries of the pleural envelope on the operated side defined the distinction between local and distant progression. All noncontiguous disease outside of the pleural cavity was considered to be distant progression. This included distinct mediastinal lymphadenopathy arising after the original operative procedure, but did not include direct invasion into the mediastinum which was considered local progression.

Statistical Analysis

Comparison of preoperative variables, patient demographics, and the distribution of the pattern of detected disease progression in the two groups at the time of investigation and according to T and N stage in the radical surgery group alone was compared with the χ^2 test. The Kaplan–Meier method [24] was used to plot survival and time to progression curves with differences assessed using the log-rank test.

Results

There were four perioperative deaths (7.5%) in the radical surgery group and seven perioperative deaths (9%) in the nonradical surgery group. The overall 30-day mortality was 8.5% (11 patients). The median follow-up in the radical surgery group was 19 months (range 2–49 months, mean 22 months) and the median follow-up in the nonradical surgery group was 30 months (range 1–49 months, mean 26 months).

Histopathology

Histologic breakdown is detailed in Table 1. Sixteen patients (12%) in the radical surgery group were found to have microscopically involved resection margins, most frequently at sites of previous drain or VATS ports, which were macroscopically completely excised at surgery. These areas were irradiated after recovery from surgery. In the nonradical surgery group, although there was no formal quantification of tumor bulk, all patients had as a minimum, sufficient tumor debulking to ensure complete expansion of the lung on the operated side at the end of the procedure.

Overall Disease Progression

Of the 132 patients, 119 were evaluable and of these, 70 patients (53%) had evidence of disease progression. Pathologic confirmation was obtained and recorded in only 4 patients (6%). Two patients were in the radical surgery group (histology from an empyema cavity in one

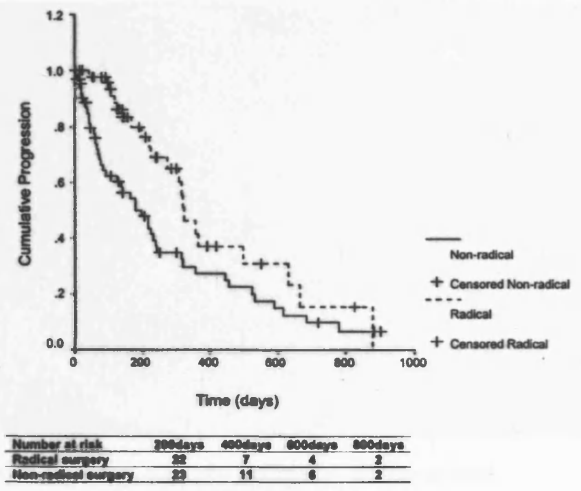


Fig 1. Time to evidence of disease progression. The median time to disease progression in the radical surgery group was 319 days compared with 197 days in the nonradical surgery group, p = 0.019.

and ascitic fluid cytology in another) and 2 patients were in the nonradical surgery group (histology from a chest wall mass in one and ascites cytology in another). Of the 119 patients, at the time of analysis, 22 patients (18%) had died without evidence of tumor progression, 27 patients (23%) were alive without progression, 10 patients (8%) were alive with progression, and 60 patients (51%) had died with disease progression. Patients who died with no evidence of disease progression included those who died in the perioperative period. Causes of death included cardiac arrest secondary to acute right ventricular failure and respiratory failure from lower respiratory tract infection and pulmonary embolism.

The median time to first evidence of disease progression was notably longer in the radical surgery group compared with the nonradical surgery group (Fig 1). This difference was maintained irrespective of the approach to debulking (thoracotomy or VATS) (Fig 2). There was a significant difference in the distribution of disease progression between the two groups ($p = 0.0071$) with a greater proportion of those patients undergoing nonradical surgery having local disease progression (Table 2).

In the radical surgery group, sites of local progression alone included thoracotomy wound (3 patients) and chest wall (4 patients) (Fig 3). Three patients had distant disease in the contralateral hemithorax and 4 patients had evidence of isolated abdominal progression (Fig 4). In the nonradical group, sites of local progression included the operated pleural cavity (18 patients) (Fig 5), the chest wall alone (11 patients), and the surgical wounds (6 patients). Two patients had evidence of disease in the contralateral hemithorax alone and 3 patients had purely abdominal progression. No patients had first evidence of disease progression anywhere other than either hemithorax or in the abdomen.

There was no significant difference in the time to evidence of distant disease progression ($p = 0.18$); how-

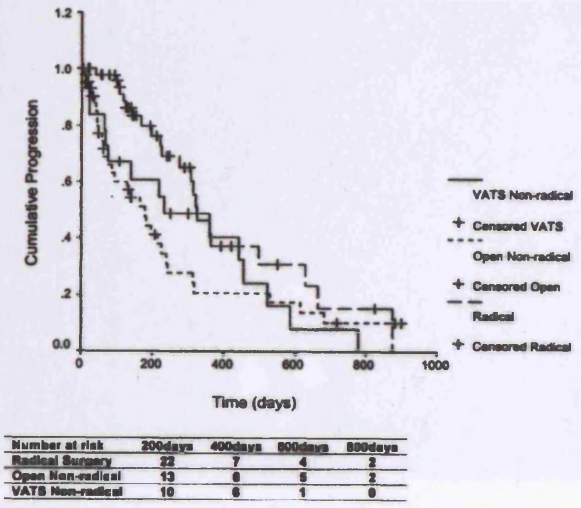


Fig 2. Time to evidence of disease progression (subdivided into three operative groups). The marked difference in time to evidence of progression was preserved when the nonradical group was subdivided into open and thoracoscopic procedures (319 days in the radical group and 177 days and 232 days, respectively, in the nonradical groups, p = 0.038). (VATS = video-assisted thorascopic surgery.)

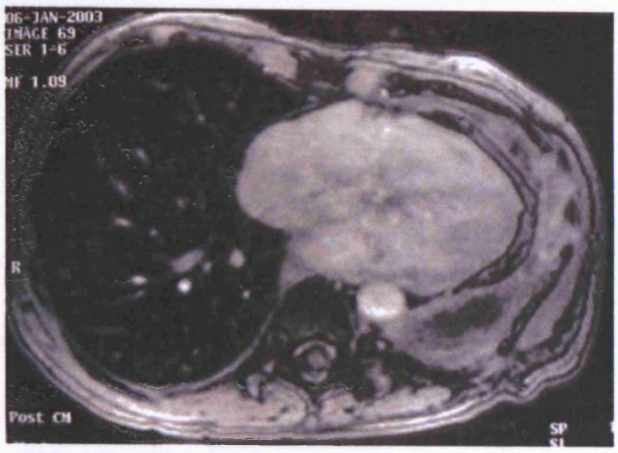


Fig 3. Ipsilateral chest wall progression after extrapleural pneumonectomy.

ever, the interval to local progression was longer after EPP compared with those in the nonradical surgery group (Fig 6). Once again, this also held true when the nonradical group was further subdivided into open or VATS approach (631 days, 177, days and 358 days, respectively, $p = 0.007$). There was no significant difference in the time to overall ($p = 0.479$), local ($p = 0.510$), or distant ($p = 0.874$) progression when comparing patients who had open debulking procedures with those who had parietal pleurectomy or a VATS approach.

Survival

Although a trend toward increased survival in the radical surgery cohort was noted, there was no significant difference between the two groups (497 days; 324 days, $p = 0.079$). The histologic subtype significantly impacted upon both survival figures and also the time to evidence of disease progression. In the radical surgery group, those patients with epithelial histology had a significantly longer survival than those with biphasic disease (538 days; 237 days, $p = 0.008$). Similarly, in the nonradical group those patients with epithelial histology had a median survival of 475 days compared with 324 days and 128 days for biphasic and sarcomatoid histology, respectively ($p < 0.0001$). There was no significant difference between the median survival of the radical and nonradical surgery groups when only those patients with epithelial disease were considered ($p = 0.23$).

The Effect of Histologic Subtype on Disease Progression

In the radical surgery group, epithelial histology was associated with delayed disease progression when compared with the biphasic subtype (364 days; 275 days, $p = 0.008$). This was also seen in the nonradical surgery group (epithelial 223 days; biphasic 162 days; sarcomatoid 62 days, $p = 0.0005$). When those patients with only epithelial disease were considered, radical surgery patients had a significantly longer median time-to-disease progression than did those from the nonradical surgery group (Fig 7).

The Effect of Operative Procedure

There was a trend to increased survival in those patients undergoing EPP when compared with those patients undergoing either pleurectomy or tumor decortication (497 days; 387 days; 260 days, $p = 0.082$). However, the median time to onset of disease progression in the radical surgery group was 319 days, in those patients undergoing pleurectomy it was 242 days, and for those having tumor

Table 2. Sites of First Disease Progression After Surgery (p = 0.0071, df = 2, χ^2)

Site of Progression	Radical Surgery		Nonradical Surgery		Total
	Number of Patients	% of Those With Progression	Number of Patients	% of Those With Progression	
Local	7	10%	34	48%	41
Distant	7	10%	5	7%	12
Both	8	11%	9	14%	17
Total	22	31%	48	69%	70

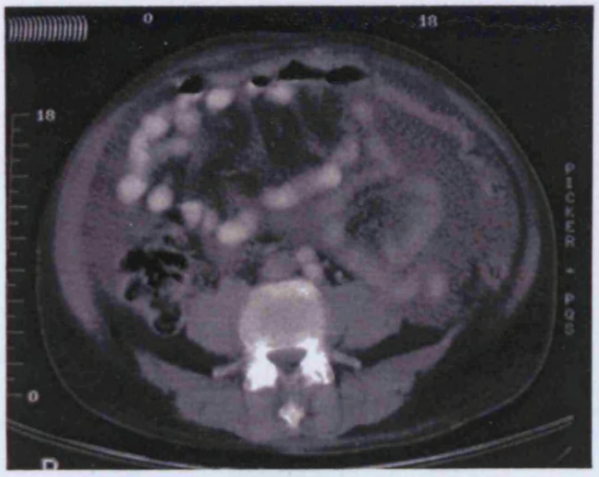


Fig 4. Abdominal progression after extrapleural pneumonectomy.

decortication it was 177 days ($p = 0.047$). When only those patients with epithelial histology were considered, although a trend did remain, it was not statistically significant (364 days; 242 days; 197 days, $p = 0.104$).

Predictors of Disease Progression

In those patients who underwent EPP, patients with N0 or N1 disease were seen to have a significantly longer median time from operation to evidence of overall disease progression than those with N2 disease (Fig 8). There was, however, no significant difference in the distribution of disease progression according to T stage ($p = 0.29$) or N stage ($p = 0.14$) in the radical surgery group at the time of analysis and N2 disease did not lead to more rapid distant disease progression ($p = 0.22$). Isolated local disease developed in only 1 out of 16 patients with incomplete resection margins as the first evidence of progression.

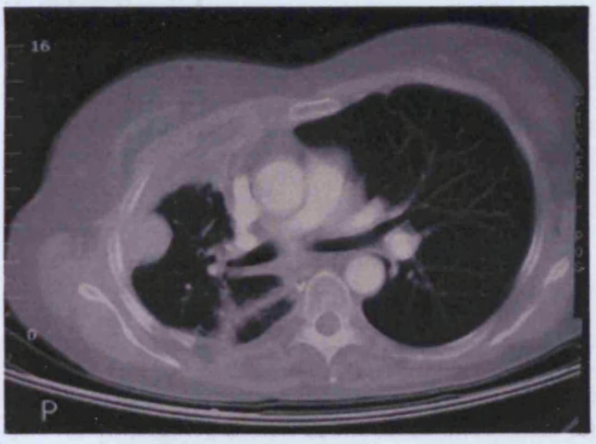
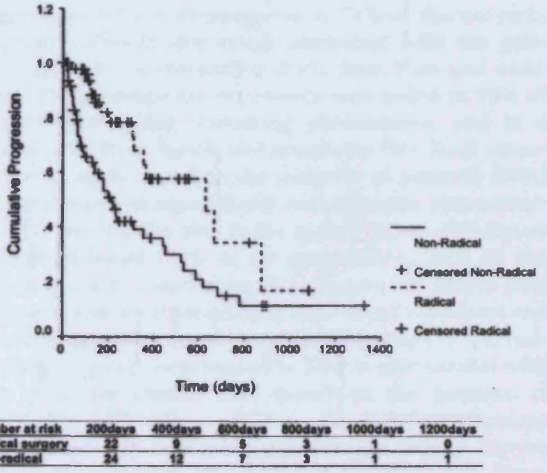


Fig 5. Pleural cavity progression after nonradical surgery.

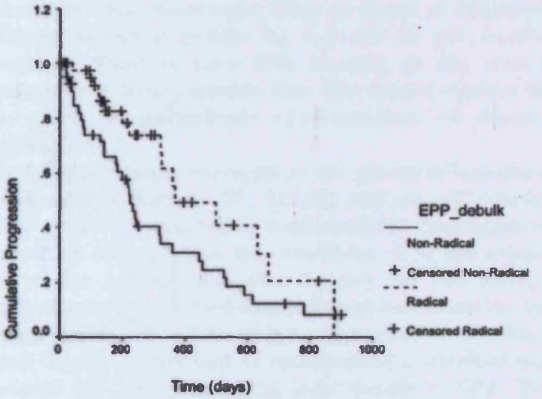


Number at risk	200days	400days	600days	800days	1000days	1200days
Radical surgery	22	9	5	3	1	0
Non-radical	24	12	7	3	1	1

Fig 6. Time to evidence of local disease progression. The median time to evidence of local disease progression in the radical surgery group was 631 days compared with 218 days in the nonradical surgery group, p = 0.0018.

Adjuvant and Neoadjuvant Treatment

All patients were referred for radiotherapy to surgical scars after recovery from surgery, but no patients underwent extensive treatments, such as radical hemi-thoracic irradiation. Eight patients undergoing radical surgery had neoadjuvant chemotherapy, all receiving a maximum of three cycles of Cisplatin and Gemicitabine before surgery. Two patients from the radical surgery group had planned adjuvant chemotherapy after the operative



Number at risk	200days	400days	600days	800days	1000days
Radical surgery	19	7	4	2	0
Non-radical	21	10	5	2	0

Fig 7. Time to evidence of disease progression (comparing radical and nonradical surgery groups with epithelial histology only). When considering those patients with epithelial histology alone, the median time to evidence of disease progression in the radical surgery group was 364 days compared with 223 days in the nonradical surgery group, p = 0.0035. (EPP = extra-pleural pneumonectomy.)

Ann Thorac Surg
2004;78:245–52

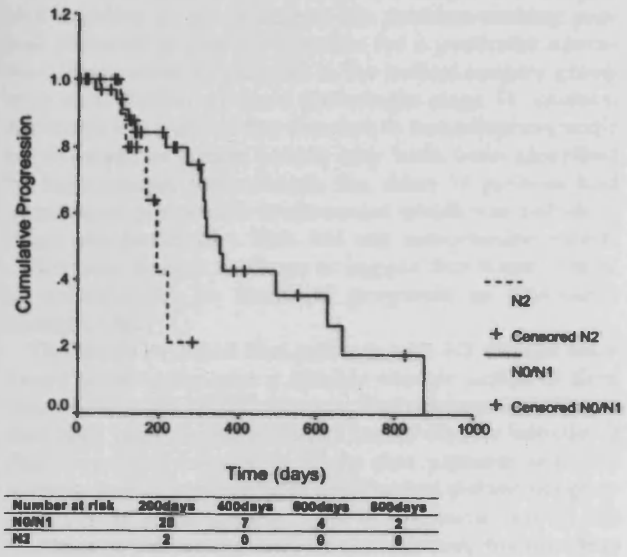


Fig 8. Disease progression within the radical surgery group (stratifying for nodal stage). Within the radical surgery group alone, the median time-to-disease progression in those patients with N0/N1 disease was 358 days compared with 197 days for those patients with N2 disease, $p = 0.02$.

procedure. In the nonradical surgery group, all patients were referred to an oncologist for consideration of adjuvant chemotherapy, but its use was largely dependent on individual patient symptoms. This situation is typical of current UK practice.

Comment

This retrospective review of the patterns of disease progression after radical and nonradical surgical procedures represents one of a limited number of direct comparisons to date [13, 25]. It demonstrates a considerable delay in time-to-clinical disease progression between the two groups of patients which was maintained for local progression alone. In addition to this, a greater proportion of patients in the radical surgery group were found to have stage 3 and stage 4 disease compared with the nonradical group (88% vs 41%, respectively). It is conceded that, had more frequent cross-sectional imaging been employed in the follow-up of these patients, it is possible that the interval-to-onset of disease progression may be different. However, clinical evidence of disease progression is far more realistic and important to the patients themselves. That was the primary reason for choosing this particular end-point.

The main aim of nonradical operative procedures was to allow complete lung expansion and prevent the reaccumulation of malignant pleural fluid in an attempt to improve quality of life by reducing dyspnea. Given the less extensive nature of the surgical procedures involved and that complete macroscopic clearance of tumor is rarely achieved in nonradical surgery for MPM, it was not surprising to find the high incidence of local disease progression (48% of all progression, 71% of the nonradical group). This is very much consistent with the published data. In a comparative study from Pass and associates [13], locoregional recurrence was noted in 90% of patients undergoing debulking pleurectomy and in a phase II trial from Rusch and associates [26], local recurrence was again noted in the majority of patients (80%) despite the use of intrapleural and systemic chemotherapy. It is noteworthy that in the radical group, this figure is much reduced (10% of all progression, 32% of the radical group). Combining the figures of those who presented with local progression and those with local and distant progression, local progression overall in the radical surgery group becomes 68%. This is comparable with three previous studies that examined the patterns of failure after EPP with or without adjuvant chemotherapy and radiotherapy, where the percentages of local disease recurrence were 67% [27], 54% [28] and 83% [29]. However, the different end-point considerations and data presentation make comparisons liable to inaccuracies. For example, in a study from Aziz and associates [30], local recurrence is given as 12% of the total number of patients undergoing EPP. It is unclear as to whether any patients suffered disease recurrence in more than one site, but considering local disease only and looking at the total number of patients in the radical group and not just those with progression, in this study the figures are again comparable with 13% of all EPP patients with evidence of local disease progression first.

One of the study limitations is the lack of histologic or cytologic confirmation of progression. This information was only available from 4 patients. Given the nature of MPM, if the attending clinician was satisfied by history and examination (coupled with diagnostic imaging) that disease progression was apparent, pathologic confirmation might never be sought. Only in times of diagnostic difficulty would a patient be exposed to yet another procedure, likely to have little bearing on the plan of management. It is probable that this would explain the low rates of pathologic confirmation of disease progression.

Despite published examples of the potential benefits of multimodality therapy [17, 31, 32], current UK practice rarely considers aggressive multimodality management of patients with pleural mesothelioma. It is the experience of the authors that, after surgery for this disease, most patients are advised that adjuvant treatment should be postponed until a time of symptom recurrence. This is based largely on the lack of randomized controlled-trial evidence for any therapy in this condition [33]. This explains the almost single-modality treatment seen in this narrative. In an attempt to encourage the use of adjuvant chemotherapy and also to assess its feasibility, neoadjuvant chemotherapy has been used in a small number of patients.

In this series, the patients in the radical surgery group experienced substantially better performance status and had better preoperative prognoses. There were also more patients with epithelial histology than in the nonradical group. Although this reflects the unavoidable bias from a

lack of randomization inherent to a retrospective study, it also explains to some degree the decision-making process in terms of patient selection for a particular operation. There were 15 patients in the radical surgery group who were found to have pathologic stage IV disease. Although in 1 patient this was due to transdiaphragmatic involvement of tumor (which may have been identified by laparoscopic assessment), the other 14 patients had transmural pericardial involvement which was not identified preoperatively. This did not compromise resectability and there is evidence to suggest that it may not be as considerable in terms of prognosis as previously thought [20].

The study revealed that patients with N2 disease were found to progress over a notably shorter period of time than those with N0/N1 disease. The obvious assertion is that they have more advanced occult disease initially. If this were the case, it is likely that patients with N2 disease would present with evidence of distant progression before local disease. This is not borne out by the findings in this study, however, at present, the numbers available for this subset analysis is small. Also, increased systemic dissemination would seem unlikely to be a reason for the observed high rates of local disease progression even in N2 disease. Previously, cross-sectional imaging determined nodal size and was relied upon before radical surgery. As work within our unit has shown that nodal size does not predict malignancy [34], it is now routine to perform cervical mediastinoscopy before radical resection. In a number of cases, this explains the pathologic finding of N2 disease.

There are limitations in the staging of the nonradical surgery patients in this series which may underestimate the presence of N2 positive disease in this group. Because of the less extensive nature of the surgery, few people in the nonradical cohort underwent lymphadenectomy and therefore pathologic correlation with preoperative cross-sectional imaging was very limited. This may mean that the true pathologic stage of the nonradical surgery group would be closer to that seen in the radical group. However, there is currently no evidence suggesting that the results can be explained solely by stage differences.

The definitions of local and distant disease progression were an attempt to separate those patients with disease progression due to tumor growth within the operated pleural cavity and those with true metastatic spread. However, the situation is less clear with abdominal progression. Through correlation of survival data with the degree of pleural involvement, Boutin and associates were able to show that the parietal and diaphragmatic pleural surfaces were the first to become involved with tumor [35]. Furthermore, the Butchart staging system describes direct penetration of tumor through the diaphragm into the peritoneal cavity as stage III [36]. There is no doubt that this does occur, but it would also seem possible to have distinct metastatic abdominal progression. In this study, 4 patients within the radical surgery group had evidence of abdominal disease progression with no radiologic evidence of disease within the pleural cavity on cross-sectional imaging (Fig 4). Interestingly, the peritoneal cavity is opened in all the patients undergoing EPP at our center without a disproportionate increase in abdominal progression which may question the protective value of the intact peritoneum.

Conclusion

The assessment and management policies within this unit have markedly evolved over time. A greater number of patients are now undergoing radical surgery than at the beginning of the study period.

This work adds further weight to the contention that maximal tumor cytoreduction by EPP in medically fit patients is preferable to debulking surgery in the management of patients with MPM because it delays the onset of overall disease progression by providing greater local disease control. Furthermore, the observation that N2 nodal involvement is associated with accelerated disease progression emphasizes the importance of this factor in operative selection. Future work should focus on the potential survival benefit offered by EPP balanced against the detrimental effect on quality of life.

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APPENDIX V

SELECTED PRESENTATIONS

The inter-relationship of hypoxia and phospho-Akt in the pathobiology of malignant pleural mesothelioma

Stewart DJ, Edwards JG, Richardson D, Jones L, Burke B, Waller DA, Zeigler-Heitbrock L, Wardlaw A, O'Byrne KJ

Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings Part I. Vol 24, No. 18S (June 20 Supplement), 2006: 17057.

The Regulation and Prognostic Significance of NF-κB in Malignant Mesothelioma

Jennions E, Swain WA, Stewart DJ, Edwards JG, O'Byrne KJ, Walker RA

Annual meeting of the British Thoracic Oncology Group, Dublin, Ireland, *25-28 January 2006 (poster).*

Median Sternotomy Versus Thoracotomy for Right Extra-Pleural Pneumonectomy in Malignant Pleural Mesothelioma

Stewart DJ, Martin-Ucar AE, Edwards JG, West KJ, Waller DA

Annual Scientific Meeting of the Society of Cardiothoracic Surgeons of Great Britain and Ireland, London, *5-8 March 2005 (oral).*

Resistance of Malignant Pleural Mesothelioma Cell Lines to Prolonged Severe Hypoxia

Stewart DJ, Edwards JG, Burke B, Waller DA, Ziegler-Heitbrock L, O'Byrne KJ, Wardlaw AJ

Annual Meeting of the British Thoracic Oncology Group, Dublin, Ireland, *26-29 January 2005 (poster).*

Phospho-akt Expression is Associated with a Favourable Outcome in NSCLC

Shah A, Swain WA, Richardson D, Stewart DJ, Richardson CM, Swinson DEB, Patel D, Edwards JG, Jones JL, O'Byrne KJ

Annual Meeting of the British Thoracic Oncology Group, Dublin, Ireland, *26-29 January 2005 (poster).*

The Impact of Active Nuclear Factor kappa B in Malignant Mesothelioma

Jennions L, Stewart DJ, Edwards JG, Waller DA, O'Byrne KJ, Walker RA

Annual Meeting of the British Thoracic Oncology Group, Dublin, Ireland, *26-29 January 2005 (poster).*

The Expression of Carbonic Anhydrase IX in Malignant Pleural Mesothelioma

Stewart DJ, Edwards JG, Jones JL, Richardson D, Swinson DEB, Waller DA, Wardlaw AJ, O'Byrne KJ.

Young Investigators Session presentation, 7th meeting of the International Mesothelioma Interest Group, Brescia, Italy, *24-26 June 2004 (oral)*.

Proc 7th Meeting Int Mesothelioma Interest Group; 104

Predictors of Complications of Extra-Pleural Pneumonectomy for Malignant Pleural Mesothelioma

Stewart DJ, Martin-Ucar A, Edwards JG, Waller DA.

SCTS Annual Scientific Meeting, Beau Sejour Centre, St Peter Port, Guernsey, Channel Islands, *6-9 March 2004 (oral)*.

Predictors of Complications of Extra-Pleural Pneumonectomy for Malignant Pleural Mesothelioma

Stewart DJ, Martin-Ucar A, Edwards JG, Waller DA.

The Midlands Cardiothoracic Surgical Meeting, Coombe Abbey Hotel, Coventry, *27 February 2004 (oral)*.

Complications of Extra-Pleural Pneumonectomy for Malignant Pleural Mesothelioma: Incidence and Management

Stewart DJ, Pilling JP, Martin-Ucar A, Edwards JG, Waller DA.

British Thoracic Society Winter Meeting, Queen Elizabeth II Conference Centre, London, *3-5 December 2003 (oral)*.

Thorax 58 (Suppl III) iii25.

Are Patterns of Disease Progression Different in Patients Undergoing Radical Surgical Procedures Compared with Debulking Procedures in Malignant Pleural Mesothelioma?

Stewart DJ, Pilling JE, Edwards JG, Martin-Ucar A, Waller DA

10th World Conference on Lung Cancer, Vancouver, Canada, *10-14 August 2003 (poster)*.

Lung Cancer 41 (Supp; 2); S273

Hypoxia Inducible Factor-1alpha in Non-Small Cell Lung Cancer: Relation to Other Biological Factors and Prognosis

Swinson DEB, Jones JL, Cox G, Stewart DJ, Richardson D, Harris AL, O'Byrne KJ

10th World Conference on Lung Cancer, Vancouver, Canada, *10-14 August 2003 (poster)*.

Lung Cancer 41 (Supp; 2); S214

The Use of Contrast-Enhanced Magnetic Resonance Imaging in the Pre-Operative Staging of Malignant Pleural Mesothelioma – The Five Year Experience of a Single Centre

Stewart DJ, Edwards JG , Entwisle JJ, Jeyapalan K, Waller DA

6th Meeting of the International Mesothelioma Interest Group, Perth, Australia, *1-4 December 2002 (oral).*

Proc 6th Meeting Int Mesothelioma Interest Group; 27

The Mulitdisciplinary Management of Malignant Pleural Mesothelioma: Changing Practice Over a Fifteen Year Period

Stewart DJ, Edwards JG, O'Byrne KJ, Waller DA

6th Meeting of the International Mesothelioma Interest Group, Perth, Australia, *1-4 December 2002 (poster).*

Proc 6th Meeting Int Mesothelioma Interest Group; 34

Changing Practice in the Surgical Management of Malignant Mesothelioma

Stewart DJ, Edwards JG, O'Byrne KJ, Waller DA

European Society of Thoracic Surgeons, 10th Annual Meeting, Istanbul, Turkey, *24-26 October 2002 (oral).*

The Influence of a Multidisciplinary Team Approach on the Management of Malignant Mesothelioma

Stewart DJ, Edwards JG, Waller DA

Midlands Cardiothoracic Surgical Meeting, Birmingham, *March 2002 (oral).*

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