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by

Kamal Chitkara

MRCP

University of Leicester

Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Abbreviations

ACT	Activated coagulation time
ADP	Adenosine diphosphate
PBS	Phosphate-buffered saline
BFGF	basic fibroblast growth factor
C7E3	Abciximab
CABG	Coronary artery bypass grafting
EDTA	EthyleneDiamineTetraacetic Acid
FDA	Food and Drug Administration
Gp	Glycoprotein
HUVEC	Human umbilical vein endothelial cell
IVUS	Intravascular ultrasound
LMWH	Low molecular weight heparin
MI	Myocardial infarction
MLD	Mean luminal diameter
LDD	Local drug delivery
LST	Late stent thrombosis
MMP	Matrix metalloproteinase
NO	Nitric oxide, endothelium derived relaxation factor
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PTCA	Percutaneous transluminal coronary angioplasty
SEM	Scanned electron microscopy
SMC	Smooth muscle cells
TBS	Tris-buffered saline
TGF-β	Tissue growth factor β
TLR	Target lesion revascularisation
TPA	Tissue type plasminogen activator
VEGF	Vascular endothelial growth factor

Chapter 1

1 Introduction

1.1 Background and historical overview of Revascularisation

Coronary heart disease (CHD) by itself is the most common cause of death in the UK accounting for around 125,000 deaths a year: approximately one in four deaths in men and one in six deaths in women. CHD by itself is the most common cause of premature death in the UK: 26% of premature deaths in men and 16% of premature deaths in women are from CHD. Nearly all deaths from CHD are because of a heart attack. Over 270,000 people in the UK suffer a heart attack each year. In about 30% of heart attacks the patient dies before reaching hospital. Death rates for CHD have been falling in the UK since the late 1970s. For adults under 65 years they have fallen by 40% in the last ten years. In year 2001 alone, CHD caused over 120,000 deaths. Over 2.5 million people suffer from angina: the most common form of CHD, costing the health care in the UK about £7.9 billion a year. The number of revascularisation procedure has doubled up in last 10 years; annually over 70000 angioplasties and 28500 bypass surgeries are carried out in UK. This represents a four-fold increase over the last decade and a 12 % increase between 2004-2005 (*www.bhf.org.uk*).

Angina pectoris is a common manifestation of coronary artery disease. Effective treatment was not available until Brunton introduced nitrate of amyl in 1867[1]. Drugs for the treatment of chronic angina became available much later, first the long acting nitrates, β blockers in the 1960s, and calcium antagonists in the 1970s.

Of the drug treatments, there is evidence that only β blockers reduce both angina and cardiac events[2]. Aspirin, coumadin, and statins have no antianginal properties but do reduce subsequent cardiovascular events[3;4]. To prevent ischaemic events, such as angina attacks and myocardial infarction in elective patients with severe coronary artery disease, coronary artery bypass grafting **(CABG)** has become a standard

treatment and its benefit has been proven for patients with severe coronary artery disease, such as left main stem disease or severe triple vessel disease with mild impairment of left ventricular function (ECSS, 1982; CASS, 1994 and VA CABS Cooperative Study Group 1984) against medical therapy. The procedure came into widespread use after Dr. David C. Sabiston Jr. performed the first coronary bypass surgery in a human in 1962. Although coronary artery bypass surgery is suitable in many cases as a treatment that will overcome this ischaemia, it is an obviously invasive procedure with significant morbidity and mortality attached to it.

General agreement prevails that patients with ongoing or recurrent ischaemia and life-threatening complications should undergo coronary angiography and revascularisation. In the past few years, follow up management by early conservative strategy or early invasive strategy has been considered for patients with unstable angina or infarction without ST elevation. Early conservative strategy involves risk stratification by using clinical data and non-invasive testing and by resorting to coronary angiography only for patients who have recurrent symptoms or a stress test result that is positive for ischaemia. In early invasive strategy, coronary angiography is routinely recommended and further revascularisation performed if anatomically appropriate.

The recent results of the Clinical Outcomes Utilizing Revascularization and Aggressive Drug Evaluation (COURAGE) trial[5] suggest that PCI plus stenting and optimal medical therapy is no better at preventing future events than optimal medical therapy alone in patients with stable coronary disease. Between 1999 and 2004, the COURAGE trial enrolled and randomized 2287 patients either to PCI plus optimal medical therapy or to optimal medical therapy alone. Over a follow-up period ranging

from 2.5 to 7.0 years, a total of 211 all-cause deaths or nonfatal MIs (the primary outcome of COURAGE) occurred in the PCI group, compared with 202 in the medical-therapy group, a statistically non-significant difference. When outcomes were analysed individually, there were no differences in rates of deaths, MI, stroke, or hospitalization for acute coronary syndromes between the PCI and medical-therapy groups. The only statistically significant difference between the two treatment strategies was reduced prevalence of angina, which was greater in the PCI group at one and three years. However, by five years in part a reflection of subsequent revascularization in the medical-therapy group, there was no significant difference in freedom from angina, with roughly 73% of both groups reporting no angina at five years. . The results of this study support the recommendations in the National Service Framework for Coronary Heart Disease that revascularisation should be considered for patients with evidence of continuing extensive ischaemia (e.g. a strongly-positive exercise test) or angina that persists despite optimal medical therapy and lifestyle advice.

Pathogenesis of Atherosclerosis

Angina is caused by atherosclerosis in the coronary blood vessels. Atherosclerosis comes from the Greek words athero (meaning flour paste) and sclerosis (hardness). It's is a slow, complex disease in which deposits of lipids, cellular waste products, and calcium build up in the inner lining of an artery. This build-up is called atheroma or plaque. Eventually, this fatty tissue can erode the wall of the artery, reduces its elasticity (stretchiness) and interferes with blood flow. Plaques can also rupture, exposing a thrombogenic surface to the blood stream causing platelet adhesion, activation and aggregation leading to platelet rich blood clot formation which can temporarily diminish or block the blood flow causing unstable angina pectoris, or lead

to a persistent blockage causing myocardial infarction. The earliest phase of atherosclerosis is fatty streak formation which is a pure inflammatory lesion, consisting only of T lymphocytes and monocyte-derived macrophages[6]. Recently there has been emphasis on the involvement of inflammation in mediating all stages of atherosclerosis[7;8]. However, in addition to inflammation, a key process of atherosclerosis involves the proliferation of VSMCs[9;10] and accumulation of VSMCs within the intima[11] causing stenosis of the arteries.

Initially endothelial denudation was thought to be as a first step in atherosclerosis, postulated by response-to-injury hypothesis[12] (Figure 1.1) but the recent version of hypothesis emphasized endothelial dysfunction[13], the cause of which is thought to be reduced production of nitric oxide and free radicals caused by smoking cigarettes, hypertension, diabetes mellitus, infectious micro-organisms such as *Chlamydia Pneumoniae* and elevated plasma homocysteine levels. Endothelial dysfunction triggers a cascade of events: inflammatory cells and vascular cells release cytokines and growth factors, upregulation of leucocyte adhesion molecules (integrins, L-selectin) and endothelial adhesion molecules (E-selectin, P-selectin, ICAM, VCAM) and migration of leucocytes into the artery wall. VSMCs migrate, proliferate and synthesize extracellular matrix components on the luminal side of the vessel wall, forming the fibrous cap of the atherosclerotic lesion. Inflammatory mediators ultimately induce thinning of the fibrous cap by expression of proteases, rendering the plaque weak and susceptible to rupture and thrombus formation.

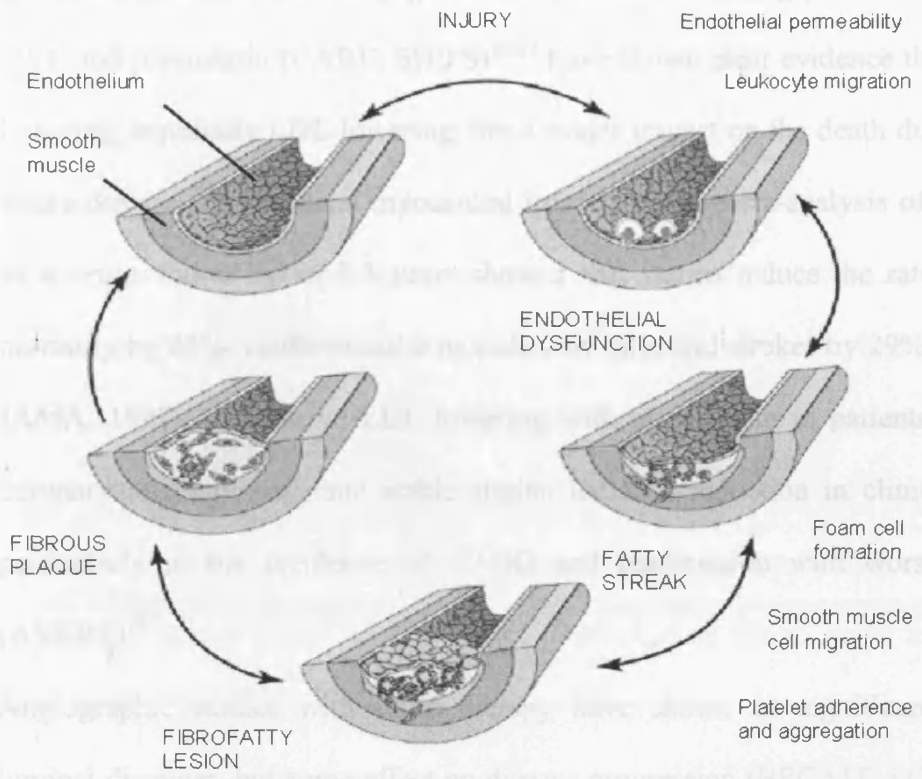


Figure 1.1: The response to injury hypothesis of atherosclerosis (modified after: Ross 1999; Encyclopaedia of Medical Images).

LDL (Low density lipoproteins) appears to be a major cause of injury to the endothelium[14], when they get oxidized and are rapidly taken up by smooth muscle cells leading to internalisation by macrophages and facilitation of the accumulation of cholesterol esters, resulting in the formation of foam cells[15] that leads to the deposition of connective tissue cells and elements. This modified lipoprotein stimulates the replication of monocyte-derived macrophages and the entry of new monocytes into lesions exacerbating the inflammatory response. This LDL remains soluble in blood plasma and passes through endothelium contributing to the development of plaques. The management of Hypercholesterolaemia and coronary artery disease has changed forever with the introduction of HMG-CoA reductase

inhibitors (statins). Secondary prevention studies comparing placebo to simvastatin (4S)[4] and pravastatin (CARE, SHIPS)[16;17] have shown clear evidence that cholesterol lowering, especially LDL lowering, has a major impact on the death due to coronary artery disease and non-fatal myocardial infarctions. A meta-analysis of 16 trials and an average follow up of 3.3 years showed that statins reduce the rate of all-cause mortality by 22%, cardiovascular mortality by 28% and strokes by 29% (Herbert PR, JAMA, 1997). Aggressive LDL lowering with atorvastatin in patients with known coronary artery disease and stable angina led to a reduction in clinical events, in particularly in the incidence of CABG and readmission with worsening angina (AVERT)[18].

Angiographic studies with statin therapy have shown no significant increase in luminal diameter, but some effect on disease progression (BECAIT, SCRIP, CCAIT, CLAS-I, PLAC-I, REGRESS)[19-24] focusing on alternative mechanisms of action by which statins improve clinical outcome. There is some evidence that statins augment the restoration of endothelial-dependent vasodilatation improving endothelial function even after a short period of treatment (6 weeks). They stabilise atherosclerotic plaques[25] and inhibit thrombus formation[26-28].

Homocysteine (amino acid) was suggested to damage cells directly by promoting oxidative stress and mural thrombosis and thus could be a major contributor to atherosclerosis[29] However, homocysteine may be merely a marker of disease and modifying it may not have an impact on disease progression. Several trials studied the effect of B-vitamin supplementation to prevent atherosclerotic disease. The results of these trials were conflicting. Results from a 3-year follow-up study (Folate After Coronary Intervention Trial-FACIT[30] found higher rates of restenosis and MACE (Major Adverse Cardiac Events) in patients randomised to receive supplemental folate

and vitamin B therapy compared with those receiving placebo. This effect was seen due to strong proliferative effect on cell growth with folate. It increases cell metabolism and promotes neointimal hyperplasia.

Chlamydia pneumoniae infection was thought to play a role in atherosclerosis by inducing monocytic matrix degradation via activation of plasminogen and the MMP activation system; this matrix disruption subsequently results in plaque rupture. It is found within the plaque, reaches high concentrations within macrophages and is rarely found in normal coronaries, but there is no direct evidence that this organism causes the lesions of atherosclerosis[31]. No reduction in cardiovascular events was seen in 2 years after 3 months of treatment with the antibiotic azithromycin, in the ACADEMIC trial[32].

Angiotensin II, a potent vasoconstrictor, is mainly present in the vascular endothelium, can contribute to atherogenesis by stimulating the growth of VSMCs[33]. It also increases smooth-muscle lipoxygenase activity, which can increase inflammation and the oxidation of LDL. Immunohistochemical studies of atherosclerotic lesions within human coronary arteries have confirmed that significant intra-lesional amount of angiotensin-converting enzyme (ACE). Angiotensin II was demonstrated within regions of inflammatory cells, especially areas of clustered macrophages and micro vascular endothelial cells[34]. Thus, ACE accumulation within the plaque may contribute to an increased production of local angiotensin II, contributing to atherosclerosis[35].

1.1.1 Percutaneous Intervention (PCI) – History

The father of interventional radiology, Charles Dotter, a vascular radiologist at the University of Oregon in Portland, introduced transluminal angioplasty in 1964, working with Melvin Judkins. Dotter used multiple catheters of increasing diameter to open blocked arteries and improve blood flow in patients with arteriosclerosis in peripheral (leg) arteries[36].

In 1977 Gruentzig *et al* reported the first use of percutaneous transluminal coronary angioplasty **(PTCA)** as a minimally invasive alternative to bypass surgery[37]. The subsequent 25 years have seen the subspecialty of interventional cardiology become one of the most exciting and rewarding fields in modern medicine. This treatment has very rapidly become the main choice of therapy for patients in whom pharmacological treatment has been insufficient to control symptoms of angina. It is also used in the context of acute myocardial infarction – so-called primary angioplasty. Although initially seen as an extremely successful treatment, PTCA has been dogged with a high complication rate. In the RITA 2 trial[38], which compared angioplasty with medical treatment alone, more patients needed bypass surgery in the PTCA group, often as an acute complication of the procedure. The incidence at 2.7-year average follow-up suggested a higher incidence death/non-fatal infarction.

Despite its drawbacks, PTCA remains at the forefront of treatments for all but in the mildest cases of angina. This is because it compares favourably with the current alternative, bypass grafting. Both techniques have similar complication rates[39].

Stents mass was concocted by Charles Thomas Stent (1807-85), an English Dentist who developed it to form an impression of the teeth and oral cavity. Stents are flexible

endovascular prostheses made from stainless steel alloys. They are designed as either metallic coils or slotted tubes. Most stents are expandable by balloon, and some are self-expanding. The stent is mounted on a balloon catheter and, with the aid of fluoroscopic screening and radiopaque markers, is positioned across the stenotic lesion, which has usually been predilated with a balloon. Inflation of the balloon results in expansion and deployment of the stent circumferentially in apposition to the endothelial surface of the coronary artery. Available stents range from 2.5 mm to 6 mm in diameter and from 8 mm to 50 mm in length.

Three conditions were considered indications for stent insertion:

- Restenosis of a segment previously treated with PTCA.
- Stenosis of aorto-coronary-bypass grafts.
- Acute coronary occlusion secondary to intimal dissection following balloon PTCA.

The first human coronary stent was implanted in 1986 by Jacques Puel (France). The idea of a balloon mounted stent for simultaneous dilatation and stent delivery was introduced by Palmaz and colleagues[40]. By early 1988, stents were placed for dilation of restenosis, acute vessel occlusion after angioplasty, and as an adjunct to primary angioplasty. Two major randomised trials compared balloon angioplasty with elective coronary stents. In the European BENESTENT[41] and the North American STRESS[42] studies, patients were randomised to conventional balloon angioplasty or to implantation of a Palmaz-Schatz stent in a primary lesion of a native coronary artery with a length of less than 15 mm and a diameter stenosis of 50% (BENESTENT) or 70% (STRESS). The incidence of restenosis, according to the 50% diameter stenosis criterion, was significantly lower after stent implantation (BENESTENT 22%,

STRESS 32%) than after balloon dilatation alone (BENESTENT 32%, p=0.02, STRESS 42%, p=0.046). One year follow-up results of the BENESTENT trial showed a continued benefit for stented patients, with a 1-year event free survival of 76.8% compared with 68.5% in the balloon angioplasty patients[43]. The advent of stents and their widespread use has undoubtedly provided a huge improvement in intravascular intervention, but at the same time it has brought to the practice of angioplasty a plethora of new complications.

Technical modifications on the angioplasty balloon had not altered the incidence of immediate or late complications, but the use of stents, metallic scaffolding devices, improved significantly the acute gain and almost abolished acute closure especially if it is due to recoil. Stents have also been shown to reduce to incidence of restenosis and they are now used routinely for the majority of interventions.

Risk of acute thrombus formation has been cut significantly by using routinely aspirin and new anti-platelet agents, such as clopidogrel and glycoprotein GPIIb/IIIa receptor inhibitors, which are not without systemic haemorrhagic side effects. These agents had little or no effect on the rate of restenosis. The stent procedure has not abolished restenosis or thrombosis completely especially in certain patient groups (diabetes mellitus, small vessel and diffuse coronary disease). Further work is necessary to improve on the overall outcome and to make this procedure universally safe and successful.

1.2 Complications of coronary stenting

1.2.1 Stent Thrombosis

Stents have improved the safety and efficacy of percutaneous coronary interventions (PCI) by reducing abrupt or threatened vessel closure[44;45] and by reducing restenosis compared with conventional balloon angioplasty. In addition, coronary vasomotion has been found intact after stent implantation and long-term clinical and angiographic follow-up have attested to the durability of their action[46].

Notwithstanding, coronary stent thrombosis has remained a serious complication of PCI. Although early aggressive anticoagulation schemes were associated with unacceptably high rates of stent thrombosis and bleeding complications[47;48], the advent of dual antiplatelet therapy had salutary effects on both adverse events. Despite dual antiplatelet therapy, stent thrombosis persists at a rate of 0.5–2%[49] in elective cases, and up to 6% in patients with acute coronary syndromes. Furthermore, longer stent length, number of implanted stents, stent malapposition[50], residual dissections, reduced TIMI flow[51], gene polymorphisms[52] and resistance to the antiplatelet effects of acetylsalicylic acid (ASA)[53] and potentially thienopyridines[54;55] have been reported to increase the risk for stent thrombosis.

The clinical sequel of stent thrombosis including death and myocardial infarction are grave and demand aggressive therapeutic interventions. Although emergency PCI is commonly employed, the efficacy of the procedure and long-term outcome of patients in this setting is not well established. Previous studies reported only a limited efficacy of PCI and intracoronary fibrinolysis[56], but a possibly favourable effect of the glycoprotein IIb/IIIa antagonist abciximab for the treatment of stent thrombosis[57]. With the current use of drug-eluting stents (DES) there still remain significant

concerns regarding the risk of late stent thrombosis (LST). Such is the concern over a possible LST risk that an advisory panel of the Food and Drug Administration (FDA) recently recommended that a warning be added to the DES labels stating that off-label use may increase the risk of thrombosis, myocardial infarction and death.

A meta-analyses[58] of data out to four years from independent trials of the Cypher (RAVEL, SIRIUS, E-SIRIUS and C-SIRIUS) and Taxus stents (TAXUS I, TAXUS II, TAXUS IV, TAXUS V and TAXUS VI); showed that a small but significant increase in late-stent thrombosis (Table1.1). In the Taxus randomized clinical trials, that increase was 0.5% between one and four years after stent implantation, a rate of approximately 0.15% per year. For the Cypher trials, the late-stent-thrombosis rate was 0.6% between one and four years, or roughly 0.2% per year. (TCT meeting 2006) The overall incidence of thrombosis from all analyzed studies was 9.3 events per 1,000 DES patients compared with 9.0 events per 1,000 BMS patients ($p = 0.91$).

The purpose of the present study was to investigate the efficacy of eptifibatide eluting stents as an anti-thrombotic and anti-restenosis agent as Local Drug Delivery.

Incidence of Early, Late and Very Late Stent Thrombosis: A Metanalysis

	Early Thrombosis <30 days Post PCI (per 1,000 patients)	Late Thrombosis >30 days Post PCI (per 1,000 patients)	Very Late Thrombosis* (per 1,000 patients)
Drug-eluting stent	4.0	5.0	5.0
Bare-metal stent	5.0	2.8	0.0
p Value	0.74	0.22	0.002
Sirolimus-eluting stent	4.2	3.5	3.6
Bare-metal stent	3.7	4.9	0.0
p Value	0.79	0.61	0.22
Paclitaxel-eluting stent	4.6	6.3	5.9
Bare-metal stent	6.3	1.1	0.0
p Value	0.51	0.034	0.049

*More than 1 year after index procedure

Table 1.1: This meta-analysis[58] included nine sirolimus trials, largely represented by the SIRIUS and RAVEL trials and five paclitaxel trials, represented by the TAXUS Trials. Increased risk with DES use was suggested as early as 1 month after revascularization, but over time this increased to four- to five-fold excess of late thrombosis associated with DES versus BMS placement. The time to late thrombosis was equally protracted for both sirolimus and paclitaxel stents, with a median thrombosis time of 15.5 to 18 months after coronary intervention, respectively, which was 11 to 14 months longer than late BMS thrombosis.

1.2.2 Pharmacological approach to prevent stent thrombosis

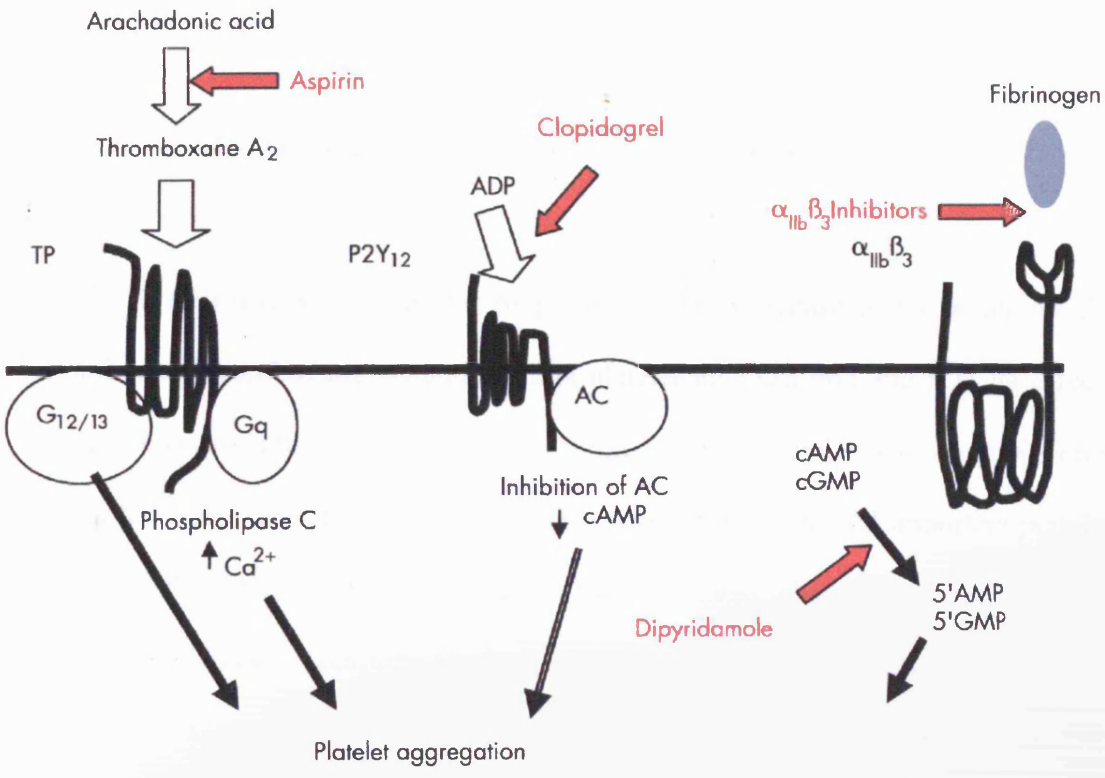


Figure 1.2: Platelet receptors and their antagonists

The ADP $P2Y_{12}$ receptor (a G-protein coupled receptor) is linked to adenylate cyclase (AC) via Gi. Upon stimulation the adenylate is inhibited, leading to a fall in cAMP and promotion of platelet aggregation. Clopidogrel irreversibly binds to the ADP $P2Y_{12}$ receptor preventing the inhibition of adenylate cyclase, therefore maintaining cAMP levels and inhibiting platelet aggregation. Dipyridamole is a phosphodiesterase inhibitor and prevents the breakdown of cAMP and cGMP. Therefore cAMP and cGMP levels are maintained inhibiting platelet aggregation. Thromboxane A_2 is produced from arachidonic acid via cyclo-oxygenase and is released from the platelet to work with the TP receptor. The TP receptor (a G-protein coupled receptor) causes platelet aggregation via both Gq and $G_{12/13}$. Aspirin irreversibly inhibits cyclo-

oxygenase, for the lifetime of the platelet (120 days), preventing thromboxane production. The $\alpha_{IIb}\beta_3$ receptor antagonists prevent fibrinogen binding to the $\alpha_{IIb}\beta_3$ receptors and therefore inhibit the "final common pathway of platelet aggregation".

1.2.2.1 Antiplatelet agents : Aspirin, Thienopyridines

Aspirin

Aspirin is an irreversible inhibitor of platelet cyclooxygenase and can inhibit the formation of thromboxane A_2 and inhibits platelet aggregation.(Fig.1.2) Its effects can be overcome in the presence of potent thrombogenic stimuli. Aspirin inhibits only the arachidonic acid pathway and has no influence on the effects of important platelet agonists such as thrombin and collagen, which can cause platelet degranulation and aggregation despite a complete blockade of the arachidonic acid pathway. With the advent of thrombolytic therapy of myocardial infarction, it became clear that treatment with streptokinase was associated with marked platelet activation, as determined by increased thromboxane A_2 production[59]. This issue was addressed by the Second International Study Of Infarct Survival (ISIS-2)[60] which involved 17187 patients with acute myocardial infarction, randomised to one of four arms of therapy consisting of placebo, aspirin, streptokinase, or streptokinase plus aspirin. Aspirin reduced mortality from 13.2% in the placebo group to 10.7% (23% relative risk reduction, $p<0.00001$) and streptokinase reduced mortality to 10.4% (25% relative risk reduction). However, the combination of aspirin and streptokinase produced the greatest benefit, reducing mortality to 8.0%, an overall relative risk reduction of 42%, thus establishing aspirin and thrombolysis as standard therapy for eligible patients with acute myocardial infarction. The benefits of aspirin are substantial and clearly defined; the antiplatelet trialist collaboration[61] demonstrated a 36% reduction in death

or myocardial infarction with antiplatelet treatment (predominantly aspirin) vs placebo in unstable angina trials.

Thienopyridines

Other than aspirin, there are few oral antiplatelet treatments with proven efficacy in patients with acute coronary syndrome (ACS). Clopidogrel and Ticlopidine are thienopyridines inhibiting platelet aggregation via the ADP dependent pathway. Ticlopidine and clopidogrel both behave in vivo as specific antagonists of P2Y12, one of the ADP receptors on platelets. Both drugs are effective orally, with about 80%–90% absorption. They require metabolism by the hepatic cytochrome P450 enzyme system to acquire their antiplatelet activity[62;63].

Ticlopidine has been established as an alternative to aspirin in the prevention of recurrent cerebral ischaemia and stroke but its use has been limited in view of its side effects, including thrombotic thrombocytopenic purpura and potentially fatal severe neutropenia[64]. Due to these side effects and its slow onset of action it has been replaced by clopidogrel, which is felt to be safer and, when a loading dose is employed, faster acting.

Clopidogrel selectively inhibits the binding of adenosine diphosphate (ADP) to its platelet receptor and the subsequent ADP mediated activation of the glycoprotein GPIIb/ IIIa complex, thereby inhibiting platelet aggregation. (Fig.2) The CAPRIE[65] trial showed that clopidogrel was more effective than aspirin in reducing ischaemic complications (ischaemic stroke, myocardial infarction, or vascular death) in patients with atherosclerotic disease but overall, the safety and tolerability of aspirin and clopidogrel were similar. Event rates of 5.32% and 5.83% were associated with clopidogrel and aspirin therapy, respectively. The CAPRIE Study therefore

established clopidogrel as an alternative antiplatelet to aspirin for secondary prevention across a wide spectrum of patients with vascular disease. The synergistic potential for aspirin and clopidogrel has been confirmed in ACS. In the CURE[66] (Clopidogrel in Unstable Angina to Prevent Recurrent Events) trial, aspirin plus clopidogrel reduced the relative risk of CVA, non-fatal MI or stroke by 20% compared with aspirin plus placebo ($p<0.001$), without significantly increasing risk of bleeding.

The PCI CURE Study was designed to test whether treatment with clopidogrel, in addition to aspirin, before PCI and continued beyond the standard course of four weeks after PCI was superior to placebo in preventing major ischaemic events[67]. In total 2658 patients who were recruited into the CURE Study and underwent PCI in response to refractory ischaemia or adverse events were examined; 1313 were assigned to clopidogrel and 1345 to placebo. A total of 1730 PCI procedures were performed during the initial hospital stay and 928 after discharge. Patients were pre-treated with clopidogrel for a median of 10 days before PCI. After PCI most (>80%) patients in both groups received open label thienopyridine for four weeks, after which the study drug was restarted for a mean of about seven months. Fewer patients in the clopidogrel group had myocardial infarction or refractory ischaemia before PCI. The number of patients with the primary endpoint of cardiovascular death, myocardial infarction, or urgent revascularisation was significantly lower in the clopidogrel than in the placebo group. Since the vast majority of patients recieved open label thienopyridine treatment for the four weeks after the procedure, the improvement in the primary endpoint was presumably caused by pre-treatment with clopidogrel prior to PCI. The results implied that in patients with non-ST elevation acute coronary syndrome in which an invasive strategy was planned, clopidogrel started on admission

before the procedure and continued afterwards was beneficial in reducing both early and late complications.

The Clopidogrel as Adjunctive Reperfusion Therapy - Thrombolysis in Myocardial Infarction 28 (CLARITY-TIMI 28)[68] trial was designed to assess the impact of pre-treatment with clopidogrel on top of thrombolytic therapy on coronary artery patency, death, and recurrent MI. The overall trial found that the addition of clopidogrel to standard fibrinolytic therapy, including aspirin in STEMI patients, resulted in a 36% reduction in the odds of an occluded infarct-related artery or death/MI by the time of angiography.

The efficacy of clopidogrel treatment both pre-PCI and subsequently was also recently addressed by the CREDO[69] (Clopidogrel for the Reduction of Events During Observation) Study. CREDO trial was a randomized, double-blind, placebo-controlled trial conducted among 2116 patients who were to undergo elective PCI or were deemed at high likelihood of undergoing PCI, enrolled at 99 centres in North America from June 1999 through April 2001. Patients were randomly assigned to receive a 300-mg clopidogrel loading dose (n = 1053) or placebo (n = 1063) 3 to 24 hours before PCI. Thereafter, all patients received clopidogrel, 75 mg/d, through day 28. From day 29 through 12 months, patients in the loading-dose group received clopidogrel, 75 mg/d, and those in the control group received placebo. Both groups received aspirin throughout the study. At 1 year, patients who received long-term clopidogrel therapy were associated with a 26.9% relative reduction in the combined risk of death, MI, or stroke (95% confidence interval [CI], 3.9%-44.4%; p =.02; absolute reduction, 3%). Clopidogrel pre-treatment did not significantly reduce the combined risk of death, MI, or urgent target vessel revascularization at 28 days

(reduction, 18.5%; 95% CI, -14.2% to 41.8%; p =.23). However, in a prespecified subgroup analysis, patients who received clopidogrel at least 6 hours before PCI experienced a relative risk reduction of 38.6% (95% CI, -1.6% to 62.9%; P =.051) for this end point compared with no reduction with treatment less than 6 hours before PCI.

1.2.2.2 Glycoprotein IIb/IIIa receptor inhibitors

The initiation of atherothrombosis requires platelet adhesion, activation and aggregation. Platelets adhere to the exposed extracellular matrix (ECM) of the blood vessel wall via adhesion receptors, which generate "outside-in" signalling, which activates the other adhesion receptor glycoprotein GPIIb/IIIa (integrin αII/βIII). (Figure 2) This integrin binds to fibrinogen resulting in the formation of platelet-rich thrombus. Several GPIIb/IIIa antagonists, such as abciximab, eptifibatide (Integrilin) and tirofiban, bind to the receptor and prevent formation of thrombus at the site of vascular injury.

Abciximab (c7E3)

Coller and colleagues in 1983 used a murine monoclonal antibody directed against the platelet GpIIb/IIIa receptor, inhibiting platelet aggregation[70]. Further development led to one antibody (7E3) that cross-reacted against dog platelets. It was cleaved into F $(ab')_2$ to reduce the immunogenicity and this fragment was shown to have antithrombotic effects in an animal model[71;72]. Removing the constant region of the original murine monoclonal antibody 7E3 and replacing it with a 'human' constant region further reduced its immunogenicity. This chimeric compound, c7E3, was then evaluated in pilot studies in patients[73].

This chimeric monoclonal antibody targets the IIb/IIIa receptor has been used in several trials including the EPIC trial of use in "high-risk" angioplasty[74]. Not only did abciximab appear to reduce the immediate thrombotic complications of intervention, but also a reduction in restenosis, as judged by the need for target vessel revascularisation, was seen. As well as blocking the IIb/IIIa receptor, it also inhibits the $\alpha_v\beta_3$ integrin in smooth muscle cells[75]. Blockade of this receptor abciximab has been shown *in vivo* to results in a reduced intimal hyperplastic response to PTCA. Abciximab has been coated onto stents and neointimal formation in a porcine coronary stent restenosis model has been assessed[76]. Follow-up quantitative coronary angiogram and histopathologic assessment was performed at 4 weeks, showed inhibition of platelet thrombus and neointimal cell proliferation. A prospective randomized trial was performed in Korea Heart Centre; to compare BMS vs abciximab coated stent in human[77]. One hundred and fifty-five patients were enrolled between August 2001 and June 2003. Follow-up coronary angiograms were performed in 62.3% (48/77) and 65.4% (51/78) of the coated and control groups, respectively. The diameter of stenosis and late loss were significantly less in the abciximab coated stent group compared with the controls (16.4 +/- 5.8% vs. 34.3 +/- 6.1%, p = 0.009; and 0.33 +/- 0.28 mm vs. 0.88 +/- 0.41 mm; p = 0.002). The restenosis and TVR rates of the abciximab-coated stent were relatively lower compared with the control stent [14.6% (7/48) vs. 29.4% (15/51), p = 0.062; and 9.2% (7/76) vs. 14.7% (11/75); p = 0.327]. The results show that abciximab coated stents may be effective in the prevention of coronary restenosis. These results warrant further investigation with a large, randomized multicenter study.

Eptifibatide

Eptifibatide is discussed in details in section 1.6 (see below).

These glycoprotein IIb/IIIa inhibitors have been shown in clinical trials to substantially decrease complications of high-risk angioplasty and to reduce the incidence of composite end-points in stented arteries by up to 50%[78].

Oral $\alpha_{IIb}\beta_3$ antagonists

So far the large trials of oral $\alpha_{IIb}\beta_3$ antagonists (sibrafiban, orbofiban, and xemilofiban), have reported no obvious benefit and there were excessive bleeding complications. Both the Orbofiban in Patients with Acute Coronary Syndromes Thrombolysis in Myocardial Infarction 16 trial (OPUS-TIMI 16)[79] and Sibrafiban Versus Aspirin to Yield Maximum Protection from Ischaemic Heart Events Post-Acute Coronary Syndromes 2 (SYMPHONY 2) trial[80] demonstrated statistically significant increased mortality in the treatment groups. Why there should be a detrimental effect compared to intravenous $\alpha_{IIb}\beta_3$ antagonists remains unclear. The increase in mortality may relate to a prothrombotic effect, unfavourable pharmacokinetics, or indeed there may be paradoxical platelet activation with oral agents.

Overall the development and use of $\alpha_{IIb}\beta_3$ antagonists have been crucial in the management of cardiovascular disease. Their benefit when used in conjunction with PCI is undoubted and now is accepted practice. Their use in the management of acute coronary syndromes without mandatory PCI has a lesser effect but has been proven in the majority of trials. Their use must be combined with risk stratification and other antiplatelet agents. Further development of these agents for the treatment of acute coronary syndromes including acute myocardial infarction is required.

1.2.2.3 Heparin

Unfractionated heparin

Heparin is composed of a range of different sized glycosaminoglycans that bind to antithrombin III and accentuate the inhibition of thrombin and factor Xa. The benefits of heparin therapy in patients with unstable angina are well established and widely accepted. A meta-analysis of six randomised controlled trials demonstrated that, in addition to aspirin, intravenous heparin conferred a relative risk reduction of 33% in the risk of death or MI[81]. This reduction, although not statistically significant, provides some support for the use of this combination in ACS.

Fractionated heparin

Unlike unfractionated heparin, low-molecular-weight heparins exert anticoagulant action mainly directed against factor Xa, and thus may limit rebound phenomenon by preventing thrombin regeneration after drug discontinuation. Other advantages of low-molecular-weight heparins include better bioavailability, longer duration of action, less binding to plasma proteins and endothelial cells. There have been four major randomised controlled trials that have compared fractionated and unfractionated heparin in patients with unstable angina – the FRIC[82], ESSENCE[83], TIMI-IIB[84] and FRAXIS[85] trials. Fractionated heparins appear to have equivalent benefits to unfractionated heparin and particularly in the case of enoxaparin, may have superior efficacy in the prevention of death, MI or reccurent angina: relative risk reduction of 15-18%[83;84]. These benfits are sustained at 1-year follow-up. There is little evidence to suggest that the continuation of fractionated heparin therapy beyond 7 days confer any additional benefits [86;87].

1.2.2.4 Direct thrombin inhibitors (DTI)

Heparin is a weak inhibitor of platelet-rich arterial thrombi because it acts via antithrombin III, which is unable to bind thrombin bound to fibrin, cells, or lipids. Patients (1-5%) receiving heparin may develop heparin-induced thrombocytopenia (HIT), which is a serious, prothrombotic allergic drug reaction. The limitations of heparin as an anticoagulant led to the development of direct acting, AT III-independent thrombin inhibitors. Thrombin bound to fibrin or fibrin degradation products is resistant to inhibition by the heparin/antithrombin complex, but is susceptible to inactivation by direct thrombin inhibitors[88;89]. Because the active site of thrombin is not involved in the interaction of the enzyme with fibrin, it remains accessible to active site-directed thrombin inhibitors, even when thrombin is bound to fibrin. Consequently, these agents inactivate fibrin-bound thrombin without displacing the enzyme from fibrin. In contrast, bivalent thrombin inhibitors, such as hirudin and bivalirudin (a semi synthetic hirudin analogue of hirudin), displace bound thrombin during the inhibition reaction by competing with fibrin for access to exosite 1 on thrombin.

Hirudin

Hirudin is a naturally occurring peptide present in the saliva of the leech (*Hirudo Medicinalis*). It is the most specific and potent inhibitor of thrombin known. Based on randomised trials[90-92], hirudin appears to be superior to heparin in patients with unstable angina, and is at least as effective as heparin in patients undergoing coronary angioplasty. Despite these data, however, the role of hirudin in acute coronary syndromes has yet to be established. In patients with unstable angina, hirudin and GPIIb/IIIa antagonists produce similar reductions in the risk of recurrent ischaemia.

The major concerns about the use of hirudin in acute coronary syndromes relate to its cost and the incidence of bleeding complications compared with heparin.

Bivalirudin

Bivalirudin is a direct thrombin inhibitor approved for use in PCI. It reversibly binds to thrombin s catalytic site and substrate recognition site and blocks both circulating and fibrin-bound thrombin. Peak concentrations occur in less than 5 minutes after bolus-dose administration, and its half-life is approximately 25 minutes. It is primarily eliminated via renal route, and dosage reduction may be required in patients with severe renal dysfunction. In the REPLACE-2[93] (Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events) study, the data showed that bivalirudin combined with provisional glycoprotein IIb/IIIa inhibitors was non-inferior to UFH with planned glycoprotein IIb/ IIIa inhibitors and superior to UFH alone with respect to ischaemic and hemorrhagic endpoints in PCI. Major bleeding with bivalirudin occurred in approximately 3% of patients. The ACUITY trial[94] evaluated the optimum upstream treatment of patients with moderate- to high-risk non-ST-elevation ACS heading to the cath lab. In this open label study, 13 819 such patients were randomized to one of three arms: unfractionated heparin (UFH) or enoxaparin plus routine GP IIb/IIIa inhibition; bivalirudin plus routine GP IIb/IIIa inhibition; or bivalirudin alone. Results showed that the bivalirudin-monotherapy group did best, with significantly less bleeding and without a significant increase in events compared with the heparin-plus-IIb/IIIa-blocker arm. 30 days results from the HORIZONS AMI trial (presented during TCT 2007 meeting) indicate that bivalirudin has now proved itself in acute-MI patients undergoing primary angioplasty. Bivalirudin was significantly better than the UFH-GP IIb/IIIa-inhibitor strategy, at 30

days, 24% reduction in net adverse clinical events and a 40% reduction in major bleeding—the primary end points of the study was seen. The cardiac mortality was significantly reduced in patients in the bivalirudin arm of the study (p= 0.035).

1.2.3 Stent Restenosis

1.2.3.1 Factors affecting restenosis

Angiographic restenosis post-angioplasty occurs much more commonly in patients with diabetes[95;96] although this effect has not always been seen in stented patients[97]. Angioplasty and stenting of restenotic lesions, as compared to *de novo* lesions, carries an even greater risk of re-restenosis. The importance of genetic influences has not yet been fully established. That there are genetic influences seems certain. For example, there is an association between high TGFβ production secondary to a TGFβ gene polymorphism and a higher incidence of restenosis[98].

1.2.3.2 Angiographic variables

Angioplasty of the left anterior descending artery has consistently been shown to have a tendency to restenose more frequently than other angioplastied arteries. This may be because of anatomical differences in the vessel e.g. its course in the interventricular septum. Restenosis would thereby be made more likely because of an initially sub-optimal result. Restenosis is also more common in patients who are stented for chronic or subtotal occlusions and in stenosed or obstructed vein grafts[99].

Longer initial lesions tend to occur in more diseased arteries. The revascularisation process is more difficult and restenosis appears to be more common[100]. Multiple interventions, either PTCA or stenting, in a single procedure are also associated with a higher restenosis rate. The size of the target vessel is closely related to the incidence of restenosis in both angioplasty and stenting[101].

To understand more fully why a patient might develop restenosis after an interventional procedure, it is important to examine the processes that underlie the phenomenon at a cellular level.

1.3 Cellular mechanisms: Pathophysiology of restenosis

The atherosclerotic plaque and the "response to injury" hypothesis

The initial stenosis found in coronary artery disease is due to the presence of atherosclerotic plaques. These plaques are complex structures that contain both cellular and acellular components. This heterogeneous composition determines the unpredictable nature of the response to angioplasty.

Angioplasty, with or without stent implantation, means the application of a great deal of force to this structure. It is this very injury to the vascular wall and to the plaque that is the stimulus to the development of intimal hyperplasia[102].

This process is thought to begin almost from the moment the injury occurs, since many of the cells and mechanisms that govern the natural healing processes will contribute to intimal hyperplasia if the same mechanisms continue in a poorly controlled fashion. This is part of the "response to injury" theory first proposed by Virchow over a hundred years ago.

Angioplasty relieves the initial narrowing of a blood vessel by forcibly fissuring or dissecting the fibrous cap over the atherosclerotic plaque. Often this force is transmitted; causing extensive fissuring that may extend circumferentially into the vessel wall. Fortunately, although very common, most of these dissections are of no clinical significance. After the fibrous cap has been breached, the plaque contents can disperse into the coronary circulation. Again, the impact of this iatrogenic burst of emboli, notably cholesterol, does not seem to be of clinical significance. After the

angioplasty balloon has successfully dilated the site of a plaque and has been withdrawn, a fissured plaque is left largely denuded of its endothelial covering.

Not only may the restenosis response be a consequence to injury, but also the severity of the injury may be directly related to the extent of restenosis seen. This has been modelled in pigs where an injury score at angioplasty was related to the resulting restenosis seen at a one month[103].

Restenosis of a vessel at the site of this fissured plaque is thought to be due to different mechanisms interacting, including:

1. Elastic recoil of the vessel wall after stretching
2. Remodeling of the vessel
3. Intimal hyperplasia
4. Thrombus formation

1.3.1 Recoil

Angioplasty relieves the obstruction partly by stretching the healthier, relatively normal parts of the vessel wall. The loss of this overstretched artery by elastic recoil is one of the factors that contribute to restenosis. Stenting overcomes this recoil. Rodriguez *et al* showed that in patients with significant angiographic recoil at 24hrs post-procedure, stent insertion changed the restenosis rate from 76% to 21%[104]. In most stent procedures a stent will be implanted with an internal diameter bigger than that of the vessel into which it is placed.

1.3.2 Remodeling

Remodeling is a term first applied to some of the changes seen in atherosclerosis. In most cases, a diseased artery will slowly increase its overall external diameter. This partially compensates for the reduction in luminal diameter due to the atherosclerotic lesion. Unfortunately, following angioplasty the opposite process often occurs. This negative remodeling causes a gradual reduction in the diameter of the vessel, compromising the luminal cross-sectional area. Mintz *et al* have looked at this process using serial intravascular ultrasound studies[105]. Of patients with restenosis, about 75% of the reduction in lumen was due to remodeling, the rest attributed to growth of plaque or to intimal hyperplasia.

It is believed that stent insertion also prevents this shrinkage of the vessel by negative late remodeling. However, in some cases, it would appear that stented vessels demonstrate some *positive* remodeling, i.e., the enlargement of the vessel diameter, often with *enlargement* of the original angioplastied plaque at the stent site. There appears to be an inverse correlation between the extent of this positive remodeling and the amount of instent restenosis[106].

1.3.3 Intimal hyperplasia

Intimal hyperplasia is the ingrowth of material into the potential space created by the angioplasty. This material is a complex mix of components, but includes vascular smooth muscle cells (SMC's) and extracellular matrix.

1.3.3.1 The vascular smooth muscle cell

The SMC's that are typically found in restenotic lesions are thought to originate in the media of the vessel wall, particularly in stented vessels[107]. Normally these cells are of a contractile nature, have a low rate of replication and do not tend to secrete large amounts of cytokines. However following injury to the vessel wall, the phenotype of these cells changes to a more proliferative or secretory one. This change is partly stimulated by the surrounding proteoglycans[108]. For example, hyaluronan forms pericellular layers around dividing cells, and blockade of this proteoglycan's receptors inhibits SMC migration and proliferation. This phenotypic change is marked by the cells beginning to produce Proliferating Cell Nuclear Antigen (PCNA).

PCNA is a 36kDa nuclear protein. In several studies, the presence of PCNA has been associated with the proliferation of cell lines, including in the restenosis process[109]. It acts as a co-factor to DNA polymerase delta. That PCNA is important in intimal hyperplasia has been demonstrated by the finding that the proliferation of smooth muscle cells in cell culture can be inhibited by the addition of an antisense oligonucleotide against the mRNA for PCNA. In a porcine model of restenosis, local delivery of a ribozyme that targeted the RNA for PCNA, showed a reduction of stent-related restenosis[110].

Cells with the secretory phenotype have lost their contractility, but have gained the ability to migrate, under the influence of chemotactic factors, to the sites of vessel injury. Here they proliferate and produce their own cytokines. These include tissue Plasminogen Activator[111], which acts via matrix metalloproteinases (MMP's).

SMC's and other cell types contribute to the restenotic area. Deep injury can rupture right through the vessel wall and allow the migration of cells from outside the vessel into the area. These may also include macrophages, which are seen in response to

angioplasty, fibroblasts and capillary endothelial or lymphatic cells. All of these cells and SMC's may differentiate to form the "restenosis cell"[112].

1.3.3.2 The extracellular matrix and matrix metalloproteinases (MMP's)

SMC's in the intima of the restenotic lesion have migrated from outside the injured part of the artery. To do this the extracellular matrix has to be broken down. This occurs under the influence of a large family of proteinases, the matrix metalloproteinases. Secretory phenotype SMC's synthesise and release MMP's to facilitate their own migration[113]. All MMP's require a zinc ion for their catalytic function; they are all secreted as pro-enzymes, cleaved by plasmin (amongst others) to an active form and growth factors, such as TNF-α, regulate their production.

Up-regulation of MMP's has been identified following angioplasty injury to a vessel within 24 hours[114].

Intimal hyperplasia, as well as depending on the presence of active SMC's, involves non-cellular material. In a restenotic lesion as much as 80% of the tissue will be of extracellular material, particularly collagen, as well as proteoglycans. The turnover of this collagen is regulated by the activities of MMP's that both break down and promote the synthesis of collagen.

The apparent contradiction inherent in this last statement can be explained in that some of the MMP's e.g. Neutral Endopeptidase (NEP) breaks down antiproliferative growth factors. Inhibition of NEP inhibits neointimal hyperplasia in the rat carotid artery model of restenosis[115].

Others have shown a beneficial effect of inhibiting the actions of MMP's. Bendeck *et al*[116] showed a 97% reduction in the number of SMC's migrating into a balloon injury site in the rat carotid. Strauss *et al* showed reduced collagen in restenotic lesions by

inhibition of MMP's in the rabbit iliac model[117]. Adenovirus-mediated transfection of the gene for TIMP-1 (an inhibitor of MMP's) resulted in a reduction in neointimal hyperplasia in a saphenous vein organ culture model[118].

1.3.3.3 *The endothelium*

In a manner analogous to the different forms of SMC's, endothelial cells also appear to have two phenotypes – "normal" or "dysfunctional"[119]. The effect of the endothelium on the underlying tissues has been much studied, including when processes such as angioplasty cause endothelial damage or denudation.

During angioplasty, the endothelium is stripped away, but this effect is combined with the extensive damage to the deeper tissues already discussed. Loss of endothelium in rabbit aorta organ culture did not cause intimal thickening. With the addition of pressure from a Teflon rod pressed onto the culture however, intimal proliferation did occur[120].

Experimental work suggests that injured intimal areas that are re-covered by an endothelial layer have less intimal thickening than bare areas. This has been seen in rat thoracic aorta[121].

This hypothesis is not uniformly supported by the data available. In atherosclerotic micropigs endothelial damage (and the extent of inflammation) after angioplasty seemed to have no relationship with the negative remodeling that contributes to restenosis[122]. Studies in a rabbit aorta model showed *worse* intimal thickening in the areas covered by endothelium than adjacent bare areas. Furthermore, the function of the new endothelial cells was affected. When the rabbits were fed either high or low lipid diets, they all showed increased lipid accumulation in the re-endothelialised areas[123]. To some extent this may be because the cells that regenerate are dysfunctional.

Dysfunctional endothelium may contribute to intimal hyperplasia. Endothelial dysfunction is characterized by an imbalance between vasodilator substances with antiproliferative properties, such as NO, and vasoconstrictors with mitogenic properties, such as endothelin. The systemic and local milieu associated with endothelial dysfunction favours cell proliferation, intimal hyperplasia, and vasoconstriction, which may contribute to the restenosis process. Indeed, there is a local release of endothelin-1 at the site of the vascular injury[124], and an endothelin receptor antagonist attenuates stent restenosis[125]. Endothelial cells are capable of producing growth factors themselves and endothelial dysfunction is seen after angioplasty. In the case of stenting, dysfunctional endothelium has been demonstrated in stented porcine arteries up to three months after the initial procedure. It has been suggested that this endothelial dysfunction may contribute to the intimal hyperplasia that is seen, especially after stent use[126].

1.3.3.4 Inflammatory response to PCI

Cells of the monocyte/macrophage line are found ubiquitously in atherosclerotic plaques as foam cells that phagocytose insoluble lipids and cholesterol. Inflammation is found within 24 hours of the initial insult that is the angioplasty dilatation of the artery in rats[127]. A cardinal feature of this inflammation is the activation and recruitment of macrophages and there is a reported correlation between hyperplasia and macrophage infiltration in the rat carotid. These cells produce the mitogen PDGF. Post-mortem studies of patients with restenotic lesions post-stenting also show abundant macrophages at the restenosis site[128]. In a porcine coronary artery stent restenosis model, the extent of inflammation in each case correlated with the degree of neointimal thickness[129].

Treatment options aimed at reducing the inflammation process have shown some promise. Hypercholesterolaemic rabbits underwent PTCA/stenting and were given systemic IL-10, a deactivator of monocyte cells. Reduced inflammation and reduced neointimal growth were noted[130]. In hyperchòlesterolaemic rabbits, local delivery post angioplasty of L-Arginine, a substrate for NO production, resulted in reduced intimal hyperplasia. This was linked to a reduction in macrophage activity[131]; the rationale being that NO inhibits the monocyte adhesiveness and accumulation at the injury site.

1.3.3.5 Role of adventitial fibroblasts

In a rat carotid model of restenosis after angioplasty it has been shown that part of the restenotic tissue is due to the migration of fibroblasts from the adventitia inwards to the neointimal layers[132]. This may occur also in stented vessels but has not been examined.

1.3.3.6 Integrins

Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix proteins of the basement membrane or to ligands on other cells. Integrins contain large (α) and small (β) subunits of sizes 120-170 kDa and 90-100 kDa, respectively. Some integrins mediate direct cell-to-cell recognition and interactions. Integrins contain binding sites for divalent cations Mg^{2+} and Ca^{2+}, which are necessary for their adhesive function and allows for the interactions between various matrix components and intracellular signalling pathways that control gene expression and a number of cellular functions including cell adhesion and cell migration[133-134]. Although extensive number of integrin receptors may be involved in migration, two ($\alpha_v\beta_3$ and $\alpha_5\beta_1$) will be addressed.

$\alpha_v\beta_3$ receptors

These receptors bind vitronectin; fibrinogen, osteopontin (OPN) and so called 'RGD' ligands, where RGD represents a peptide sequence of arginine-glycine-aspartic acid containing glycoproteins. This particular integrin receptor is highly expressive in angiogenesis and following vascular injury, mediates platelet adhesion to OPN and platelet mediated thrombin generation, and plays a role in cell adhesion and spreading. These receptors have been implicated in VSMC and inflammatory cell migration[135-37]. VSMCs demonstrate marked upregulation of these receptors following arterial injury[138]. Antibodies to the β_3 integrin block VSMC migration after balloon injury[139]. There is important interaction between integrin receptors and MMP activity[140]. The $\alpha_v\beta_3$ integrin receptors on endothelial cells can bind and activate MMP-2 at the cell surface, promoting cell invasion. SMCs that are positive for $\alpha_v\beta_3$ expression are able to produce MMP-1. This upregulation of proteolytic activity by increased metalloproteinase expression appears to be crucial factor in stimulating cell migration by modulating the ECM environment around the cell.

$\alpha_5\beta_1$ receptors

$\alpha_5\beta_1$ receptors (fibronectin receptors) are expressed in injured, but not normal, rat carotid arteries and localised to the neointimal SMC subjacent to the lumen. PDGF-BB stimulated SMCs have an increased expression of surface $\alpha_5\beta_1$ integrins[141].

1.3.3.7 *Extracellular matrix (ECM) glycoproteins*

The principal ECM components are glycoproteins, collagen, elastin and proteoglycans. ECM formation ultimately contributes ~90% of the intimal lesion volume[142]. These ECM components not only provide mechanical stability to the vessel wall but also cellular-extracellular matrix interactions that signal the cell to control proliferation, migration, differentiation and survival.

Osteopontin (OPN)

OPN is a secreted RGD-containing glycoprotein that has been implicated in vascular injury by promoting cell adhesion, migration and chemotaxis. Although OPN binds to several integrins and CD44, the adhesive and migratory effects are particularly dependent on $\alpha_v\beta_3$ integrin cell receptors[143]. In cultured human coronary SMCs and the SMC layer of coronary atherosclerotic tissues express both OPN mRNA and protein at significantly elevated levels compared with controls. Interaction of OPN with $\alpha_v\beta_3$ integrin receptors, expressed on VSMCs, causes migration, ECM invasion and proliferation[145-45]. Application of antibodies against OPN or $\alpha_v\beta_3$ abolished these effects.

Fibronectin (FN)

Fibronectin is a multifunctional, cell-interactive glycoprotein with multidimer structure. The tissue form of FN is present in normal connective tissue, but its deposition by SMCs is augmented in atherosclerosis[146]. It has been shown that injured rat carotid arteries synthesize an FN matrix at their luminal surface, and an *in vitro* study demonstrated that this process requires $\alpha_5\beta_1$ integrin. FN supports cell migration in response to PDGF and bFGF induction[147].

Collagen

Once the non-thrombogenic surface of the vessel is breached during an angioplasty procedure, exposed collagen fibres induce the adhesion and aggregation of platelets, and the release of proteases, chemoattractants and growth factors. Collagen synthesis is associated with SMC migration and is required for integrin clustering and linkage to the cytoskeleton[148]. The $\alpha_v\beta_3$ integrin on the surface of VSMCs has important interactions with collagen that contribute to the formation of migratory phenotype[149]. The degraded collagen exposes cryptic RGD sequences, which allow binding to the

cell surface $\alpha_v\beta_3$ integrin receptors. This interaction can be induced by PDGF-BB and results in SMC motility[150].

1.3.4 Thrombus formation and restenosis

Angioplasty and stenting produce a situation where all of Virchow's Triad exists, i.e. abnormal flow, intimal injury and hypercoagulability.

After angioplasty/stenting, the fissured plaque presents a highly thrombogenic surface to the constituents of the blood. The surface is no longer smooth, producing turbulent, abnormal flow over the damaged plaque.

Intimal injury causes the loss of the endothelium, so the antithrombotic effects of the endothelial cells are lost as well. These cells naturally produce compounds such as tissue plasminogen activator and prostacyclin that inhibit thrombus formation and propagation.

Hypercoagulability can be attributed to the presence of exposed tissue factor in the vessel wall activating the extrinsic coagulation cascade resulting in the production of thrombin that activates platelets and cleaves fibrinogen to fibrin. Fibrinogen binds to activated platelets via the activated glycoprotein IIb/IIIa integrin, leading to platelet aggregation. Fibrin binds together platelets and red blood cells to form thrombus.

The underlying tissues exposed contain molecules that act as ligands for the receptors on platelets that allow them to adhere to surfaces. These include:

- von Willebrand Factor, to which platelet glycoprotein Ib attaches to allow platelet adhesion.
- Collagen, to which the platelet integrin Gp1a-IIa attaches, again allowing platelets to adhere to the tissue. Collagen will also activate platelets directly[151].

A formed clot is potentially of haemodynamic significance as a partial obstruction in the vessel lumen, causing by its presence an element of restenosis. Its physical

presence also provides a scaffold for the migration of other cells that produce the fibrocellular matrix that causes restenosis. The formed clot is an active structure since the cells within it continue to exert influence over the surrounding microenvironment. Pre-eminent amongst the cells within the thrombus is the activated platelet. This produces a long list of bioactive molecules including serotonin, ADP and thromboxane A_2. All of these products are known to be mitogenic[152]. Platelets also release growth factors like basic fibroblast growth factor, TGF-β or PDGF. These molecules are directly implicated in the development of neo-intimal hyperplasia and thus restenosis.

1.3.4.1 Platelet-derived Growth Factor (PDGF)

Platelets are known to produce PDGF. This is a potent mitogen and chemo-attractant for vascular smooth muscle cells (SMC's). It is one of the main factors responsible for the phenotypic shift of SMC's described earlier.

The production of PDGF has been demonstrated in animal models of restenosis. In atherectomy patients, levels of PDGF in the coronary sinus rose acutely following angioplasty. The PDGF detected was also shown to be mitogenic for SMC's cultured from the atherectomy specimens[153]. Both PDGF and its receptors have been shown at the sites of angioplasty in the coronary arteries of patients that had died days or weeks after the procedure[154]. In organ culture of human vein grafts[155], not only was PDGF detected, but also the addition of specific antibody to PDGF led to a reduction by 66% of neointimal thickness. Anti-PDGF antibodies inhibited neointimal smooth muscle growth in a rat carotid artery model. Similar reductions in neointima formation were found when the action of PDGF through its tyrosine kinase receptor was blocked *in vivo* in a porcine model of carotid artery angioplasty[156]. It has therefore been suggested that PDGF is an integral part of the restenosis process.

1.3.4.2 Cyclic flow variation and intimal hyperplasia

The phenomenon of cyclic flow variation (CFV) is discussed fully in chapter 6. In brief, variations in the observed flow of blood through a damaged blood vessel occur when thrombi accumulate. Inhibition of platelets greatly reduces the occurrence of these variations and the frequency of CFV's is correlated with the extent of intimal hyperplasia seen much later. This has been demonstrated in a canine restenosis model[157].

1.4 Local drug delivery for restenosis

1.4.1 Local drug delivery balloons

Initial work looked at a double-balloon catheter. Although successful at delivering agents to the vessel wall, long periods of instillation are required for the drug to be taken up in significant amounts. This precludes their use in coronary arteries where prolonged occlusion of the artery can be fatal.

It is accepted that the small calibre holes of local delivery balloons cause the fluid instilled to form fluid jets. These jets cause local trauma to the vessel itself, somewhat negating the healing properties of the drug instilled. Much of the fluid instilled does not remain in the vessel wall. As little as 1%[158] of the drug instilled will remain in the vessel wall, opening the possibility that there will be a significant amount of the drug found in the bloodstream, certainly further downstream in the coronary circulation and even systemically. Finally, local trauma can be quite significant, as the angioplasty balloon has to be inflated to allow the drug to be delivered. Infusion pressure seems to be related to development of intimal hyperplasia[159]. Even saline infusion is associated with increased intimal hyperplasia in one study in a porcine coronary stent model[160].

The advantages and drawbacks of these local delivery balloons can be illustrated briefly. Antisense oligonucleotides to c-myb, a proto-oncogene expressed after angioplasty injury in vessels, were infused locally with the transport catheter. The authors noted exacerbated intimal hyperplasia in the control group, reflecting the trauma caused by the local instillation of fluid[161]. In a small (ten patient) study, locally delivered urokinase was very successful in lysing intracoronary thrombus following complicated angioplasty. The authors note however that as little as 0.4% of the urokinase was delivered to the vessel wall in their animal model, with around 90% lost into the systemic circulation and about 10% remaining on the balloon even after it had been used[162].

All these balloon catheters have similar weaknesses. They rely on the instillation directly into the vessel wall and this often leads to trauma above and beyond that found with angioplasty alone. Nevertheless, an enormous number of agents have been used in various animal models to test local drug delivery by one or other balloon type (Table 1.2). As well as direct application of the target agent, various adaptations have been made to the basic balloon principle to try and improve on either delivery or retention of the agents. These include microparticle suspensions, liposomes and targeted drug therapy. These are briefly discussed.

1.4.1.1 Microparticles

To overcome the loss of liquid drug away from where it was intended, Wilensky *et al* showed that agents might be injected into the vessel wall not as a free solution, but in a suspension of 5μm diameter microparticles. These, when injected into the femoral arteries of atherosclerotic rabbits, were retained locally up to 14 days later in the majority of cases[163]. These biodegradable microparticles can carry drugs and keep them in the area where they are needed.

1.4.1.2 Liposomes

Liposomes containing agents felt to be beneficial in limiting the restenosis process have been used with some success. These nanoparticulates are infused into the angioplastied vessel through a delivery balloon. Using liposomes as a vehicle, oestradiol has been shown to reduce neointimal hyperplasia in a rat carotid model[164].

1.4.1.3 Targeting

A refinement of the use of local drug delivery balloons has been the use of targeted agents to aid retention at the intended target site. Targeting means the fusion of the active moiety of an agent with a suitable agent that binds to structures in the target site, for example the injured arterial wall after angioplasty. This theory has been tested by Hogrefe *et al*[165] who showed that a bispecific antibody fragment could be produced combining the binding sites for the GPIIb/IIIa receptor and for tissue factor. This bispecific antibody showed increased affinity for the angioplasty site because of the exposed tissue factor there. *In vivo*, a trend towards reduced thrombus formation at the site was observed over that seen with either agent alone.

Agent	Model used	Result
Local Photodynamic therapy		
Photofrin[166]	Pig	Reduced neointimal hyperplasia
Anticoagulants and antiplatelets		
Enoxaparin[167]	Human	Reduced restenosis
Nadroparin[168]	Human	No benefit
Fraxiparin[169]	Human	Not assessed
Reviparin[170]	Rabbit	No benefit
Urokinase[171]	Pig	No benefit
Heparin[172]	Human	No benefit
Argatroban[173]	Rabbit	Reduced neointimal hyperplasia
Antisense oligonucleotides		
To MAPK[174]	Pig	Reduced neointimal hyperplasia
To c-myb[175]	Pig	Reduced neointimal hyperplasia
To c-myc[176]	Rabbit/Human	Reduced neointimal hyperplasia/No benefit
Anti-proliferatives		
Mitomycin C[177]	Rabbit	No benefit

Methotrexate[178]	Pig	No benefit
Paclitaxel[179]	Rabbit	Reduced neointimal hyperplasia
Green tea catechins[180]	Rat	Reduced neointimal hyperplasia
Hormones and cytokines		
Lipoxygenase inhibition[181]	Rat	Reduced neointimal hyperplasia
VEGF[182]/VEGF [183]	Rat/ Human	Successful /no benefit (not stented)
Hepatocyte Growth Factor[184]	Rabbit	Reduced neointimal hyperplasia
Angiopeptin[185]	Rabbit	Reduced neointimal hyperplasia
I-kappa-B[186]	Rabbit	Reduced re-occlusion
Interferon Beta[187]	Pig	Not available
Tyrosine/Protein Kinase inhibitors		
ST 638[188]	Pig	Reduced stenosis arteries given drug from outside
Antioxidants		
HA1077[189]	Rat	Reduced neointimal hyperplasia
Gene therapy		
Adenovirus/Retrovirus		
GAX[190]	Pig	Reduced neointimal hyperplasia
NOS3[191]	Pig	Equivocal
Antisense cyclin G[192]	Rat	Reduced neointimal hyperplasia
Rb[193]	Rat	Reduced neointimal hyperplasia
Plasmid		
VEGF[194]	Rabbit	Reduced neointimal hyperplasia
Others		
Suramin[195]	Mouse	Reduced neointimal hyperplasia
Chimeric ribozymes[196]	Pig	Reduced neointimal hyperplasia
Ethanol[197;198]	Pig/human	Reduced neointimal hyperplasia/NA
Dexamethasone[199]	Rat	Reduced neointimal hyperplasia
L-Arginine[200]	Rabbit	Not determined

Table 1.2: Table of drugs administered locally to try and reduce restenosis. This is not an exhaustive list but illustrates the very wide range of agents used to try and treat the restenosis process.

1.4.2 Local Drug Delivery Stents

1.4.2.1 Stent surface characteristics

The roughness of the stent surface is thought to determine the extent of early thrombus deposition. Irregularities as small as 1μm have shown increase thrombogenic potential in a study of stainless steel rings implanted into the inferior vena cava of experimental dogs. In this same work, the impact of surface charge was also assessed. It has been felt intuitively that negatively charged surfaces might be antithrombogenic in that they might repel the predominantly negatively charged proteins that form blood clots. However, no such relationship was seen in this study. It is therefore not at all certain that the charge *per se* of a stent influences its thrombogenicity.

Irregularities can easily be seen in the surface of stents under scanning electron microscopy. This is demonstrated using the polymer-coated stents used in this work (Figure1.3).

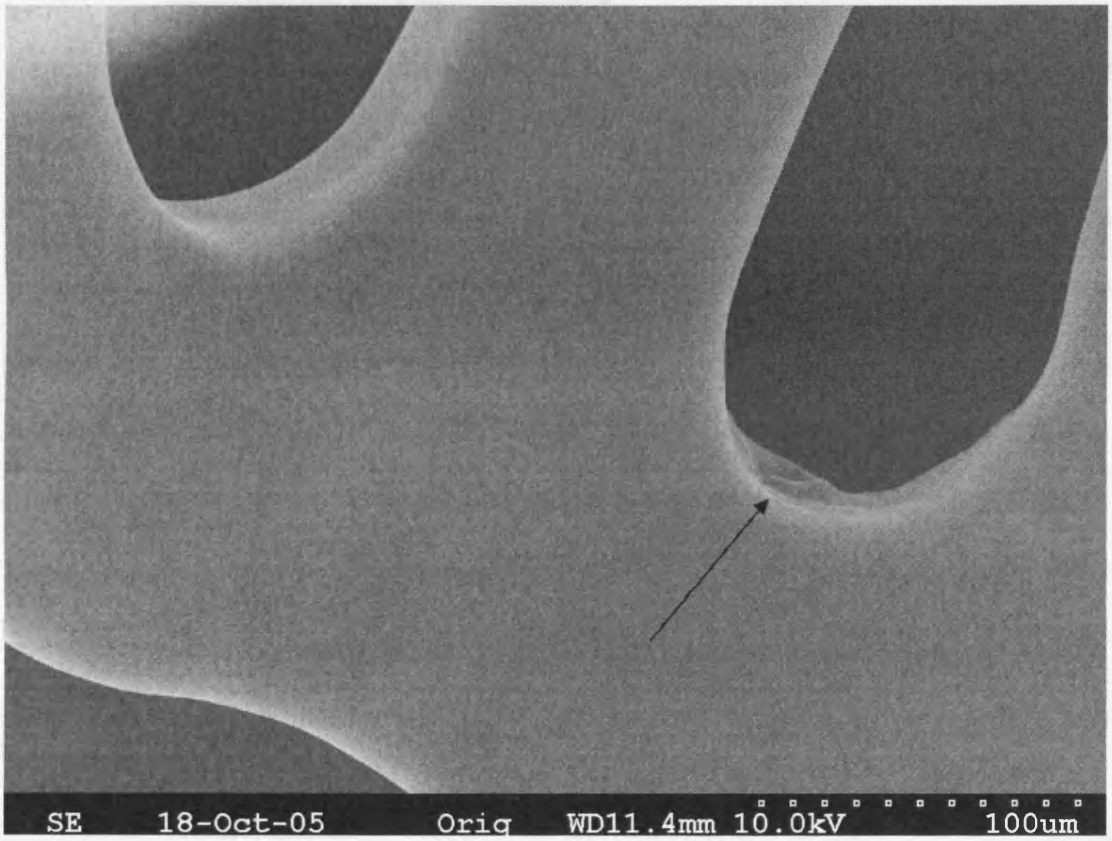


Figure 1.3: Irregular surface on polymer-coated stent can increase risk of thrombogenecity. (SEM)

1.4.2.2 Coated stents

As it has been felt that many or all of the complications of stents are related to their non-physiological surface, attempts on different fronts have been made to coat the surface of stents with different materials that make them more biocompatible.

1.4.2.2.1 *Plating stents with other metals*

Experimental models using stents coated with platinum, copper or gold initially suggested that these might prove to have less adverse effects on the blood vessels into which they were placed. The effect of ion charge of the metals was not found to be an indicator of extent of intimal hyperplasia or thrombus formation. A randomised control trial of gold-plated intracoronary stents proved disappointing however, with a higher rate of complications than the control group[201]. Iridium Oxide stent coatings have been shown *in vitro* to reduce the concentration of Reactive Oxygen Species in the vicinity of a stent to which leucocytes had been adhered[202]. This is thought to be due to a catalase-like ability of the coating to reduce these free radicals. In theory, such stents would reduce the amount of inflammation related to stent insertion. In a porcine model, reduced restenosis has been seen[203].

Titanium-nitride-oxide (TiNOX) has been shown to resist platelet adhesion and stainless steel stents coated with this alloy have been tested in a porcine model. At six weeks, a 44% reduction in neointimal area was seen ($p<0.02$) compared to plain, stainless steel stent[204].

1.4.2.2.2 *Covered stents*

Stents covered with an artificial membrane have been promoted as they were thought to hold the potential to reduce restenosis simply by acting as a barrier to ingrowth of SMC's. The Jostent, which is covered with PTFE, has been used for patients with aneurysms or perforations of the coronaries. No controlled trial of their use exists[205-8]. Animal studies of covered stents have shown that they inhibit the recovery of the endothelium, not surprisingly, compared to bare metal stents[209].

A different clinical trial in a small number (9) of patients with femoral artery stenosis, showed an *increased* stenosis rate with Dacron covered nitinol stents[210].

1.4.2.2.3 *Tissue coated/covered stents*

The use of endothelial cells bound onto stents is discussed elsewhere (1.6.1.1) as it is relevant to the concept of re-endothelialisation. Stefanidis *et al*[211-212] have published a series of reports of using stents covered with sections of either vein or artery derived from the patient. All these reports show promise but represent a handful of cases, in an uncontrolled trial, with no published long-term results.

1.4.2.2.4 *Semiconductor-coated stents*

A semiconductor coating of Silicon Carbide has shown promise as an inhibitor of thrombus formation on stents in high-risk patients[213-214]. Semiconductors are thought to deter the adherence of platelets and fibrinogen. In rabbit studies, these stents showed a reduced incidence of intimal hyperplasia and complete re-endothelialisation[215]. These stents are now in clinical use as the Carbostent.

1.4.2.3 Bioabsorbable stents

Concerns about the long-term effects of intracoronary metal stents have been raised. These concerns included the potential risk that the persistent presence of a stent might itself be the stimulus to ongoing intimal hyperplasia. A permanent stent was thought to be a potential focus for infection. Mechanical failings were anticipated that might cause trauma to the vessel wall sufficient to cause aneurysm or vessel perforation.

As a result of concerns regarding these hypothetical problems, some researchers have explored the use of temporary, biodegradable or bioresorbable stents. These stents should ideally also be of a material that as it degraded released a bioactive drug i.e. the biodegradable stent would be a local drug delivery device. The Duke bioabsorbable stent was pioneered in the early 1980's, although problems were experienced developing a copolymer with adequate radial hoop strength. Stents degraded over approximately 18 months. The Igaki-Tamai stent, made of Poly-l-Lactic Acid (PLLA), is not appreciably weaker than typical Palmaz-Schatz stents when compared in a porcine model[216]. These stents have been introduced into early clinical practice by the same group, who report no significant recoil with this stent when assessed early by IVUS[217]. At six months, a restenosis (and target vessel revascularisation) rate of 10.5% was seen in a non-randomised series. Another class of biodegradable stent is that where the stent gradually degrades despite being entirely metal. Magnesium alloy stents have been developed that in an uncontrolled porcine study showed low thrombosis and restenosis rates[218].

Although an enticing concept, technical difficulties concerning the structural characteristics (e.g. radial strength), radio-opacity and delivery, have proven almost insurmountable.

Furthermore, although metallic stents do have complications, these all tend to be early after implantation, up to six months or so with the problem of restenosis. Late complications after this point, like aneurysm formation or infection, are not common and there appears even to be a late improvement in luminal diameter. Therefore there is not a need to have stents with a short lifespan, merely that they should biodegrade quickly.

1.4.2.4 Radio-active stents

Vascular brachytherapy (VBT) is the local delivery of radiation to the coronary arteries to prevent complications post-angioplasty, particularly restenosis due to intimal hyperplasia. Two approaches to this have been taken. Radiation can be delivered post-angioplasty by passing a radioactive wire that emits either beta or gamma irradiation. This approach has proven to significantly reduce the incidence of intimal hyperplasia in some, but not all, animal models[219]. Alternatively, stents themselves can be made radioactive. In some animal studies, a reduction in neointimal growth was seen, although others showed a less clear response. Different doses of radiation from the stents caused differing intimal reactions, including *stimulated* hyperplasia[220-21]. In humans, the IRIS trial of beta-emitting stents has been reported. Restenosis at six months was still 31%[222].

Both approaches to delivering radiation seem to have unwanted effects in the longer term with reports of an "edge effect" complicating outcome in several patients. This is the finding of good patency and continued inhibition of hyperplasia where the radiation is delivered, but stimulated hyperplasia and consequent stenosis in sections of artery that immediately abut it.

1.4.2.5 Fibrin-coated stents

Fibrin is the natural end product of enzymatic cleavage of fibrinogen in the coagulation cascade. It can be polymerised *in vitro* with thrombin. This biopolymer has been coated onto stents to form a kind of synthetic film of thrombus on the metal. The principle behind this is that this synthetic thrombus is relatively inert and will protect the stent from the components of the coagulation cascade. It may also act as a framework onto which endothelial cells can regrow, encouraging the passivation of the stent. Schwartz *et al* have used these stents in a porcine model successfully. A reduction in both stent thrombosis and restenosis was seen[223-224]. In addition, the fibrin coating is biodegradable and could be combined with drugs to act as a local delivery method.

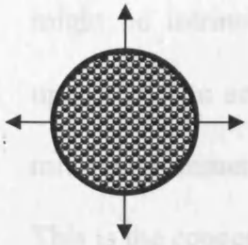
1.4.2.6 Polymer coated stents

Although there are problems with the use of polymer stents alone, the use of polymers still has some attractions. An ideal polymer would be biocompatible and non-thrombogenic. The extent to which a polymer is hydrophilic is inversely related to the extent to which it attracts fibrin and so thrombus formation, at least in terms of acute thrombus. *In vivo* assessment is essential, as the polymer will usually interact in some fashion with the components of the blood or coagulation cascades. Albumin will often adsorb onto or coat a polymeric surface, although studies with albumin-coated polyethylene did not decrease thrombosis rates.

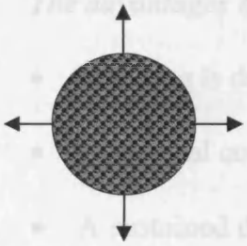
Thus the idea of a hybrid stent, combining the proven mechanical benefits and long term success rate of metal stents with the biocompatibility of polymers, has been proposed. The development of such a stent has not proven easy. Many different polymers have been applied to stents with mixed success. The thrombogenicity of

polyurethane-coated, tantalum stents was examined in an *ex vivo* porcine model using stented polytetrafluoroethylene grafts. A reduction in thrombogenicity was seen[225]. In separate work, five different polymers were used to coat stents in porcine arteries. These were a mixture of both biodegradable and non-biodegradable polymers, including polyurethane. All polymers had previously shown evidence that they were biocompatible *in vitro*. All induced a significant inflammatory response followed by neointimal hyperplasian of platelets[226]. This same heparin-bound polymer coated stent was used clinically in the BENESTENT II trial[227]. Although this study was not a randomised control trial, the results using this stent were at least as favourable as historical comparisons. The trial restricted the use of the stents to fairly large arteries (≥3.0mm) with a single, short (<15mm) stenosis. The Cook GRII stent used a cellulose polymer coating. It has been trialled in a randomised, controlled trial against uncoated Palmaz-Schatz stents in patients. Poor results were obtained in the GR II group with higher incidences of both Target Vessel Revascularisation and MACE[228]. The authors defend this disappointing result on the basis of poor deployment of the GR II and differences between lesions treated. Nevertheless, this stent is no longer marketed.

1.4.2.6.1 Mechanism of Drug Delivery from Polymers

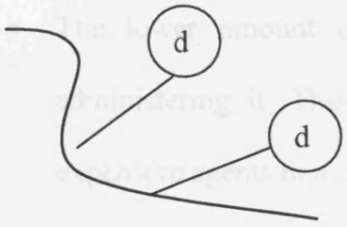


A. Reservoir system in which drug diffuses through a polymer membrane.

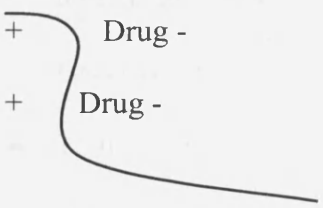


B. Matrix system in which the drug is evenly distributed through the polymer.

- The drug can be released by diffusion through the polymer
- Drug release can occur through a combination of drug diffusion and polymer erosion (degradation)



C. Polymer drug conjugates. The curved line represents the polymers and the bonds connecting the drug (d) and polymer are cleavable within the body.



D. Polymer drug ionic complexes. Drug adsorbed to the polymer and the elution occurs via displacement and diffusion of the drug.

Figure 1.4: Mechanism of drug release from polymer

1.4.2.6.2 Polymer coated stents as drug delivery vehicle

The development of hybrid stents has been driven not only by the concept that they might be intrinsically more biocompatible than bare metal. They also provide an opportunity to act as a reservoir of drugs or molecules that are released into the local microenvironment.

This is the concept of the stent as a local delivery device.

The advantages of this concept are that:

- The drug is delivered specifically to the area where it is required.
- High local concentrations can be achieved.
- A sustained delivery of drug can be achieved, dependent on the pharmacokinetics of the individual drug and polymer used.
- Less systemic side effects can be anticipated as the total amount of drug delivered will be much less than that required for a clinical effect when administered systemically.
- The lower amount of drug required will substantially reduce the cost of administering it. This may well allow more widespread use of newer, more expensive agents in a cash-constrained health service.

There are disadvantages as well:

- All stents that deliver a drug loosely bound to its surface will lose a considerable proportion of the drug as soon as the stent is exposed to the blood flowing in the target vessel.
- Transiently, a small amount of drug will wash off the stent in a very short period of time, creating high local concentrations. This is the "bolus effect".

- It is therefore important to ascertain that the drug used will not have any adverse effects if relatively high concentration of it will be delivered into the coronary circulation, albeit for very short periods.
- Stents because of their small surface area can only carry small quantities of an agent; so to be effective it must be very potent.
- Similarly, the stent does not completely cover the intimal surface of an artery, so agents delivered must be able to diffuse around the area covered by the stent to be uniformly effective. Stents cover only 5 -12% of the arterial surface area, so drug transfer into the disrupted area is unlikely to be uniform since diffusion of the drug over a relatively large distance may be necessary.
- As discussed, a polymer coating used to deliver the drug may produce adverse effects.

The practicality of using stents as a local drug delivery device experimentally is supported by the existence in other fields of cardiology of devices that fulfil this same role. Diphosphonates have been successfully bound to polymeric matrices on bioprosthetic heart valves *in vitro* and shown to reduce the extent of calcification on the valve[229].

Various groups have exploited this potential use for stents (Table 1.3).

Drugs delivered using coated stents	Model	Reduction in thrombosis rate	Reduction in intimal hyperplasia
Heparins[230-232]	Rabbit Pig Human	Y Y Y	N N NA
Methotrexate[233]	Pig	NA	N
Dexamethasone[234-236]	Rat Dog Human	N N NA	N N NA
Methylprednisolone[237]	Pig	NA	Y
DNA[238]	Pig	NA	Y
Forskolin[239]	Rabbit	Y	NA
Argatroban[240]	Pig	Y	NA
Angiopeptin[241-242]	Pig	NA	N
NO donors[243]	Pig	Y	Y
Urokinase[244]	Pig	Y	N
Prostacyclin analogues[245]	*In vitro*	Y	NA
PEG-Hirudin[245]	*In vitro*	Y	NA
Activated Protein C[246]	Rabbit	Y	NA
ST638 (tyr. Kinase inhibitor)[247]	Pig	NA	Y
Paclitaxel[248-253]	Rat Rabbit Pig Human	NA NA NA NA	Y Y Y Y
QP-2[254-255]	Rabbit/ Human	NA/NA	NA/Y
Sirolimus[256]	Pig	NA	Y
Actinomycin[257]	Human	NA	N
Batimastat[258-259]	Pig Human	NA NA	N N

Table 1.3: Drugs used for local stent delivery (NA=Not Assessed)

1.4.2.6.2.1 Paclitaxel (Taxol)

In 1963 an extract from the bark of *Taxus brevifolia* was found to have cytotoxic activity against tumours by its effect on the micro tubular function. Paclitaxel specifically inhibits microtubules by inhibiting their depolymerization resulting in an inhibition of cellular replication at the $G_0/G_1/M$ phases. This means that paclitaxel blocks a cells ability to break down the mitotic spindle during mitosis.

The TAXUS stent system has now established an excellent long-term safety record as demonstrated by clinical trial data now reporting out to four years. TAXUS I, the longest running TAXUS clinical trial to date, reported excellent safety results out to four years with no cases of stent thrombosis, death or myocardial infarction since patient enrolment. The TAXUS clinical trials have reported excellent long-term efficacy at three- to four-year follow-up as demonstrated by low target lesion revascularization (TLR) rates reported from TAXUS I, TAXUS II, TAXUS IV and TAXUS VI trials (Table 1.4). There have been no cases of TAXUS TLR for the TAXUS I clinical trial out to four years. The long-term TAXUS II trial results suggest that the TAXUS stent system inhibits restenosis as demonstrated by significant reductions in TLR. TAXUS II trial reported no new cases of TLR in the TAXUS stent groups from two to three years with a low overall TLR rate of 5.4 percent in the slow-release formulation and 3.7 percent in the moderate-release formulation, as compared to 15.7 percent in the control (bare metal stent) group ($p=0.0001$), resulting in an absolute reduction of 10.3 percent versus control for the slow-release formulation and a 12 percent absolute reduction versus control for the moderate-release formulation. TAXUS IV trial featured markedly reduced restenosis resulting in lower rates of bypass graft surgery and repeat percutaneous interventions. The three-year TLR rate for TAXUS IV trial was 6.9 percent for the TAXUS stent group, as compared to 18.6

percent for the control group (P<0.0001), resulting in an absolute reduction of 11.7 percent versus control. In TAXUS VI, which used the moderate-release formulation, patients in the TAXUS stent group experienced a 54 percent reduction in TLR out to two years. The overall TLR rate in the TAXUS stent group of TAXUS VI was 9.7 percent, as compared to 21 percent for the control group out to two years (p=0.0013). TAXUS IV and TAXUS VI are studying a more complex patient population than TAXUS I and TAXUS II.

PACLITAXEL COATED STENTS TRIALS	**CLINICAL FOLLOWUP**	**RESTENOSIS** R_X **VS CONTROL**		**MACE**
TAXUS-I[260] (May 2003)	48 mths n=61	0%	11%	3.3%vs.10% in control group (p<0.001)
TAXUS II[261] (March2005)	36 mths n=536	2.3% in slow release 4.7% in moderate release	18% 20%	8.8% vs. 20% in control group (p=0.004)
TAXUS III[262] (Feb 2003)	6 mths n=28	4%	NA	29%
TAXUS IV/V/VI[263-264]	Ongoing			
ELUTES[253] (March 2004)	1 year n=190	3%	21%	11% vs. 11% in control group (p=NS)
ASPECT[265] (Feb 2003)	6 mths n=177	4%	27%	Not available ever

Table 1.4: Various trials with Paclitaxel.

1.4.2.6.2.2 Sirolimus (Rapamycin)

Rapamycin is a naturally occurring macrolide antibiotic produced by *Streptomyces Hygroscopicus.* Rapamycin is a macrocylic lactone agent that interacts with cell-cycle regulating proteins and inhibits cell division between phases G1 and S1, thereby inhibiting cellular proliferation (Figure 1.5). The immuno-modulating antibiotic sirolimus has been shown to have a significant effect in reducing intimal hyperplasia. Its action is mediated by binding to an intracellular receptor the FK506 binding protein (FKBP12). The complex rapamycin-FKBP12 then inhibits the activity of a specific kinase named mammalian target of rapamycin (mTOR), which prevents mitogen-induced down-regulation of p27 (KIP1) by an unknown mechanism. It up-regulates p27 levels and inhibits the phosphorylation of retinoblastoma protein (pRb) with blockage of cell cycle progression at the G_1-S phase. In *in-vitro* and *in-vivo* studies, sirolimus showed inhibition of proliferation[266-267] and migration[268] of VSMCs. Various multicenter, double-blind, placebo-controlled randomized studies have been conducted with the Sirolimus-Coated Bx Velocity Balloon-Expandable Stent in the Treatment of Patients with De Novo Native Coronary-Artery Lesions (RAVEL), the Sirolimus-Eluting Balloon-Expandable Stent in the Treatment of Patients with De Novo Native Coronary-Artery Lesions (SIRIUS), and the smaller European and Latin American (E-SIRIUS) and Canadian (C-SIRIUS) trials (Table 1.5). Each of these four trials compared a sirolimus-eluting stent with a bare-metal stent of identical design (Bx Velocity), but without polymer and drug coatings, implanted in single, previously untreated lesions in native coronary arteries, using a double-blind study design with a 1:1 randomization process. In summary, RAVEL included patients in clinically stable condition with relatively low-risk lesions, whereas the three SIRIUS trials involved patients with higher-risk and more complex lesions. A pooled analysis of four

randomized trials comparing sirolimus-eluting stents and bare-metal stents in 1748 patients with 4 years of follow-up, found no evidence of a significantly higher rate of death, myocardial infarction, or stent thrombosis in the patients treated with sirolimus-eluting stents.

SIROLIMUS COATED STENTS TRIALS	CLINICAL FOLLOWUP	RESTENOSIS R_X VS CONTROL		MACE *
First In Man sirolimus-coated stent implantation (FIM) Trial[269] (March 2003)	3 years n=45	0%	NA	10% vs. 20% in control group (p<0.001)
RAVEL [270-271] (March 2003)	2 years n=238	0%	26%	10% vs. 20% in control group (p<0.001)
SIRIUS[272] (September 2002)	1 year n=1058	3%	35%	8% vs. 23% in control group(p<0.001)
C-SIRIUS[273] (March 2004)	9 months n=100	0%	41%	4% vs. 18% in control group (p=0.05)
E-SIRIUS[274] (October 2003)	8 months n=352	4%	42%	8.1% vs. 22.8% in control group(p<0.001)

MACE* (Major Adverse Cardiac Events which includes Death, Myocardial Infarction and Revascularization)

Table 1.5: Various trials with Sirolimus

1.4.2.6.2.11 Heparin

Heparin has been given in humans as a local drug infusion into the coronary artery wall by a modified angioplasty balloon in either angioplasty alone or with stenting[275]. Although safe, with a ~10% restenosis rate, no control group existed in these trials. Heparin-coated stents have been developed that show-reduced incidence of thrombosis, but not restenosis, in animals[276]. Stents where the heparin is covalently bound to the stent surface had an extremely low incidence of thrombosis in humans when used in the Benestent-II trial. However the results were only compared to

previous studies, as there was no control group with uncoated stents. Doubts exist over the effectiveness of locally delivered heparins. Use of the low molecular weight heparin, reviparin delivered using the Dispatch catheter has not shown effectiveness in rabbits[277]. In a human pilot study, a 28% restenosis rate was seen. No control group was used[278].

1.4.2.6.2.12 Glycoprotein IIb/IIIa receptor antagonists

Work in our group has shown that the cellulose polymer-coated Cook GR II stent quickly absorbs and slowly releases drugs. Abciximab, the most widely studied of the glycoprotein IIb/IIIa inhibitors has successfully been shown to bind to and elute from this polymer[279]. *In vitro*[280], this drug has shown antithrombotic effects. In the rabbit iliac artery, deep arterial injury, reduced flow model of angioplasty, the same reduction in thrombosis was demonstrated. The effects of abciximab were still detected *in vitro* after sterilisation of the coated stents.

Tirofiban is another non-peptide GPIIb/IIIa receptor blocker. Clinical studies in unstable angina have suggested a beneficial effect, at least over short follow-up periods[281]. An analogue of tirofiban, L-703081, has been tested in a canine model. When bound to a polycaprolactone-coated stent, the drug showed a significant reduction in platelet deposition[282].

The concept of combining two active drugs and absorbing both, as a conjugate, was also developed in this department. Abciximab-urokinase conjugates were successfully absorbed onto the same stent[283], again showing beneficial effects *in vitro*.

1.4.2.6.2.13 Other antithrombotics

The effectiveness of the polymer-coated GR II stent has been demonstrated using the antithrombotic agent Activated Protein C[284]. In this study, fibrinogen deposition was

reduced by 96% and flow was increased tenfold in APC-eluting stents compared to controls. Similarly, urokinase, a thrombolytic agent, has been absorbed successfully to stent[285].

PEG-hirudin and prostaglandin analogues have both demonstrated an antithrombotic effect *in vitro* when bound to polylactic acid coated stents[286].

1.5 Summary of agents targeted at the restenosis process

The many different mechanisms that play a role in the proliferative response after angioplasty have been reviewed. From an understanding of the underlying mechanisms, many researchers have attempted to reduce the response therapeutically. The table below summarises the agents used and their effectiveness or otherwise in various models for intimal hyperplasia (Table 1.6).

Agent	Model used	Result
Antibiotics		
Rapamycin[287]	*in vitro*	Inhibits growth
Lipid lowering agents		
Fluvastatin – FLARE study[288]	Human	No benefit
Lovastatin – CLAPT study[289]	Human	No benefit
Bezafibrate[290]	Human	Reduced neointimal hyperplasia
NO donors		
Linsidomin/misoldomine – ACCORD trial[291]	Human	No clinical benefit
Cytokines and hormones		
Il-10[292]	Rabbit	Inhibits stent restenosis.
Prostaglandin E1[293]	Human	Reduced neointimal hyperplasia
Prostacyclin[294]	Human	No benefit
Oestradiol[295]	Rabbit	Reduced neointimal hyperplasia
Activin[296]	Mice	Reduced neointimal hyperplasia
Angiopeptin[297]	Pig	Reduced neointimal hyperplasia
Angiotensin II/ACE inhibitors		
Losartan[298]	Pig	Reduced neointimal hyperplasia
Fosinopril[299]	Human	No benefit

Calcium antagonists		
Verapamil[300]	Human	Reduced neointimal hyperplasia (Peripheral vessels)
Amlodipine-CAPARES tria[301]	Human	No reduction in restenosis
Diltiazem[302]	*In vitro*	Reduced neointimal hyperplasia
Y-27632 (Rho-Kinase inhibitor)[303]	Rat	Reduced neointimal hyperplasia
Antiallergens		
Tranilast – TREAT studY[304]	Human	Reduced neointimal hyperplasia
Permirolast[305]	Human	Reduced neointimal hyperplasia
Tyrosine kinase inhibitors		
Epigallocatechi[306]	*In vitro*	Reduced neointimal hyperplasia
PP1/AGL1872[307]	*In vitro*	Reduced neointimal hyperplasia
Anticoagulants		
Ardeparin[308]	Human	No benefit
Nadroparin –FACT trial[309]	Human	No benefit
Anti-thrombin III/heparin[310]	Pig	Borderline benefit
Clivarine/PEG hirudin[311]	Pig	Reduced neointimal hyperplasia
r-hirudin[312]	Pig	Reduced neointimal hyperplasia
Heparin[313]	Human	No benefit
Hirudin – HELVETICA trial[314]	Human	No benefit
Warfarin[315]	Human	Mixed results
Antiplatelets, anti-PDGF & TXA2 antagonists		
Trapidil[316]	Human	No benefit
Cilostazol[317]	Human	Reduced neointimal hyperplasia compared to aspirin
Sulotraban[318]	Human	No benefit
GR32191B – CARPORT study[319]	Human	No benefit
Abciximab – ERASER trial[320]	Human	No benefit
Non-specific		
Suramin[321]	Rabbit	Reduced neointimal hyperplasia
U-86983[322]	Rat	Reduced neointimal hyperplasia
Fatty Acids		
N-3 Fatty Acids – CART study[323]	Human	No benefit
Fish Oil[324]	Human	No benefit
Endothelin Antagonism		
ABT147627[325]	Pig	Reduced neointimal hyperplasia
Antioxidants		
Probucol-MVP trial[326]	Human	Reduced neointimal hyperplasia
Beta-carotene[327]	Rabbit	No benefit
IRFI 042[328]	Rat	Reduced neointimal hyperplasia
Mithramycin[329]	Rat	Reduced neointimal hyperplasia

Table 1.6: Agents used for restenosis

1.6 Models of restenosis

Various models have been developed to investigate the problem of restenosis. These all have strengths and weaknesses of their own. It is helpful to explain the different models, as there is no model yet described is an accurate predictor of outcome in humans. Indeed the transfer of results in animals of various treatments to clinical trials has often been disappointing.

Some of the trials using treatments with proven benefit in animals were used in doses that were considerably lower per kilogram in the human trials. Although this can be seen as a fault of the trial, often the equivalent dose in humans would have prohibitive side effects. This then can often mean that it is the animal trials that are unhelpfully conducted. An agent tested must be tested at a dose usable in humans or the animal work is of dubious worth. Alternatively, the agent could be delivered locally, bypassing its systemic side effects. Perhaps if some of the agents rejected in clinical trials were to be administered locally, a benefit would be seen.

Finally, it can be argued that it is the animal models themselves that give the unreliable results. It may well be that differences between species stop animal results being applicable to humans. For example, cyclosporin A, an immunosuppressant, showed reduction of neointima formation in rats but not rabbits[330].

Part of the problem with all animal models is the difficulty of modelling complex coronary disease. In humans this occurs over years or even decades, giving an organized, fibrous and even calcified, atherosclerotic plaque. Laboratory animals are young and healthy, with completely normal blood vessels. Several animal models have been used to model restenosis.

Various animal models of stent restenosis

2.4.2.1 Canine model

Folts *et al*[331-332] describe a model using coronary vessels. It involves the placing of constricting rings around the dogs' coronaries and has been used to study thrombosis and restenosis. However, for the evaluation of stent-related complications it is not ideal. First, deployment of balloon expandable stents is associated with deep vessel injury, in particular disruption of the internal elastic lamina and penetration of stent wires into the deep media as has been confirmed in human necropsy specimens. Constricting rings may cause a stenosis, but do not reproduce the deep vessel injury. Second, the stent itself should be well expanded to correspond to clinical practice, so use of a plastic constrictor to produce a stenosis within the stented area of the vessel is inappropriate.

2.4.2.2 Rabbit iliac model

Work in our laboratory has been based around a rabbit iliac model. This model was described by More *et al.* In brief, balloon angioplasty injury is produced in the common iliac artery via the superficial femoral artery. This produces a deep arterial injury. The balloon is withdrawn and the superficial femoral artery ligated. The effects on thrombosis and restenosis following angioplasty were studied up to three months later. Reliable intimal thickening caused by myointimal hyperplasia is seen with gradual re-endothelialisation over 14 days. This model is relatively simple to perform and rabbits are easily kept. The technique is also similar to that used in the rabbit hindlimb model of ischaemia for angiogenesis. Many other groups have used similar rabbit models with or without hypercholesterolaemic diets[333-335].

2.4.2.3 Rat carotid artery model

This long established model was initially developed to study plaque formation rather than restenosis. Its strength lies in the comprehensive knowledge of rat cellular biology available. Rats are easy to keep and inexpensive. However, neointimal formation following balloon angioplasty in the rat is compensated for by increases in vessel diameter, i.e. remodeling. This means functional stenosis does not occur despite intimal hyperplasia. Furthermore, significant thrombosis does not occur in this model[336].

2.4.2.4 Porcine coronary model

This is the main model that studies coronary vessels rather than limb vessels. This may be important in that coronary vessels are quite different to other vessels studied. They fill in diastole rather than systole; they are not elastic arteries like carotid or iliac arteries; functionally important restenosis occurs. The restenosis that occurs is similar to that seen in humans. A hypercholesterolaemic diet exacerbates the lesions seen.

The porcine model is not perfect however. The animals are still young and the lesions formed are produced in healthy arteries over a period of days rather than years. The animals are difficult and costly to look after. A separate animal licence is required for their use in Britain. Positive studies in pigs have not always translated into a reduction of restenosis in humans[337].

1.7 Why study eptifibatide as drug-eluting stent?

Eptifibatide is a cyclic heptapeptide[338-339] (figure 1.6) containing modified KGD sequence, mimicking a part of the structure of a glycoprotein IIb/IIIa inhibitor barbourin derived from the venom of the pigmy rattlesnake Sistrurus barbouri. Chemically it is N_6 -(aminoiminomethyl)-N_2 -(3-mercapto-1-oxopropyl-L-lysylglycyl-L-a-aspartyl- L-tryptophyl-L -prolyl-L-cysteinamide, cyclic (1Ø6)-disulfide. Like barbourin, eptifibatide is a strong inhibitor and has high affinity to the GP IIb/IIIa receptor[340]. Furthermore, it also binds to αvβ3 integrin, but with much lower affinity. There is very little data on effect of eptifibatide on cells via interactions with αvβ3 integrin. It is expressed in human vascular smooth muscle cells (SMCs) and is implicated in SMC adhesion, migration, proliferation and response to growth factors.

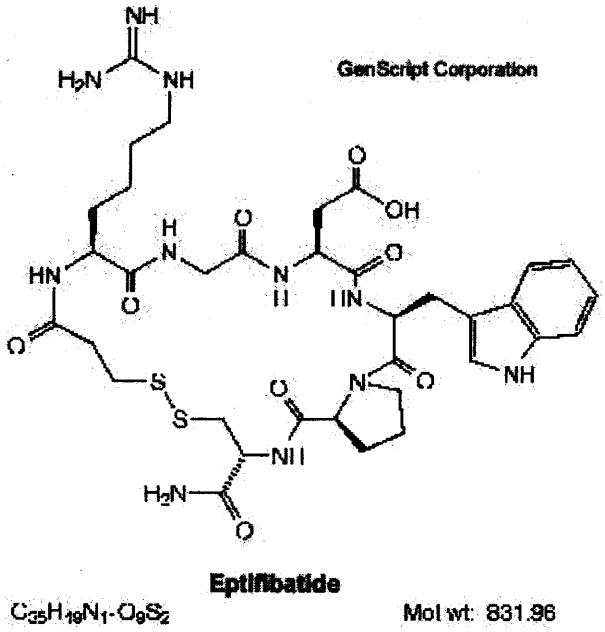


Figure 1.5: Structure of eptifibatide

Eptifibatide, like other antagonists of platelet receptor GP IIb/IIIa, functions by blocking the binding of the adhesive proteins fibrinogen and von Willebrand factor to GP IIb/IIIa on the surface of activated platelets (Figure2). It is a potent antithrombotic because the binding of these proteins to GP IIb/IIIa is the event that precipitates platelet aggregation and subsequent arterial thrombus formation. Eptifibatide is a specific inhibitor of GP IIb/IIIa, and because GP IIb/IIIa is confined to platelets and their precursors, the pharmacologic action of eptifibatide is essentially confined to those cells, thus minimizing the potential for side effects and making the drug more attractive for clinical use. The effectiveness of eptifibatide as an inhibitor of GP IIb/IIIa has been confirmed in a variety of preclinical and clinical studies.

Two large clinical trials (IMPACT II and PURSUIT)[341-342] have demonstrated that eptifibatide significantly reduced coronary events in patients without increasing the risk of bleeding complications. Significant clinical benefits have been demonstrated with eptifibatide as adjunctive therapy in patients undergoing selective PCI with stent implantation in the ESPRIT[343] trial and in patients with ACS in the PURSUIT trial. In the ESPRIT trial patients were randomly assigned to receive placebo & eptifibatide (two 180-[micro] g/kg boluses, 10 minutes apart, with a continuous infusion of 2.0 [micro] g/kg per minute; n = 1040), started immediately before stent implantation and continued for 18 to 24 hours. A novel higher-dose, double-bolus regimen of eptifibatide in coronary intervention attains and maintains >90% inhibition of platelet aggregation in >90% of patients. Of all regimens evaluated, the eptifibatide regimen inhibited platelet aggregation most consistently throughout both the early and late periods compared to abciximab or tirofiban at doses used in the Evaluation of Platelet IIb/IIIa Inhibitor for stenting (EPISTENT)[344]. These studies showed a rapid inhibition of platelet aggregation within 15 minutes of eptifibatide treatment, and at appropriate

dosages this inhibition was sustained for the duration of the drug infusion.

Interest in eptifibatide as a potential agent began following publication that eptifibatide also specifically inhibits $\alpha_v\beta_3$ mediated binding of human smooth muscle and endothelial cells, to the same degree as abciximab[345]. Eptifibatide inhibited $\alpha_v\beta_3$-mediated attachments of HASMCs to thrombospondin (TSP) and prothrombin but had no effect on $\alpha_v\beta_5$ or β_1 mediated HASMC attachment to vitronectin-, collagen-, or fibronectin-coated or uncoated tissue culture plates. In cell proliferation assays, eptifibatide inhibited $\alpha_v\beta_3$-mediated responses to soluble TSP by HASMCs and β_3 integrin-expressing HEK cells. This study suggested that it might be possible to inhibit neointimal hyperplasia with an anti-platelet drug, if the local dose is sufficient (perhaps through local stent based delivery), simultaneously reducing the risk of both restenosis and thrombosis. With the recent findings on the effectiveness of eptifibatide on the vitronectin receptor, we felt the potential value of a GPIIb/IIIa inhibitor-eluting stent needs to be re-visited.

This research aimed to demonstrate local delivery of an established antithrombotic drug with proven clinical benefits in preventing thrombus formation in man, and with potential to influence restenosis.

1.4 Summary

Percutaneous coronary intervention is a rapidly expanding and developing field. Drug eluting stents have been part of the procedure since 2002 as they reduce the risk of in-stent restenosis. However, thrombotic complications are not abolished and systemic haemorrhagic complications are still observed. During percutaneous coronary intervention, balloon inflation and stent deployment injures the endothelial layer of the vessel wall. Endothelial recovery takes about four weeks with bare metal stents, but it can take several months with drug eluting stents because of bystander eluted drug inhibition. The risk of stent thrombosis may therefore increase because of prolonged exposure to the stent strut. The association between late thrombosis with delayed stent coverage and cessation of dual antithrombotic therapy strongly suggest the need for a longer period of combination therapy. On the other hand, longer administration of dual therapy is hampered by its higher cost, patient compliance and the possibility of increased bleeding complications. What seems to be clear is that a longer period of dual anti-platelet therapy is needed for patients undergoing DES implantation than for those receiving a bare metal stent. The current recommendations from the AHA/ACC suggest 12 months of dual antiplatelet therapy.

As discussed in the introduction, thrombus formation at the angioplasty site is due to exposure of the subendothelial surface and a complex interaction between the vessel wall and blood stream. Platelets play a central role in the formation of mural thrombus and have been the target of various pharmacological interventions especially after developing anti-GpIIb/IIIa receptor antagonists. Local drug delivery could increase target tissue drug saturation with little systemic exposure enhancing is efficacy and safety. Only stent-based drug delivery has shown a significant effect on vessel wall

behaviour after injury overcoming the problem of tissue retention by prolonged elution. Localising an anti-platelet agent to the angioplasty site could increase delivery efficacy and retention.

This project examines the hypothesis that a stent can be coated with eptifibatide and has dual anti-platelet and anti-restenosis properties.

The more common methods, such as adsorbtion-elution, radiolabelling, platelet aggregometry, cell culture, and angioplasty of iliac vessel are described in Chapter 2. Chapter 3 deals with the results of adsorbtion-elution studies with eptifibatide-polymer coated stent, chapter 4 explains effect of eptifibatide eluting stents on platelet deposition, chapter 5 gives in-vitro cell culture results and chapter 6 explains results of in-vivo experiments with eptifibatide eluting stents.

Chapter 2

2 Materials and methods

2.1 Background

GPIIb/IIIa (αIIb/β3) is closely related to the vitronectin receptor (αvβ3), which binds matrix proteins deposited on injured tissues, including vitronectin, VWF, fibrinogen, fibronectin, collagen and thrombospondin. This receptor mediates a number of processes, which include smooth muscle cell adhesion, migration, and proliferation. Until recently only abciximab was thought to have any potential benefit, through inhibition of this receptor, on smooth muscle cell function. Recent studies[345] have however shown that eptifibatide also specifically inhibits αvβ3 mediated binding of human smooth muscle and endothelial cells, to the same degree as abciximab. Eptifibatide was selected for this work, since it has demonstrated the effectiveness of eptifibatide on the vitronectin receptor. We felt that the potential value of a GPIIb/IIIa inhibitor-eluting stent needs to be re-visited. It has not however been investigated as a stent-based therapy. The aim of this MD thesis was to investigate the effects on the in-stent restenosis process of drug eluted from the stent itself.

2.2 Materials

The polymer polyvinyl butyrate (PVB) was used as a delivery vehicle for eptifibatide on the bare metal stents. Polyvinyl butyrate is a biocompatible resin and contains only carbon, hydrogen, and oxygen. Acetals, such as polyvinyl butyral, are formed by the well-known reaction between aldehydes and alcohols. The addition of one molecule of an alcohol to one molecule of an aldehyde produces a hemiacetal. Hemiacetals are rarely isolated because of their inherent instability, but rather are further reacted with another molecule of alcohol to form a stable acetal.

Polyvinyl acetals are prepared from aldehydes and polyvinyl alcohols. Polyvinyl alcohols are high molecular weight resins containing various percentages of hydroxyl and acetate groups produced by hydrolysis of polyvinyl acetate. PVB resins are soluble in alcohols, glycol ethers and certain mixtures of polar and non-polar solvents and can withstand heating up to 200°F for prolonged periods. When an alcohol is the only solvent, the viscosity of a PVB solution increases as the molecular weight of the alcohol increases. PVB is used in a wide variety of metal coating and can improve coating uniformity, minimize cratering, improve adhesion, and increase coating toughness and flexibility. Solutia Inc. has assessed the toxicity of polyvinyl butyral/polyvinyl alcohol/polyvinyl acetate co-polymers in oral and dermal acute toxicity tests, eye and skin irritation, and genetic toxicity studies (Ames mutagenicity and chromosomal aberration assays). The available data indicate that polyvinyl butyral/polyvinyl alcohol/ polyvinyl acetate co-polymers have a low order of toxicity.

The eptifibatide was supplied at a concentration of 2 mg/ml by Schering-Plough and was freeze-dried and later radiolabelled with tritium (Pepceuticals Ltd.).

Human vascular SMCs (HVSMCs) originated from primary explants culture of vein fragments. The fragments of veins with normal morphology were obtained from healthy margins of veins removed from patients undergoing varicose vein surgery.

New Zealand White rabbits were used for the *in vivo* experiments. Animals were housed and cared for in the Biomedical Services Unit, Leicester University and all procedures were undertaken in accordance with the Animals (Scientific Procedures) Act 1986 under licence from the Home Office in London.

Leicester Research Ethics Committee approved these experiments. All investigations conformed to the principles outlined in the Declaration of Helsinki[346].

2.3 in- vitro Methods

2.3.1 Eptifibatide radiolabelling

Radiolabelling was chosen as the means to quantify the absorption and subsequent rate of release of the agent from the respective polymer. In each case a small amount of labelled agent was mixed with a known quantity of unlabelled agent. This "spiked" solution was used to determine the amount of radioactivity associated with a known quantity of unlabelled agent. This allowed the calculation of amounts of drug represented by samples of a known radioactivity associated with the stents.

In the case of eptifibatide, the radiolabel used was tritium (H^3), a beta-emitting isotope. Radiochemical purity was determined and found to be 94.26% by using High-performance liquid chromatography (HPLC) method. HPLC profile showed that radiolabelled eptifibatide co-elutes with cold eptifibatide. (Pepceuticals Limited)

2.3.2 Eptifibatide adsorption and elution in-vitro

2.3.2.1 Passive adsorption

Passive adsorption is the adherence of peptides or other molecules to artificial surfaces. This is a non-covalent interaction, i.e. without chemical change of the agent used. It is a method that has been used to bind proteins onto polymeric or other surfaces to facilitate drug delivery.

This means that the molecules used for absorption will remain intact during delivery, although some conformational change is possible depending on how large the size of any pores in the polymer are. It is a very simple technique to load an object, like a stent, with drug but is limited by the finite volume of the coating on the stent. Furthermore, it is inevitable that a significant amount of the protein is washed off very easily from the surface of the stent. Nevertheless, previous work has shown that appreciable amounts of a protein will remain adherent to the polymer for long periods of time, up to many days.

The pharmacokinetics of eptifibatide was studied in relation to the polymer-coated (PVB) stent. The absorption and elution kinetics *in vitro* were studied to ascertain whether this combination of drug and stent was likely to prove useful in further *in vivo* work. The polyvinyl butyrate-coated stent has been designed specifically to be suitable for rapid absorption and release of agents of use in the restenosis process. The absorption and release of drugs into the polymer coating of a stent depends on the property of passive absorption.

All bare metal stents (3x18mm) were weighed prior to coating. The bare metal stents were sprayed with hydrophobic polymer (PVB). Polymer and unlabelled drug (15%) was dissolved in methanol/chloroform (50%w/w), 80 µl of H3-radiolabelled eptifibatide (specific activity 20ci/mmol)) was added to the solution and used as a 'spike' to allow detection of the drug. The stents were sprayed using ultrasonic micro spray (SONOTEK ultrasonic generator) to allow uniform distribution of the drug on the stent (by author at Polybiomed Limited). Adsorption is by diffusion, in a hydrophobic solvent such as ethanol, into the substance of the polymer, where a drug is held by hydrophobic–hydrophobic interaction between the drug and the polymer.

Each stent had six drug/polymer coating passes, dried in a vacuum oven for 1 hr (to remove any remaining solvent) and then weighed to obtain approximate drug loadings. Each stent had then a top coating with pure polymer (2 passes) to retard drug elution. The stents were stored under vacuum desiccant over 2 days.

2.3.2.2 Eptifibatide elution in-vitro

Simple Immersion method:

Eptifibatide coated stents were immersed in polypropylene tubes containing PBS solution at room temperature. The tubes were pre-treated with 1% bovine serum albumin (BSA) in PBS for 48 h, and then rinsed. This reduced non-specific binding of radiolabelled eptifibatide from adhering to the surface of tubes. Three stents were tested under each set of conditions. At specific time points, stents were removed from solution, air-dried and immersed in new polypropylene tubes containing PBS solution. Radioactive drug eluted from stent in each PBS solution was determined in a beta-counter (Tricarb® Packard Liquid Scintillation Analyzer).

Perfusion method:

Eptifibatide coated stents were also perfused continuous[347] at 25 ml min^{-1} in a closed-loop circuit with PBS containing 1% bovine serum albumin (BSA) (Fig.2.1), for upto a month. The bottles were pre-treated with 1% bovine serum albumin (BSA) in PBS for 48 h, and then rinsed. Three stents were tested under each set of conditions. The PBS was changed from the reservoir and the eptifibatide eluted in PBS was quantified by beta counting. The perfusate was maintained at 37°C, and was changed every 24 hours.

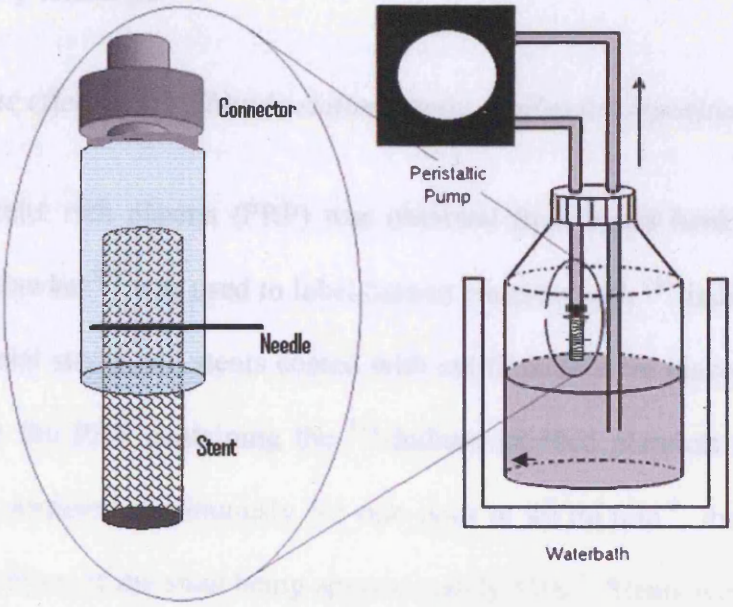


Figure 2.1: *In vitro* perfusion circuit for elution of eptifibatide from polymer-coated stents. The standard flow rate used was 25ml min^{-1}. In the platelet deposition experiments, the PBS was replaced by blood containing ^{111}Indium labelled platelets.

2.3.3 Platelet aggregometry

2.3.3.1 Platelet aggregation

Samples from the perfusate were taken after elution of the eptifibatide-coated stents, and the ability of the platelets to aggregate to ADP (4μM) was assessed using Born aggregometry technique[348].

2.3.3.2 The effect of eptifibatide eluting stents on platelet deposition

Human platelet rich plasma (PRP) was obtained from blood bank and the standard method of Hawker[349] was used to label human platelets with ^{111}-indium oxine (^{111}-In). The bare metal stents and stents coated with eptifibatide were placed in the perfusion circuit, with the PRP containing the ^{111}-indium labelled platelets as perfusate. The stents were perfused continuously for one hour at 40 ml min^{-1}, the calculated sheer rate at the surface of the stent being approximately 850s^{-1}. Stents were then rinsed and the radioactivity associated with each stent was counted in gamma-counter (Packard Cobra series Auto-gamma counting system, 15-75 keV window).

2.3.4 The effect of eptifibatide on smooth muscle cell function

Human plasma fibronectin, human vitronectin, human thrombospondin, PDGF and basic FGF were purchased from Sigma-Aldrich; rat osteopontin was kindly provided by SmithKline Beecham Pharmaceuticals. Eptifibatide was provided by Schering-Plough.

Human vascular SMCs (SMCs) originated from primary explants culture of vein fragments. The fragments of veins with normal morphology were obtained from healthy margins of veins removed from patients undergoing varicose vein surgery. Human SMCs used in migration assay originated from umbilical artery. The Leicester

Research Ethics Committee approved their use. Cells were cultured in RPMI 1640 medium with 15% foetal bovine serum (FBS) and used at passages 4 to 6. SMC phenotype was verified by a characteristic hill-and-valley growth pattern. The rat PAC1 cell line derived from pulmonary arterial smooth muscle was cultured in DMEM medium with 10% FBS.

2.3.4.1 The effect of eptifibatide on SMC adhesion

The assay was performed with some modifications. [^{3}H]-thymidine-labelled SMCs were detached, washed in the presence of trypsin inhibitor (Sigma-Aldrich), re-suspended in DMEM containing 1% bovine serum albumin (BSA) and allowed to adhere (10^4 cells/ well) to 96 well plates (ViewPlate-96, Packard) for 1 hour at 37^0C, followed by gentle washing with phosphate buffered saline (PBS). Plates were pre-coated with ECM proteins fibronectin, vitronectin, thrombospondin and osteopontin (1000 ng/cm^2) overnight. Adhered cells were dissolved in Microscint-40 (Packard) and counted in a Top Count (Packard).

2.3.4.2 Thrombospondin binding to cells

Thrombospondin was labelled with [^{125}I] (Amersham Pharmacia Biotech). Human SMCs (10^5) were incubated with [^{125}I]-labelled thrombospondin (750 ng/11x10^6 cpm) in the absence or presence of 10 and 50 μg/ml of eptifibatide in 200 μl of RPMI medium at 37°C for 40 min, washed 3 times with PBS and counted in β-counter (Packard Bioscience). To measure a background integrin-independent binding, 5 mM of EDTA was added.

2.3.4.3 The effect of eptifibatide on SMC migration

Migration was measured using migration assay kit (Chemicon International) according to manufacturer's recommendations. SMCs originated from umbilical artery were starved for18 hrs in Earls medium 199, detached with trypsin, washed in the presence of trypsin inhibitor (Sigma-Aldrich), re-suspended in Earls medium 199 containing 5% BSA. Cells ($5x10^4$ cells/well) were placed in the upper 96 well migration chamber. The feeder tray was pre-coated with vitronectin, thrombospondin, osteopontin, PDGF and FGF (each protein with concentration of 350 ng/well) with and without treatment with eptifibatide, abciximab and GRGES peptide. Abciximab and GRGES peptide were used as positive and negative controls. Cells migrated for 18h in the medium 199 at 37°C in a 5% CO_2 incubator. The cells migrated through the membrane (8 μm pore size) were detached, collected, lysed and stained with fluorescent dye CyQuant. Equal aliquots of each sample were transferred to a 96 well plate and fluorescence was read with a fluorescence plate reader using 480/520 nm filter set.

2.3.4.4 The effect of eptifibatide eluting stents in cell culture proliferation

Human smooth muscle cells were cultured in medium 199 with Earl's salt and 10% foetal bovine serum (FBS). Cells were passaged 4 to 6 times. SMC phenotype was verified by a characteristic hill-and-valley growth pattern and were 80% confluent prior to assay. Cells were starved for 18 hrs in Earl's medium 199 prior to assay. SMCs were detached with trypsin, washed in the presence of trypsin inhibitor (Sigma-Aldrich), re-suspended in Earls medium 199 containing 5% BSA and centrifuged for 5 minutes at 1500 rpm. Cells pellet was resuspended in Earls medium 199 in 5% BSA and then cells were plated at a density of 3×10^5 cells per 60 mm dish. Each of bare

metal and eptifibatide-loaded stents were fixed in the centre of dishes with sterile surgical bone cement in separate experiments. All culture dishes were incubated at 37°C in 5% CO_2 incubator. Culture media was replaced every 48 hours. The Zone of cell growth inhibition was measured at 7 days after initial plating with a micrometer inserted into the eyepiece of a standard inverted microscope (Olympus). In a subset of the cultures (at 7 days), the cells were stained with toluidine blue to allow clear-cut demarcation of the zone of cell growth inhibition at low levels of magnification.

2.4 In vivo model methods

2.4.1 Loading of stents with eptifibatide for in vivo work

2.25x13 mm bare metal Guidant Pixel stents were used to test the effects of eptifibatide delivery in the animal model. These were gently removed from the delivery balloon and coated with PVB/eptifibatide as outlined in 2.3.2.1. The stents were then replaced on the delivery balloon with minimal handling of the stent itself. Delivery balloons and stents were then put through a cold cycle (37°C) sterilisation program using ethylene oxide gas. This is the usual means for sterilising stents and delivery balloons. Control stents without eptifibatide were treated identically. After sterilisation, the stents were stored in sterile packs at 4°C until required for animal use. The longest they were kept was for one week.

2.4.2 Angioplasty and stent model

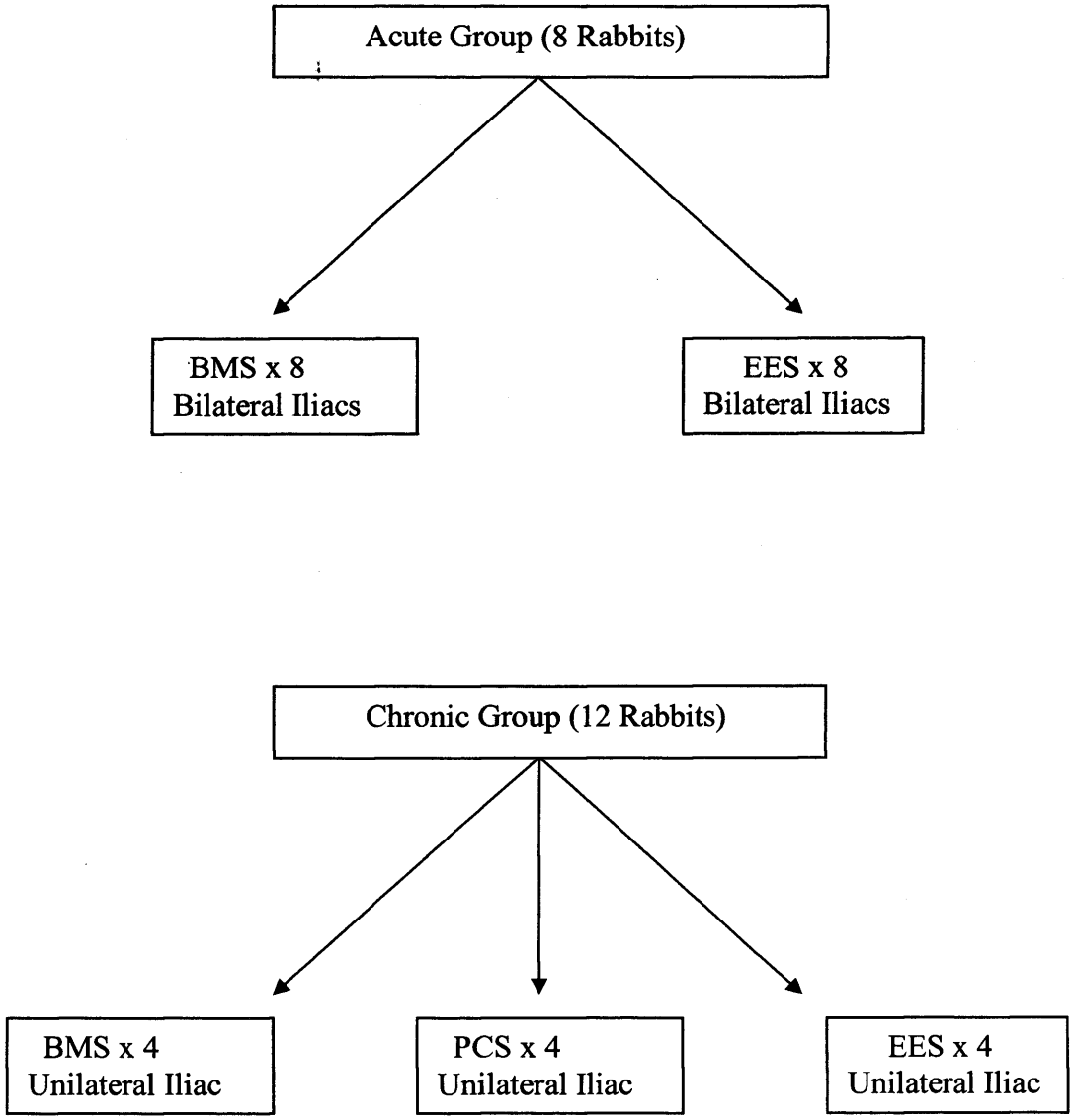
In this work, the rabbit iliac artery model was used after consideration of its advantages and disadvantages. Rabbits used underwent one of two different procedures. These were the acute and chronic groups (Figure 2.2).

Male New Zealand White rabbits were used to develop a suitable model. Animals were housed and cared for in the biomedical services unit; Leicester University and all procedures were undertaken in accordance with the Animals (Scientific Procedures) Act 1986 under licence from the Home Office in London.

Animals were prepared for the angioplasty and stent implantation by the pre-administration in their drinking water of aspirin 1mg/kg/day for five days and clopidogrel 1mg/kg was given 1 day prior to the procedure. This is similar to the situation in clinical practice where patients have antiplatelet medications peri-procedurally and for up to a month afterwards.

Animals aged 4-8 months and weighing 3.0 - 4.5kg were used. General anaesthesia was induced 30 minutes after premedication with Domitor 0.2 mg/kg i/m, Ketamine 10 mg/kg i/m and Torbugesic 0.05 mg/kg i/m. Inhaled halothane (2 - 3%) was used for induction and a combination of halothane (0.5 - 3%) and oxygen (0.5 - 3l/min) for maintenance of anaesthesia. Animals were spontaneously ventilating for all studies; all were placed on a heating pad (38°C) and had continuous intraoperative monitoring of heart rate, respiratory rate and rectal temperature.

All animals were given 1000IU-unfractionated heparin before the procedure(s) were performed within the artery. This was after the induction of anaesthesia and dissection to expose the relevant vessels.



BMS = Bare Metal Stent

PCS = Polymer Coated Stent

EES = Eptifibatide Eluting Stent

Figure 2.2: Acute and Chronic group of rabbits.

2.4.3.1 Acute model

The inner (ventral) aspect of both thighs, groin area and abdomen were shaved and cleaned thoroughly with betadine and 2.5% chlorhexidine prior to sterile draping of the animal to maintain asepsis. The neurovascular bundle, composed of femoral artery, nerve and vein, was exposed by blunt dissection and the superficial femoral artery carefully freed of surrounding adventitial connective tissue. Both right and left femoral arteries were exposed in this manner. Next a midline abdominal incision was used to open the peritoneum; the abdominal contents were deflected to the left lateral position and the iliac arteries exposed and dissected free of surrounding tissues to facilitate placement of a perivascular flowprobe on each side.

An arteriotomy was fashioned between two ligatures placed loosely around the superficial femoral artery distal to its lateral circumflex branch (Figure 2.2 an illustration from MD thesis by Rajesh Aggarwal)[350]. Arterial bleeding was controlled by tension on the proximal ligature. A 2x12mm diameter, Maverick monorail angioplasty balloon catheter (Boston Scientific) was advanced to the proximal part of the common iliac artery 0.5 - 1cm distal to the aortic bifurcation and inflated to eight atmospheres for 60 seconds using a standard balloon inflation device. This was done three times, with a 30 second recovery period between balloon inflations. The balloon catheter was then removed. A 2.25x13mm Guidant Pixel stent was placed using a further delivery balloon into the iliac artery. These were either a control or an eptifibatide-loaded stent (single blinded). The stent was introduced into the femoral artery, advanced to the proximal common iliac artery and deployed at the site of arterial injury by inflating the balloon to eight atmospheres for 20 seconds.

Following stent deployment, the balloon catheter was removed and the superficial femoral artery ligated (using 2/0 mersilk) at the arteriotomy site.

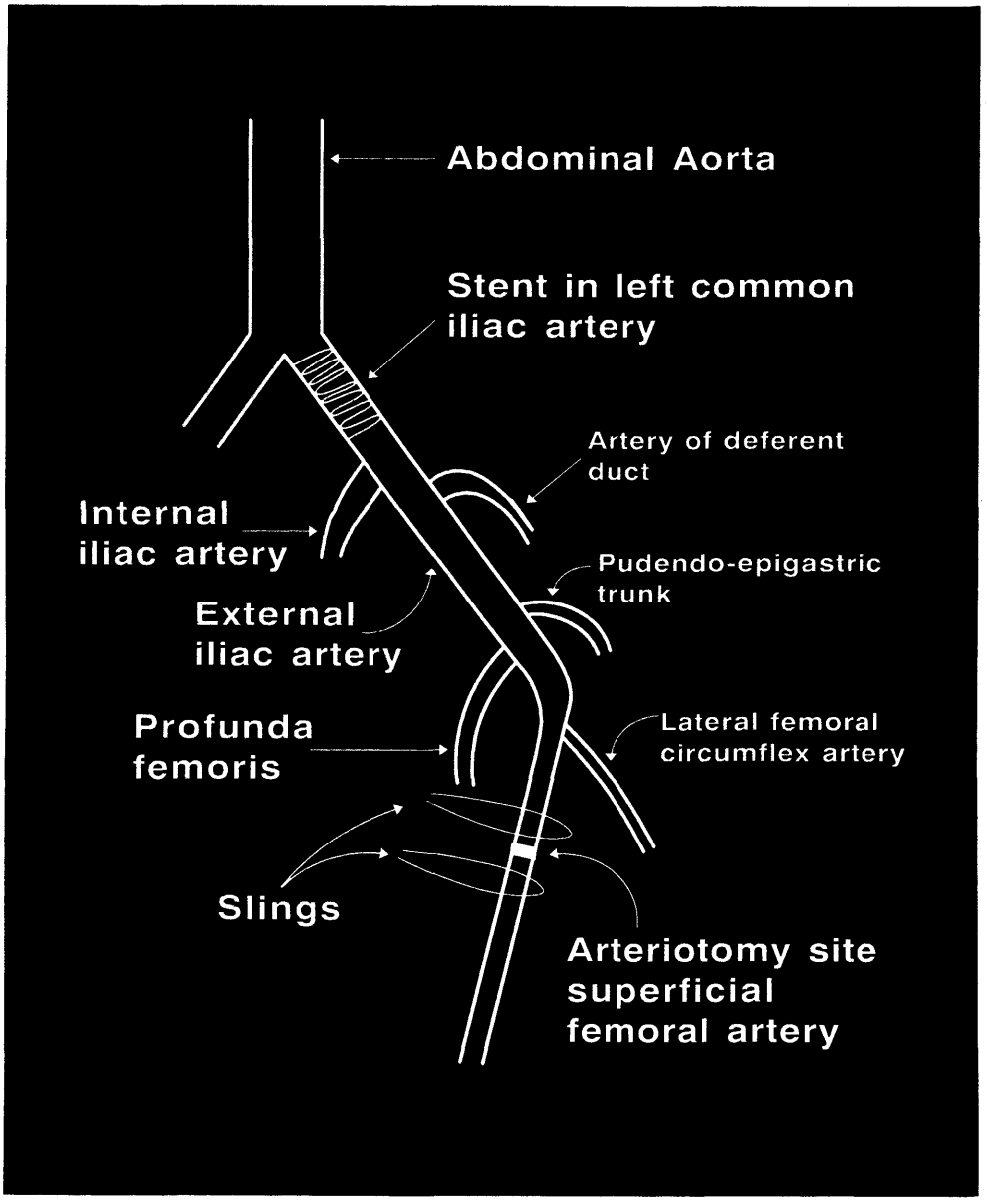


Figure 2.3: Schematic illustration of operative anatomy, from MD thesis by Rajesh Aggarwal.

2..4.3.1.1 Indium Labelling

The method of Hawker *et al*[351] was used to label rabbit platelets with indium[111]. Seventeen millilitres of rabbit blood were obtained by cannulating an ear artery. This was drawn up into a syringe containing 3ml of acid citrate as anticoagulant (see appendix). A further 9ml was drawn up in a separate syringe with 1ml of sodium citrate. A large bore needle (21G) was used to minimise the possibility of platelet activation or red cell haemolysis. The first sample was centrifuged at 180g (850rpm) in a Wifug laboratory centrifuge (Eltex of Sweden) for 15 minutes; 5-7.5ml of supernatant, containing platelet rich plasma (PRP), were then transferred using a "Kwill" into a 15ml tube (tube A). The total amount of PRP was calculated by subtracting the residual volume in the tube that was not PRP. 10µl of PRP was removed, to be used to count the total platelet concentration (see below). The blood from the other syringe was centrifuged at 640g (2100rpm, Wifug centrifuge) for 10 minutes to obtain platelet poor plasma (PPP) for later use. The PRP in tube A was mixed with Tyrode's buffer (Appendix) to give a final volume of 10ml and then centrifuged at 640g for 10 minutes, leaving a platelet pellet in the bottom of the tube. The supernatant (diluted plasma) was decanted and retained. The platelet pellet was then thoroughly washed using about 5ml of buffer. Washed platelets were resuspended in 2.5ml Tyrode's buffer. 10MBq Indium (^{111}In) oxine solution (Amersham Health Science, Bucks, UK) was then added drop wise to the tube whilst gently swirling it and the tube incubated for two minutes. Approximately 7.5ml of diluted plasma were then added to the platelet suspension to give a final volume of 10ml and the mixture centrifuged at 640g for ten minutes. The supernatant was then decanted and used to measure labelling efficiency. The platelet pellet was resuspended in 3ml of platelet poor plasma. The total radioactivity present was

measured by counting 10μl samples of labelled platelets and supernatant in a gamma well counter using a 90keV symmetrical window around the 245keV peak (Cobra II auto-gamma counting system, Packard Instruments, Meriden, CT). Labelled platelets were reinjected into rabbits one hour before operation. At the end of the procedure, stented vessels were dissected out and the radioactivity associated with the stents calculated in the gamma counter.

2.4.3.1.2 Cyclic flow variation

After the anatomy of the iliac vessels had been exposed, baseline blood flow before any angioplasty or stent placement was recorded for 2-3 minutes through each common iliac artery using perivascular transit time flowprobes (T206 small animal blood flow meter with 2.5SB probes, Transonics Inc., Ithaca, NY, USA). Blood flow through the vessels after they had been stented was recorded immediately after superficial femoral artery ligation and continuously for one hour thereafter with the flowprobe placed immediately distal to the stent.

2.4.3.1.3 Thrombosis

To calculate the number of platelets represented by a single radioactive count, platelets per ml were counted. 10μl of PRP taken before the labelling process and this was diluted with 990μl PBS to make 1ml. A 50μl sample of this dilute sample was taken and further diluted to 1ml in PBS again to dilute further. This solution was counted in a haemocytometer and the initial number of platelets per ml of PRP calculated. Knowing the total volume of PRP obtained, which contained all the platelets from that blood sample, allowed calculation of the total platelet count per ml of blood. The total number of platelets in each animal was estimated assuming 60ml

blood per kilogram body weight (based on Home Office (1991) Antibody production guidelines) and the number of platelets in the suspension re-injected was estimated. From this a count per platelet was derived that allowed the estimation of the amount of platelets adhering to the stents, based on their radioactivity.

2.4.3.1.4 Termination of animals and recovery of tissues

Two hours after stent deployment, animals were sacrificed using an intravenous overdose of pentobarbitone (140 mg/kg body weight). An intravascular cannula was then introduced into the abdominal aorta, advanced distally and used to flush stented vessels with 0.9% sodium chloride solution. Vessels were removed and formalin fixed.

2.4.3.2 Chronic model

Animals that were allowed to recover after stent deployment for 28 days were also anaesthetised and prepared for an aseptic procedure as detailed above. Operative dissection was however restricted to right-sided groin incisions for superficial femoral exposure. Particular care was taken to avoid trauma to nerves during dissection of the neurovascular bundle since limb paralysis is associated with a reduced likelihood of post-operative recovery. Animals were closely observed post-operatively for signs of limb ischaemia. Elective sacrifice was undertaken at 28 days after stent implantation using an intravenous overdose of pentobarbitone. Vessels were then exposed and dissected free of surrounding tissues, flushed with 0.9% sodium chloride, and assessed macroscopically for the presence of thrombus prior to perfusion-fixation and removal as detailed above.

2.4.3.2.1 Processing of stented arteries for restenosis

Each stented specimen was explanted with at least 1cm of artery either side of the stent. Sections were formalin-fixed in 4% formalin for at least 48-72hours. Thereafter each specimen was transferred into PBS and stored at 4°C. When all the samples were ready, they were sent, still in PBS by courier to Sheffield Northern General Hospital for tissue processing in collaboration with Dr. J Gunn.

The technique used to resin-embed the sections is described fully in published work[352]. The author of this work supervised the processing of the tissues used in this work.

2.4.3.2.2 Resin embedding of tissue

The technique allows the preservation of the arterial architecture *with the stent in situ,* while still preserving the antigens that are used to identify the proliferative response on the smooth muscle cells in the tissue.

Stented tissue was dehydrated in 100% acetone and immersed in infiltrating solution (50% benzoylperoxide with hydoxyethyl methacrylate) for 24 hours at 4°C. Blocks were then transferred into an embedding solution (infiltrating solution with tetramethyl aniline) and orientated in polythened tubes. The tubes were hermetically sealed and left at 4°C for 24hr. This allowed the formation of glycol methacrylate resin (T8100).

A high-speed precision saw (Isomet 2000) was used to cross-section the resin-embedded tissue blocks. This produced 100μm thick sections which were then further thinned by use of a Metaserv 200 grinder with increasing fineness of grinding paper used to produce sections between 10-20μm in thickness. These sections were then affixed to Perspex slides with Super-attack adhesive (Sheffield).

2.4.3.2.3 H&E staining of T8100-embedded sections

Two slides from each stented vessel were used for H&E staining. Slides were stained using a standard protocol. Wax-embedded slides were dried in an incubator at 37°C overnight before progressing to H&E staining. Slides were processed to remove wax by immersing in xylol, and then the xylol was removed with immersion in industrial methylated spirits (IMS). Slides were rehydrated in decreasing concentrations of IMS. Haematoxylin staining was followed by dipping briefly in 1% acid/alcohol to remove any excess. Eosin staining was used before dehydrating in increasing concentrations of IMS. Finally slides were immersed again in xylol. This was to ensure that the tissues were in an organic solvent to optimise the mounting of the slides. Adaptations were made to the method, particularly with regard to the length of time needed to clearly delineate histology by an initial stage with varying length of time to produce the best results.

2.4.3.2.4 Digital morphometry of resin embedded stent sections

To assess qualitatively the extent of neointimal hyperplasia that had developed in the stented iliac vessels, the resin-embedded cross-sections were examined under a microscope (Nikon Eclipse E800) equipped with a digital video camera. This allowed the imaged stent sections to be analysed using a digital morphometry package (Scion Image 1.62a) on an Apple Mac computer. All the H&E stained slides had identifying marks concealed to ensure an unbiased analysis. A single blinded observer made all the measurements. Two slides were chosen from each stent. Thus 24 separate slides were examined (4 animals per group, two slides from each, control (bare metal, polymer-coated stents) and eptifibatide coated stents.

2.5 Sputter coating

Sputter coating is a method used to coat samples for SEM (scanned electron microscopy) examination with an extremely thin and even coating of atoms of a conducting metal, usually gold. The gold is bombarded under a high voltage and atomises. This cloud of atomised gold will then thinly coat the target sample. When the sample is swept by the beam of electrons from the microscope, it becomes charged and will emit secondary electrons. These are detected and imaged by the microscope.

In this work, the stent was mounted on a pedestal and sputter coated with a 15nm layer of gold in a Polaron sputter coating unit E5150. (Figure 2.4) and assessed the polymer on the unexpanded (Figure 2.5) and fully expanded stents to make sure the polymer was evenly distributed over the stent. There was no tear in the polymer when the stent was fully expanded. (Figure 2.6)

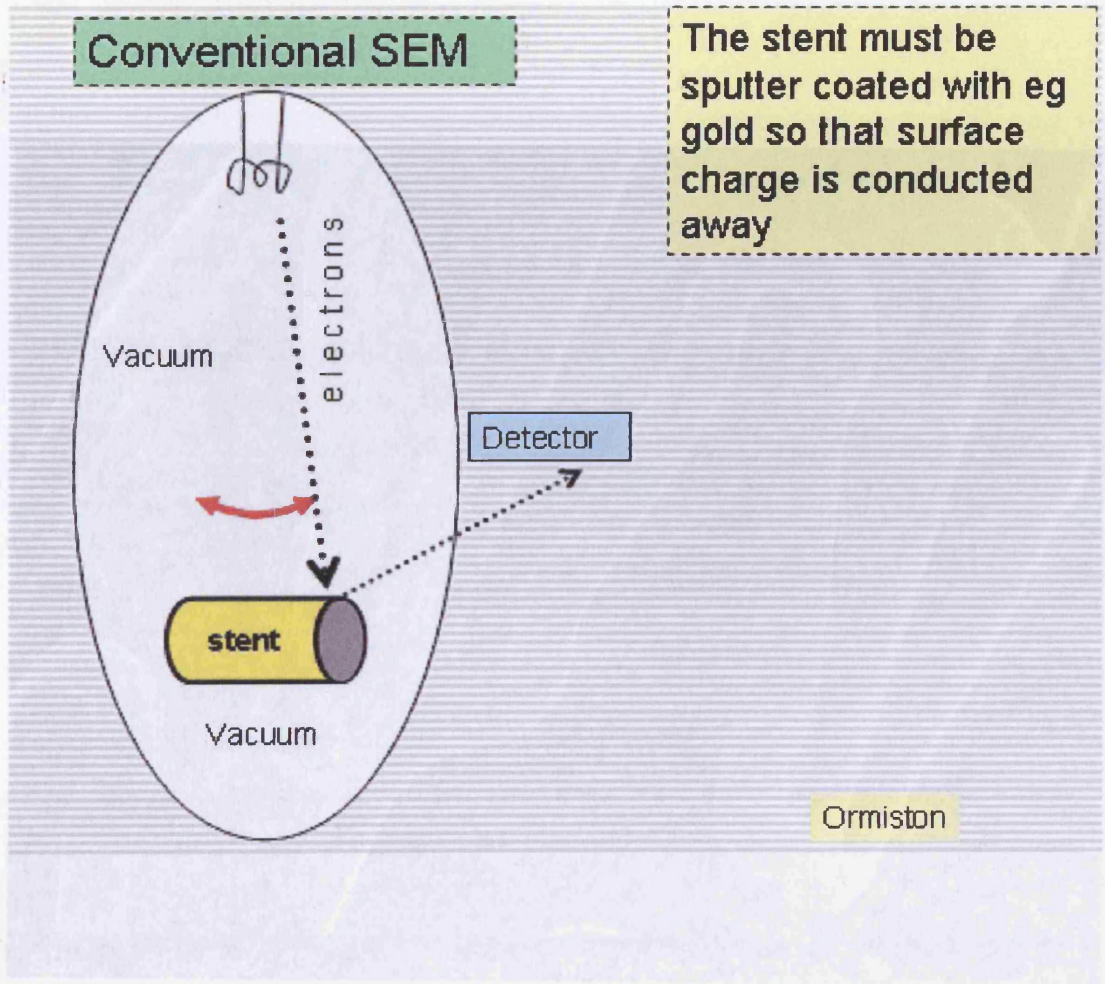


Figure 2.4: Sputter coating of stent for SEM examination

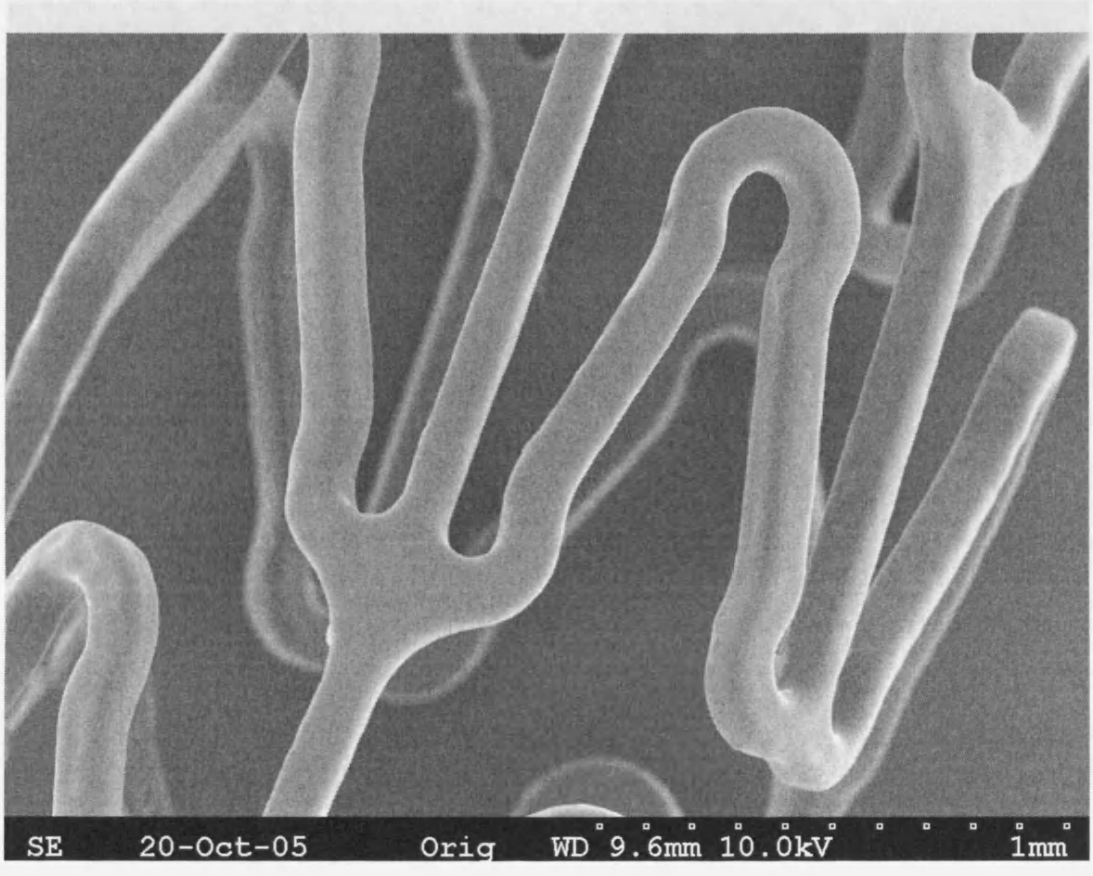


Figure 2.5: Unexpanded polymer-coated stent

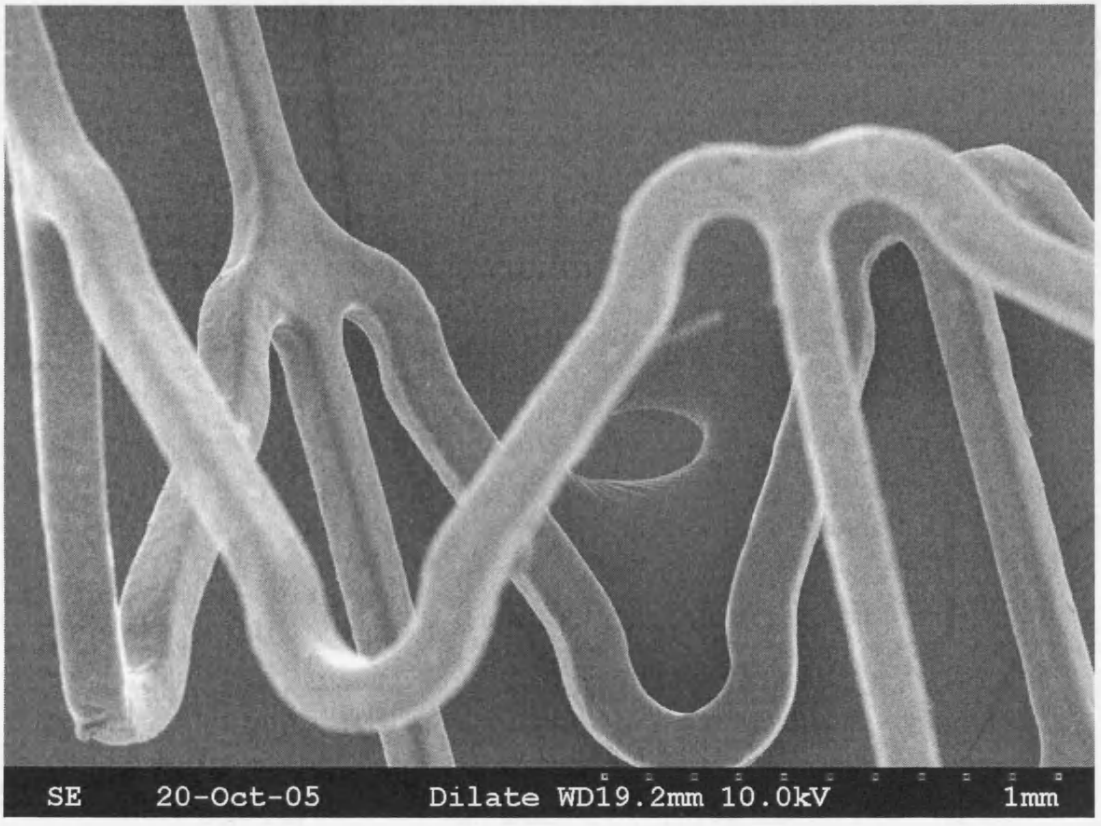


Figure 2.6: Expanded polymer-coated stent

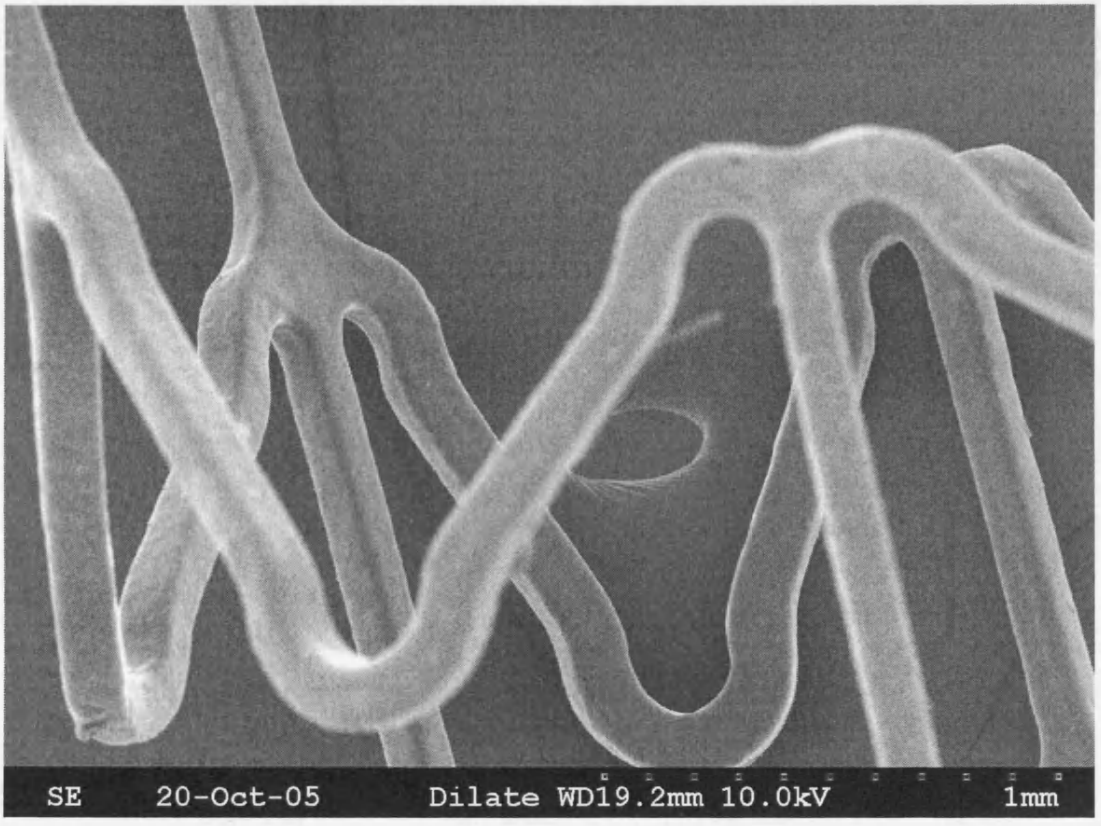


Figure 2.6: Expanded polymer-coated stent

Chapter 3

3 Results of adsorption and elution studies with eptifibatide

3.1 Eptifibatide adsorption

Attempts were made to maximise the absorption of drug into the polymer. It was important to quantify the amount of drug that could be reliably absorbed onto the stent structure. Eptifibatide was successfully loaded onto bare metal stents using polyvinyl butyrate polymer. A maximum of 111μg of eptifibatide was loaded onto 3.0 x 18mm bare- metal stents with less total amount of eptifibatide loaded on shorter stents (Figure3.1).

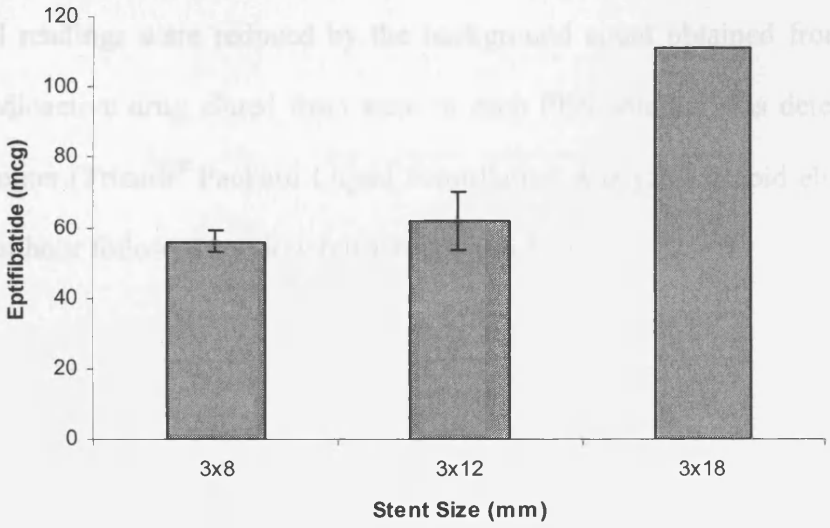


Figure 3.1: Eptifibatide absorption onto polymer coated stents by ultrasonic spraying method. Experiments were repeated three times to verify a reproducible result.

P value<0.001.

3.2 Eptifibatide elution in-vitro

Simple immersion method

In this preliminary experiment, 3 tritium labelled eptifibatide-polymer coated stents of known weight were immersed in PBS for 24 hours at room temperature. Each solution (PBS) was contained in a 1.5 ml polypropylene (Eppendorf) tube. Stents were placed vertically and totally immersed in each solution. PBS was changed every 24 hours at room temperature for up to four weeks. At the end of this time the stents were removed, excess eptifibatide solution shaken off and surface eptifibatide solution removed by running the stent over filter paper until dry. This was to try and eliminate variation amongst the stents due to amounts of solution adhering to the surface.

All readings were reduced by the background count obtained from an empty tube. Radioactive drug eluted from stent in each PBS solution was determined in a beta-counter (Tricarb® Packard Liquid Scintillation Analyzer). Rapid elution was noted at first hour followed by slow release. (Figure 3.2)

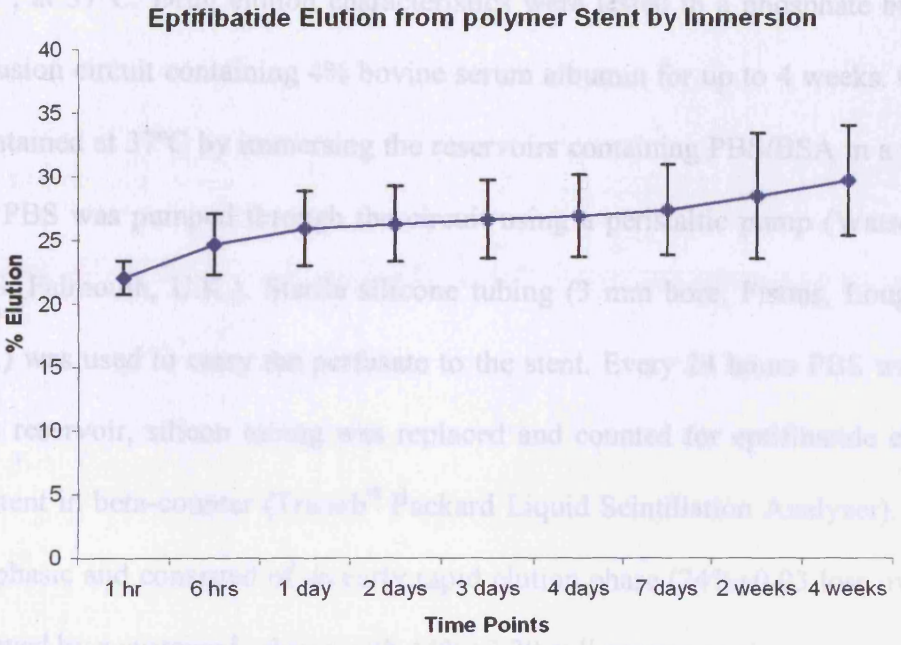


Figure 3.2: Eptifibatide elution by simple immersion showed initial rapid elution and then slow elution up to four weeks. N=3.

Perfusion Circuit

Elution of eptifibatide from polymer-coated stents in the perfusion circuit is shown in Figure 3.3. These stents were perfused continuously in a closed loop circuit at 25ml min^{-1}, at 37°C. Drug elution characteristics were tested in a phosphate buffer saline perfusion circuit containing 4% bovine serum albumin for up to 4 weeks. Circuit was maintained at 37°C by immersing the reservoirs containing PBS/BSA in a water bath. The PBS was pumped through the circuit using a peristaltic pump (Watson-Marlow 302S, Falmouth, U.K.). Sterile silicone tubing (3 mm bore, Fisons, Loughborough, U.K.) was used to carry the perfusate to the stent. Every 24 hours PBS was changed from reservoir, silicon tubing was replaced and counted for eptifibatide eluted from the stent in beta-counter (Tricarb® Packard Liquid Scintillation Analyzer). The curve is biphasic and consisted of an early rapid elution phase (24%±0.03 loss over 1 hour) followed by a sustained release with 44%±2.30 still present on the stent after 30 days.

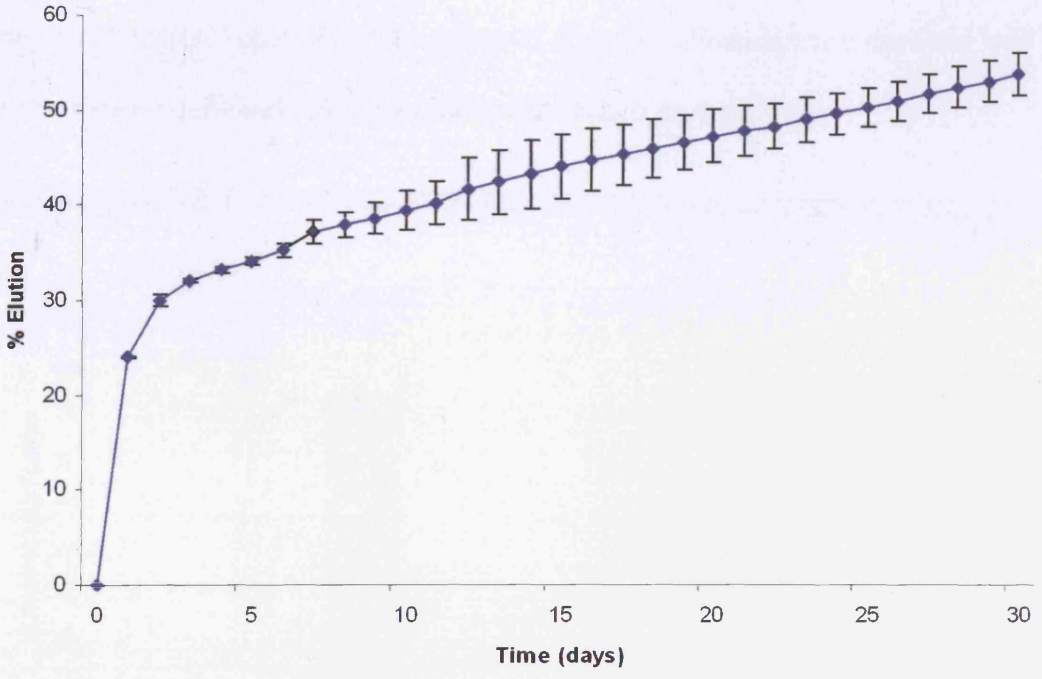


Figure 3.3: The elution of eptifibatide coated stents in an *in vitro* perfusion circuit. The stents were continuously washed with phosphate buffer saline+1%bovine serum albumin at 25ml/min. at 37°C. Experiments were repeated three times to verify a reproducible result. p value<0.05. N=3.

Chapter 4

4 Platelet studies results

4.1 Platelet Aggregometry

Aggregometry undertaken during the platelet deposition experiment was carried out to confirm both the platelet viability, and that the eptifibatide eluted from the stent had retained antiplatelet properties in the perfusate. Eluted eptifibatide significantly inhibited platelet aggregation by 95%±0.70 in response to ADP (4μMole) at 10 minutes ($p<0.01$). Figure 4.1. The effect of eluted eptifibatide from the stent was the same as pure eptifibatide from injection vial when compared.

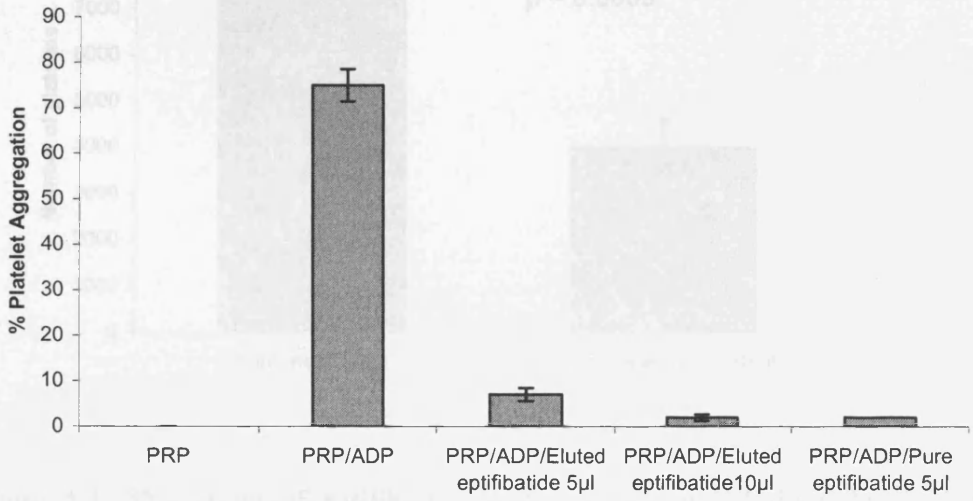


Figure 4.1: In platelet rich plasma (PRP), effect of eluted eptifibatide on platelet aggregation in response to adenosine diphosphate (ADP). Effect of eluted drug was compared with pure eptifibatide from injection vial as positive control. Experiments were repeated three times to verify a reproducible result. p value=0.01.

4.2 *The effect of eptifibatide eluting stents on platelet deposition*

There was a significant reduction in platelet deposition onto polymer-coated stent treated with eptifibatide as compared with controls. Figure 4.2. Platelet deposition on stents eluting eptifibatide was significantly reduced by 48%±6 compared with controls (p=0.0065), when stents were perfused continuously in blood containing the ^{111}In labelled platelets for one hour at 40ml min^{-1}.

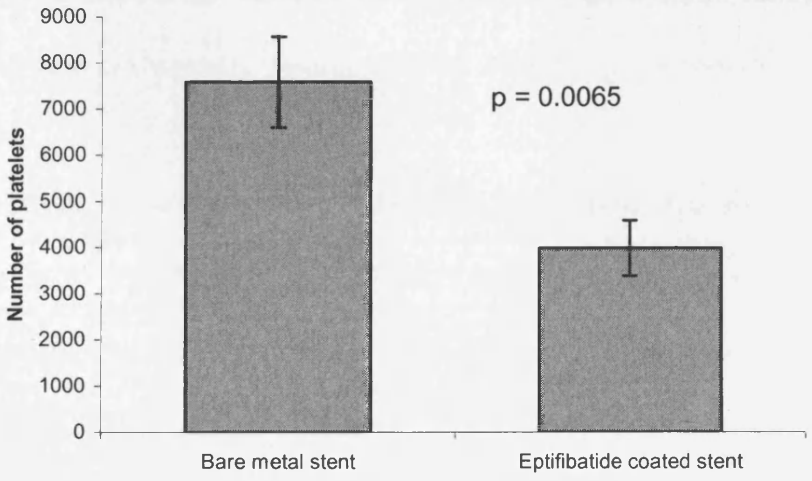


Figure 4.2: The effect of eptifibatide coated stents on ^{111}Indium labelled platelet deposition versus bare metal stents in an *in vitro* perfusion circuit for one hour at 37°C. Experiments were repeated three times to verify a reproducible result. P value=0.0065.

Chapter 5

5 Cell culture results

The effect of eptifibatide on smooth muscle cell adhesion

In this study we used human venous SMCs, as well as rat pulmonary arterial PAC1 cells, since the latter express SM differentiation markers, which are not normally expressed in cultured vascular SMCs. Despite differences in expression for ECM proteins and integrins, adhesion profiles of PAC1 cells and human SMCs to the ECM proteins were very similar (Figure 5.1). Human SMCs and PAC1 had increased adhesion to fibronectin and vitronectin compared to plastic. Thrombospondin did not increase adhesion of either human SMCs or PAC1 SMCs compared to plastic. Osteopontin consistently caused reduced adhesion of human SMCs from different sources.

Adhesion to osteopontin was inhibited by eptifibatide in a dose-dependent manner (Figure5.2). Eptifibatide significantly reduced adhesion of human SMCs to osteopontin (by 27%); thrombospondin (by 25%), vitronectin (by 12%) and plasma fibronectin (by 13%), but it did not affect adhesion to plastic (Figure5.3A). Adhesion to thrombospondin and osteopontin was inhibited the most. In contrast, only adhesion of PAC1 cells to thrombospondin (up to 18%) was affected by eptifibatide (Figure5.4B). Since reduced of adhesion may be caused by decreased binding of ECM proteins to cells, the effect of eptifibatide on binding of thrombospondin to cells was investigated (Figure 5.5). The addition of EDTA to inactivate integrin-dependent binding reduced it by half. Eptifibatide reduced binding of thrombospondin to cells, but it was not as effective as inactivation of integrins by EDTA.

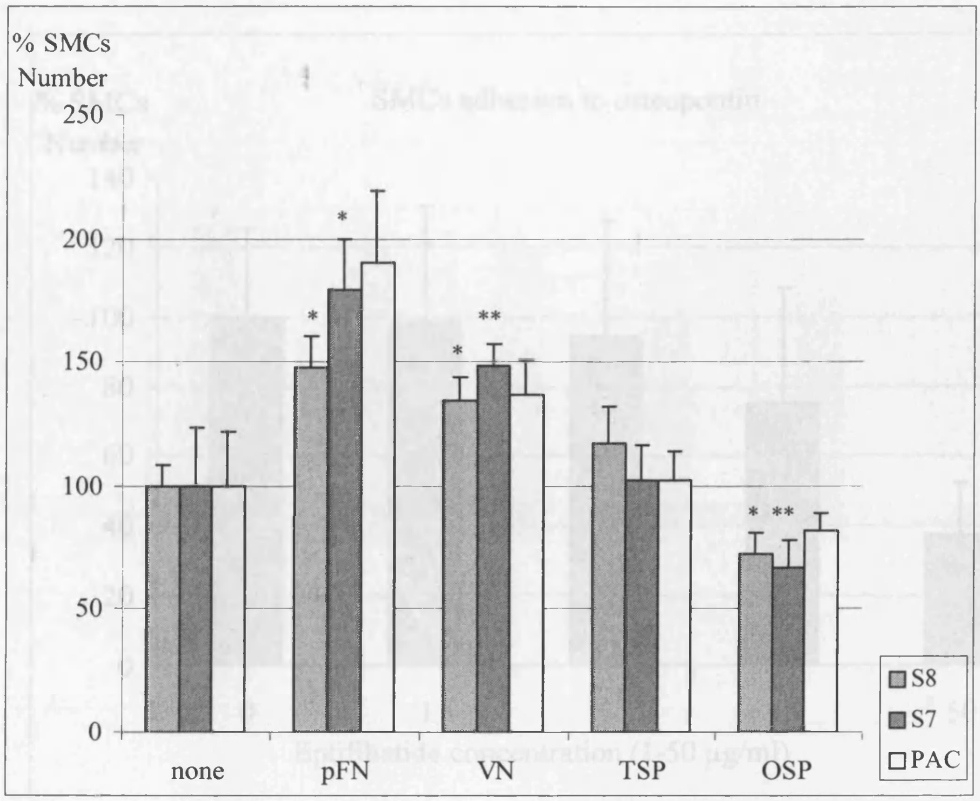


Figure 5.1: Cell attachment to ECM proteins [^{3}H]-thymidine-labelled PAC1 cells (white) and human SMCs from different sources S7 and S8 (shades of grey) adhered to plastic coated with ECM proteins: fibronectin (pFN), vitronectin (VN), thrombospondin (TSP) and osteopontin (OSP). Cell attachment is shown as a percentage of cells attached to plastic. All data are shown as the means ± SD. *P<0.05 versus plastic (n=8).

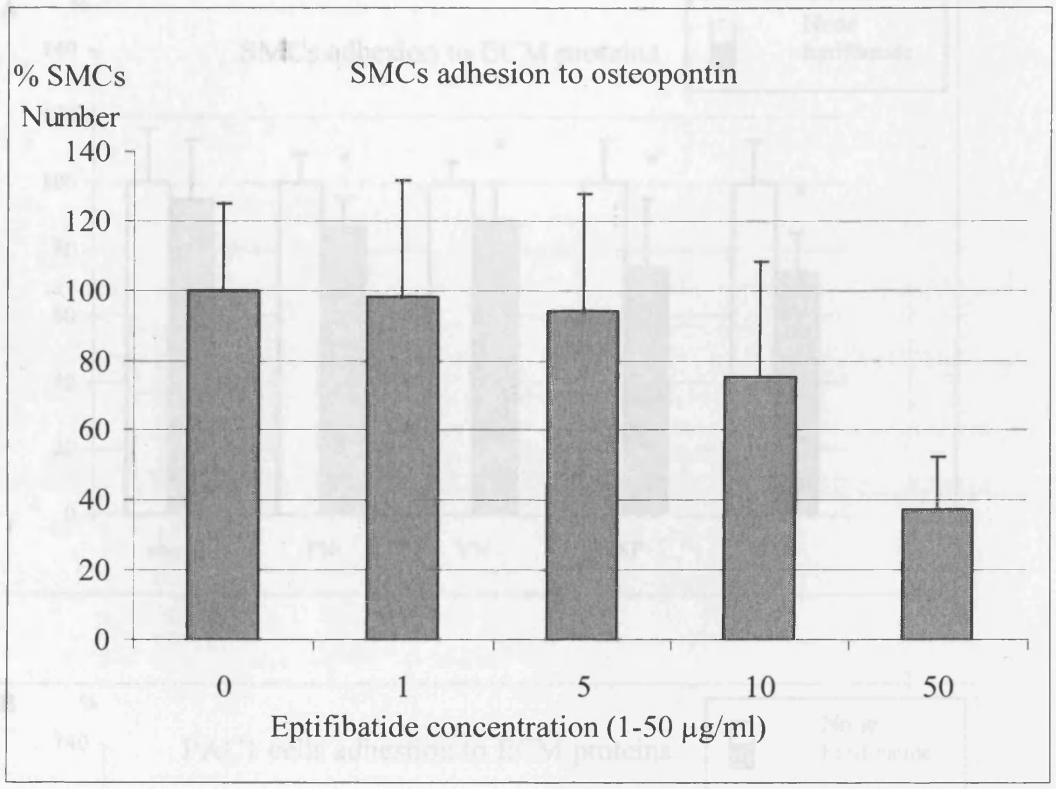


Figure 5.2: Eptifibatide decreases adhesion of human SMCs to osteopontin. Human SMCs adhered to osteopontin-coated plastic in the presence of eptifibatide (1-50 μg/ml). Cell attachment is shown as a percentage of control in the absence of eptifibatide. The data are presented as the means of eight ± SD. *P<0.05 versus control.

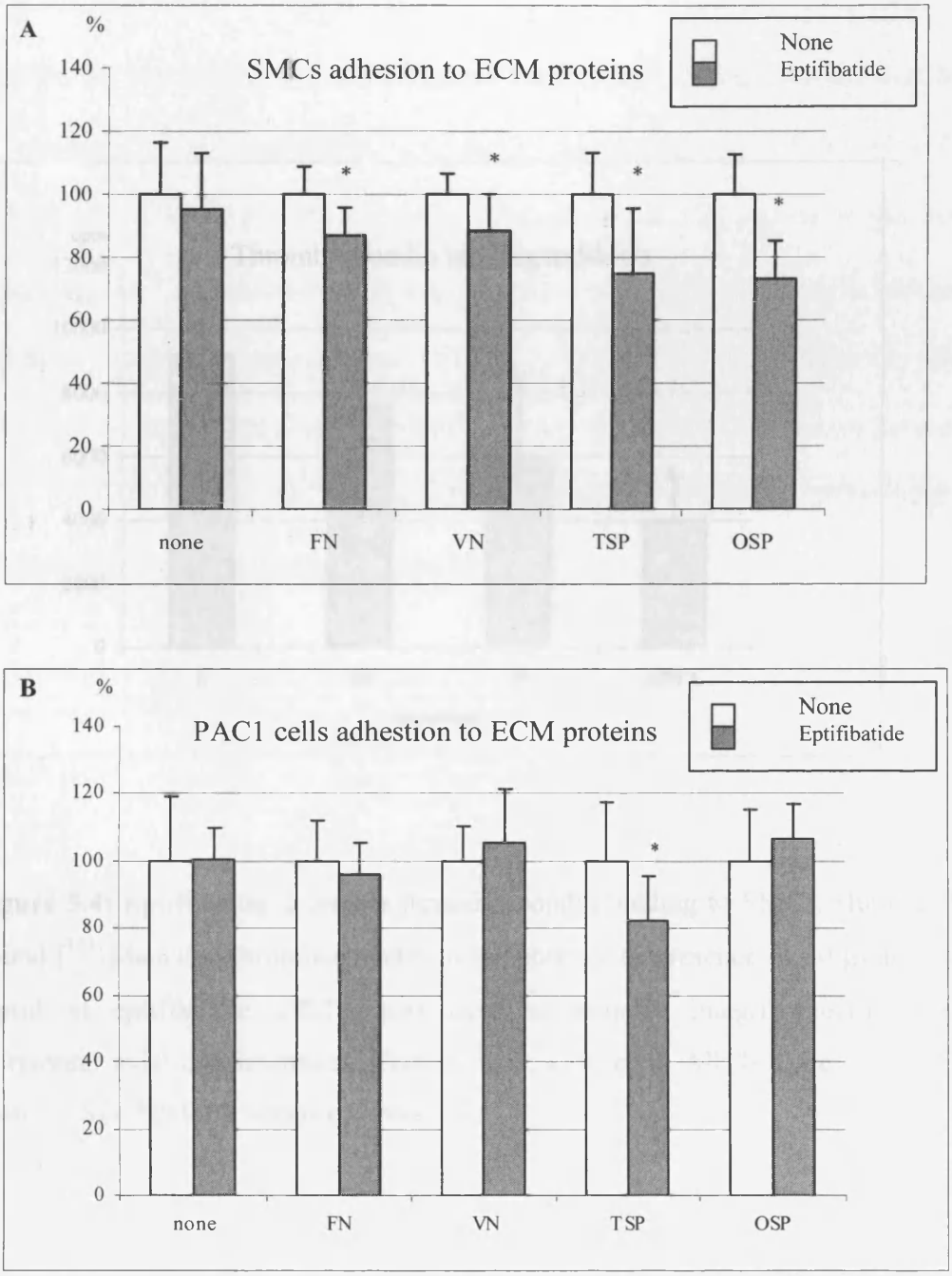


Figure 5.3: Eptifibatide inhibits SMC adhesion Human SMCs **(A)** and PAC1 cells **(B)** adhered to plastic coated with ECM proteins: plasma fibronectin (pFN), vitronectin (VN), thrombospondin (TSP) and osteopontin (OSP), in the presence of 10 μg/ml of eptifibatide. Cell attachment is shown as a percentage of cells attached to plastic. The data are shown as the means ± SD. *P<0.05 versus plastic (A. n=8, B. n=16).

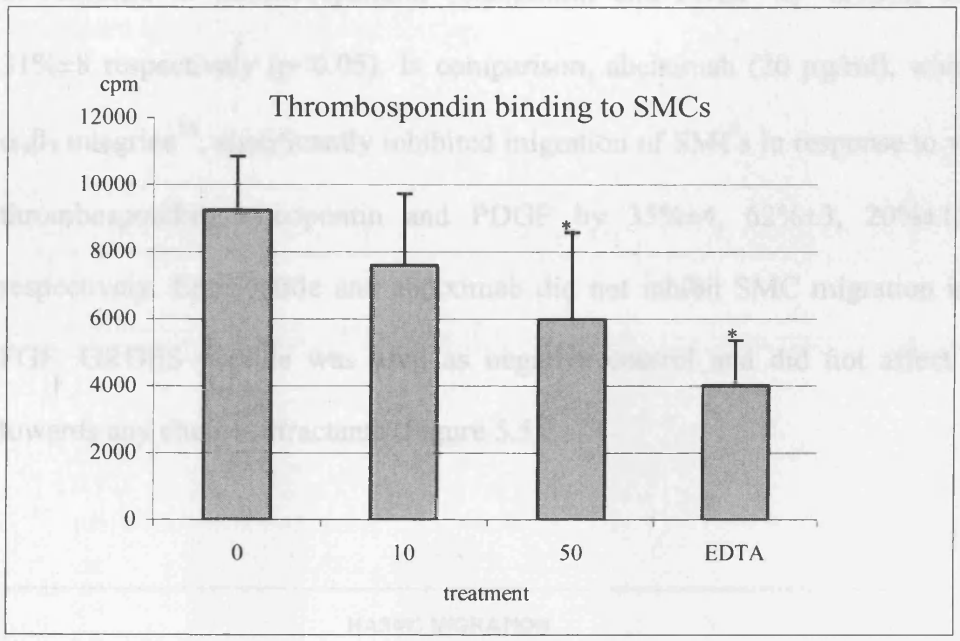


Figure 5.4: Eptifibatide decreases thrombospondin binding to SMCs. Human SMCs bound [^{125}I]-labelled thrombospondin in the absence or presence of 10 μg/ml and 50 μg/ml of eptifibatide. EDTA was used to estimate integrin-specific binding. Horizontal axis: cell treatment. Vertical axis: x10^3 cpm. All data are shown as the means ± SD. *P<0.05 versus control (n=4).

The effect of eptifibatide on smooth muscle cell migration

Eptifibatide in a concentration of 50 μg/ml significantly inhibited migration of SMCs in response to thrombospondin, osteopontin and PDGF by 42%±6, 49%±5 and 31%±8 respectively ($p<0.05$). In comparison, abciximab (20 μg/ml), which inhibits $\alpha_v\beta_3$ integrins[75], significantly inhibited migration of SMCs in response to vitronectin, thrombospondin, osteopontin and PDGF by 35%±4, 62%±3, 20%±12, 26%±8 respectively. Eptifibatide and abciximab did not inhibit SMC migration induced by FGF. GRGES peptide was used as negative control and did not affect migration towards any chemo-attractants (Figure 5.5).

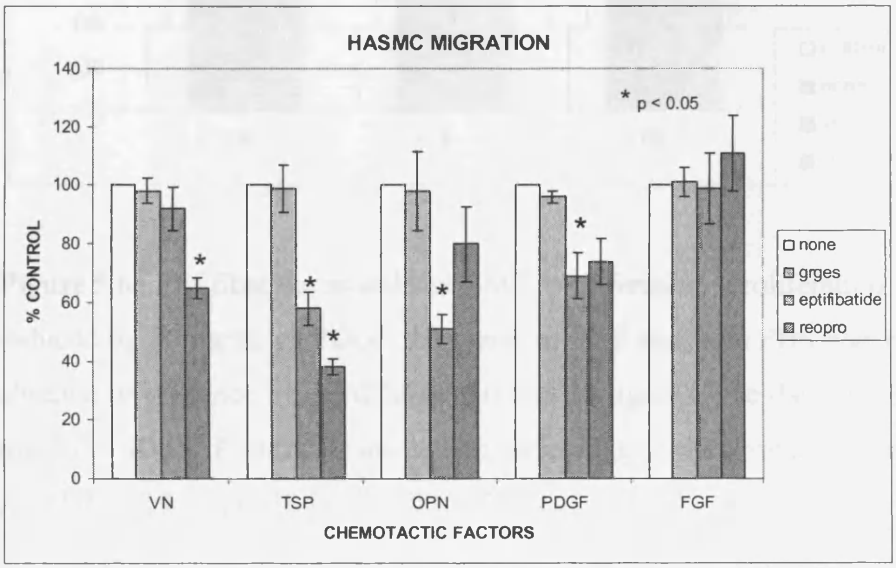


Figure 5.5: Eptifibatide inhibits SMC migration. Migration of human SMCs towards chemo-attractants thrombospondin (TSP), osteopontin (OPN), FGF and PDGF was measured in the absence or presence of eptifibatide (50 μg/ml), Abciximab (ReoPro) and GRGES peptide. Cell migration as a percentage of migration in the absence of any additives. The data are shown as the means ± SD. *, $P<0.05$ (n=4).

5.3 *The effect of eptifibatide eluting stents in cell culture proliferation*

PAC1 cell proliferation, induced by 10% FBS and FGF, was inhibited by 50 μg/ml of eptifibatide (Figure 5.6). In contrast, PDGF-induced cell proliferation was slightly stimulated by 10 μg/ml of eptifibatide.

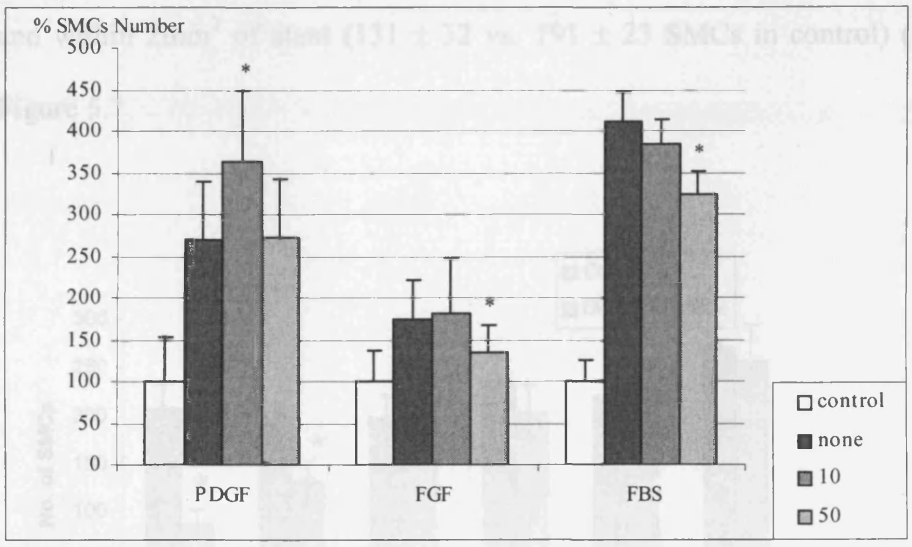


Figure 5.6: Eptifibatide modulates SMC proliferation. Proliferation of PAC1 cells induced by 10 ng/ml of PDGF, 20 ng/ml of FGF and 10% FBS was measured in the absence or presence of eptifibatide (10 and 50 μg/ml). The data are presented as the means ± SD. *, $P<0.05$ versus cells proliferating in the absence of eptifibatide (n=8 for FGF and FBS, n=16 for PDGF).

5.4 *The effect of eptifibatide eluting stents in cell culture proliferation*

In the cell culture experiment, SMC proliferation was measured around the stents and the cells were stained with toluidine blue to allow clear-cut demarcation at 7 days. Eptifibatide loaded stents (50μg/stent) placed in SMC culture showed a distinct zone of cell growth inhibition within $1mm^2$ of stent (88 ± 22 vs. 208 ± 23 SMCs in control) and within $2mm^2$ of stent (131 ± 32 vs. 191 ± 23 SMCs in control) (both $p<0.01$). Figure 5.7

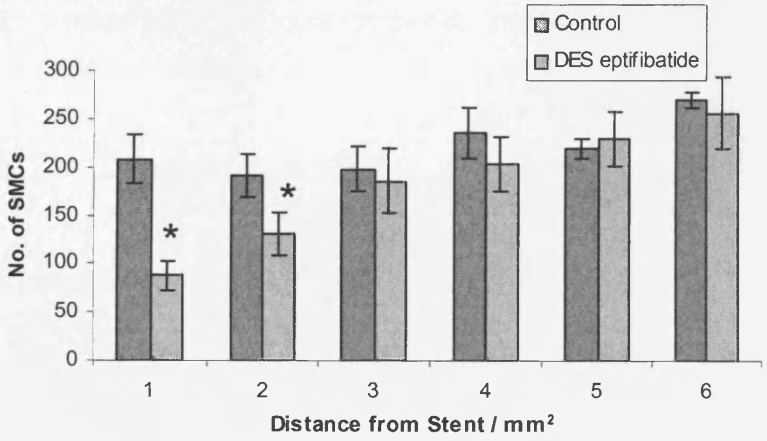


Figure 5.7: Human smooth muscle cell proliferation inhibition from centre to periphery/mm^2 of culture dishes by eptifibatide coated stents. Experiments were repeated three times to verify a reproducible result. P value=0.002.

Chapter 6

6 In-vivo results

6.1 Introduction

The *in vitro* studies discussed in chapters 2,3,4&5 were used as a preliminary assessment of the absorption and elution and physiological effects of eptifibatide eluting polymer-coated stent. Testing of eptifibatide as an anti-restenosis agent further required a more complex model. The work in chapter 5 had developed a model for smooth muscle cell proliferation in response to stents and the response to various chemotactic proteins. However, this model did not allow the examination of effects on the neo-intimal hyperplasia. To test the apparent beneficial effects of eptifibatide - eluting stents further, they were tested *in vivo*. A rabbit iliac artery model was chosen, after consideration of alternative models available.

6.2 The acute model

The aim of this model was to supply various data. Firstly, it provided control results of the intima/media composition immediately after an angioplasty. Secondly, it showed the extent of thrombus formation early on after stent implantation in this model. Finally, it assessed whether the eptifibatide eluted from a stent might have some effect on the thrombosis process early on.

The techniques used in the acute model are described fully in chapter 2 (2.4.3.1.4). This model was a termination procedure, i.e. the rabbits were never recovered from anaesthesia. These rabbits had both iliac vessels exposed under anaesthesia. The abdomen was also opened and the main abdominal vessels carefully dissected free. This allowed stents to be placed into both iliac arteries.

At the outset of the experiment, Indium-radiolabelled platelets were infused into the animal (section 2.4.3.1.1). Perivascular flow probes were used to monitor the cyclical

flow variations (CFV's) through the stented vessels. The importance of CFV's is discussed below (6.2.2.). Flow was monitored for up to two hours and then the rabbit was killed. The stented vessels were dissected free of the surrounding tissue and examined macroscopically for thrombus. The vessels were counted in a gamma counter to assess how much platelet deposition there had been.

6.2.1 Indium labelling of platelets

^{111}Indium has a half-life of 67.2 hours and decays by emission of γ radiation with two peaks of energy, 171 and 245keV. Indium oxine complex is neutral and lipid soluble and thus penetrates cell membranes. Within the cell, Indium becomes firmly attached to cytoplasmic components and the liberated oxine is released by the cell. There is negligible release of ^{111}Indium from cells. ^{111}Indium is thus ideal for labelling platelets and quantifying platelet accumulation at sites of arterial injury. ^{111}Indium labelling of autologous platelets is commonly employed in both research and clinical practice for detection of venous and arterial thrombi, determination of the thrombogenicity of prosthetic devices such as arterial grafts and measurement of platelet survival.

6.2.2 Cyclic flow variations

Cyclic Flow Variation (CFV) is the observation that flow down a vessel, especially one that has recently been damaged by, for example, angioplasty, varies. Typically, flow gradually reduces and then is abruptly restored to normal. This has been shown to be due to the gradual accumulation of thrombus and its sudden dislodgement[353-354]. The stimuli to CFV's occurring include adrenaline, which promotes platelet

aggregation by enhancing the action of agonists such as ADP, thrombin, serotonin and thromboxane A_2. Although ADP is an important *in vivo* mediator of platelet aggregation, inhibition of ADP-induced aggregation alone does not completely prevent adrenaline-enhanced platelet aggregation in aspirin-treated animals. This is because of accumulation of other important mediators of platelet aggregation such as serotonin and thromboxane A_2 at arterial sites with stenosis and endothelial damage. Broad-spectrum antiplatelet agents such as clopidogrel can prevent adrenaline-enhanced cyclic flow variation in canine coronary arteries[355].

CFV's also occur in human coronary arteries. Eichhorn and colleaguE[356] found spontaneous variations in coronary blood flow velocity in 3 of 13 patients undergoing angioplasty for severe angina. Flow variations occurred before angioplasty in one patient, after angioplasty in a second and both before and after angioplasty in the third. The authors concluded that flow variations were related to platelet aggregation, vasoconstriction, or both at the site of angioplasty induced-injury.

There appears to be a correlation between CFV measurements and coronary ischaemia and infarction. In a canine study[353], cyclic flow variations were produced in endothelial-injured coronary arteries. Transient coronary occlusion during CFV's induced electrocardiographic ST segment changes, which returned to baseline after reflow. In those dogs, which developed persistent coronary occlusion, histological examination showed thrombus formation at the stenotic site and evidence of myocardial infarction.

That this phenomenon is of importance in humans was demonstrated by Sunamara *et al*[357] who showed that although CFV was rare (~5% of cases), it was an important predictor of imminent, clinically significant thrombus formation or even acute occlusion.

In summary, cyclic flow variations occur in stenosed, endothelial-damaged arteries in both animal models and in man following coronary angioplasty and are associated with vessel occlusion.

6.3 The chronic model

The chronic model (2.4.3.2) was used to examine in-stent restenosis to the eptifibatide-eluting stent. The extent of instent restenosis was measured at 28 days.

6.3.1 Injury score

It can be argued that the implantation of eptifibatide or control stents may have unconsciously been performed differently, introducing bias. Implantation pressure and technique may themselves have an impact on the restenosis process. In this work, the results at 28 days were observed to determine whether the original injury suffered by the stented vessels were similar in the two groups. This injury scoring, which was done by a single blinded observer, was done using the previously published and validated scoring system of Schwartz *et al*[103].

This work graded the injury seen in porcine stented arteries, using wire stents. Stented vessels were cross-sectioned and stained. For each wire site, a histopathologic score proportional to injury depth and the neointimal thickness at that site were determined. A mean score for that vessel was calculated by dividing the total of all the injury scores for that site by the number of wire sites seen.

The scoring system that was described in this paper and was used in the work of this thesis is as follows. Two slides from each stented vessel were examined. Each vessel was examined microscopically and the number of stent/vessel contacts was counted.

Each contact point was then scored as follows to indicate how much injury that stent section had caused to the adjacent vessel wall:

Score	Description of injury
0	IEL intact, media compressed slightly
1	IEL lacerated, media compressed
2	IEL lacerated, media compressed >50%, EEL compressed but intact
3	IEL and EEL lacerated, stent section visible in adventitia.

(IEL = internal elastic lamina, EEL = external elastic lamina)

The value obtained for each stent section was added up and the total divided by the number of stent struts visible in that cross-section. An average injury score for each slide was calculated. The two slides for each stent were then averaged and the values obtained compared between the eptifibatide coated stent and baremetal/polymer coated-stent groups.

The typical features in each case are illustrated using examples taken from the specimens used in this research. (Figure 6.1-6.4)

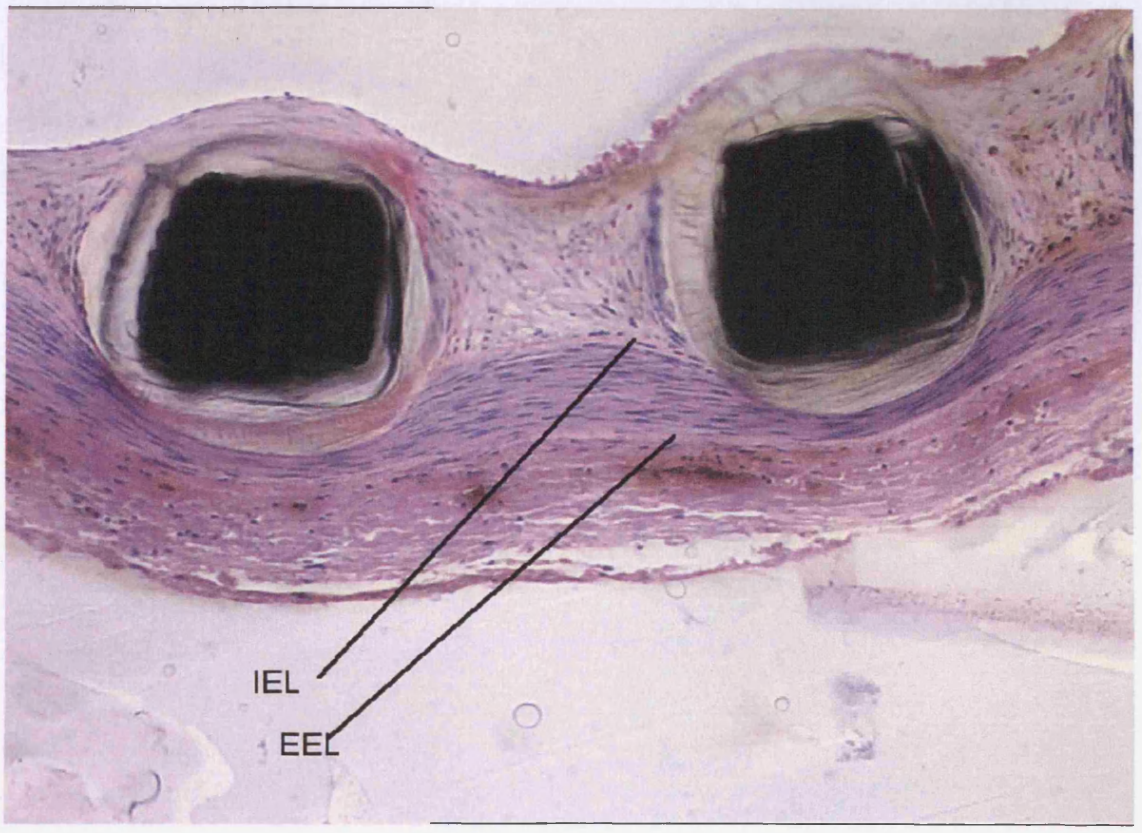


Figure 6.1: Grade 0 injury

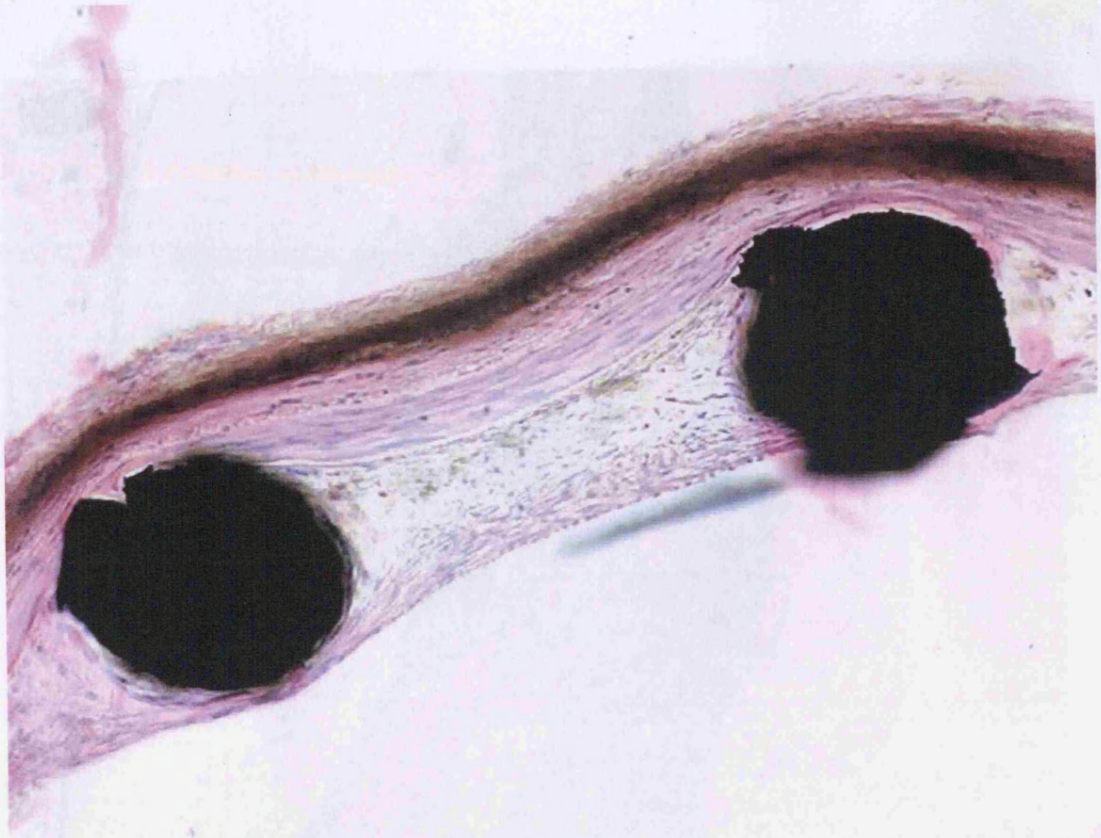


Figure 6.2: Grade 1 injury

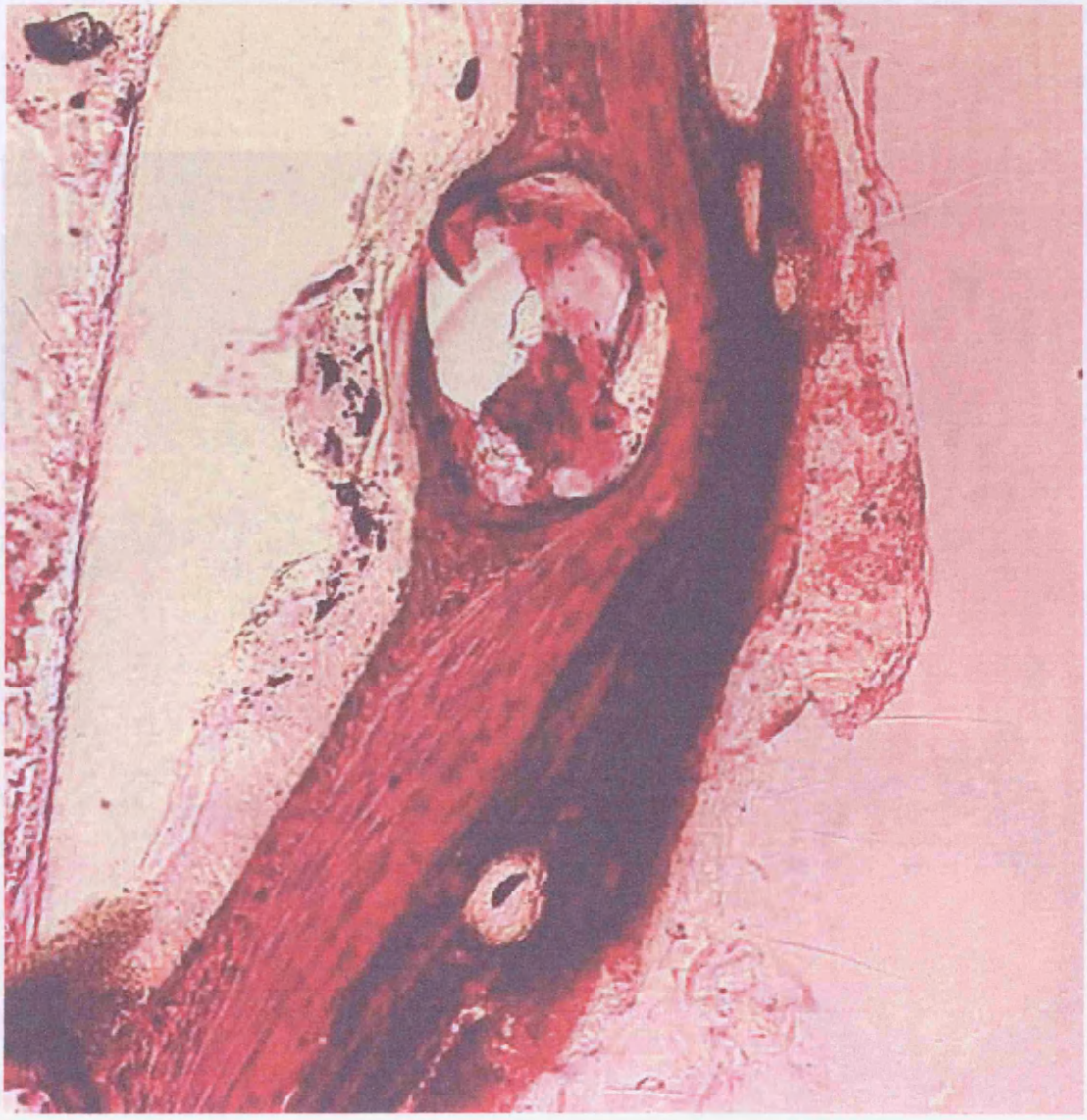


Figure 6.3: Grade 2 injury

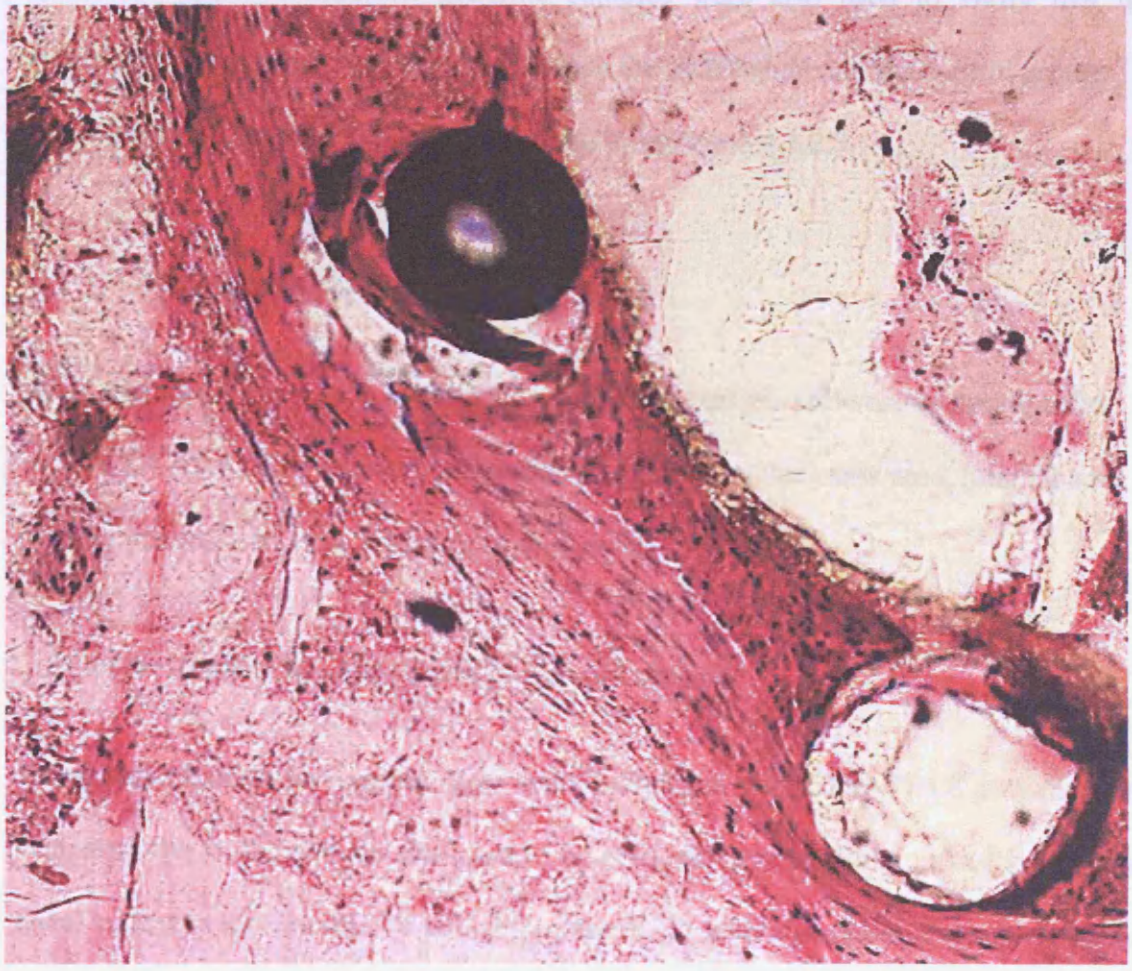


Figure 6.4: Grade 3 injury

6.3.2 Tissue section staining for neo-intimal hyperplasia

All stented vessels taken from the animals killed at 28 days were processed as in section 2.4.3.2.3. In brief, the vessels were fixed in glycol methacrylate resin (T8100). The sections were then cut into thin slices between 10-20μm and slide-mounted. The slides were then stained with haematoxylin & eosin in order to allow quantitative estimation of intimal thickness. The results at 28 days were compared with the normal vessel thickness at baseline. Baseline samples were also used to verify that no significant differences were evident between the eptifibatide and the control groups. The techniques used for H&E are described in section 2.4.3.2.3.

6.3.3 Image analysis

H&E stained sections were examined using image analysis software as described in section 2.4.3.2.4. This allowed calculation of neointimal thickness/area, luminal area, media thickness and injury scoring of the vessels.

6.3.4 Statistical analysis and power calculations

Power calculations for sample sizes were calculated using MINITAB software. In designing the study, sample sizes had to be compatible with Home Office guidelines about reducing the number of animals in the study where possible. 2-sample t-Tests were used with a predicted power of 90% and a significance value of $p<0.05$.

6.4 Results

6.4.1 Acute model

6.4.1.1 Cyclic flow variations

Flow measurements were obtained from six animals (i.e. 12 vessels), three controls and three given eptifibatide-coated stents. One animal died intraoperatively. This occurred before the stents were implanted and it was felt therefore that the stent assigned was not related to the cause of death. Flow was measured before stent implantation with and without occlusion of the distal vessel. This allowed calculation to be made of the reduction in flow due to the tying off of the distal femoral artery. The results of these measurements are shown below (Figure 6.5a.), together with an example of the flow seen during this procedure (Figure 6.5b).

In all the animals that survived long enough to have stent implantation, flow was preserved at a rate similar to that seen after temporary femoral artery occlusion prior to stenting. Cyclical flow variation was not seen in vessels with bare metal or EES (Figure 6.6a&b).

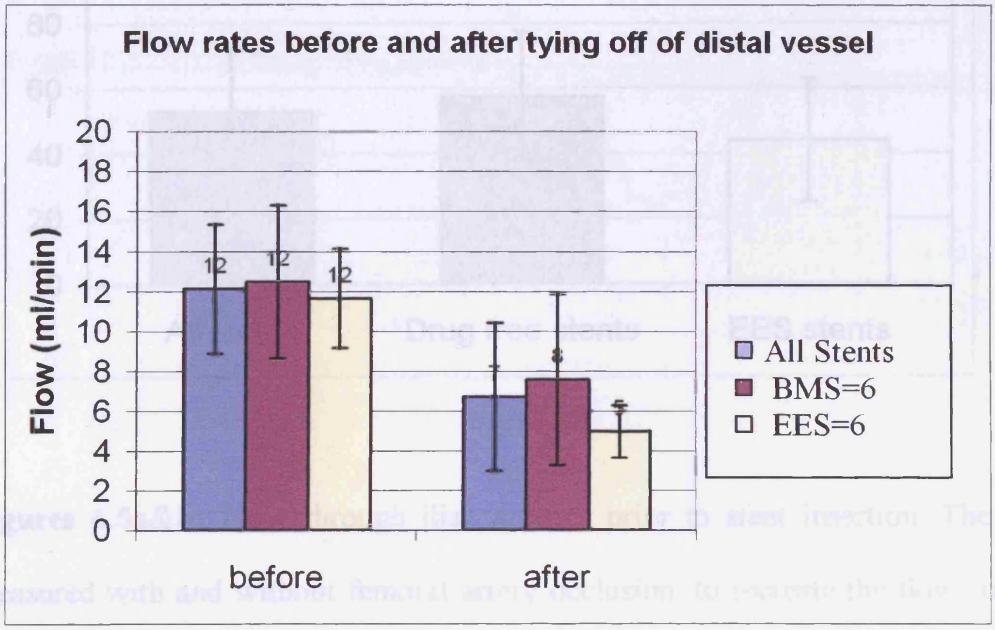
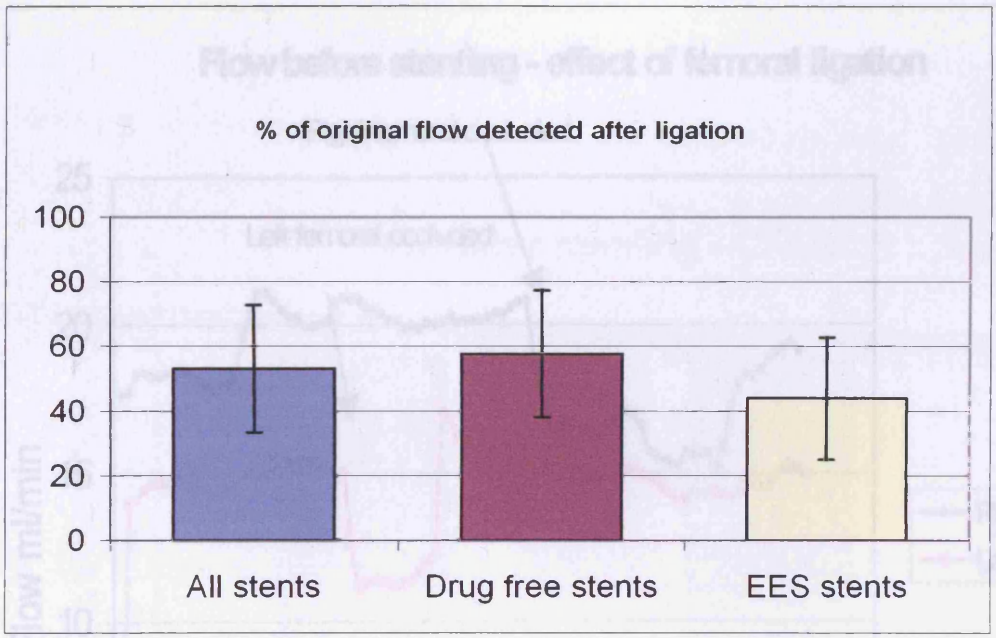

Flow rates before and after tying off of distal vessel
Flow (ml/min)
20
18
16
14
12
10
8
6
4
2
0
12
12
12
before
after
All Stents
BMS=6
EES=6

Figure 6.5a



Figures 6.5a&b: Flow through iliac arteries prior to stent insertion. The flow is measured with and without femoral artery occlusion, to recreate the flow conditions that will exist after stent implantation and femoral artery ligation. After ligation, flow was still about 53% of that prior to tying the vessel (53.1±19.7 %). No significant differences existed between the two groups prior and after stenting.

N.b. Although results are given for both "EES" and "control" stents, this differentiation is arbitrary, as at the time of flow recording stents had not yet been implanted.

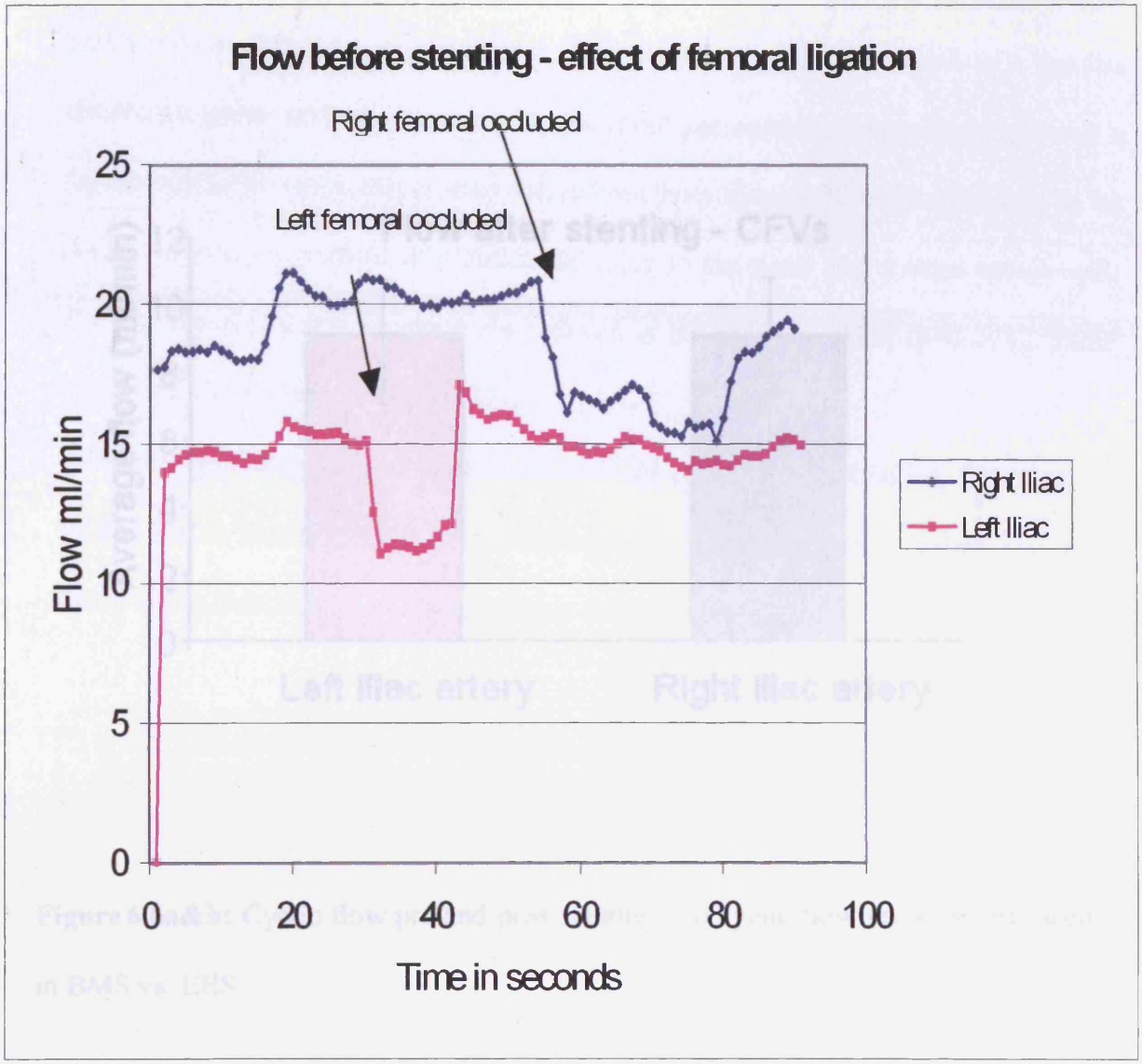

Flow before stenting - effect of femoral ligation
Right femoral occluded
Left femoral occluded
25
20
15
10
5
0
Flow ml/min
0
20
40
60
80
100
Time in seconds
Right Iliac
Left Iliac

Figure 6.6a

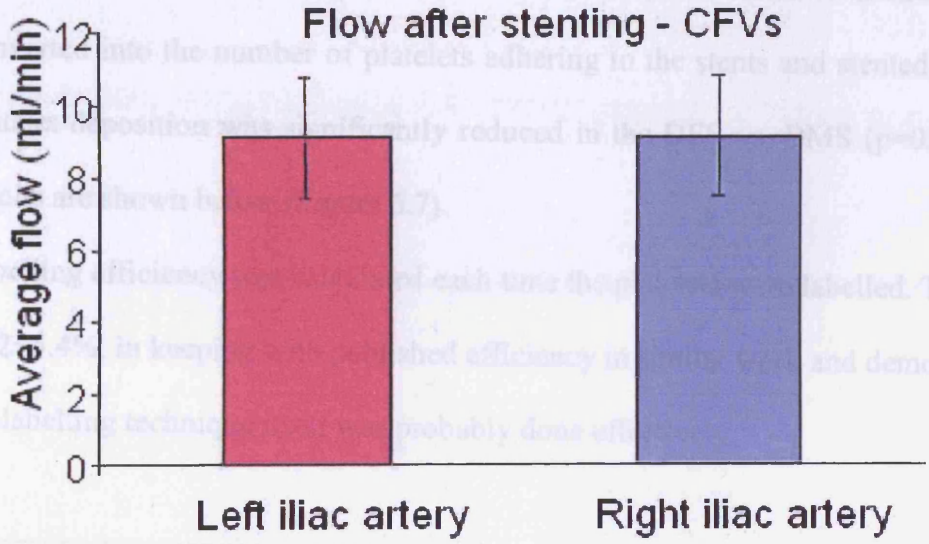


Figure 6.6a&b: Cyclic flow pre and post stenting. No cyclic flow variation was seen in BMS vs. EES.

6.4.1.2 Deposition of indium labelled thrombus

After the two hour flow observation period had been completed, stented vessels were dissected free. They were flushed with normal saline. Any adherent fat or adventitia was removed from the stented vessel. The vessels were then counted in a gamma counter, together with the known platelet count per animal; these counts allowed a calculation to be made that related the radioactivity associated with each stent to be converted into the number of platelets adhering to the stents and stented vessel wall. Platelet deposition was significantly reduced in the DES vs. BMS (p=0.014). These results are shown below (Figure 6.7).

Labelling efficiency was calculated each time the platelets were labelled. This was 88.2±5.4%, in keeping with published efficiency in similar work and demonstrate that the labelling technique itself was probably done effectively.

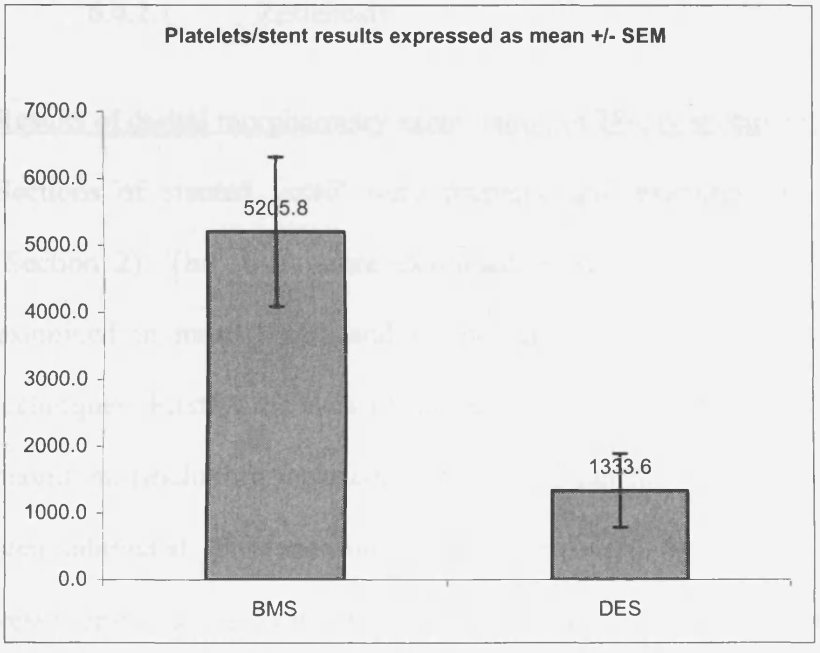


Figure 6.7: Results of indium-labelled platelet deposition on stented iliac vessels. (p=0.014)

6.4.1.3 Thrombus formation

A macroscopic assessment of the stented arterial segment was made for each vessel. Thrombus within this segment was graded according to the following criteria:

Occlusive - completely occupying and occluding the arterial lumen

Luminal - visible encroachment into the lumen without complete occlusion

Minor - minimal thrombus visible on stent struts or endothelial surface.

None - no visible thrombus

This grading has been used in previous work (Rajesh Aggarwal, Neil Swanson, MD Thesis, University of Leicester)[347].

No macroscopic thrombus was seen on microscopic examination in either group.

6.4.2 Chronic model 28 days results

6.4.2.1 Restenosis

<u>Results of digital morphometry examination of 28-day specimens:</u>

Sections of stented vessel were prepared and examined as discussed elsewhere (Section 2). The slides were examined in several ways. The 28-day slides were examined in more detail, and intimal growth was quantified using two different techniques. Firstly, the area of the lumen was calculated. Then the area within the neointima (including the stent sections and lumen) was measured and the lumenal area subtracted. This measurement was done at a low power magnification of the vessel cross-section to allow capture by the digital camera of the whole vessel. This had the benefit of measuring all of the neointimal area, but may have been prone to inaccuracies due to the low magnification (Figure 6.8-10). To overcome this potential problem, further measurements were made of the lumenal area in four quadrants of

the vessel, captured at a higher magnification. This allowed more precise tracing of the boundary between media and intima, but gave only an estimate of the average intimal thickness, since the results for the four quadrants measured were averaged and extrapolated to the whole circumference of the vessel.

To ensure the results were reproducible, three randomly selected vessels were re-measured and the various measurements plotted against the first reading to demonstrate that they were essentially the same. These internal control results are shown in graph form (figure 6.11). The results of the measurements of neointimal area and thickness are summarised below (figure 6.12-6.14).

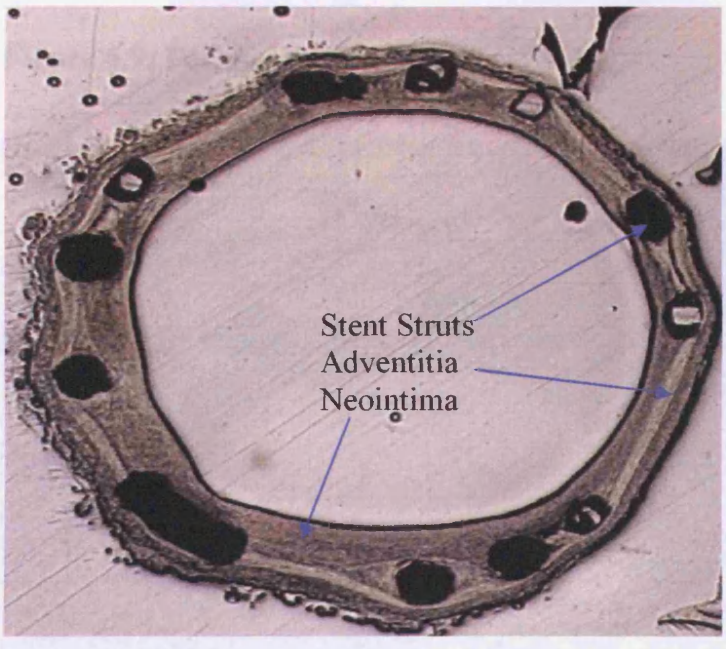


Figure 6.8: Bare Metal Stent section

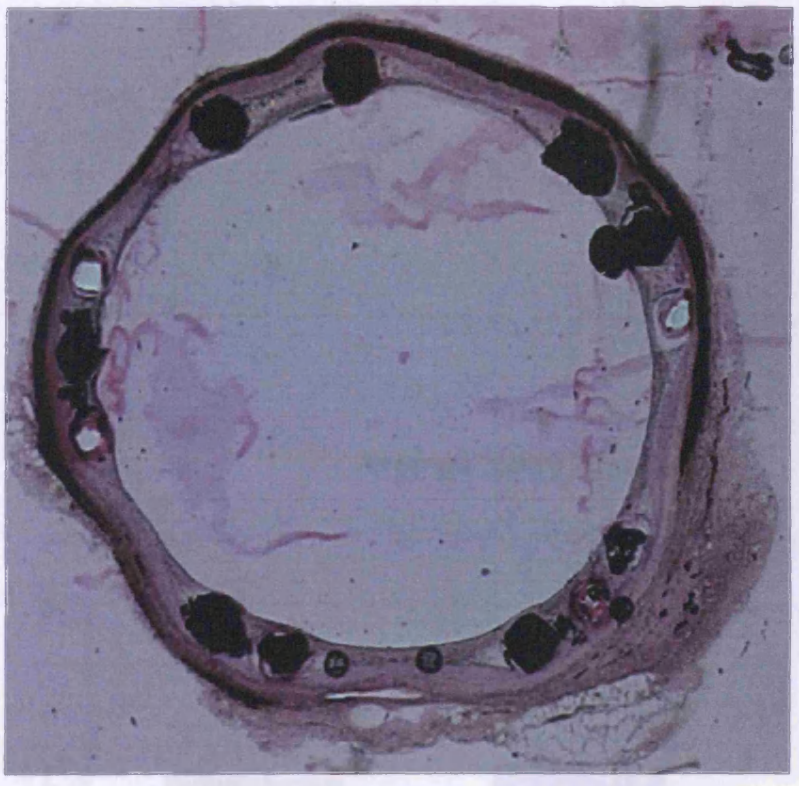


Figure 6.9: Polymer Coated Stent section

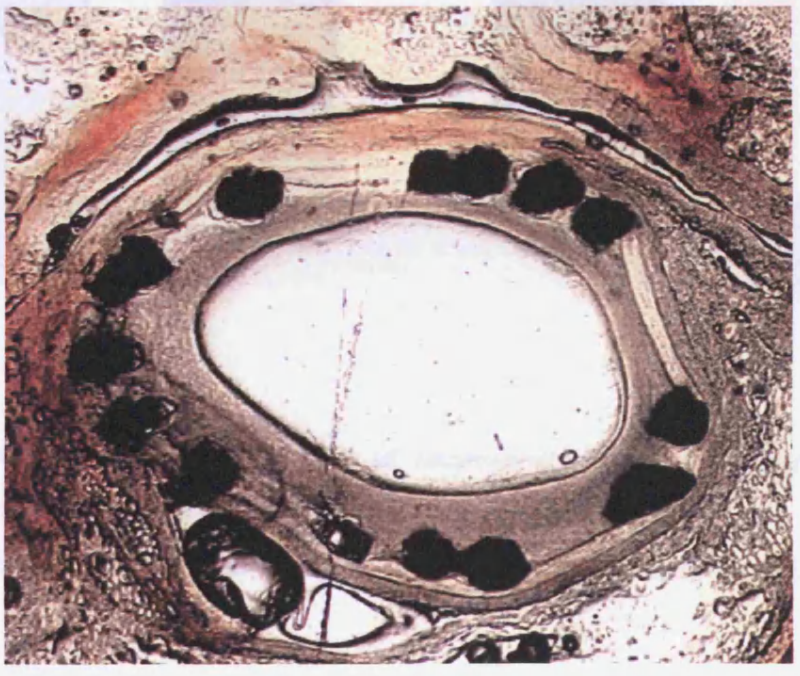


Figure 6.10: Eptifibatide Coated Stent section

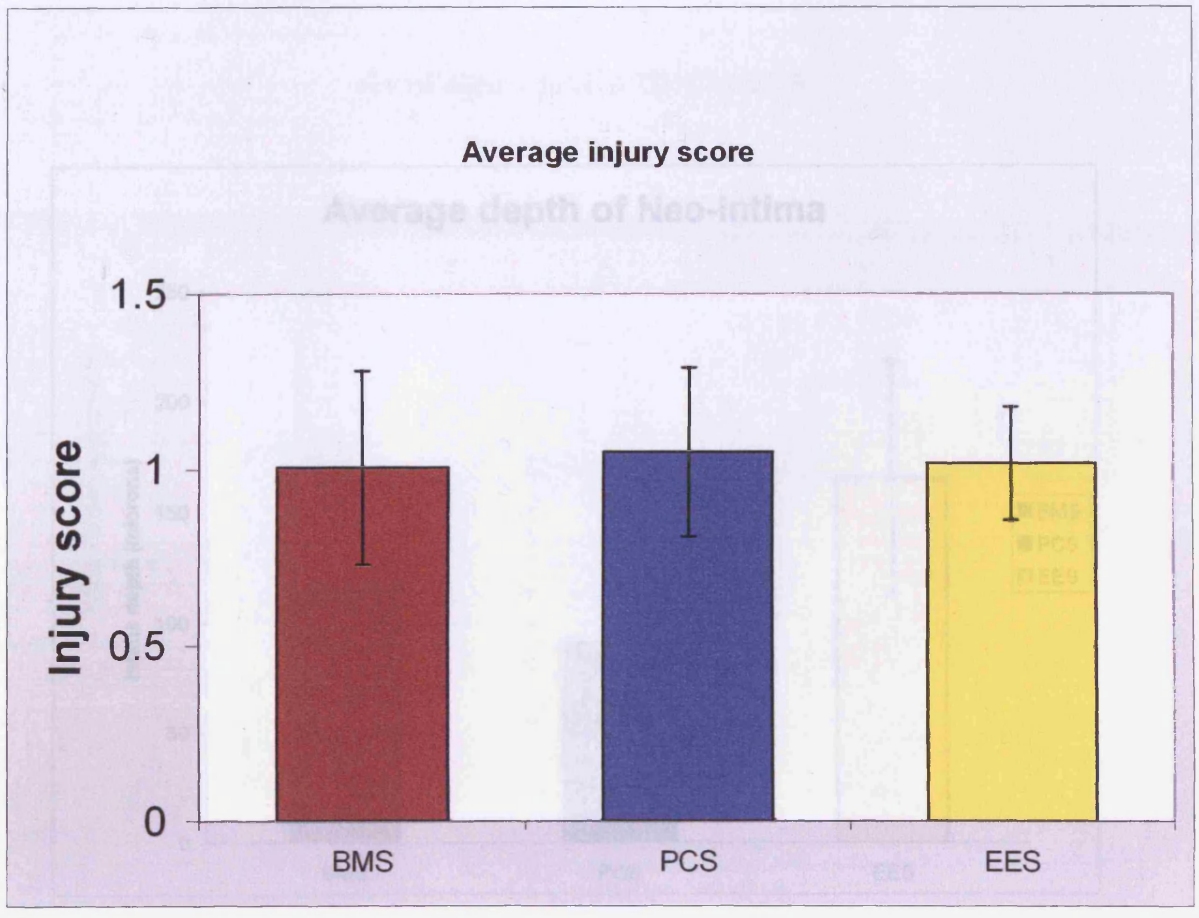


Figure 6.11: Mean injury scores of sections examined at 28 days. No significant difference was detected, suggesting that the initial injury, and thus stimulus to neointima formation, was similar in both the control and EES groups.

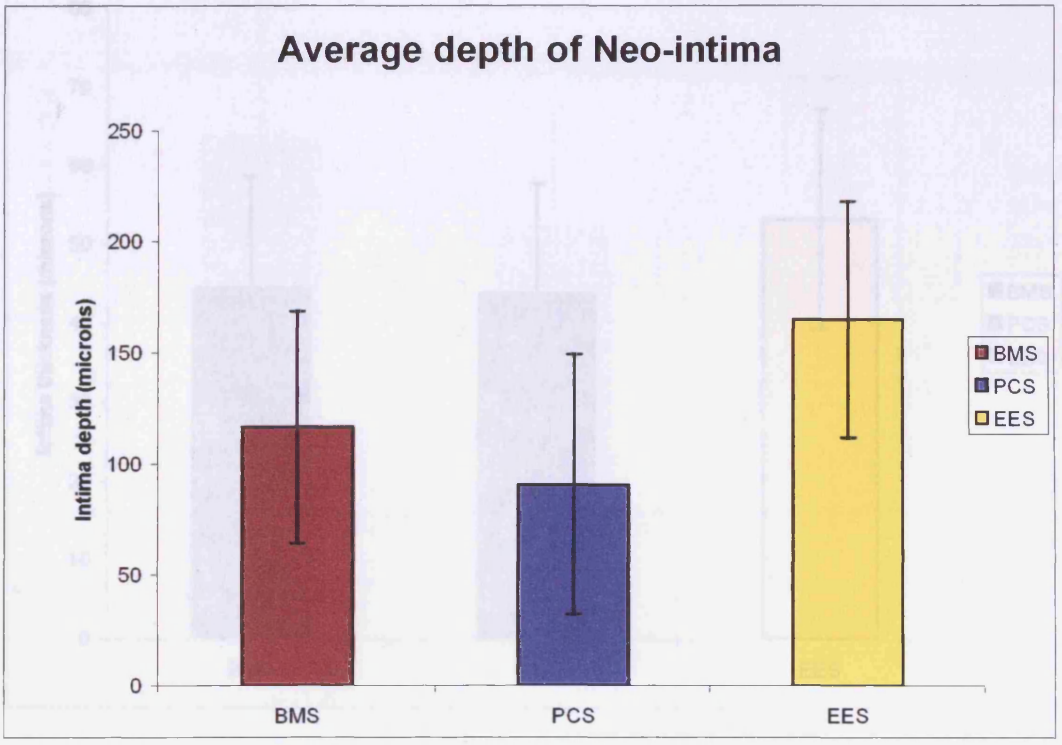


Figure 6.12: Comparison between the measured average depths of neointima in the three groups. Significant reduction in neointimal thickness was seen in polymer coated stents group (p=0.05 in PCS vs. EES).

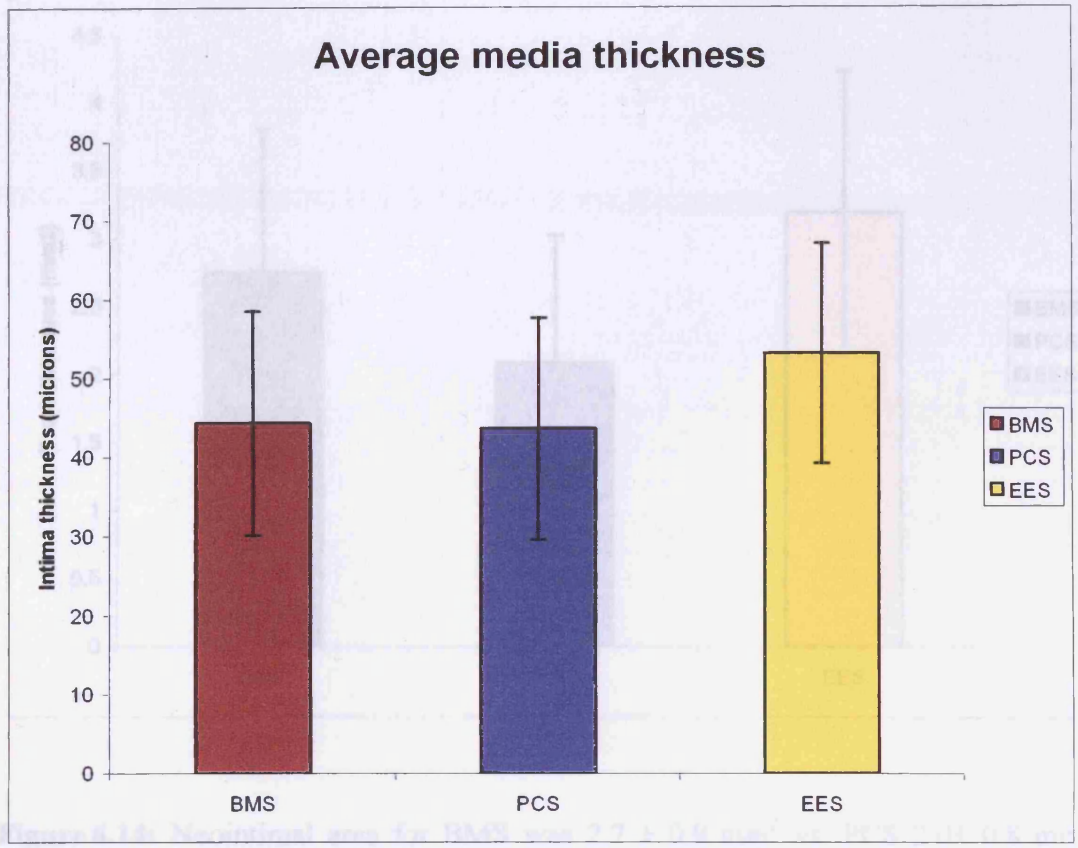


Figure 6.13: Comparison between the measured average media thickness in the three groups. No significant reduction in media thickness was seen in any stent group.

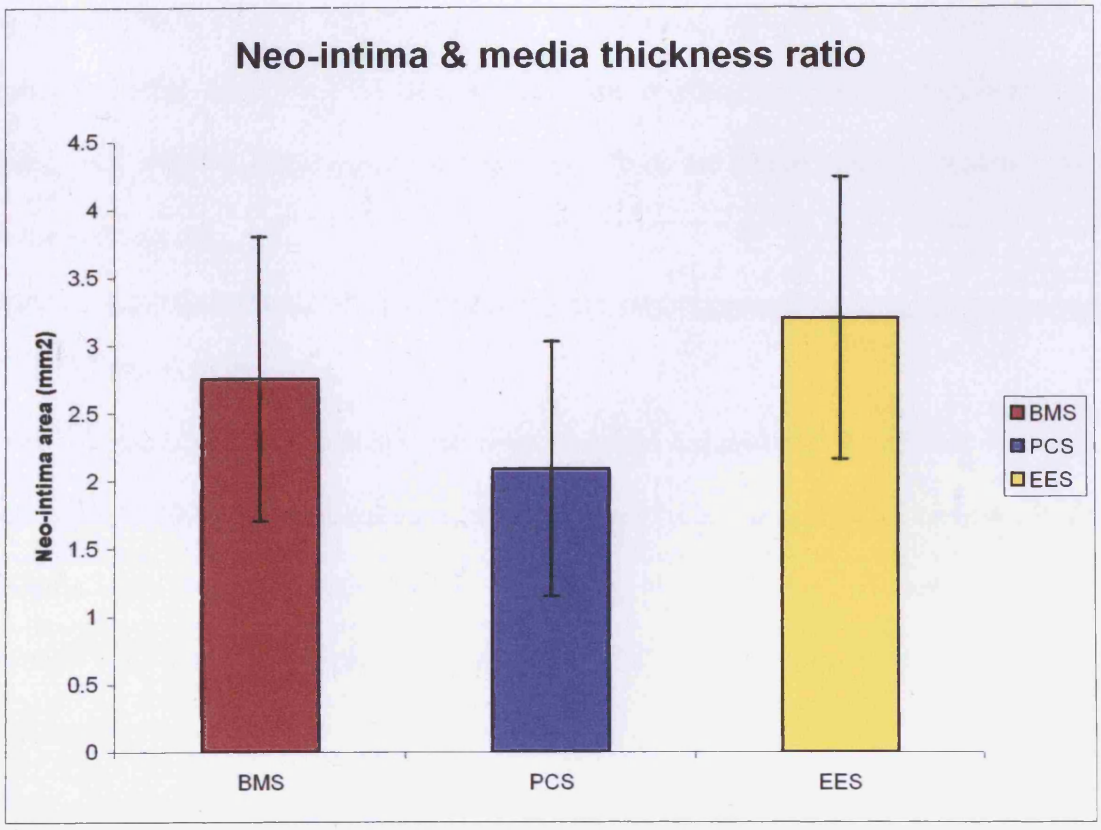


Figure 6.14: Neointimal area for BMS was 2.7 ± 0.9 mm^2 vs. PCS 2.0± 0.8 mm^2 (p=0.31) vs. EES 3.2±1.4 mm^2 (p = 0.08).

Chapter 7

7 Discussion and conclusion

7.1 Introduction

In this chapter the results of all the work done with eptifibatide will be summarized. Eptifibatide has formed the core of the work of this MD and the place of eptifibatide-eluting stents in humans will be explored in particular, including the limitations on their use. The scope for further studies with eptifibatide eluting stent will be discussed. Finally, conclusions will be drawn from the whole body of experiments described thus far.

7.2 Discussion

Stent implantation has become the new standard angioplasty procedure. In-stent restenosis (ISR) is not uncommon, affecting approximately 20% of patients at 6 months with Benestent type lesions and rising to almost 40% in high-risk sub-groups[358]. Current antiplatelet regimens, combined with improvements in stent design and deployment, have reduced the incidence of sub-acute thrombosis[359]. As medium term aspects of stenting are resolved with new drug eluting stents (rapamycin, paclitaxel)[360-361], the acute and long-term outcomes are the focus of current attention. There is little experimental and only preliminary clinical knowledge of the long-term effects of drug eluting stents in coronary arteries. The concerns include delayed wound healing and reduced endothelialization. This could increase thrombogenecity and the complication of late stent thrombosis. Further potential side effects could be late positive remodelling and aneurysmal formation. There is paucity of data on the drug distribution with in the vessel wall and tissue concentration, which are crucial parameters for biological effects. The search for ideal stent resulted in a huge variety of stents, differing in design, material, surface and coatings[362]. Polymer coatings have shown conflicting results and can themselves be responsible for local inflammation

and excessive neo-intimal proliferation[363]. A number of other coatings like phosphorylcholine[364], inert polymer or heparin[365], demonstrated a reduction in sub-acute stent thrombosis rate and neo-intimal hyperplasia. Therefore, the choice of coating is important. The polyvinyl butyrate is a biocompatible resin, has shown to have no adverse effects on the vessel wall in animal studies. We have demonstrated polyvinyl butyrate as a delivery vehicle for eptifibatide elution from stent.

7.2.1 Radiolabelling experiments

Initially, attempts were made to maximise the absorption of drug into the polymer. It was important to quantify the amount of drug that could be reliably absorbed onto the stent structure. Small amounts of drug can be absorbed into the finite volume available within the polymer structure and future experiments depend on knowing that a quantity of drug was absorbed that might credibly be expected to have a significant physiological effect.

Attempts were also made to recreate the physiological conditions the drug-coated stents would be exposed to *in vivo* – flow, pH, temperature and plasma proteins. This was important, as one of the key advantages of a drug-eluting stent would be its capacity to release the active drug in a sustained fashion. The sustained release with drug-eluting stents may be contrasted with other forms of local delivery, especially local drug delivery balloons. In these forms of delivery, drug delivery can only occur for as long as the delivery device is left in the target vessel. In practical terms in clinical practice, this can only mean a delivery period of minutes.

A biphasic elution curve is characteristic of the release of drugs from many polymer-coated stents, including eptifibatide-eluting stent. This is thought to be due to a very rapid wash-off of very lightly adherent drug molecules on or near the surface of the polymer, followed by a slower release of drug from within the substance of the

polymer. Elution seen with other drug/stent combinations, including the anti-thrombotic medications APC[246] and Abciximab[279], also followed a similar rapid initial release and then a slower, more sustained release over several days. The elution characteristics seen can only be considered as an approximation of those that may occur in vivo, since the model used is clearly not identical to the conditions found in an atherosclerotic human coronary artery. In particular, the stent is not perfused with blood and has not been deployed into an artery segment to examine stent vessel wall interaction. This model has, however, been used previously to predict in vivo effectiveness. The radiolabelling experiments showed that about 50μg of eptifibatide could be absorbed per stent. This is within the same range that had been delivered with beneficial effects in similar experiments. More importantly, the radiolabelling experiments showed that although a large quantity of eptifibatide (24μg) was eluted from the stent surface very quickly after it was placed in the perfusion circuit, a more sustained release was also seen for more than four weeks.

The results obtained here may be contrasted with the much longer elution times seen in previous work with an abciximab-eluting stent. Work by Baron *et al*[279] showed elution over 14 days with 53% retention at this time point.

7.2.2 Cell culture work

Up regulation of the $\alpha_v\beta_3$ integrin following arterial injury has been shown to be greatest within the first 2 weeks and inhibition of the $\alpha_v\beta_3$ integrin using peptides significantly reduces neointimal formation following arterial injury in animal models[366]. With approximately 55% of eptifibatide still available on the stent at 15 days, it should be present locally throughout the period of time that SMCs are more active. Our data on SMC proliferation assay has shown that eptifibatide eluting stent inhibits SMC proliferation with in 2 mm^2 of stent, which subsequently can have effect on neo-intimal hyperplasia. Additional effects may be mediated through the prevention of non-occlusive platelet thrombus deposition locally. Animals rendered thrombocytopenic have a reduced neointimal response to vessel injury; presumably through reduction in the release of growth factors and a reduction in the amount of thrombin present[367].

It is well established that atherosclerosis is a slow, complex disease in which deposits of lipids, cellular waste products, calcium and other substances build up in the inner lining of an artery. The earliest phase of atherosclerosis is fatty streak formation which is a pure inflammatory lesion, consisting only of T lymphocytes and monocyte-derived macrophages. Recently there has been emphasis on the involvement of inflammation in mediating all stages of atherosclerosis. However, in addition to inflammation, a key process of atherosclerosis involves the proliferation of VSMCs and subsequent accumulation of SMCs within the intima causing stenosis of the arteries. The SMC express $\alpha_v\beta_3$ integrin, which are up regulated following vascular injury and mediate cell-to-matrix binding. Comparison of two types of SMCs indicated that these cells express distinctive sets of integrins and varied levels of expression of integrin subunits and ECM proteins.

Diverse integrin expression in two SMC types correlated with slightly changed patterns of adhesion of these cells to ECM proteins. Osteopontin decreased the adhesion of human SMCs, but not PAC1 cells, which may be attributed to variations in integrin expression. Osteopontin is known for its anti-adhesion properties, although the mechanism of anti-adhesion has not been elucidated. Thrombospondin was not an adhesive substrate for SMCs in contrast to fibronectin or vitronectin, and was produced in considerable amounts particularly by human SMCs. Noteworthy, thrombospondin displays unusual adhesion properties: it is adhesive for some cell type and anti-adhesive for the others. It is often associated with vascular diseases and neointima formation.

Based on previous studies which demonstrated the inhibitory effect of eptifibatide on $\alpha_v\beta_3$ integrin binding of SMCs to thrombospondin[345], we investigated, further effects of eptifibatide on other ECM proteins and interestingly found that in our experimental settings, eptifibatide inhibited HSMCs attachment to vitronectin, osteopontin, thrombospondin and fibronectin. Further dose response using higher concentration of eptifibatide showed reduction of HSMCs adhesion to osteopontin. The inhibitory effects of eptifibatide on HSMC adhesion were specific for $\alpha_v\beta_3$. It has frequently been observed that expression of the$\alpha_v\beta_3$ integrin varies in HSMC in culture and *in-vivo*. Our results provide novel evidence that $\alpha_v\beta_3$ exist in multiple conformations and are subject to modulation as Scarborough et al[368] found that eptifibatide did not block vitronectin binding to purified $\alpha_v\beta_3$, whereas we found that eptifibatide does block vitronectin adhesion to $\alpha_v\beta_3$ on HSMCs. In our migration assay experiment, we found that eptifibatide inhibits migration of SMCs in response to thrombospondin, osteopontin and PDGF but no effect was observed towards vitronectin and FGF.

7.2.3 Eptifibatide-eluting stents to reduce stent thrombosis

Eptifibatide-eluting stents may find a niche role as an anti-thrombotic stent based on the results described in chapter 6. Stent thrombosis is in general a rare occurrence since the introduction of antiplatelet and anticoagulant agent's post-stenting such as clopidogrel, GIIb/IIIa antagonists, weight-adjusted heparin and aspirin. However, in certain situations, stent thrombosis remains a relatively common, and potentially very serious, complication. One such high-risk group would be diabetic patients with small vessel disease. Eptifibatide is a selective high affinity inhibitor of the platelet GPIIb/IIIa receptor. It produces dose-dependent *ex-vivo* inhibition platelet aggregation induced by adenosine diphosphate (ADP) by preventing the binding of fibrinogen, von Willebrand factor and other adhesive ligands to GPIIb/IIIa. We have demonstrated that eptifibatide eluting stents significantly inhibit platelet adhesion onto stents and eluted drug from the stent effectively inhibits platelet aggregation in response to ADP.

Overall, the inhibitory results of eptifibatide on SMC adhesion, migration to various ECM proteins, on platelet deposition, SMC proliferation and involvement of $\alpha_v\beta_3$ in the restenotic process show that local drug delivery of eptifibatide may be the way forward to ensure higher local concentration, local inhibition of platelet deposition and SMC activity reducing the need for antiplatelet agents post procedure. In order to establish whether eptifibatide can be an effective anti-restenotic agent, further studies were done to demonstrate the effect of eptifibatide eluting stents in an in-*vivo* model.

7.2.4 *In-vivo* experiments

The animal model experiments gave results of various aspects of the interaction between the artery and the eptifibatide -coated stents. The results from the *in vivo* experiments described leave some doubts as to whether there is any place for an eptifibatide eluting stent. Eptifibatide has not been shown to reduce intimal growth in this small animal model. It could be argued that there are important differences in the effects seen in a peripheral artery, especially in abnormal flow conditions. To test the eptifibatide eluting stent in a coronary model would require the use of a larger animal, e.g. the porcine model. The work done in this thesis with drug-eluting stents is predicated on the assumption that stent delivered agents of some kind will be effective methods for reducing the restenosis or stent thrombosis rates.

7.3 Study limitations

There are important limitations in both the *ex vivo* experiments and *in vivo* studies.

- Some assumptions have been made in conducting these experiments: It is assumed that radiolabelled eptifibatide will absorb to and elute from a coated stent in the same way as unlabelled eptifibatide. Only one concentration of eptifibatide was tested and a plateau of eptifibatide absorption was not demonstrated. It might be postulated that higher still concentrations of eptifibatide would result in even higher absorption but this has not been tested. Furthermore, higher concentrations of peptide would tend to form peptide aggregates that would not behave in a comparable method to the isolated peptide.

- For drug elution study, the perfusion circuit was filled with normal saline and not whole blood or platelet containing perfusate, which was technically impractical.

- The antiplatelet drugs given to the animals in this work may have greatly reduced the chances of vessel thrombosis. Flow was maintained in all the vessels throughout the recording and no vessels occluded in the two-hour observation period prior to the animal being killed.
- A bolus dose of 1000IU of heparin was given to all animals. Practical considerations limited the ability to monitor coagulation parameters intraoperatively.
- It is worth noting that local delivery studies performed in animals often, as in this case; use vessels that do not closely resemble the coronary arteries in humans. Firstly, the coronary arteries have a high number of small side branches. This means a great deal of the drug will be lost down these branches where it is not needed. Secondly, in clinical practice, it is patients with significantly atherosclerotic arteries who require these procedures. An atherosclerotic plaque is highly vascular and in these lesions a considerable proportion of drug delivered will pass down the *vasa vasorum*, away from the intended site of action[163]. It is not clear that local delivery of an agent in animals will have similar effects in patients.

7.4 New drug-eluting stents -Is stent thrombosis still an issue?

Since completing this research, there have been major advances in the management of stent restenosis. Stent thrombosis (ST) is a rare but remains a feared complication following percutaneous coronary intervention (PCI) that can occur at various time periods following intervention. For example, acute stent thrombosis usually occurs while the patient is still in the hospital and its effects are quite dramatic, whereas subacute stent thrombosis usually occurs within 30 days after the patient has been discharged home. Because the event occurs out of the hospital setting in the latter group of patients, rapid restoration of normal coronary blood flow is often not possible, and the patient may sustain an acute myocardial infarction with high morbidity and mortality rates. Thrombosis occurring after this 30-day period, termed late thrombosis, is a rare phenomenon that became a cause of concern after an increased rate of the event was observed in patients treated with intracoronary brachytherapy for the prevention of restenosis. Keeping these concerns in mind, long-term administration (at least 3 months) of dual antiplatelet therapy following intervention is now the recommended regimen for patients. Both paclitaxel and sirolimus-eluting stents have been unequivocally shown to reduce angiographic restenosis compared with bare metal stents (BMS), thereby reducing recurrent ischaemia resulting in the need for repeat hospitalization and revascularization procedures (see section 1.2.1). Although their efficacy in reducing neointimal hyperplasia and clinical restenosis has been maintained in a broad spectrum of clinical conditions, there is an emerging safety concern regarding the risk of ST motivated by a number of reports of late ST in real-world patients, particularly after the discontinuation of double-antiplatelet therapy. Little is known about the incidence of

ST in patients who continue thienopyridine treatment beyond 6 months after stenting compared with patients who receive only aspirin. This uncertainty has prevented physicians from establishing the appropriate duration of dual-antiplatelet therapy after DES implantation. There is possibility that the greater prevention of restenosis-related adverse events by DES compared with BMS occurring within the first year might offset some or all of the excess risk from stent thrombosis with DES occurring after the first year.

7.5 Conclusion

Although the ultimate results refuted the initial hypothesis under test, the methods used are robust and will enable other research to be performed of a similar nature to test other combinations of drug, polymer and stent.

Some interesting results have been produced in the course of the studies. Eptifibatide appeared to inhibit smooth muscle cell growth in a particular subset of cells, i.e. older passage cells in adverse culture conditions. Secondly, eptifibatide-eluting stents reduced thrombus formation on the stents when assessed at two hours. The *in vivo* work is completely original since eptifibatide has never been delivered bound onto a stent and so delivered in a sustained fashion in such an animal model. Clear results have been obtained from this section of work. No benefit to restenosis was seen in the rabbit iliac model. The study was powered sufficiently to detect any clinically meaningful result and so this negative result may be confidently said to be a true conclusion. The result was obtained from soundly designed experiments that predominantly used well-established methods that have been the subject of published work in the past. Despite the research limitations, this research gave valuable information that eptifibatide eluting stents works as anti-thrombotic stent and

therefore provides a starting point for further research into eptifibatide in the future especially stent laden with dual combination of eptifibatide and an established restenotic agent such as paclitaxel or rapamycin.

In conclusion, the future of the drug-eluting stent appears to be very bright, with the promise of a therapy that may spare many thousands of patients the morbidity and mortality associated with the problem of stent restenosis and thrombosis.

Appendix

Composition of buffers, and reagents

Enzyme buffer – pH 4.5

Sodium Acetate	(0.1 mol/L)	0.820g
Sodium Chloride	(0.1mol/L)	5.84g

Adjust pH to 4.5 using conc. HCl and dilute to 1 litre with deionised water

Phosphate-buffered saline – pH 7.4 (PBS)

Na_2HPO_4	[anhydrous]	10.65g
NaH_2PO_4	[anhydrous]	3.0g
NaCl	(0.1mol/L)	5.84g

Dilute to 1l with deionised water – check pH 7.4 +/- 0.2

Sodium Phosphate buffer – pH 7.2 (coating buffer)

Na_2HPO_4	[anhydrous]	1.065g
NaH_2PO_4	[anhydrous]	0.3g
NaCl	(0.15mol/L)	8.474g

Dilute to 1l with deionised water – check pH 7.2 +/- 0.3

Acid-Citrate Solution

Trisodium citrate dihydrate	2.5g
Citric acid monohydrate	1.49g

Dilute to 100ml in deionised water. Dispense in 5ml aliquots into sterile containers via a 0.22μm membrane filter.

Trisodium Citrate Solution

Trisodium citrate dihydrate	3.2g

Dilute to 100ml in deionised water. Dispense in 5ml aliquots into sterile containers via a 0.22μm membrane filter.

Tyrode's Buffer

To 450ml sterile, pyrogen-free water add:

Sodium Chloride	8.0g
Potassium chloride	0.1g
NaH_2PO_4, $2H_2O$	0.025g
$MgCl_2$, $6H_2O$	0.2g
D-glucose	0.5g
Sodium heparin	12,500u
Prostaglandin E_1	0.15 mg

Adjust pH to 6.5 with 1M HCl; dilute to 500 ml total volume. Dispense in 25 ml aliquots into sterile universal containers via a 0.22 μm membrane filter and store at -20° C.

Cell culture media

BAEC Cell culture Medium – 20% FCS in DMEM

500ml of DMEM, warmed to 37°C
Add 120ml of FCS
Add 15ml of Gentamicin

HUVEC culture medium

500ml Gibco M199 with Earl Salts with Glutamine (Ref 31150-022)
20% FCS
1% Penicillin/streptomycin
Heparin 2500iu (Monoparin)

1% Acid/Alcohol (1l)

10ml Conc. HCl
300ml Distilled Water
700ml 99% alcohol

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Eptifibatide-Eluting Stent as an Antiproliferative and Antithrombotic Agent: *In Vitro* Evaluation

Kamal Chitkara, MRCP, Kai Hogrefe, MD, Mariuca Vasa-Nicotera, MD, Neil Swanson, MD, Anthony H. Gershlick, FRCP

ABSTRACT: Objective. Stent thrombosis and in-stent restenosis remain problematic despite drug-eluting stents (DES), especially in diabetic patients and in those with small-vessel disease. Eptifibatide inhibits the platelet glycoprotein IIb/IIIa and smooth muscle cell (SMC) αvβ3 receptor, thus potentially influencing both thrombosis and proliferation. The aim of the present studies was to examine the absorption and elution characteristics of eptifibatide on polymer-coated stents and investigated their effect on SMC proliferation, platelet deposition and platelet aggregation *in vitro*. **Methods and Results.** Polymer-mixed eptifibatide and H3-labeled eptifibatide were loaded onto bare metal stents. A maximum of 111 μg of eptifibatide was loaded onto 3.0 x 18 mm stents. Drug elution characteristics were tested in a PBS perfusion circuit. Elution profile consisted of an early rapid phase (24% ± 0.03 loss over 1 hour) followed by a sustained release with 44% ± 2.30 still present on the stent after 30 days. Eluted eptifibatide significantly inhibited adenosine diphosphate (ADP)-induced platelet aggregation by 95% ± 0.70 ($p < 0.01$). Efficacy of stents eluting eptifibatide for antiplatelet effect was determined by measuring deposition of 111indium-labeled platelets on stents. Platelet deposition was significantly reduced by 48% ± 6 in comparison to controls ($p = 0.0065$). Finally, drug-loaded stents were placed in SMC culture and showed a distinct zone of cell growth inhibition within 1 mm^2 (88 ± 22 vs. 208 ± 23 SMCs in control), and within 2 mm^2 of stent (131 ± 32 vs. 191 ± 23 SMCs in control) (both $p < 0.01$). **Conclusions.** Eptifibatide can be successfully loaded onto stents. It elutes in a predictable manner, significantly inhibiting platelet deposition, aggregation and SMC proliferation *in vitro*. These studies pave the way to developing stent-based delivery of a potent antiplatelet agent, which additionally may inhibit SMC activity.

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Coronary stent placement is the most commonly employed technique for percutaneous treatment of atherosclerotic heart disease. It accounts for about 75% of the procedures performed worldwide.[1] In comparison to conventional balloon angioplasty, stents have improved the efficacy of percutaneous coronary interventions (PCI) by reducing abrupt or threatened vessel closure[2,3] and by reducing restenosis.[4,5] Notwithstanding, coronary stent thrombosis has remained an uncommon, but serious complication of PCI.[6] The newer drug-eluting stent (DES) inhibits restenosis by inhibiting SMC proliferation, but there are concerns that the drug eluted from the stents may delay the endothelialization process[7] around the stent struts and thus increase the risk of prolonged thrombogenicity of the stent leading to late stent thrombosis.[8] Therefore, stent thrombosis may be more common after DES use, with consequent danger to the patients. For this reason, patients who have received DES are often prescribed aspirin and clopidogrel therapy for at least 6 months. Despite dual antiplatelet therapy, stent thrombosis persists at a rate of 0.5–2% in elective cases,[9–12] and up to 6% in patients with acute coronary syndromes.[10,13]

Eptifibatide[14] is a synthetic cyclic heptapeptide inhibitor of the platelet glycoprotein (GP) IIb/IIIa receptor. It is derived from the structure of barbourin, which has been isolated from the venom of the rattlesnake. The GP IIb/IIIa receptor is a member of the integrin super-family of cell surface adhesive protein receptors.[15] The GP IIb/IIIa receptor binds specifically to one anchorage region on each end of the fibrinogen protein, facilitating cross-links and platelet aggregation.

Preclinical pharmacological studies have established that eptifibatide can inhibit thrombosis effectively when given intravenously. Pharmacokinetics and pharmacodynamic studies[16] in both animal models and humans have shown that the antiplatelet effect of eptifibatide has a rapid onset of action, > 80% inhibition of platelet aggregation within 15 minutes and a short plasma life (2.5 hours), as well as being rapidly reversible. It is licensed for use in patients with acute coronary syndromes and for use in PCI.[17–19]

The GP IIb/IIIa receptor is closely related to the αvβ3 receptor[20–23] which binds matrix proteins deposited on injured tissues, including vitronectin, von Willebrand Factor, fibrinogen, fibronectin, collagen and thrombospondin. This receptor mediates a number of processes, which include smooth muscle cell adhesion, migration and proliferation.

Previous studies[24] have shown that eptifibatide alone can inhibit αvβ3-mediated attachments of human arterial smooth muscle cells (HASMCs) to thrombospondin (TSP) and prothrombin. In cell proliferation assays, eptifibatide compound inhibited αvβ3-mediated responses to soluble TSP by HASMCs and β3 integrin-expressing human embryonic kidney cells. With these findings on the effectiveness of eptifibatide on the αvβ3 receptor, it was considered that it might be possible to inhibit neointimal hyperplasia with such an antiplatelet drug if the local dose were sufficient (which might not be safely achievable using systemic administration) and reduces the risk of both restenosis and thrombosis.

We hypothesized that a polymer-coated stent could deliver eptifibatide locally and for a sustained period. To test this, the

From the University of Leicester, Cardiovascular Medicine, Clinical Sciences, Glenfield Hospital, Leicester, United Kingdom.

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Address for correspondence: Kamal Chitkara, MRCP, Specialist Registrar, Cardiology, Clinical Sciences Wing, Cardiovascular Medicine, Glenfield Hospital, Leicester LE3 9QP, United Kingdom. E-mail: kc29@le.ac.uk

absorption and elution characteristics of eptifibatide on this stent were studied, as well as its effects on platelet aggregation and SMC proliferation. The current studies extend the concept of DES to eptifibatide, a drug with established clinical use, in order to assess its potential in man as a DES agent with dual properties, both antithrombotic and possibly antirestenotic as well.

Materials and Methods

The polymer polyvinyl butyrate (PVB) is a derivative of polyvinyl alcohol, used as a delivery vehicle for eptifibatide on the bare metal stainless steel stents. PVB is a biocompatible resin and contains only carbon, hydrogen and oxygen. PVB is nonthrombogenic and has neutral effect on the platelets.[25-29]

All bare metal stainless stents were expanded at low atmospheric pressure (6 atm). Eptifibatide was supplied at a concentration of 2 mg ml by Schering-Plough and was freeze-dried and later radiolabeled with tritium *(Pepceuticals Ltd.)*. Human vascular SMCs (HVSMCs) originated from primary explants culture of vein fragments. Fragments of veins with normal morphology were obtained from healthy margins of veins removed from patients undergoing varicose vein surgery.

Leicester Research Ethics Committee approved these experiments. All investigations conformed to the principles outlined in the Declaration of Helsinki.[30]

Absorption and elution studies with eptifibatide — Radiolabeling experiment.

Eptifibatide absorption. All bare metal stents (3 x 18 mm) were weighed prior to stent coating. The bare metal stents were sprayed with hydrophobic PVB. Polymer and unlabeled drug (15%) were dissolved in methanol/chloroform (50% w/w), and 80 µl of 3H-radiolabeled eptifibatide (specific activity 20 ci/mmol)) was added to the solution and used as a "spike" to allow detection of the drug. The stents were sprayed using ultrasonic micro spray to allow uniform distribution of the drug on the stent. Absorption was by diffusion, in a hydrophilic solvent such as ethanol, into the substance of the polymer, where the drug is held by hydrophilic-hydrophobic interaction between the drug and the polymer.

Each stent had 6 drug/polymer coating passes, dried in a vacuum oven for 1 hour (to remove any remaining solvent), and was then weighed to obtain drug loadings. Each stent then had a top-coating with pure polymer (2 passes) to retard drug elution. The stents were stored under vacuum desiccant over 2 days.

Eptifibatide elution *in vitro*. Radiolabeled eptifibatide-coated stents were perfused continuously (as previously described in detail)[31] at 25 ml min-1 in a closed-loop circuit with PBS solution (Figure 2). The reservoirs were pretreated with 1% bovine serum albumin (BSA) in PBS for 48 hours and then rinsed. This reduced nonspecific binding of radiolabeled eptifibatide from adhering to the surface of the reservoir. The perfusate was maintained at 37°C and was changed every 24 hours. The radioactive drug eluted from the stent in each PBS solution was quantified by beta counting in a beta-counter *(Tricarb® Packard Liquid Scintillation Analyzer)*. Three stents were tested under each set of conditions.

Platelet aggregometry. Samples from the perfusate were taken after elution of the eptifibatide-coated stents, and the ability of the platelets to aggregate to ADP (4 µM) was assessed using a whole blood impedance aggregometry technique.[32]

The effect of eptifibatide-eluting stents on platelet deposition. Human platelet-rich plasma (PRP) was obtained from the blood bank and the standard method of Hawker[33] was used to label human platelets with indium oxine (111In). The bare metal stents and stents coated with eptifibatide were placed in the perfusion circuit, with the blood containing the 111In-labeled platelets as perfusate. The stents were perfused continuously for 1 hour at 40 ml min-1, the calculated shear rate at the surface of the stent being approximately 850 s-1. Stents were then rinsed and the radioactivity associated with each stent was counted in a gamma counter *(Packard Cobra series Auto-gamma counting system, 15–75keV window)*.

The effect of eptifibatide-eluting stents in cell culture proliferation. Human smooth muscle cells were cultured in medium 199 with Earl's salt and 10% fetal bovine serum (FBS). Cells were passaged 4 to 6 times. SMC phenotype was verified by immuno-precipitation with anti-SM alpha-actin antibody (1A4, Sigma) and by a characteristic hill-and-valley growth pattern, and were 80% confluent prior to assay. Cells were starved for 18 hours in Earl's medium 199 prior to assay in order to "equilibrate" them to stop the proliferation of cells and then reach the same cell cycle point. SMCs were detached with trypsin, washed in the presence of trypsin inhibitor (Sigma-Aldrich), resuspended in Earl's medium 199 containing 5% BSA and centrifuged for 5 minutes at 1,500 rpm. Cells pellet were resuspended in Earl's medium 199 in 5% BSA and then cells were plated at a density of 3 x 105 cells per 60 mm dish. Each of the bare metal and eptifibatide-loaded stents were fixed in the center of the dishes with sterile surgical bone cement in separate experiments. All culture dishes were incubated at 37°C in a 5% CO_2 incubator. Culture medium was replaced every 48 hours. The zone of cell growth inhibition was measured at 7 days after initial plating with a micrometer inserted into the eyepiece of a standard inverted microscope *(Olympus)*. In a subset of the cultures (at 7 days), the cells were stained with toluidine blue to allow clear-cut demarcation of the zone of cell growth inhibition at low levels of magnification.[34]

Statistical evaluation. Results are presented as mean ± standard deviation (SD). Analysis of statistical significance was performed using the Student's t-test for parametric and the Mann-Whitney U-tests for nonparametric results. A p-value of < 0.05 was considered statistically significant.

An unpaired t-test or ANOVA was used to analyze the adsorption and elution data. This was used to analyze the platelet deposition data for the segments of stent. Because of the variability in absolute platelet deposition, the results are presented as a percent reduction in 111In counts on treated stents compared to control stents exposed to labeled platelets during the same experiment. The Wilkinson matched-pairs signed ranks test was used to analyze the data for the whole stent platelet deposition experiments.

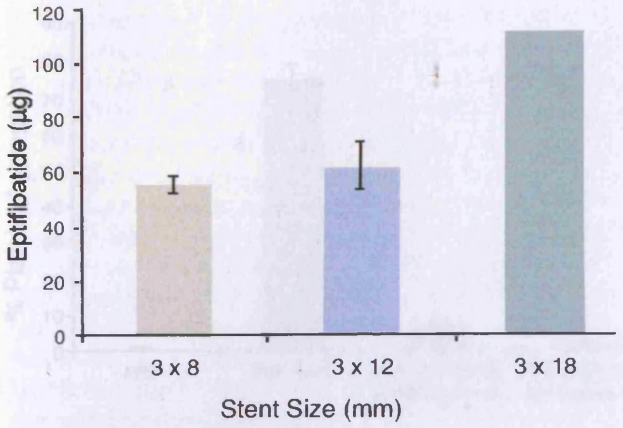

Figure 1. *Eptifibatide absorption onto polymer-coated stents by the ultrasonic spraying method. Experiments were repeated three times to verify a reproducible result. P-value < 0.001.*

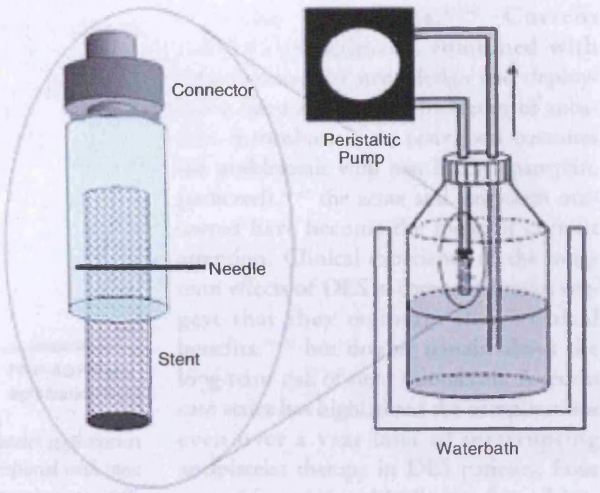

Figure 2. *In vitro perfusion circuit for elution of eptifibatide from polymer-coated stents. The standard flow rate used was 25 ml min-1. In the platelet deposition experiments, the PBS was replaced by blood containing 111Indium labeled platelets.*

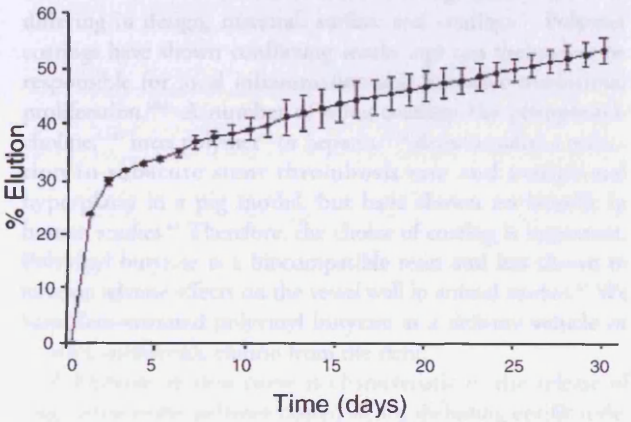

Figure 3. *The elution of eptifibatide coated stents in an in vitro perfusion circuit. The stents were continuously washed with phosphate buffer saline +1% bovine serum albumin at 25 ml/minute at 37°C. Experiments were repeated three times to verify a reproducible result. P-value < 0.05.*

Results

Loading of eptifibatide onto polymer-coated stents. Eptifibatide was successfully loaded onto bare metal stents using polyvinyl butyrate polymer. A maximum of 111 µg of eptifibatide was loaded onto 3.0 x 18 mm bare metal stents, with less total amount of eptifibatide loaded on shorter stents (Figure 1).

Elution *in vitro*. Elution of eptifibatide from polymer-coated stents in the perfusion circuit (Figure 2) is shown in Figure 3. These stents were perfused at 25 ml min-1 at 37°C. Drug elution characteristics were tested in a phosphate buffer saline perfusion circuit for up to 4 weeks. The curve is biphasic and consisted of an early rapid elution phase (24% ± 0.03 loss over 1 hour), followed by a sustained release with 44% ± 2.30 still present on the stent after 30 days.

Platelet aggregometry. Aggregometry undertaken during the platelet deposition experiment was carried out to confirm both the platelet viability and that the eptifibatide eluted from stent had retained antiplatelet properties in the perfusate. Eluted eptifibatide significantly inhibited platelet aggregation by 95% ± 0.70 in response to ADP (4 µM) at 10 minutes ($p < 0.01$) (Figure 4). The effect of eluted eptifibatide from the stent was the same as pure eptifibatide from the injection vial when compared.

Effect of eptifibatide-coated stents on platelet deposition. There was a significant reduction in platelet deposition onto polymer-coated stent treated with eptifibatide as compared with controls (Figure 5). Platelet deposition on stents eluting eptifibatide was significantly reduced by 48% ± 6 compared with controls ($p = 0.0065$), when stents were perfused continuously in blood containing the 111In-labeled platelets for 1 hour at 40 ml min-1. Unlike the control stents, none of the treated stents had visible thrombus attached to them.

Effect of eptifibatide-eluting stents on SMC proliferation. In the cell culture experiment, SMC proliferation was measured around the stents and the cells were stained with toluidine blue to allow clear-cut demarcation at 7 days. Eptifibatide-loaded stents (50 µg/stent) placed in SMC culture showed a distinct zone of cell growth inhibition within 1 mm^2 of stent (88 ± 22 vs. 208 ± 23 SMCs in control), and within 2 mm^2 of stent (131 ± 32 vs. 191 ± 23 SMCs in control) (both $p < 0.01$) (Figure 6).

Discussion

Stent implantation has become the standard treatment during PCI.[35-37] This is because it has overcome most of the major complications of a standard balloon angioplasty procedure which include acute coronary occlusion secondary to intimal dissection following balloon angioplasty and a superior long-term outcome in comparison to balloon angioplasty.[38-41] In-stent restenosis (ISR) is not uncommon, affecting approximately 20% of patients at 6 months with BENESTENT-type lesions, and rising to almost 40% in

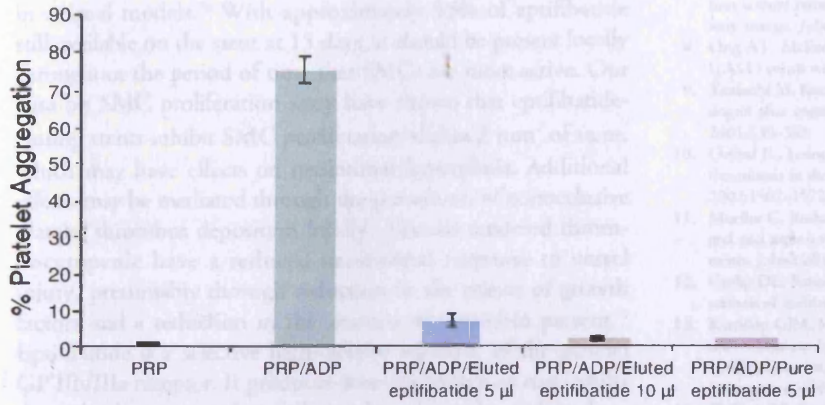

Figure 4. *In platelet-rich plasma (PRP), the effect of eluted eptifibatide on platelet aggregation in response to adenosine diphosphate (ADP). The effect of eluted drug was compared with pure eptifibatide from injection vial as positive control. Experiments were repeated three times to verify a reproducible result. P-value = 0.01.*

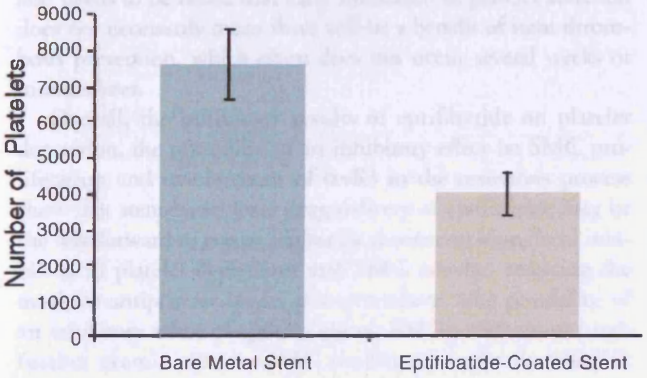

Figure 5. *The effect of eptifibatide coated stents on 111Indium labeled platelet deposition versus bare metal stents in an in vitro perfusion circuit for 1 hour at 37°C. Experiments were repeated three times to verify a reproducible result. P-value = 0.0065.*

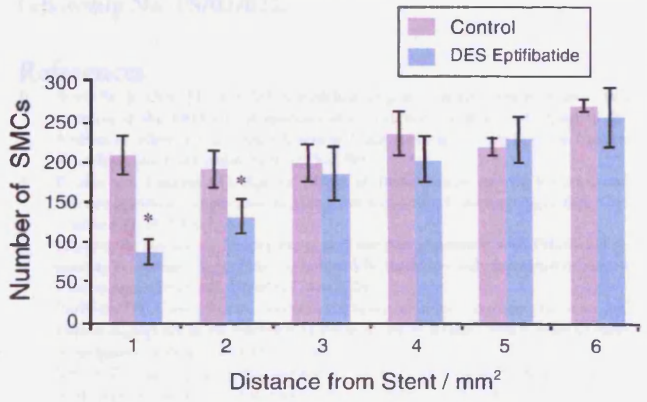

Figure 6. *Human smooth muscle cell proliferation inhibition from center to periphery/mm² of culture dishes by eptifibatide coated stents. Experiments were repeated three times to verify a reproducible result. P-value = 0.002.*

high-risk subgroups.[42-44] Current antiplatelet regimens, combined with improvements in stent design and deployment, have reduced the incidence of subacute thrombosis.[45] As restenosis becomes less problematic with new DES (rapamycin, paclitaxel),[46-49] the acute and long-term outcomes have become the focus of current attention. Clinical experience of the long-term effects of DES in coronary arteries suggest that they maintain their clinical benefits,[50-57] but doubts remain about the long-term risk of stent thrombosis. A recent case series has highlighted the complications even over a year later of interrupting antiplatelet therapy in DES patients. Four cases of angiographically confirmed late thrombosis were reported between 335 and 442 days after DES implantation, resulting in myocardial infarction.[58] Further potential side effects could be late positive remodeling and aneurysmal formation. The search for an ideal stent has resulted in a huge variety of stents, differing in design, material, surface and coatings.[59] Polymer coatings have shown conflicting results and can themselves be responsible for local inflammation and excessive neointimal proliferation.[60,61] A number of other coatings like phosphorylcholine,[62,63] inert polymer[64] or heparin,[65,66] demonstrated a reduction in subacute stent thrombosis rate and neointimal hyperplasia in a pig model, but have shown no benefit in human studies.[66] Therefore, the choice of coating is important. Polyvinyl butyrate is a biocompatible resin and has shown to have no adverse effects on the vessel wall in animal studies.[67] We have demonstrated polyvinyl butyrate as a delivery vehicle *in vitro* for eptifibatide elution from the stent.

A biphasic elution curve is characteristic of the release of drugs from many polymer-coated stents, including eptifibatide-eluting stent. This is thought to be due to a very rapid wash-off of very lightly adherent drug molecules on or near the surface of the polymer, followed by a slower release of drug from within the substance of the polymer. Elution seen with other drug/stent combinations, including the antithrombotic medications activated protein C and abciximab,[68,69] also followed a similar rapid initial release and then a slower, more sustained release over several days. The elution characteristics seen are an approximation of those that occur *in vivo*, since the model used is not identical to the conditions found in an atherosclerotic human coronary artery. In particular, the stent is not perfused with blood and has not been deployed into an artery segment to examine stent-vessel wall interaction. The model used has been used previously to predict *in vivo* effectiveness.

It was found that eptifibatide elutes rapidly in the first hour from the stent in a perfusion circuit followed by sustained release over days. Up-regulation of the $\alpha v\beta 3$ integrin following arterial injury has been shown to be greatest within the first 2 weeks, and inhibition of the $\alpha v\beta 3$ integrin-using peptides significantly reduces neointimal formation following arterial injury

in animal models.[70] With approximately 55% of eptifibatide still available on the stent at 15 days, it should be present locally throughout the period of time that SMCs are more active. Our data on SMC proliferation assay have shown that eptifibatide-eluting stents inhibit SMC proliferation within 2 mm^2 of stent, which may have effects on neointimal hyperplasia. Additional effects may be mediated through the prevention of nonocclusive platelet thrombus deposition locally. Animals rendered thrombocytopenic have a reduced neointimal response to vessel injury, presumably through reduction in the release of growth factors and a reduction in the amount of thrombin present.[71] Eptifibatide is a selective high-affinity inhibitor of the platelet GP IIb/IIIa receptor. It produces dose-dependent *ex vivo* inhibition platelet aggregation induced by adenosine diphosphate (ADP) by preventing the binding of fibrinogen, von Willebrand factor and other adhesive ligands to GP IIb/IIIa.[72,73] We have demonstrated that eptifibatide-eluting stents significantly inhibit platelet adhesion onto stents and eluted drug from the stent effectively inhibits platelet aggregation in response to ADP. It also needs to be noted that early inhibition of platelet adhesion does not necessarily mean there will be a benefit of stent thrombosis prevention, which often does not occur several weeks or months later.

Overall, the inhibitory results of eptifibatide on platelet deposition, the possibility of an inhibitory effect on SMC proliferation and involvement of αvß3 in the restenosis process show that stent-based local drug delivery of eptifibatide may be the way forward to ensure higher local concentration, local inhibition of platelet deposition and SMC activity, reducing the need for antiplatelet agents post-procedure. The possibility of an inhibitory effect of eptifibatide on SMC proliferation needs further examination in SMC studies. In order to establish whether eptifibatide can be an effective antirestenotic agent, further studies to demonstrate the effect of eptifibatide-eluting stents in an *in vivo* model have now been commenced.

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