Vascular endothelial growth factor-eluting, polymer-coated, coronary stents. An *in vitro* and *in vivo* evaluation.

Neil M.G. Swanson

Doctor of Medicine The University of Edinburgh 2003

Declaration of originality

I confirm that the work contained in this thesis has been composed by myself and that the work is my own. I further confirm that this work has not been submitted for any other degree or professional qualification.

THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

Name of candidate Neil Maxwell Grenville Swanson
Address .....
Degree MD ***Date*** 23rd September 2003
Title of Thesis: Vascular endothelial growth factor-eluting, polymer-coated coronary stents. An *in vitro* and *in vivo* evaluation.
No. of words in main text of Thesis 38,591

Introduction: Percutaneous intervention (PCI) is complicated by restenosis, stent thrombosis and delayed endothelial recovery at the PCI site. One approach to reduce these complications is to deliver potent agents directly to the PCI site. This local drug delivery can be achieved by absorbing drugs into a polymer coating applied to the stent itself. Vascular Endothelial Growth Factor (VEGF) has been shown to accelerate the recovery of endothelium over a stent, reducing intimal hyperplasia and thrombosis. It has not previously been delivered bound onto the stent itself. VEGF-eluting stents were tested *in vitro* and in a rabbit model.

Original Hypothesis: Polymer coated stents may absorb and gradually release Vascular Endothelial Growth Factor. This released VEGF may reduce the stent complications of thrombosis and neointimal hyperplasia, primarily by accelerating the recovery of denuded endothelium. This was tested *in vitro* and in an animal model.

Methods: Studies were performed to determine optimum stent loading with VEGF, which was radiolabelled. Loaded stents were perfused and the drug release kinetics measured. VEGF's potential to stimulate growth was assessed in endothelial cell culture. The VEGF-eluting stents were placed in rabbit iliac arteries and their effects on flow through the artery and acute platelet deposition were determined. Other animals were used to show longer-term effects on endothelial recovery and stent thrombosis (at 7 days) and intimal hyperplasia (at 28 days).

Results: 21.7μg of VEGF was absorbed. This was released with a bi-exponential release curve with 20% remaining at 9 days. In arterial tissue, 11% of the VEGF was detectable in the tissue at 24hr. VEGF-eluting stents stimulated endothelial cell growth by 11% over 5 days, with effects that were sustained beyond the initial rapid VEGF release. The animal studies showed a trend (p=0.07) towards reduced platelet deposition early after PCI, with reduced thrombus formation at 7 days (0mg in VEGF stents *vs.* 12.5mg in controls). No benefit of VEGF stents was seen on the re-endothelialisation process or on intimal hyperplasia.

Conclusions: VEGF can be delivered by polymer-coated stents. Prolonged drug release to the injured vessel wall can be shown. However, VEGF did not fulfil the potential suggested by previous work and by the cell culture experiments when tested *in vivo*. It did appear to reduce thrombus formation and so may have potential benefits in clinical practice as a stent-based therapy.

Table of Contents

Chapter 1 Introduction to the use of VEGF-eluting stents.

Chapter 2. Absorption and elution studies of VEGF

Chapter 3. VEGF-eluting stents - assessment in cell cultures.

Chapter 4. VEGF-eluting stents - *In vivo* assessment.

Chapter 5. Discussion and Conclusions.

Appendices

Table of figures

Chapter 1.

Introduction to the use of VEGF-eluting stents.

1.1 Thesis introduction overview.

Coronary artery disease is commonly treated by interventional means, often meaning the use of a coronary stent to maximally open a stenosed, atherosclerotic vessel. After four to six months, a proportion of patients undergoing stenting suffer from restenosis. This is the re-narrowing of the artery, often with important adverse clinical consequences.

This thesis examines the use of polymer-coated coronary stents as a means of delivering a drug locally to the site of the intervention. The agent selected, vascular endothelial growth factor (VEGF), has been shown to reduce restenosis and thrombosis in animal models when delivered by other means.

In this introduction the clinical importance of restenosis is discussed. The causes and mechanisms underlying restenosis are explained. The use of stents and modification to their design is considered. The use of stents as a local delivery device is compared to other existing local drug delivery methods. The properties and clinical uses of VEGF are reviewed. Finally, the main target of VEGF, the endothelium, is considered in relation to stent restenosis.

1.2 Introduction – Restenosis and coronary heart disease.

Ischaemic Heart Disease is the greatest single contributor to death in the industrialised world, including the United Kingdom. These deaths are often at a relatively early age, accounting for about a third of all deaths in men and one fifth of all deaths in women aged under 65 years in the UK (*Our Healthier Nation, 1998*). In Scotland, 1 in 1,000 men under 65 will die every year of the disease[1]. 11,914 Scottish people died of IHD in 2001. As well as causing a high mortality, IHD places a heavy burden of morbidity on the country. 20% of the Scottish population aged between 65 and 74 report symptomatic angina[1]. The prevalence of IHD continues to rise as the population ages. Angina resulting from IHD leads to a large number of hospital admissions. Over 10% of acute hospital admissions are for chest pain[1]. Chronic illness leads to early retirement from the workplace.

Angina is caused by atherosclerosis in the coronary blood vessels. Atherosclerosis is a progressive disease thought to be provoked initially by damage to the vascular endothelium, e.g. from oxidised LDL, hypertension or smoking. This is the "response to injury hypothesis". The response of the vessel is complex but essentially involves a thickening of the wall with cells and extracellular material. This can encroach into the vessel lumen, often to the extent where it causes significant haemodynamic compromise. Poor bloodflow distal to this point may cause relative ischaemia to part of the myocardium, which manifests as angina.

Although coronary artery bypass surgery is suitable in many cases as a treatment that will overcome this ischaemia, it is an invasive procedure with significant morbidity and mortality. In 1979 Gruentzig *et al* reported the first use of percutaneous transluminal coronary angioplasty (PTCA) as a minimally invasive alternative to bypass surgery. 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. 23,700 PTCA procedures were performed in 2000/2001 in the NHS in England, 3370 in 2001/2002 in Scotland (*NHS Hospital Episode Statistics, Information and statistics division, NHS Scotland.*). Although initially seen as an extremely successful treatment, PTCA has a high complication rate. In the RITA 2 trial, which compared angioplasty with medical treatment alone, more patients needed bypass surgery in the PTCA group, often as an acute complication of the procedure. At 2.7-year average follow-up a higher incidence of death/non-fatal infarction was found[2]. The problem with analysing these sorts of data is that

the pace of change in interventional cardiology is fast. The techniques, equipment and adjunctive therapies used now are very different to those in RITA-2 or similar trials and the presumption is that PTCA is becoming safer, especially with growing operator experience. Despite its drawbacks, PTCA remains at the forefront of treatments for all but the mildest cases of angina. This is because it compares favourably with the current alternative, bypass grafting. Both techniques have similar complication rates, but PTCA is less invasive, is cheaper and can be more easily repeated.

1.2.1 Intra-coronary stents

This development in interventional therapy forms much of the core of this research and stent developments and modifications are discussed later (*section 1.5*).

1.3 Restenosis.

Restenosis is the process of re-narrowing of a vessel after initially successful dilation with an interventional procedure, typically angioplasty.

1.3.1 Clinical importance of restenosis.

Despite the advent of intra-coronary stenting, restenosis remains the most common complication following coronary intervention. Approximately a third of patients undergoing PTCA will show angiographic evidence of restenosis. There is some difficulty in reaching an exact estimate, since angiographic follow-up in clinical trials is incomplete (*fig.1*). Furthermore, the definition of angiographic restenosis is varaible. The calculation of restenosis is derived from the relationship between the luminal diameter and the dimater of the refernece vessel. Unfortunately, the reference vessel can be measured before, within or after the restenotic lesion, which will change the resulting %restenosis. The usual binary cutoff is a ≥50% narrowing. The incidence of angiographic restenosis in several of the major trials of PTCA and stenting is shown below (*fig. 1.1*).

Study Name/ Authors	Year	Variables studied	Angiographic Follow-up (%)	Angiographic in-stent restenosis rate (%)	Vessel occlusion/ thrombosis rate (%)
BENESTENT[3]	*1994*	≥3mm vessels	93	22	1
STRESS[4]	*1994*	≥3mm vessels	88	31.6	1.5
Kastrati *et al*[5]	*1997*	DM, multiple or small stents.	80	16-59	NA
STRESS[6] substudy	*1998*	2.7mm±0.2 vessels	88	34	3.6
BENESTENT II[7]	*1996*	≥3mm vessels	92	13	0
Van Belle *et al*[8]	*1997*	DM	NA	27	NA
		Non-DM		25	NA
Lau *et al.*[9]	*1997*	≥2.5mm vessels	NA	33	0
		≤2.5mm vessels		47	0

Figure 1.1. The extent of restenosis in various stent trials. Note that in trials dealing with non – 'Benestent' lesions, stent restenosis rates may be considerably higher (DM=diabetics. NA=not assessed or not available).

More than 500,000 angioplasties are performed annually in the US, a quarter of them are for patients with restenosis following previous PTCA or stenting. This is estimated to cost around $3 billion in the US alone[10]. Of course these re-do procedures are not without risk to the patients that have them. An estimate of approximately 1-2% mortality or other major adverse event is commonly accepted. These figures are likely to rise year upon year as the use of PTCA has steadily grown in Europe and the US.

However the processes that underlie this process are complex. Angiographic measures of restenosis, the most common means to quantify the problem, differ greatly in clinical trials to the clinical results. The variable correlation between angiographic and clinical results in a few trials is shown below (*fig.1.2*):

Trial	Difference between treatment and control of final minimal luminal diameter (mm)	Clinical event rate	
		Control %	Treatment %
CAVEAT-I[11]	0.11	30	28 (p=NS)
STRESS[4]	0.20	27	22 (p>0.05)
BENESTENT[3]	0.12	33	23 (p<0.05)
Angiopeptin[12]	0.01	30	22 (p<0.05)

Figure 1.2: Differences in angiographic and clinical outcomes of restenosis trials (Adapted from Topol *et al* [13]).

Angiography is, by virtue of its worldwide familiarity and daily use, the present standard by which interventions to alter restenosis are judged. However, this does not necessarily mean it is a gold standard giving accurate assessments of restenosis. It has a poor correlation with clinical outcome, due to technical failings of a system that gives two-dimensional representations of the lumen of arteries affected by atherosclerosis. It has difficulty with distinguishing features smaller than about 0.2mm with even the best resolution. Intravascular ultrasound overcomes many of these failings and may well be much closer to giving a true representation of the extent or otherwise of restenosis, but is not commonly performed. Interestingly, the correlation between these techniques is as low as 0.30[13].

1.3.2 Factors affecting restenosis rates.

1.3.2.1 Patient characteristics

Angiographic restenosis post-angioplasty occurs approximately 1.3 times more commonly in patients with diabetes[14] although this effect has not always been seen in stented patients[8]. 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[15].

1.3.2.2 Angiographic variables.

Angioplasty of the left anterior descending artery has been shown in one study to have a tendency to restenose more frequently than other angioplastied arteries[14]. This may be because of anatomical differences in the vessel e.g. its course in the interventricular septum. It is also suggested that the large territory at risk if the LAD is lost may make interventionists slightly less aggressive in their attempts to open fully a stenosis at this location. Restenosis would thereby be made more likely because of an initially sub-optimal result[14]. Restenosis is also more common in patients who are stented for chronic or subtotal occlusions and in stenosed or obstructed vein grafts[16; 17]. Longer initial lesions tend to occur in more diseased arteries. The revascularisation process is more difficult and restenosis appears to be more common[16]. Multiple interventions, either PTCA or stenting, in a single procedure are also associated with a higher restenosis rate[18], although this does not correlate exactly with clinical recurrence of symptoms. As seen above (*fig.1.1*), the size of the target vessel is closely related to the incidence of restenosis in both angioplasty and stenting, as is the diameter of the residual stenosis following angioplasty. This latter variable, the mean luminal diameter (MLD), is the strongest single predictor of restenosis.
Stent and balloon design is thought by many to be influential in the incidence of restenosis. This is discussed elsewhere (section 1.5).

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.

1.3.3 Cellular mechanisms – the pathophysiology of restenosis.

Coronary stenoses are due to atherosclerotic plaques, complex structures that contain both cellular and acellular components. Angioplasty means the application of force to this structure and this injury to the vascular wall and plaque is the stimulus to restenosis. This process begins early after injury, since the natural healing process will also lead to restenosis if it continues in a poorly controlled fashion. This is part of the "response to injury" theory. Angioplasty relieves the initial narrowing of a blood vessel by forcibly fissuring or dissecting the fibrous cap over the atherosclerotic plaque. Most of these dissections are not of clinical significance[19]. 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. 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 hyperplasia seen at one month[20].
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. Remodelling of the vessel
3. Intimal hyperplasia
4. Thrombus formation

1.3.3.1 Recoil

Angioplasty relieves the obstruction partly by stretching the healthier, relatively normal parts of the vessel wall. After angioplasty, much of the apparent gain in luminal diameter is lost as the stretched vessel recoils. 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%[21].

1.3.3.2 Remodelling

Remodelling is a term orginally applied to changes in vessel size caused by atherosclerosis. In some cases, a diseased artery will slowly increase its overall external diameter. This partially compensates for the reduction in luminal diameter due to the atherosclerotic plaque.

Unfortunately, following angioplasty the opposite process often occurs. This negative remodelling 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. Of patients with restenosis, about 75% of the reduction in lumen was due to remodelling the rest attributed to growth of plaque or to intimal hyperplasia[22].
It is believed that stent insertion also prevents this shrinkage of the vessel by negative remodelling.

1.3.3.3 Intimal hyperplasia

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

1.3.3.3.1 The vascular smooth muscle cell

The SMCs in restenotic lesions are thought to originate in the media or adventitia of the vessel, particularly in stented vessels[23]. Normally these cells are of a contractile nature, have a low rate of replication and do not tend to secrete large amounts of cytokines. Following injury to the vessel wall, the phenotype of these cells changes to a more proliferative or secretory one. 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. 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 include macrophages, fibroblasts and capillary endothelial or lymphatic cells. All of these cells and SMCs may differentiate to form the "restenosis cell"[24].

1.3.3.3.2 The extracellular matrix and matrix metalloproteinases (MMPs).

SMCs in the intima of the restenotic lesion may 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 SMCs synthesise and release MMPs to facilitate their own migration[25]. Up-

regulation of MMPs has been identified following angioplasty injury to a vessel within 24 hours[26].

Intimal hyperplasia, as well as depending on the presence of active SMCs, 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[27]. The turnover of this collagen is regulated by the activity of MMPs that both degrade and promote the synthesis of collagen. Strauss *et al* showed reduced collagen in restenotic lesions by inhibition of MMPs in the rabbit iliac model[28]. Adenovirus-mediated transfection of the gene for TIMP-1 (an inhibitor of MMPs) resulted in a reduction in neointimal hyperplasia in a saphenous vein organ culture model[29].

1.3.3.3.3 The endothelium

In a manner analogous to the different forms of SMCs, endothelial cells also appear to have two phenotypes – "normal" or "dysfunctional"[24]. The effect of the endothelium on the underlying tissues has been much studied, including when processes such as angioplasty cause endothelial damage or denudation. Endothelial removal had been thought to be the sole requirement for the stimulation of intimal hyperplasia. In work using the rat aorta however, careful experiments showed that gentle denudation of the endothelium did not lead to intimal thickening. There was evidence of SMC proliferation nevertheless[30]. 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[31]. 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[32]. 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 remodelling that contributes to restenosis[33]. 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[34]. To some extent this may be because the cells that regenerate are dysfunctional.

Dysfunctional endothelium may contribute to intimal hyperplasia. Endothelial cells are capable of producing growth factors themselves[24] 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[35]. "Normal" ECs produce heparan sulphate and NO[36]. Cell culture studies of endothelial cells have shown that cerivastatin, an HMG CoA reductase inhibitor, increases endothelial cell NO production and inhibits smooth muscle cell growth[37].

1.3.3.3.4 The macrophage/inflammation

Cells of the monocyte/macrophage line are found in atherosclerotic plaques as foam cells. Inflammation, involving such cells, is found within 24 hours of angioplasty in rats[38] and there is a correlation between hyperplasia and macrophage infiltration in the rat carotid[38]. Post-mortem studies of patients with restenotic lesions post-stenting also show abundant macrophages at the restenosis site[39]. 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[40].

1.3.3.3.5 The adventitia/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[41].

1.3.3.4 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. 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. The exposed underlying tissues contain molecules, including von Willebrand Factor and collagen, that act as ligands for the

receptors on platelets that allow them to adhere to surfaces. Blockade of platelet adherence with recombinant leech antiplatelet protein has been shown, in the rat carotid model, to reduce intimal hyperplasia[42].

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.

Activated platelets produce many bioactive molecules including serotonin, ADP, basic fibroblast growth factor, TGF-β or PDGF and thromboxane A_2. All of these products are known to be mitogenic[43].

1.3.3.5 Apoptosis in the restenotic process.

Apoptosis, or programmed cell death, is a particular form of cell death. Unlike necrotic cell death, cells which undergo apoptosis do not usually excite an inflammatory reaction[44]. Malik *et al* have shown that, in a porcine coronary angioplasty model, apoptosis and cell proliferation are both stimulated in comparison to quiescent, undamaged vessel[45]. Apoptosis affected all cellular layers of the artery, but the time course amongst different layers varied. In all, however, apoptosis occurred before cell proliferation. Atherectomy specimens taken from patients with atherosclerosis and, particularly, with restenosis, have shown immunological evidence of apoptosis[46]. It seems reasonable, therefore, to see the restenotic process as a combination of cell proliferation and cell death, mediated by apoptosis. In concept, being able to tilt the balance in favour of apoptosis for proliferating smooth muscle cells, and against it for endothelium, might be beneficial both in influencing the course of atheroma and in controlling post angioplasty restenosis. Steg *et al,* using the atherosclerotic rabbit model, locally delivered gene therapy using an adenoviral vector. Systemic gancyclovir was given. The gene synthesised an enzyme that activated gancyclovir to a toxic metabolite. This caused the cells transfected with the gene to undergo apoptotic cell death. Restenosis was reduced at the target site[47].

1.3.3.6 Allergy as a contributor to in-stent restenosis

An interesting small study has investigated the hypothesis that differences between the allergic responses to the metals in coronary stents may determine which patients develop

restenosis. In a retrospective study, patch testing for nickel allergy was strongly correlated to restenosis. All patients with nickel sensitivity (9/131) developed restenosis angiographically at six months[48].

1.4 Models of restenosis.

Various models have been developed to investigate the problem of restenosis. These all have strengths and weaknesses of their own. The transfer of results in animals of various treatments to clinical trials has often been disappointing. Despite their shortcomings it is accepted that animal studies are the gold standard in testing the safety of agents of proposed use in clinical trials. It may be the animal models themselves that gave the unreliable results and 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[49]. All animal models fall short of modeling 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.

1.4.1 Animal models of stent restenosis.

1.4.1.1 Canine coronary artery.

Folts *et al*[50] describe a model using coronary vessels. It involves the placing of constricting rings around the dogs' coronaries and has been used to study thrombus 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[51]. 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. Third, on a practical level, use of dogs for research purposes is increasingly difficult in this country because of public antipathy and correspondingly tight regulatory controls.

1.4.1.2 Rabbit iliac model.

A rabbit iliac model was described by More *et al*[52]. Balloon angioplasty injury was produced in the common iliac artery via the superficial femoral artery. This produced a deep arterial

injury. 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 was seen with gradual re-endothelialisation over 14 days. This model was relatively simple to perform and rabbits are easily kept. Many other groups have used similar rabbit models with or without hypercholesterolaemic diets[40; 53-57].

1.4.1.3 Rat carotid artery model.

This 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. remodelling, so functional stenosis does not occur. Significant thrombosis does not occur in this model[58].

1.4.1.4 Porcine coronary vessels

Coronary vessels differ from other vessels. They fill in diastole; they are not elastic arteries like carotid or iliac arteries; in pigs, functionally important restenosis occurs, which is similar to that seen in humans[59]. 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[12; 60]. Conversely, porcine models of the use of sirolimus to reduce restenosis significantly underestimated the efficacy in human trials[61; 62].

1.4.2 Power calculations in clinical trials of restenosis.

Unless adequate numbers of similar patients are recruited a trial may be underpowered and fail to demonstrate small but significant reductions in restenosis rates with a certain agent i.e. type II error. When 57 restenosis trials were examined for this fault, only five were powered sufficiently to be 80% confident that there was no type II error[63]. These trials all reported negative results. It is possible that useful agents with clinical benefit have already been found and erroneously discarded.

1.4.3 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 systemic agents used and their effects in various models of intimal hyperplasia (*fig. 1.3*).

Agent	Model used	Result
Antibiotics		
Rapamycin[64]	*in vitro*	Inhibited growth
Lipid lowering agents		
Fluvastatin – FLARE study[65]	Human	No benefit
Bezafibrate[66]	Human	Reduced neointimal hyperplasia
NO donors		
Linsidomin/misoldomine – ACCORD trial[67]	Human	No clinical benefit
Cytokines and hormones		
Il-10[40]	Rabbit	Inhibited stent restenosis.
Prostaglandin E1[68]	Human	Reduced neointimal hyperplasia
Prostacyclin[69]	Human	No benefit
Oestradiol[70]	Rabbit	Reduced neointimal hyperplasia
Activin[71]	Mice	Reduced neointimal hyperplasia
Angiopeptin[72]	Pig	Reduced neointimal hyperplasia
Angiotensin II/ACE inhibitors		
Losartan[73]	Pig	Reduced neointimal hyperplasia
Cilazopril[74]	Human	No benefit
Calcium antagonists		
Verapamil[75]	Human	Reduced neointimal hyperplasia (Peripheral vessels)
Amlodipine-CAPARES trial[76]	Human	No reduction in restenosis
Y-27632 (Rho-Kinase inhibitor)[77]	Rat	Reduced neointimal hyperplasia
Antiallergens		
Tranilast – TREAT study[78]	Human	Reduced neointimal hyperplasia
Permirolast[79]	Human	Reduced neointimal hyperplasia
Tyrosine kinase inhibitors		
PP1/AGL1872[80]	*In vitro*	Reduced neointimal hyperplasia
Anticoagulants		
Enoxaparin – ERA trial[81]	Human	No benefit
Anti-thrombin III /heparin[82]	Pig	Borderline benefit
Clivarine/PEG hirudin[83]	Pig	Reduced neointimal hyperplasia
Heparin[84]	Human	No benefit
Hirudin – HELVETICA trial[85]	Human	No benefit
Warfarin[86]	Human	Mixed results
Antiplatelets, anti-PDGF & TXA2 antagonists		
Trapidil /STARC study[85; 87]	Human	No benefit/Reduced stenosis
Cilostazol[88]	Human	Reduced neointimal hyperplasia compared to aspirin
Sulotraban[89]	Human	No benefit
GR32191B – CARPORT study[90]	Human	No benefit
CV-4151[90]	Human	No benefit
Abciximab – ERASER trial[91]	Human	No benefit
Metalloproteinase inhibitors		
Batimastat[92]	Pig	Reduced neointimal hyperplasia

Non-specific		
Suramin[93]	Rabbit	Reduced neointimal hyperplasia
U-86983[58]	Rat	Reduced neointimal hyperplasia
Fatty Acids		
N-3 Fatty Acids – CART study[94]	Human	No benefit
Endothelin Antagonism		
ABT147627[95]	Pig	Reduced neointimal hyperplasia
Antioxidants		
Probucol-MVP trial[96]	Human	Reduced neointimal hyperplasia
IRFI 042[97]	Rat	Reduced neointimal hyperplasia
Inhibitors of DNA synthesis		
Mithramycin[98]	Rat	Reduced neointimal hyperplasia
Others		
Octreotide[99]	Human	No benefit
Carvedilol[100]	Human	No benefit in atherectomy patients
Colchicine[101]	Human	No benefit
Methylprednisolone	Human	No benefit
ATF.BPTI[102]	Rat	Reduced neointimal hyperplasia

Figure 1.3. Table of systemic agents used for restenosis

1.5 Local drug delivery for restenosis

In the search for suitable agents to prevent the problem of restenosis post-angioplasty, a number of promising animal trials have led to disappointing results in humans. Clinical trials of cilazipril, enoxaparin, angiopeptin and trapidil - all promising in animal studies - had negative results[12; 74; 103; 104]. It is suggested that part of the reason that these and other agents have failed to translate to human studies is that they used relatively low doses of the drug under trial compared to the animal work. This is usually because of concerns about the likely side effects of high doses systemically. In the cilazipril trials the dose given had a high incidence of postural hypotension, despite the fact that the dose was roughly 70 times lower per kilogram than the doses used in animal work. Thus it may be that the ideal agent(s) for the prevention of restenosis has already been found but that at systemic doses its side-effect profile in humans makes it useless.

It seems reasonable to find a way to minimise the dose of the drug given, yet retain its efficacy at the point where it is needed; the angioplasty site. This is the rationale behind the concept of local drug delivery (LDD). By finding a way to deliver small doses of the drug to where it is needed, the drug could achieve very high local concentration. Even if a drug is normally inactivated *in vivo* by, for example, first pass metabolism in the liver, it will remain an effective local treatment as it bypasses this biological inactivation. Less drug is required, saving money. One approach has been to combine the angioplasty procedure with a LDD technique, using modified angioplasty balloons:

1.5.1 Delivery balloons

The double-balloon catheter has a balloon at the tip of the catheter and one several centimetres more proximally. In between there are holes through which drug solution can be expressed, after both balloons have been inflated to prevent blood flow. Long periods of instillation with vessel occlusion are required[103], which precludes their use in coronary arteries. The small holes of local delivery balloons cause the fluid instilled to form fluid jets. which cause local trauma to the vessel itself[103]. As little as 1%[105] of the drug instilled remains in the vessel wall, the remainder is released into the bloodstream, further downstream in the coronary circulation and even systemically. 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[106].

Several modifications of the basic balloon design have been made. These include the Transport, Wolinsky, Despatch, Channel and hydrogel balloons. 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. Neointimal growth was reduced. The authors note exacerbated intimal hyperplasia in the control group, reflecting the trauma caused by the local instillation of fluid[107]. In a small (ten patient) study, locally delivered urokinase was very succesful in lysing intra-coronary thrombus following complicated angioplasty. The authors note that only 0.4% of the urokinase was delivered to the vessel wall in their animal model[108]. These balloon catheters have similar weaknesses. They rely on the instillation of large volumes of fluid compared to the volume of space available in the wall, causing trauma. Nevertheless, an enormous number of agents have been used in various animal models to test local drug delivery by one or other balloon type (*fig. 1.4*). Various adaptations have been made to the basic balloon principle to try and improve on either delivery or retention of drugs. These include adenoviruses containing DNA coding for the desired agent, microparticle suspensions, liposomes and targeted drug therapy. These are briefly discussed.

1.5.1.1 Adenoviruses.

Although DNA can be transfected into cells using various vectors, adenoviruses tend to prove the most efficient method[109]. They carry the risk of severe hypersensitivity reactions being provoked by their use and of transfecting cells accidentally with rogue viral DNA. Adenoviruses have been used to deliver endothelial nitric oxide synthase in stented porcine arteries, with equivocal results. Angiography failed to show a benefit, despite reduced intimal hyperplasia. GAX (growth-arrest specific homeobox) transcription factor has been shown to be an inhibitor of SMC proliferation and furthermore stimulates SMC apoptosis. Adenoviral transfection delivered by channel balloon to overexpress GAX in stented porcine arteries produced encouraging reductions in instent restenosis[110].

1.5.1.2 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 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[111]. These biodegradable microparticles can carry drugs and keep them in the area where they are needed.

1.5.1.3 Liposomes

Liposomes containing agents felt to be beneficial in limiting the restenosis process have been infused into the angioplastied vessel through a delivery balloon. Using liposomes as a vehicle, oestradiol reduced neointimal hyperplasia in a rat carotid model[112].

1.5.1.4 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. Hogrefe *et al* showed that a bi-specific antibody fragment could be produced combining the binding sites for the Gp IIb/IIIa receptor and for tissue factor. This bi-specific 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[113].

1.5.2 Polymeric Endoluminal Gel Paving

Slepian *et al* approached local drug delivery using a different concept. They have experimental polymers that can be moulded onto the interior surface of an angioplastied vessel. The polymers acted as a barrier between the exposed intima and the bloodstream, as wall supports and as depots for LDD. Initially, thermoplastic polymers were used to reinforce the wall of the artery. Their work also tested hydrogels. These are not suitable for supporting the vessel wall but will act as a barrier and as a vehicle for drug delivery. They delivered the gel to the vessel and moulded it onto the surface by heating the gel with the catheter. The gels biodegraded over weeks and released drug slowly. These studies, in rabbit carotid models, showed favourable reductions in neointimal growth. In pigs, a similar hydrogel containing radiolabeled heparin was used. The technique proved technically successful and unlike some of the techniques described for LDD, no downstream delivery of drug was seen. However, after only four hours, only 3% of the heparin remained in the coronary vessel wall[114].

Agent	Model used	Result
Local Photodynamic therapy		
Photofrin[115].	Pig	Reduced neointimal hyperplasia
Anticoagulants and antiplatelets		
Enoxaparin[51]	Human	Reduced restenosis
Reviparin[116]	Rabbit	No benefit
Urokinase[117]	Pig	No benefit
Heparin[118]	Human	No benefit
Dipyridamole[119]	Rabbit	Reduced neointimal hyperplasia
Argatroban[120]	Rabbit	Reduced neointimal hyperplasia
Antisense oligonucleotides		
To MAPK[121]	Pig	Reduced neointimal hyperplasia
To c-myb[107]	Pig	Reduced neointimal hyperplasia
To c-myc[122; 123]	Rabbit/Human	Reduced neointimal hyperplasia/No benefit
Anti-proliferatives		
Doxorubicin[124]	Rabbit	No benefit
Mitomycin C[124]	Rabbit	No benefit
Methotrexate[125]	Pig	No benefit
Paclitaxel[126]	Rabbit	Reduced neointimal hyperplasia
Green tea catechins[127]	Rat	Reduced neointimal hyperplasia
Hormones and cytokines		
Lipoxygenase inhibition[128]	Rat	Reduced neointimal hyperplasia
Oestradiol [112]	Rat	Reduced neointimal hyperplasia
VEGF[129]/VEGF [130]	Rat/ Human	Successful /no benefit (not stented)
Hepatocyte Growth Factor[131]	Rabbit	Reduced neointimal hyperplasia
Angiopeptin[132]	Rabbit	Reduced neointimal hyperplasia
I-kappa-B[133]	Rabbit	Reduced re-occlusion
Proteasome/Tyrosine/Protein Kinase inhibitors		
ST 638[134]	Pig	Reduced stenosis arteries. Given drug from outside
MG132[135]	Rat	Reduced neointimal hyperplasia
Antioxidants		
HA1077[124]	Rat	Reduced neointimal hyperplasia
Gene therapy		
Adenovirus/Retrovirus		
GAX[110].	Pig	Reduced neointimal hyperplasia
Antisense cyclin G1[136]	Rat	Reduced neointimal hyperplasia
Rb[137]	Rat	Reduced neointimal hyperplasia
Tk[124]	Rabbit	Reduced neointimal hyperplasia
Protein Kinase G[138]	Rat	Reduced neointimal hyperplasia
Plasmid		
VEGF[57]	Rabbit	Reduced neointimal hyperplasia
Others		
Suramin[139]	Mouse	Reduced neointimal hyperplasia
Chimeric ribozymes[140]	Pig	Reduced neointimal hyperplasia
Ethanol[141]	Pig	Reduced neointimal hyperplasia

Dexamethasone[124]	Rabbit	Reduced neointimal hyperplasia
Apolipoprotein A-I_{MILANO}[142]	Rabbit	Reduced neointimal hyperplasia
AtRA[143]	Rabbit	Reduced neointimal hyperplasia
Thrombospondin blockade[144]	Rat	Reduced neointimal hyperplasia

Figure 1.4 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.

1.5.3 Local Photodynamic therapy in stented arteries

Photodynamic therapy (PDT) involves the systemic administration of a light-excitable photosensitiser that is taken up preferentially by rapidly proliferating cells. During laser irradiation, light energy is transferred from the photosensitiser to oxygen generating the highly reactive oxygen radical. This causes severe cellular damage to the proliferating tissue. Several different photosensitisers, for example photofrin[115], have been used with success in reducing restenosis in both animal and human models.

1.5.4 Stents as local drug delivery devices.

Since stenting and local drug delivery by stent is the main focus of this MD thesis, stents and their variations are now discussed in depth.

1.6 Stenting and stent modifications

Since coronary angioplasty was introduced by Gruentzig[145] in the late 1970s, it has been apparent that although successful at relieving the symptoms of angina, it has some complications. Intra-coronary stents are supportive devices that are placed inside the lumen of a vessel to prevent collapse or elastic recoil of a dilated arterial segment after angioplasty. Intra-coronary stents were first used in humans in the mid 1980s by Sigwart *et al* who used them, initially, for bailout after an intimal dissection had been caused by balloon angioplasty[146].

1.6.1 In-stent restenosis

Two major trials have established the effectiveness of intra-coronary stents as a means of reducing restenosis, at least in patients with low-risk, so-called "BENESTENT" type lesions[3; 4]. Despite these encouraging findings, there remains a considerable minority of patients who suffer a major adverse cardiac event (MACE) or require subsequent target lesion revascularisation. Studies suggest that restenosis occurs in up to 15-20% of cases. This figure is higher in patients with diabetes, multiple stents and in stented, small, non-BENESTENT vessels where restenosis rates approaching 45% have been documented (*see fig 1.1*). Restenosis in stented vessels has proven difficult to treat. Current therapies include the use of irradiation (brachytherapy), transluminal rotablation and atherectomy (cutting balloon), to increase lumen diameter.

1.6.2 In stent thrombosis

Stents, which do appear to reduce the incidence of restenosis, may also have adverse effects. In-stent thrombosis, although rare in BENESTENT lesions, has been reported in 2-3% in small vessels[147]. Some of the incidence of instent subacute thrombosis can be attributed to suboptimal stent deployment. Colombo *et al* demonstrated, using intravascular ultrasound, that even when good angiographic deployment was seen, there was often poor apposition of parts of the stent to the vessel wall. They showed that the use of antiplatelet rather than anticoagulant treatment was effective in reducing thrombosis rates if the stent was fully deployed to begin with[148]. This often meant repeated, high-pressure balloon inflations, although more recent stents are more deformable, improving apposition.

Thrombosis is currently reduced also by the prescription of antiplatelet therapies including aspirin and ADP receptor antagonists like ticlopidine or clopidogrel. In addition to their natural thrombogenicity, stents appear also to cause other problems. One study in a porcine model has found that stents adversely affect endothelial function as assessed by vascular permeability and cell morphology, compared to angioplasty alone[35].
It is postulated that both the thrombogenic potential and the stimulus to intimal hyperplasia from a stent are secondary to the defect in the endothelium at the stent site. Until the vascular endothelium fully regenerates therefore, the risks of these complications remain present[149].

1.6.3 Modifications to intravascular stents.

The growing recognition that stents were not without complications led to the search for modifications to the original concept that might reduce the incidence of problems. The physical properties of stents have been examined to determine which of these is of importance in the generation of complications of either thrombosis or restenosis. Stents are generally stainless steel, tantalum or nitinol (a nickel-titanium alloy). There do not seem to be great differences in the complication rates amongst these different metals[150]. They have different structural designs although typically can be classed as either a self-expanding stent (e.g. Wallstent) or balloon-expandable stents, which are further divided into coiled wire (e.g. Wiktor) type or a slotted tube (e.g. Palmaz-Schatz). In some animal models there are suggestions that the greater the degree of coverage of the vessel wall by the stent – or the greater the number of struts it has – the less restenosis is seen[151]. In contrast, another group suggest that closed stents, i.e. those without gaps between struts, have a worse restenosis rate. In other words, they show that the less metal in contact with the vessel, the better[152]. Furthermore, the stents without gaps also, unsurprisingly, had an increased rate of side-branch occlusion[153]. Other factors including stent geometry and the uniformity of tension within a stent have also had claims made as to the importance they have on the incidence of restenosis[154]. Confusing the issue also is the extent to which qualities of the balloon used may affect vessel wall injury and restenosis. The compliance of the balloon and the extent to which it is inflated do seem to have some impact on these events[155].

There is no clear picture as to whether there are important differences in clinical terms amongst the many different stents on the market. It is difficult to demonstrate any difference between stents because of the number of similar patients needed to power a study to show a small difference in outcome. In ostial LAD stenoses, one very small study (55 patients) suggested an advantage of the slotted tube style over coil stents. However, only 80-85% of

patients in the trial returned for six month angiography[156]. It does not seem reasonable to base major decisions as to stent selection on trials as small and as selective as this.

1.6.3.1 Stent surface characteristics

The roughness of the stent surface may determine the extent of early thrombus deposition. Irregularities as small as 1μm have shown increased thrombogenic potential in a study of stainless steel rings implanted into the inferior vena cava of experimental dogs[157]. In this same work, the impact of surface charge was also assessed. Negatively charged surfaces might repel the predominantly negatively charged proteins that form blood clots and so be antithrombogenic. However, no such relationship was seen in this study. 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 (*fig. 1.5*).

1.6.3.2 Coated stents

Attempts have been made to coat the surface of stents with different materials that make them more biocompatible.

1.6.3.2.1 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[158]. A randomised control trial of gold-plated intra-coronary stents proved disappointing however, with a higher rate of complications than the control group[159]. 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. 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[160]. 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 stents[161].

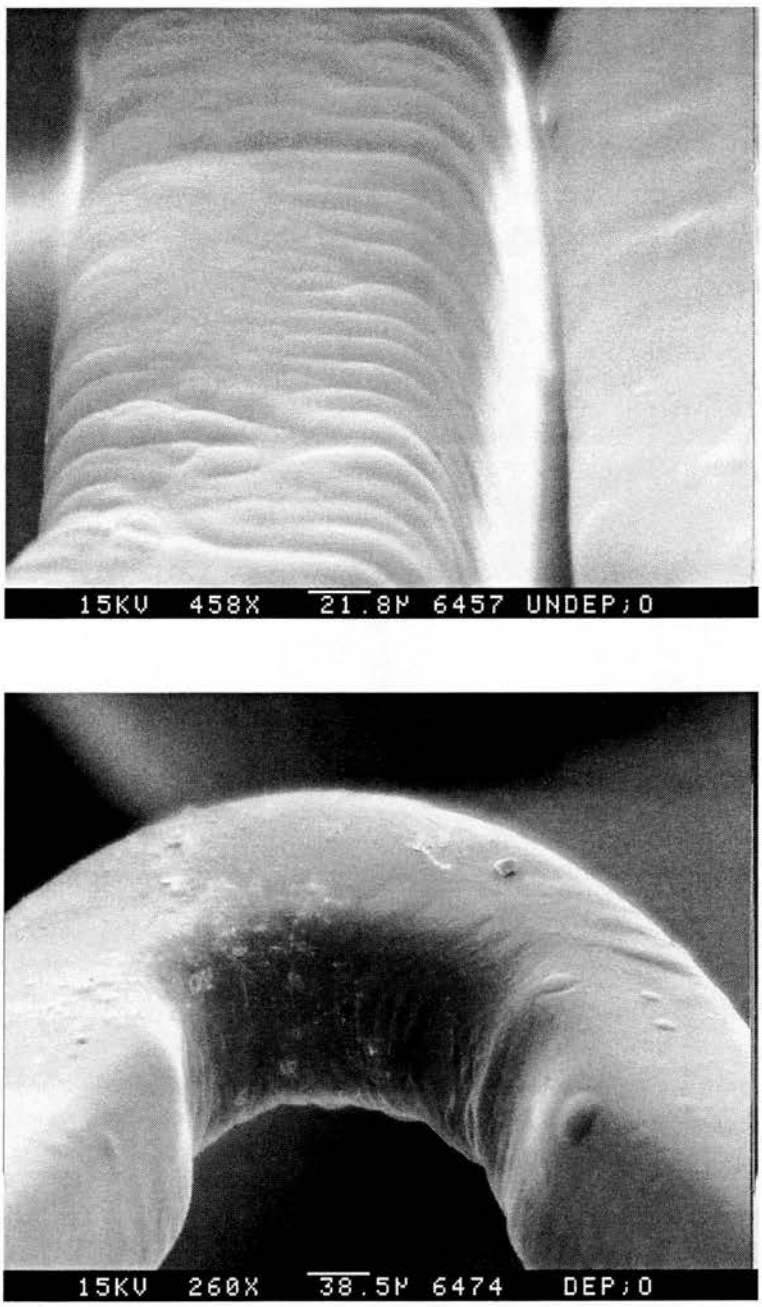


Figure 1.5. Scanning electron micrographs of the surface of the polymer-coated Supra G stent. This shows the surface irregularities present on this type of stent. The white line at the foot of each picture indicates the scale. Irregularities larger than 1μm have been shown to be thrombogenic in vivo.

1.6.3.2.2 Covered stents.

Stents covered with an artificial membrane have been promoted as they hold the potential to reduce restenosis simply by acting as a barrier to ingrowth of SMCs. The Jomed, which is covered with PTFE, has been used for patients with aneurysms or perforations of the coronaries. No controlled trial of their use exists. Animal studies of covered stents have shown that they inhibit the recovery of the endothelium, not surprisingly, compared to bare metal stents[162]. 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[163].

1.6.3.2.3 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. Stefanadis *et al*[164; 165] have published 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.

1.6.3.2.4 Semiconductor-coated stents.

A semiconductor coating of Silicon Carbide has shown promise as an inhibitor of thrombus formation on stents in high-risk patients[166]. 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[167].

1.6.3.2.5 Diamond-like coating.

Diamond-like carbon films, including Dylyn™, have been tested for their biocompatibilty as a stent coating in a porcine model. In this study, two variations of the coating were tested against identical, uncoated stents. Both coated groups showed reduced thrombus formation at six weeks. Reduced neointima formation was noted, but did not reach statistical significance[168].

1.6.3.3 Radioactive 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. Human trials, with small numbers of patients, have shown promising results when used to treat *de novo*[169] or restenotic lesions[170]. Alternatively, stents themselves can be made radioactive. In humans, the IRIS trial of beta-emitting stents has been reported. Restenosis at six months was 31%[171]. 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[172]. Furthermore, several case reports have highlighted a phenomenon of late thrombosis in patients receiving VBT. Across all reported VBT trials, 9.1% of patients have suffered acute occlusion of the treated area, 40% of these with resultant myocardial infarction, as long as nine months post-treatment[173]. This concern has led to prolonged use of antiplatelet agents such as clopidogrel for up to a year post brachytherapy.

1.6.3.4 Bioabsorbable stents.

Concerns about the long-term effects of intra-coronary metal stents have been voiced[174]. 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 these concerns, some researchers have explored the use of temporary, biodegradable or bioresorbable stents. These stents would 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 1980s. Stents degraded over approximately 18 months. The Igaki-Tamai stent, made of Poly-l-Lactic Acid (PLLA), has been introduced into early clinical practice. At six months, a restenosis (and target vessel revascularisation) rate of 10.5% was seen in a non-randomised series[175]. Ye and colleagues[176] have also reported the use of biodegradable stents in a rabbit carotid model as a delivery device for gene therapy. An adenovirus carrying a reporter gene was successfully transfected

into the vessel wall. Magnesium alloy stents have been developed that in an uncontrolled porcine study showed low thrombosis and restenosis rates[177]. Technical difficulties concerning the structural characteristics (e.g. radial strength), radio-opacity and delivery of biodegradable stents, have proven almost insurmountable. Furthermore, although metallic stents do have complications, these all tend to be early after implantation. Late complications like aneurysm formation or infection are not common. Therefore there is not a need to have stents with a short lifespan, merely that they should be biocompatible quickly.

1.6.3.5 Polymer coated stents.

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[178]. Fibrin deposition is an early stage in thrombus formation. *In vivo* assessment is essential, as the polymer may interact with the components of the blood or coagulation cascades. 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[179]. 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 hyperplasia[180]. This study is disappointing, although technical aspects of it that may have contributed to the negative results have been identified[181]. The layers of polymer used were not applied uniformly, which would stimulate more turbulent flow over the surface and encourage the deposition of activated platelets. Furthermore, the coating in each case was relatively thick and other workers have reported more favourable results with thinner coatings. Finally, stents used were not sterilised prior to implantation and therefore could have been contaminated with pyrogenic organisms.

Phosphorylcholine (PC) is a phospholipid that is naturally found as part of the cell membrane bilayer. PC-coated stents proved to be biologically neutral in rabbits and pigs, but showed no reduction in restenosis at six months[55]. Other polymer coatings have shown more promise, not just *in vitro* but *in vivo*, including in clinical trials. Canine studies of fluorine-acryl-styrene-urethane-silicone coated stents implanted in iliac veins did show both a reduction in thrombus formation and, at four weeks, reduced neointimal hyperplasia[182]. A

polyamine/dextran sulphate trilayer stent has been studied in porcine arteries and shown to have a reduced incidence of thrombosis when covalently bound to heparin[183]. Heparin-adsorbed polymer surfaces have long been known to reduce the adhesion of platelets[184]. This same heparin-bound polymer coated stent was used clinically in the BENESTENT II trial[7]. Although this study was not a randomised control trial, the results using this stent were at least as favourable as historical comparisons. The Cook GRII stent used a cellulose polymer coating. In a randomised, controlled trial against uncoated Palmaz-Schatz stents, poor results were obtained in the GR II group with higher incidences of both target vessel revascularisation and MACE[185]. 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 being marketed.

1.6.3.6 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. This synthetic thrombus was relatively inert and protected 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[186]. In addition, the fibrin coating was biodegradable and could be combined with drugs to act as a local delivery method.

1.6.4 Polymer-coated stents as drug delivery devices.

The development of polymer-coated stents has been driven not only by the concept that they might be intrinsically more biocompatible than bare metal, but also to 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[187]. 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[103].
- The 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[188].
Various groups have exploited this potential use for stents (*see fig.1.6*).

Drugs delivered using coated stents	Model	Reduction in thrombosis rate	Reduction in intimal hyperplasia
Heparins[183; 189]	Pig Rabbit	Y Y	N N
Methotrexate[125]	Pig	NA	N
Dexamethasone[190 191]	Dog Pig Pig	N N NA	Y N N
Methylprednisolone[192]	Pig	NA	Y
DNA[193]	Pig	NA	NA
Forskolin[194]	Rabbit	Y	NA
Argatroban[195]	Pig	Y	NA
Angiopeptin[117]	Pig	NA	N
NO donors[196]	Pig	Y	Y
Urokinase[170]	*In vitro*	Y	NA
Prostacyclin analogues[197]	*In vitro*	Y	NA
PEG-Hirudin[197]	*In vitro*	Y	NA
Activated Protein C[198]	Rabbit	Y	NA
VEGF plasmid[199]	Rabbit	NA	Y
ST638 (tyr. Kinase inhibitor)[200]	Pig	NA	Y
Paclitaxel[117] [201; 202]	Rat Rabbit Pig	NA NA NA	Y Y Y
QP-2[203]	Rabbit	NA	NA
Sirolimus[204]	Pig	NA	Y
GIIb/IIIa blockers[205; 206]	Rabbit Dog	Y Y	NA NA

Figure 1.6. Drugs used for local stent delivery in preclinical studies. (NA=Not Assessed).

1.6.4.1 Corticosteroids

Strecker *et al*[190] used stents coated in a biodegradable polymer membrane containing dexamethasone in a canine model of restenosis. With small numbers they were able to show reduced rates of restenosis at follow-up. 4 out of 14 dogs developed in-stent thrombosis. Similar work in pigs[191] showed a marked inflammatory response with the polymer coating used to deliver dexamethasone. No beneficial effect was seen on intimal hyperplasia in the treated group, despite high levels of the drug locally.
Methylprednisolone–eluting, polymer-coated stents were implanted in pigs and a reduced amount of stenosis recorded. 96% of the drug had eluted from the stent within 24 hours[192].

1.6.4.2 Sirolimus (Rapamycin)

Rapamycin, a macrocyclic lactone antibiotic, has shown immunosuppressive properties in animal studies, acting via inhibition of cell cycle-dependent kinases[64]. It has shown a reduction in intimal hyperplasia when delivered using a stent implanted in porcine coronaries[204].

1.6.4.3 Trapidil, valsartan, ibuprofen, methylprednisolone

Fluorinated polymethacrylate polymer coated stents (PFM-P75) were used in a series of porcine coronary experiments. All four of the above agents were impregnated into the polymer prior to it being spraycoated onto the stent[207]. Ibuprofen, trapidil and valsartan had no beneficial effect. Methylprednisolone did produce reduced neointimal hyperplasia, at the highest dose used.

1.6.4.4 DNA

Stents coated with a biodegradable volatile polymer emulsion mixed with DNA for a marker protein, have been implanted into porcine coronaries. A transfection rate of ≈1% was seen[193]. DNA oligonucleotides have also been loaded onto PC-coated stents *in vitro*[208].

1.6.4.5 Forskolin

Forskolin is an adenylate cyclase activator with vasodilatory and anti-platelet effects. In rabbit carotids[194], this drug was delivered using a novel, removable stent. This showed that the drug could be applied in high concentrations to the tissues adjacent to the stent, with biological effects measured by increases in bloodflow through the stented vessels. Prolonged time before the onset of cyclical flow variability was seen, suggesting less intraluminal thrombus formation[194]. The effects on restenosis were not assessed. 95% of the drug had eluted within 24hrs. The influence of the thermoplastic stent itself, requiring to be heated to 55°C, was not discussed.

1.6.4.6 Tyrosine Kinase inhibitors

Growth factors tend to exert their proliferative effects via tyrosine kinase receptors. ST638 causes blockade of these receptors and PLLA polymer stents coated with ST 638 have been implanted into porcine coronaries. Reduced intimal proliferation was seen at three weeks post-procedure[200].

1.6.4.7 Paclitaxel

Paclitaxel is a diterpenoid compound, which polymerises the tubulin components of a cell's microtubules into defective structures. Without normal microtubule function, cells cannot divide or migrate. Paclitaxel has been shown to inhibit smooth muscle cell proliferation and migration *in vitro* and *in vivo* after angioplasty in the rat[209]. Locally delivered paclitaxel in the rabbit carotid artery angioplasty model resulted in reduced neointima formation[210]. Heldman *et al* placed paclitaxel-eluting stents in minipigs. Stents with 90µg paclitaxel resulted in significant reduction in the angiographic lumen loss index and histological neointima area after four weeks[211].

1.6.4.8 QP-2

QP-2, an alternative microtubular inhibitor, has also been delivered via a polymer sleeve stretched over a stent to the iliac arteries of rabbits. Sustained local delivery was demonstrated[203].

1.6.4.9 Argatroban

This directly-acting, antithrombin drug was loaded onto polymer-coated stents in the porcine coronary model and an inhibition of platelet deposition was seen[195]. This was a fairly short study and so no data are available as to any effect on the restenotic process. It has been used in humans both as an adjunct for thrombolysis and as an alternative to heparin during stent placement in patients with thrombocytopaenia.

1.6.4.10 NO donors

Nitric Oxide (NO), which naturally is a product of the intact endothelium, is known to inhibit, *in vitro*, the growth of vascular smooth muscle cells[212]. The delivery of exogenous nitric oxide to the area of artery at risk of developing intimal hyperplasia might well be beneficial. This has been achieved by modifying bovine albumin to carry covalently bound S-NO groups. This polynitrosated albumin has been applied to angioplasty-damaged rabbit arteries both alone, or loaded onto stents. The stents coated with this compound showed antithrombotic effects, above and beyond that attributable to the albumin alone[213]. Restenosis, although present in half the animals studied, was less than in the uncoated group[196].

1.6.4.11 Heparins

Heparin-coated stents have been developed that show reduced incidence of thrombosis, but not restenosis, in animals[189]. Use of the low molecular weight heparin reviparin delivered using the Dispatch catheter has not shown effectiveness in rabbits[116].

1.6.4.12 Glycoprotein IIb/IIIa receptor antagonists.

In the rabbit iliac artery, stent delivery of an abciximab analogue led to a reduction in thrombosis[205]. 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[206].

1.6.4.13 Other antithrombotics

Activated Protein C loaded stents[198] reduced fibrinogen deposition by 96% and flow was increased tenfold compared to controls. Urokinase, a thrombolytic agent, has been absorbed successfully to stents[214]. PEG-hirudin and prostaglandin analogues have both demonstrated an antithrombotic effect *in vitro* when bound to polylactic acid coated stents[197].

1.6.5 Clinical trials of drug-eluting stents

1.6.5.1 Clinical trials of anti-thrombotic stents.

1.6.5.1.1 Heparin.

Whilst heparin-coated stents have been used in large clinical trials, heparin delivery was not itself under study in a randomised fashion. A heparin-bound polymer coated stent was used clinically in the BENESTENT II[7]. Although this study was not a randomised control trial, the results using this stent were at least as favourable as historical comparisons.

1.6.5.1.2 GIIb/IIIa antagonists.

Abciximab-coated stents have been used in a small group of patients (*Dr G. Roubin, personal communication*). The GRII stent was used to deliver abciximab, without complications. This stent was withdrawn from the market because of high restenosis rates in patients receiving the uncoated, drug-free stent. No clinical trial has been done with an alternative stent for an abciximab-eluting stent, or indeed with any other GIIb/IIIa antagonist.

Drug	Study name	Trial	Comments	Results
Immunosuppressant antibiotics				
Sirolimus	FIM	Reg.		
	Ravel		2 year results	97.5% vs 86% EFS
	Sirius	RPB		TVF 21% vs. 8.6%
	E-Sirius	RPB	9 month, allows direct stenting	92% vs. 77% EFS
	C-Sirius	RPB	High risk lesions 9 month results	18% vs. 4% MACE
Everolimus	Future I		6 month results	9.1% vs. 0% restenosis
	Future II			
	Vision-E		Not yet started	
Tacrolimus	PRESENT III			
ABT 578	Endeavor		In progress	
Actinomycin-D	ACTION		Trial abandoned	high restenosis
Paclitaxel/derivative				
Paclitaxel	Aspect	R,PB	6 month	4% v 27%
	Elutes	RPB	12 month	5% v 16%
	Taxus I	RPB	6 month	11% v 0% restenosis
	Taxus II	R,P	6 month results	19.5 vs 8.5 MACE
	Taxus III		ISR trial	30 day benefit
	Taxus IV		In progress	
	Taxus V	High risk lesions	Not yet started	
	Taxus VI	Long lesions	Not yet started	
	Deliver	P	9 month results	No benefit
	Simple-1	Reg	6 month, small vessels	4.4% restenosis
QP-2	SCORE	RPB	Trial abandoned high thrombosis rate	36.9% v 6.4% restenosis
Steroids				
Dexamethasone	STRIDE	Reg	6 month	3.3% restenosis
Dexamethasone	Emperor			
Others				
Mycophenolic acid	IMPACT	RP	Six month results	No benefit
Heparin	BENESTENT -II	Reg		
Heparin	Jostent trial	RPB	6 month	No benefit.
CD-34 antibody	Case reports			No adverse events
Batimastat	BRILLIANT	Reg	Trial abandoned	Poor restenosis rates
Abciximab	Series			No adverse events
Angiopeptin	Trial in progress			
Oestrogen	EASTER	Reg	12 month results	90% EFS

Figure 1.7 Drug-eluting stent trials. EVF – Event-free survival MACE – Major adverse cardiac event. TVF- Target vessel failure RPB – Randomised, Placebo-controlled, blinded study Reg – Registry ISR In stent restenosis trial

1.6.5.2 Clinical trials of anti-restenosis stents.

1.6.5.2.1 Sirolimus

The **RAVEL** (**Ra**ndomized study with the sirolimus coated BX **Ve**locity balloon-expandable stent in the treatment of patients with *de novo* native coronary artery **l**esions) study randomised 120 patients to the sirolimus-coated stent and 118 patients to the bare, non-coated stent.0% restenosis was seen in the sirolimus group *vs.* a 26% restenosis rate in the control arm ($p<0.0001$). No "edge effect" was detected (Morice *et al.* Presented at the XXIII Congress of the European Society of Cardiology. Sep 4, 2001). The large-scale SIRIUS clinical trial was reported in 2002. A 2% rate of angiographic in-stent restenosis was seen, 91`% less than that seen in the control arm.

1.6.5.2.2 Paclitaxel

ASPECT (**AS**ian **P**aclitaxel-**E**luting Stent **C**linical **T**rial). The stent was coated with paclitaxel using a process which does *not* involve a polymeric layer, i.e. bare metal. QCA results at six-month showed a dose-dependent reduction in binary restenosis (4% *vs.* 12% *vs.* 27%, $p<0.001$). (Park S-J *et al.* Presented at the Transcatheter Cardiovascular Therapeutics 2001 meeting. Sep 12, 2001).

TAXUS-I enrolled 61 patients with *de novo* lesions <12mm in length and 3.0mm in diameter. Thirty-one patients were randomised to the NIRx stent, coated with paclitaxel and thirty to the NIR uncoated stent. At six months the binary restenosis rate was higher in the control than the paclitaxel treated arms (11% *vs.* 0% (respectively), $p=0.1062$) (Grube *et al*). A further trial (TAXUS-III) with the same stent in restenosis patients was reported at the American Heart Association meeting in 2001 (Grube *et al*) with favourable results at 30 day follow-up (7%MACE),

The **ELUTES** (**E**va**lu**ation of the pacli**t**axel-**e**luting **s**tent) trial evaluated the safety and effectiveness of the Cook Inc. paclitaxel-coated, V-Flex coronary stent to reduce in-stent restenosis. The study was a dose-finding study and 192 patients with single type A/B1 *de novo* lesions less than 15mm long were randomised to receive stents coated with one of four

doses of the drug or to a placebo stent arm. At 12 months, patients who received the highest dose of stent-borne paclitaxel experienced a TLR rate of 5%, compared to 16% in the patients treated with the non-drug-coated stent (Gershlick, A. et al, presented 2002 Paris Course on Revascularization.)

1.6.5.2.3 QP-2.

Studies with the Quanam drug-delivery stent used QP-2, similar in structure to paclitaxel. The **SCORE** trial has been suspended after completing only 275/400 patient randomisations. The results were presented at TCT 2001 by G.W. Stone. Binary restenosis rate was significantly lower in the Taxane group (36.9 *vs.* 6.4%, $p<0.001$). However, high stent thrombosis rates 9.4% *vs.* 0%, including five deaths, caused the early termination of the trial.

1.6.5.2.4 Heparin

The BENESTENT II trial showed apparent benefits of the use of a stent with heparin covalently bound to it. However this trial did not compare heparin-coated stents with bare metal stents. Wohrle *et al*[215] reported a clinical study of 277 patients with 306 stented lesions. Heparin-coated or bare Jostents were implanted in a blinded fashion. No differences were seen at six-month follow-up in clinical endpoints, subacute stent thrombosis or angiographic restenosis between the two groups. Very small doses of heparin, were bound onto the surface of the stent.

1.6.5.2.5 Dexamethasone

The **STRIDE** (**ST**udy of anti-**R**estenosis with the B**I**o*divYsio* **D**examethasone **E**luting stent) trial is a small 60 patient registry, rather than a randomised trial. Results reported by de Scheerder at the ACC in 2002 showed a 3.3% MACE at six months. A larger trial is planned.

1.6.5.2.6 Oestrogen.

Oestradiol may have multiple modes of action in the vasculature. It is known to inhibit SMC migration and proliferation and promotes re-endothelialistion of vessel wall after injury. It also inhibits adventitial fibroblast cell migration, a process involved in negative remodeling.The **EASTER** (**E**strogen **A**nd **S**tents **T**o **E**liminate **R**estenosis) trial is a 90

patient trial of oestrogen-coated stents in *de novo* lesions. The primary endpoint is binary restenosis, secondary endpoint are MACE and IVUS measures of restenosis.

1.6.5.2.7 Batimastat

The MMP inhibitor batimastat has been selected as a stent-delivered agent because it is active at nanomolar concentrations. The **BRILLIANT** (**B**atimastat anti-**R**estenosis tr**I**a**L** uti**L**iz**I**ng the BiodivYsio loc**A**l drug Delivery PC-ste**NT**) study was abandoned in March 2002, because of poor restenosis rates, although these have not been published.

1.6.5.2.8 Actinomycin-D.

Actinomycin-D, an antibiotic with known pro-apoptotic properties was tested as a stent-delivered agent in the. **ACTION** trial. This was abandoned in March 2002 due to poor results, which have not been made public.

1.6.5.2.9 Angiopeptin

A small (50pts) trial of an angiopeptin-eluting trial is planned in Hong Kong. Six month angiographic and IVUS follow-up will be performed. The primary endpoint is binary restenosis, secondary endpoint are MACE and IVUS measures of restenosis.

1.7 Re-endothelialisation.

The concept of re-endothelialisation is central to this thesis. The importance of the endothelium in the response to injury is discussed above (1.3.3.3.3). At the time of PCI, the endothelium covering the stenotic area of artery is severely disrupted or even destroyed. In animal models of stent placement, re-endothelialisation begins to occur in the first two to seven days post placement. Full endothelialisation can take at least three or four weeks[216]. It is thought that a similar or slightly longer time-course occurs in humans, although this has been difficult to quantify from the rarity of available autopsy specimens from patients post stent insertion[217]. Radiation causes endothelial dysfunction even six months after exposure[218]. Groups working with radioactive stents have showed either delayed or incomplete endothelial regrowth over the stent[219; 220]. Late thrombosis[221] in patients receiving brachytherapy is felt to be due to adverse effects of radiation on the re-endothelialisation process.

Over time endothelium will grow back over the PCI site. When this occurs over a stent, this has been called "stent passivation". Several approaches have been tried to improve re-endothelialisation.

1.7.1 Exogenous endothelial cell seeding to promote re-endothelialisation

In a rabbit hindlimb model[222], endothelial cell (EC) suspension was seeded onto an angioplasty site. Platelet deposition, a marker of endothelial integrity, was significantly reduced at thirty minutes. This demonstrated the feasibility of introducing ECs onto a denuded site. *In vitro,* human umbilical vein endothelial cells (HUVECs) were attached to stainless steel stents. The ECs continued to grow after stent expansion until a complete endothelial coating was seen[223]. In porcine stented arteries, the stents grew an endothelial coat within six days of implantation, with no evidence of thrombus formation. In a later refinement of this work, ECs were genetically modified to deliver t-PA[224]. Genetically modified, immortalised ECs have been coated onto a stent. Vascular endothelial growth factor (VEGF) has been used as the agent produced by genetically-engineered ECs seeded onto a stent. No report has been made as to the effectiveness of such stents in reducing stent complications[225]. Cell seeding has several disadvantages. It requires delicate handling and would require harvesting and culturing ECs from the patient in advance of the stent placement. The use of cultured cells raises the possibility of introducing infection e.g. of a

fungal species into the coronary circulation. It is not clear how these cells could be adequately sterilised for human use without damaging them. It is unclear to what extent endothelial cells will remain on a stent *in vivo* or how long they will remain viable. The long-term effects of genetically altered cells are unknown, raising concerns that these cells may not function normally, even if they express the protein that they were modified to produce. The use of cell seeding is not currently practical in human trials.

1.7.1.1 Cytokines and hormonal manipulation to promote re-endothelialisation.

Rather than directly administering ECs, agents that promote EC growth and recovery have been tested in models of angioplasty.

1.7.1.1.1 Prostacyclin synthase (PCS).

PCS gene transfer has been performed using a balloon delivery to stented rabbit iliac arteries. This led to more rapid stent endothelialisation and reduced neointimal area[226]. VEGF was found to be expressed more in the PCS treated arteries, suggesting that this gene treatment acts through up-regulation of VEGF.

1.7.1.1.2 Hepatocyte Growth Factor (HGF).

HGF is a heterodimeric protein, which also belongs to a group of highly specific endothelial cell mitogens. As well as promoting endothelial cell growth it promotes angiogenesis and wound healing. It suppresses apoptosis. Systemic delivery of HGF failed to demonstrate reduced intimal hyperplasia in a rat carotid artery model of restenosis. However, in the rabbit iliac model, increased re-endothelialisation and reduced intimal thickness was seen when the drug was delivered locally with a delivery balloon[131].

1.7.1.1.3 TNF-alpha blockade.

Krasinski *et al*[227] have shown that TNFα induces apoptosis in human endothelial cells. Blockade of TNFα might be expected to favour the growth and recovery of endothelial cells post-angioplasty. This has been demonstrated in the rat carotid model with systemic administration of soluble TNF receptor molecules that block TNFα. At one week,

endothelialisation was improved significantly in the treated group. Intimal hyperplasia was not studied in this experiment.

1.7.1.1.4 Oestrogen.

Women suffer atheroclerotic narrowing of the coronary arteries at later age than men. This difference may be related to differences in the effects of oestrogens on the vessel wall. Oestradiol has been shown to inhibit myointimal hyperplasia and it is suggested that this is due to its protective effect on endothelial regrowth[228]. In rats, a dose-dependent increase in re-endothelialisation was seen with systemic oestradiol administration post-angioplasty[229].

1.7.1.1.5 Thrombospondin blockade.

Thrombospondin is a matrix glycoprotein secreted by activated platelets and known to stimulate vascular SMCs whilst inhibiting endothelial cell growth and migration. An antibody to this glycoprotein, when delivered locally to the angioplastied rat carotid artery shows significantly increased re-endothelialisation associated with decreased neointimal hyperplasia[144].

1.7.1.1.6 IRFI 042.

This vitamin E-like antioxidant has been given systemically to rats with angioplastied carotid arteries. Less intimal hyperplasia was seen compared to controls in conjunction with improved endothelial function[97].

1.7.1.1.7 C-type Natriuretic Peptide (CNP)

CNP is known to inhibit smooth muscle cell growth. Work with rabbit jugular vein grafts dipped in solutions containing adenovirus coding for CNP has shown that this peptide leads to improved re-endothelialisation. Reduced thrombus and neointima formation were also seen[230].

1.7.1.1.8 VEGF-C.

VEGF-C is a member of the VEGF family, which particularly affects the lymphatic system. However, it has shown beneficial effects on re-endothelialisation when given locally to balloon angioplastied rabbit aortae[231]. The resulting reductions in neointima formation were not statistically significant.

1.7.1.1.9 Vascular Endothelial Growth Factor (VEGF).

The structure and function of this cytokine, which is the focus of this MD thesis, with particular regard to the effects on re-endothelialisation, will now be reviewed.

1.8 Vascular Endothelial Growth Factor (VEGF)

VEGF is a protein initially identified as Vascular Permeability Factor[232] (VPF) or Vasculotropin[233]. It appears to be essential to the growth, maintenance and repair of vascular structures. Initially, the relevance of this protein to clinical medicine lay in its role in the progression of cancers. It also appears crucial to our understanding of the repair or regeneration of blood vessels in ischaemic tissues. It has also been recognised as important in other clinical diseases.

1.8.1 Structure of VEGF.

VEGF is a heparin-binding, endogenous 46kD homo-dimeric peptide. It is one of a family of peptides (VEGF-A to E). The VEGF (i.e. VEGF-A) form is by far the most thoroughly studied. It occurs in five isoforms of 121, 145, 165, 189 and 206 amino acids, which are formed by alternative splicing. VEGF121 and 165 are diffusible in the extracellular medium, whilst VEGF 189 and VEGF 206 bind heparin more avidly, and tend to be more tightly bound to proteoglycans in the extracellular matrix. All the isoforms are preceded by a 26 amino acid residue signal-protein that allows the protein to be secreted from cells. All also possess an N-terminal glycosylation site and so the protein *in vivo* is found as a glycoprotein, although removal of the carbohydrate moiety does not appear to alter its physiological effects. VEGF is synthesised in smooth muscle cells and macrophages (amongst others). It is almost exclusively bound by endothelial cells. Importantly however, both key receptors for VEGF have been detected in human atherosclerotic artery tissue[234]. This suggests that VEGF is not solely active on the endothelial cell and may have effects on other cells within the vascular wall.

1.8.2 Receptors for VEGF

Three different receptors have been isolated for VEGF:

The flt-1 receptor (VEGFR-1) is a tyrosine kinase trans-membrane receptor. It has high affinity for VEGF[235]. The flk-1 (VEGFR-2) receptor is another of the tyrosine kinase family[236]. It stimulates the Protein Kinase C and Map kinase pathways for protein

synthesis[237]. The flt-4 (VEGFR-3) receptor has a similar structure to the other VEGF receptors. It does not bind VEGF, but does however actively bind VEGF-C and D[238; 239]. In addition to these receptor proteins, a subset of co-receptors has been identified that selectively bind the VEGF-165 isoform[240]. These are Neuropilin 1 and 2. Neuropilins are proteins originally identified as being involved in neuronal cell guidance. It is thought that binding to these receptors modulates the effect of VEGF when bound to the VEGFR-2 receptor. To varying degrees, VEGF binds to heparan-sulphate proteoglycans on the cell membrane. This is thought to affect its bioavailability. The interactions of these receptors and their ligands are shown below (*fig.1.8*)

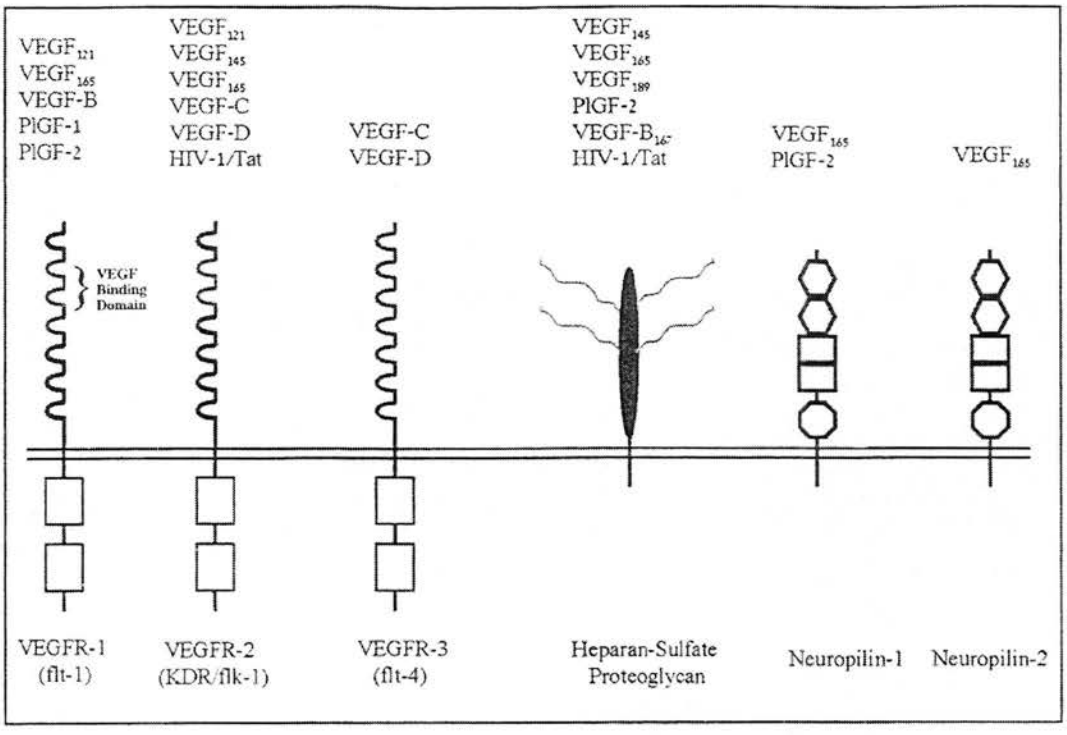


Figure 1.8: The VEGF receptor family and their specific ligands. HIV-1/tat is a protein produced by HIV infection. It may be that this is the mechanism whereby HIV produces the lesions seen in Kaposi's sarcoma. PlGF is Placental Growth Factor, see below (1.8.13.6).(adapted from Neufeld et al [241]*).*

1.8.3 VEGF and nitric oxide (NO).

Adequate endothelial function, via the nitric oxide synthase release of NO (EDRF) is essential for the action of VEGF[242]. In mice the effects of VEGF can be abolished where the animal is deficient in nitric oxide synthase and its effects replicated by dietary augmentation with L-Arginine, which increases NO synthesis[242]. NO may act in a negative feedback system to reduce the production of VEGF[243]. The actions of VEGF can be abolished by removing calcium from models of the action of the protein or by adding NO Synthase inhibitors such as N-monomethyl-L-arginine[244]. Intimal hyperplasia caused by a silicone collar around the carotids was attenuated by local VEGF delivery in an NO-dependent manner[245].

1.8.4 VEGF and thrombosis.

NO and PGI_2, which are both produced in endothelial cell cultures by VEGF, inhibit platelet aggregation. As well as stimulating these factors, VEGF increases the expression and activation of the serine proteases, urokinase and tissue-type plasminogen activator, which cleave plasminogen to generate the key thrombolytic enzyme, plasmin[246]. VEGF is released by aggregating platelets[247] and increased levels of VEGF have been found within clots in humans. Conversely, VEGF induces von Willebrand factor (vWF) and tissue factor in endothelial cells. vWF promotes platelet adhesion to subendothelial collagen, whilst tissue factor expression and activation are essential for the extrinsic pathway of coagulation and clot formation[246]. *In vivo*, reduced clot formation has been seen on stents implanted in rabbit iliac arteries when washed with VEGF by a channel balloon system[57].

1.8.5 Actions of VEGF.

The actions of VEGF are summarised below (*fig. 1.9.*)

- It is essential for normal embryonic development. It is an integral agent for vasculogenesis, the development of a normal vasculature during embryonic life. Deletion of even one allele for VEGF has proven to be uniformly fatal in animal studies[248].

- It is implicated in the process of angiogenesis. It has mitogenic activity that is specific for vascular endothelium[249].
- It also appears to have a role in the neovascularisation seen in diabetic retinopathy. Although many growth factors have been identified in patients with this sight-threatening condition, VEGF is the most closely correlated factor to it[250].
- It causes an increase in vascular permeability mediated via the VEGFR-1 receptor[251] and by increasing Platelet Activating Factor[252]. It seems likely that its proven effect of increasing vascular permeability is an integral part of the process of angiogenesis. As plasma proteins exude into the extracellular matrix, they may form the core around which migrating endothelial cells develop into new vessels. It is also found in the permeable membranes of the islets of Langerhans, suggesting a role in the control of insulin production[253].
- It upregulates the expression of receptors for similar growth factors including basic Fibroblast Growth Factor and for endothelin-1 mRNA[254].
- It causes vasodilatation[255]. This effect is likely to be due to the release of NO/EDRF, mediated by the VEGFR-2 receptor[256].
- Via VEGFR-1[241], it is chemoattractant for monocytes[257], which when activated to become macrophages themselves produce VEGF.
- Like other angiogenic growth factors[258], it has been linked to haematopoiesis. VEGFR-2 deficient mice fail to form blood islands, the progenitors of the haematopoietic system[259].
- It modulates the function of endothelial cells, promoting the release of the endothelial cell products plasminogen and PAI-1[260]. VEGF upregulates the expression of endothelin-1 mRNA[254].
- It improves endothelial function when the vessels are damaged[261] or in new collaterals[262]. VEGF increases the resistance of endothelial cells to oxidative stress[263].

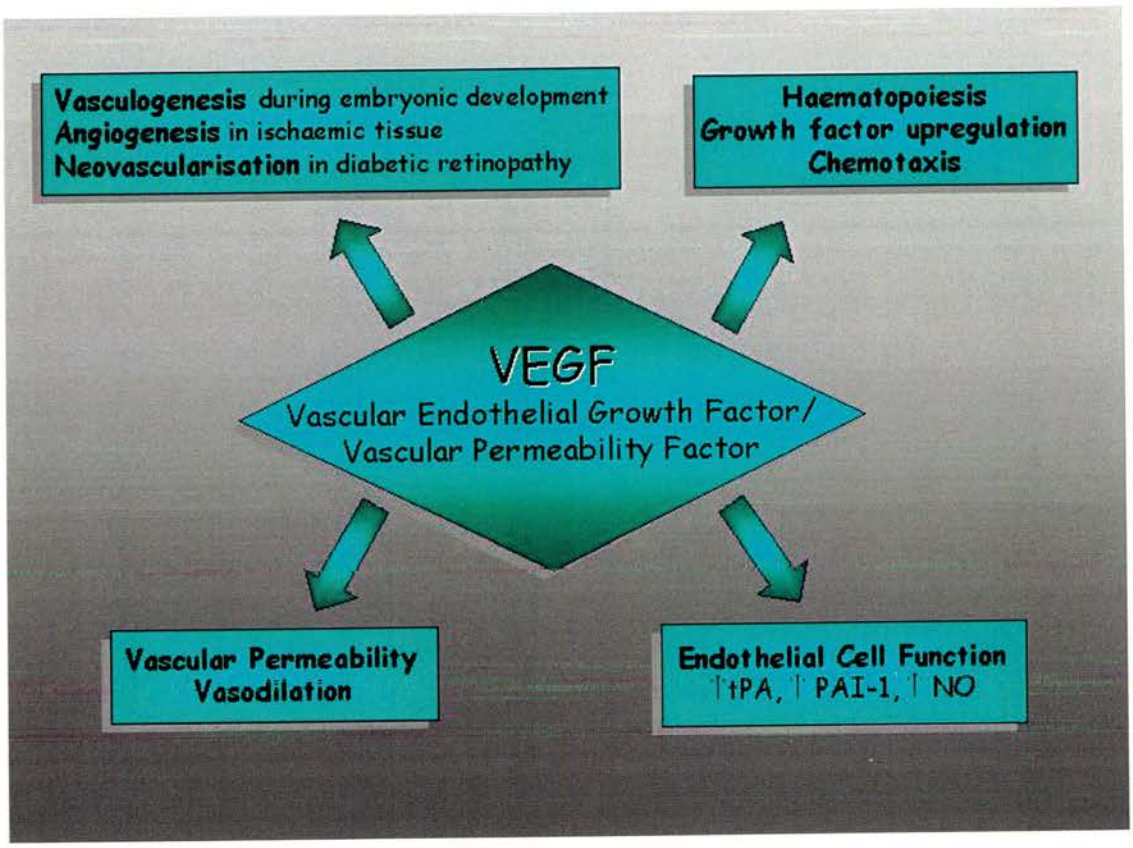


Figure 1.9 Diagram of the main actions of VEGF in vivo.

1.8.6 Regulation of VEGF.

The release of VEGF from cells is under the influence of a number of humoral, chemical and physical factors. Cytokines and hormones including IL-6[264], IGF-1[265], Endothelin-1[266], FGF-4[267], TGF-β[268], PDGF[269] and angiotensin-II have all been shown to up-regulate the expression of VEGF. bFGF[270] and TNFα[271] up-regulate not the protein itself, but the VEGFR-2 protein. Conversely TGFβ has been shown to down-regulate VEGFR-1 and 2, reducing the levels of mRNA for these proteins in a dose-dependent fashion[272].
Chemical conditions, *in vitro* at least, have shown effects on VEGF production in cardiac myocytes as well. Hypoxia appears to be of importance in the stimulation of VEGF production. VEGF is often found in the relatively ischaemic and hypoxic microenvironment of the interior of a solid tumour. *In vitro*, hypoxia induces endothelial cells to produce VEGF[273]. A transcription promoter for the VEGF gene has been identified that upregulates VEGF synthesis in response to hypoxia-inducible factor (HIF-1)[274]. HIF-1 is also responsible for the upregulation of erythropoietin, emphasising the close functional similarities between the angiogenic and haematopoietic growth factors.
Other stimulatory factors for VEGF production include phorbol (a stimulator of protein Kinase C), glucose[275], veratridine (a promoter of calcium influx) and the transition metals cobalt and manganese[276]. Reactive oxygen species[277] and oxidised-LDL[278], which promote the progression of atherosclerotic plaques, both potentiate the production of VEGF.
Electrical stimulation of skeletal muscle, either directly[279], or via chronic motor nerve stimulation[280] both demonstrate increased VEGF production that led to angiogenesis *in vivo*.
Mechanical stimulation, via cyclical mechanical strain, also produces VEGF[281]. Such stretch-induced VEGF release seems likely to be mediated by the release of TGF-β and resulting protein kinase C activation[282].

1.8.7 VEGF in oncology.

For a tumour to grow it requires a rich blood supply and this entails angiogenesis. Tumour cell lines release VEGF and VEGF over-expression has been identified in almost all human tumours[283]. The richness of expression has been linked to the aggressive nature of these tumours. In the case of both breast and stomach cancers, VEGF-rich tumours had the worst prognosis[284]. Attention has been paid to the potential to inhibit the effects of VEGF in

tumours in an attempt to stop their growth. Selective loss of new blood vessels by apoptosis is seen in even established tumours following removal of VEGF[285].

1.8.8 VEGF in re-endothelialisation.

1.8.8.1 Experimental work using VEGF to stimulate re-endothelialisation.

Endogenous VEGF and VEGFR-2 receptors begin to be expressed in increasing amounts within 48 hours of an angioplasty injury in a minipig model[286]. Exogenous VEGF has been reported to accelerate re-endothelialisation of damaged rat carotid artery[129]. In this study, a 30-minute infusion of VEGF was given directly into the carotid artery at the site of balloon denudation of the endothelium. Not only did the group report superior re-endothelialisation in the treated group of rats, but also that intimal hyperplasia at this site had been attenuated. Antibodies against VEGF seemed to inhibit this effect on re-endothelialisation[243]. When vein grafts were dipped in VEGF prior to implantation in rabbit carotid arteries, EDRF-dependent relaxation was seen to occur, whereas controls showed no relaxation. This study also found a decreased incidence of intimal hyperplasia[287]. As well as a direct effect on local endothelial cells, VEGF appears to stimulate the activity of circulating endothelial progenitor cells (EPCs). In the mouse hindlimb ischaemia model, EPCs transfected with the VEGF gene were adminstered and showed an improvement in neovascularisation[288].

Other groups have not replicated these results, however. Six-hour infusions of VEGF into the aortic arch of rats with denuded endothelium in the carotid artery or aorta showed no evidence of endothelial regrowth, nor indeed the presence of any VEGFR-2 receptors in large vessels[289]. These include the carotid vessel that Asahara *et al*[129] had reported as demonstrating endothelial recovery. These data were replicated in rats given either two or six week infusions of VEGF. Furthermore, in the porcine restenosis model bolus, delivery of 1mg of VEGF as a local infusion into the coronary arteries failed to demonstrate any inhibition of restenosis, raising the possibility that different species had different responses to the same method of administering VEGF[290]. In this study the total dose of VEGF given was small especially in a fairly large animal and was not given for longer than five minutes and it may be that a more sustained delivery is required. One study has been reported that suggests a deleterious effect of VEGF administration on the progression of atherosclerotic lesions (rather than restenotic ones), albeit in grafts rather than native vessels[291]. Indeed, this study reported that blockade of the effect of VEGF led to *reduced* intimal thickening. A mechanism for this effect is suggested by Zhao *et al*[292] who reported that L-NAME, which

induces inflammation through NO inhibition, depended for its action on the VEGF-1 receptor. This suggested that VEGF is required for the inflammatory reaction that led to murine atherosclerosis.

VEGF protein was also used, by Van Belle *et al,* in a rabbit hind-limb model of endothelial damage. In this study direct transfer of recombinant VEGF was performed with a channel balloon catheter. At the same site a metallic stent similar to those used on human coronary artery disease was implanted, causing endothelial damage. The rate of endothelialisation of this stent was compared to controls without VEGF administration. A clear increase in the rate of re-endothelialisation was shown[293] (*see fig. 1.10*). In a further study, naked plasmid DNA coding for VEGF was used in place of VEGF itself and administered locally using a hydrogel-coated balloon. Stent "passivation" occurred quickly and completely in the rabbits given VEGF DNA. This effect was associated with a decreased incidence of both intimal hyperplasia and thrombus formation on the stent[57]. A further study using the same techniques showed that not only was the endothelial integrity restored, but also that the regenerated endothelium was functional, at least as far as restoring vasomotor responsiveness and thromboresistance[261].

VEGF plasmid has been delivered bound to the stent and appeared to reduce intimal hyperplasia, (29+/-18% reduction in intimal thickness ($p<0.016$)) in a rabbit iliac model) although this study did not examine the speed with which the stent was covered by the endothelium[199]. This group gave the rabbits in both cohorts a low molecular weight heparin throughout the experiment. As has been discussed elsewhere, these are under investigation for their own possible effects on reducing intimal hyperplasia.

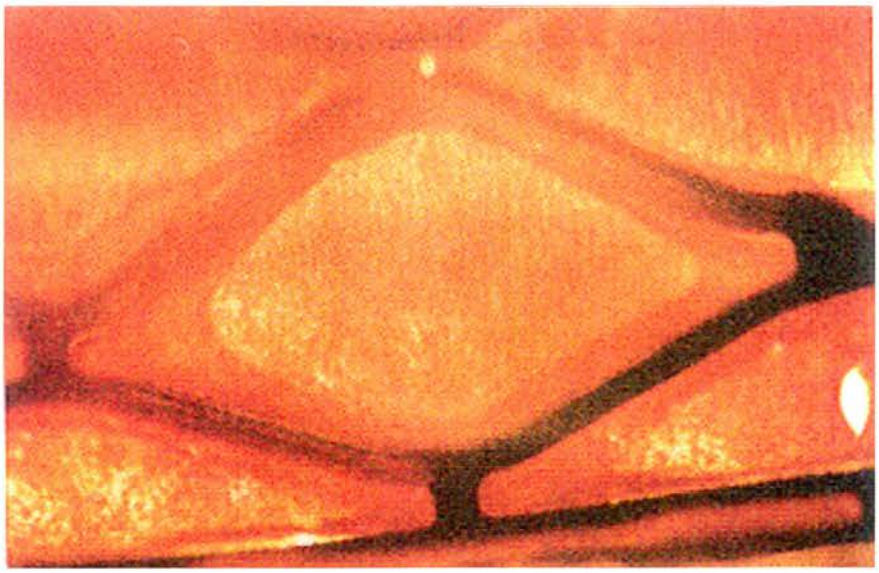


Figure 1.10a Micrograph of the surface of a partially endothelialised stent. From Van belle et al

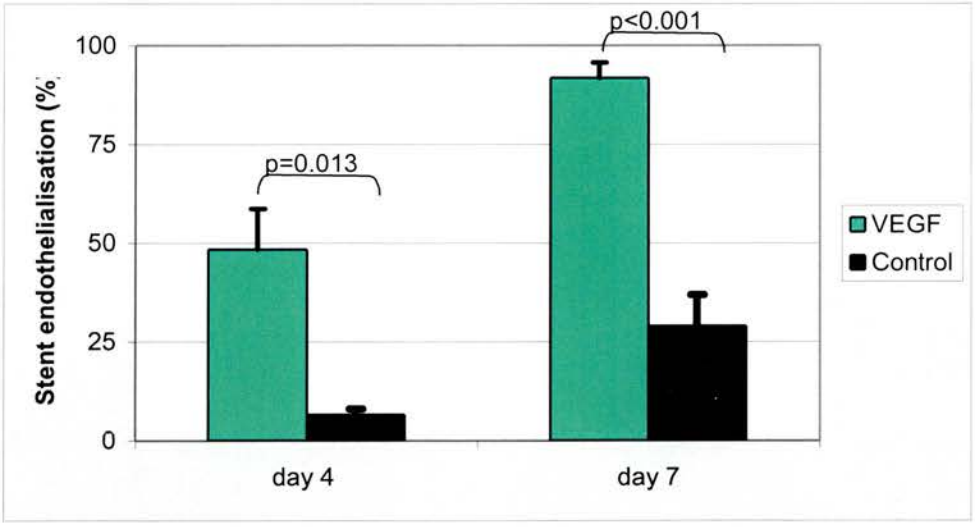


Figure 1.10b Quantification of stent endothelialisation after local delivery of VEGF or PBS (control). From Van belle et al.

1.8.9 VEGF and angiogenesis.

VEGF *in vitro* and *in vivo* has been shown to have mitogenic effects on the vascular endothelium. The VEGFR-2 receptor appears to mediate this effect[237]. As well as direct effects on the endothelial cell, VEGF promotes the process of angiogenesis indirectly through the production of matrix metalloproteinases such as interstitial collagenase. *In vitro*, VEGF has been shown to stimulate this enzyme's production from HUVECs[294]. Breakdown of the interstitial matrix will facilitate angiogenesis. Neovascularisation/angiogenesis is influenced by the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, collagen receptors. Blockade of these receptors by antibodies has largely abolished the angiogenic effects of VEGF[295].

VEGF in normal arteries is not expressed in detectable quantities. In angioplastied vessels, it is expressed and correlates with the adventitial angiogenesis that causes the growth of *vasa vasorum*[296]. These new vessels may be of importance in the repair of the damaged vessel. It is also detectable in coronary sinus blood samples from patients with complete coronary artery occlusions. VEGF levels in patients with severe stenosis are much lower. It is felt that this may represent the natural angiogenic response to an ischaemic stimulus in the total occlusion group[297]. Similarly, VEGF may have a role in the neovascularisation of atherosclerotic plaques[298]. This raises the possibility that VEGF, including exogenously delivered VEGF, may contribute to an accelerated atherosclerotic process.

VEGF may have a beneficial role in improving the blood supply to ischaemic areas of the myocardium. VEGF may stimulate the growth into these ischaemic territories of new collateral vessels. A single intra-arterial bolus of 500-1000μg has been shown to promote collateral growth and improve ankle-brachial blood pressure in rabbit hindlimb ischaemia[299]. The increased number of collaterals was associated with physiological benefits in that significantly higher bloodflow was seen in rabbits given the VEGF bolus[300]. These initial results have been confirmed using a rabbit model, in which severe ischaemia was created by ligation of the femoral artery and all its branches. After ischaemia had been established in the hindlimb, VEGF was infused over a 28-day period using an osmotic pump into the iliac artery[301], improving neovascularisation. Gene therapy with VEGF increased collateral growth and increased blood flow in the rabbit hindlimgbmodel when delivered locally on a hydrogel balloon[302].

After ameroid constrictor placement to the left circumflex artery (LCx) 2μg VEGF was administered extraluminally. VEGF animals showed higher bloodflow in the LCx territory[303]. Separately, 2mg VEGF was instilled into the left coronary ostium 28 days after ameroid constrictor placed around LCx. Half of these animals died due to extreme hypotension. The survivors showed improved perfusion [304]. Yorkshire pigs underwent placement of an ameroid occluder to LCx. Three weeks later the animals were randomised to with either VEGF (20μg) via an intracoronary delivery system, intracoronary bolus infusion, epicardial implantation of an osmotic delivery system, or intracoronary saline. Three weeks later, the animals were tested for myocardial perfusion and ventricular function. All three VEGF treatment groups, but not the control animals, demonstrated a significant increase in collaterals and myocardial bloodflow[305]. Intracoronary VEGF in pigs produced a dose-dependent increase in coronary blood flow in the absence of significant changes in epicardial artery diameter[306].

In a canine[307] models of chronic coronary ischaemia, VEGF showed enhanced collateral vessel density and blood flow when delivered as a local infusion, although not when administered systemically[220]. VEGF has been implicated in the angiogenic proliferation seen following transmyocardial laser revascularisation (TMR). The effects of TMR are augmented by the addition of VEGF in ischaemic pig hearts[308]. Studies have suggested that the effect of collateral growth may be most marked when VEGF is co-administered with bFGF both *in vitro*[309] and *in vivo*[310]. VEGF has been delivered injecting it directly into ischaemic muscle. VEGF protein injected daily for ten days in high doses showed a dose-dependent improvement of collateral growth in the rabbit model[311]. Intramuscular injections for the plasmid encoding for VEGF show successful transfection of the DNA into striated muscle cells. Synthesis of VEGF was demonstrated for up to two weeks following the procedure despite only 2% of cells having incorporated the plasmid[312]. Conflicting with these results is the finding that injection of an entirely unrelated gene into the myocardium of pigs also shows an upregulation of VEGF expression[313]. It may not be that gene therapy using VEGF DNA is occurring but merely that the trauma of injection is itself a stimulus for VEGF production, undermining the scientific rationale for performing the treatment. Other workers have tried to replicate the results obtained in the rabbit hindlimb model of ischaemia and have found that while VEGF plasmid injection did seem to be correlated with increased angiogenesis, no actual VEGF could be detected in the tissues[314]. Angiogenesis due to VEGF has been seen in relation to interventional treatments of restenosis. VEGF expression was highest in vessels where the vasa vasorum were proliferating following earlier injury in a porcine model[296].

1.8.10 VEGF in apoptosis.

VEGF inhibits apoptosis by the upregulation of apoptosis inhibitors including survivin[315], Bcl-2 and A1[316]. In a tumour model of immature blood vessels, removal of VEGF led to selective apoptosis of endothelial cells forming immature blood vessels. More mature vessels, with a layer of periendothelial cells did not undergo apoptosis[285].

1.8.11 VEGF in other diseases.

VEGF expression has been identified in several human conditions that are characterised by vascular growth or permeability. Synovial fluid in rheumatoid arthritis, where a highly vascularised pannus erodes the joint surfaces, contains VEGF[317]. The lesions associated with some skin conditions, including psoriasis, pemphigoid and dermatitis herpetiformis, all express VEGF. Elevated levels of VEGF are found in peritoneal fluid of patients suffering from endometriosis. Graves' disease, which tends to produce a highly vascular goitre, stimulates VEGF production[283].

1.8.12 Clinical trials of VEGF.

A small study of plasmid-derived VEGF showed collateral vessel growth and improved outcome in patients with critical limb ischaemia[318]. A different group, again in a very small sample, showed angiographic evidence of improved collateralisation and perfusion scan evidence of increased tissue perfusion when intra-coronary VEGF was given to patients with ischaemic myocardium[319].

Preliminary phase II trials of VEGF protein administered by intra-coronary bolus followed by intravenous infusions have had disappointing results in patients with coronary arterial disease. No significant angiogenic effect was seen in the vascular endothelial growth factor in ischemia for vascular angiogenesis (VIVA) trial[320]. It has been suggested that the route of administration, by intra-coronary bolus and subsequent i.v. infusion, meant a poor local concentration of the drug was achieved. The amounts of drug administered were significantly less than the doses per kilogram found to be effective in animal models.

It may be that small doses of VEGF are all that can safely be given into the coronary circulation. When doses of 2mg were given as a bolus into the coronaries of pigs with experimental ischaemia, half the animals died abruptly of severe hypotension[304].

A human study used a local delivery of VEGF plasmid into angioplastied arteries. At six months, no improvement in restenosis was seen[130]. This study was not in stented patients, where the benefit of VEGF might be expected to be most noticeable. It also used a local delivery balloon, which may cause intimal hyperplasia and so obscure any beneficial response from the drug. An adenovirus vector to deliver VEGF-121 cDNA was administered by direct intramyocardial injection during surgery in patients with severe coronary artery disease. This Phase I trial suggested a subjective benefit for the treatment although no statistically significant improvement in symptoms related to augmented angiogenesis occurred[321]. A study using VEGF-165 plasmid delivery into the myocardium of inoperable coronary disease patients showed objective improvements in perfusion as well as subjective improvement in anginal symptoms in a small group of non-randomised patients[322]. A randomised, double-blind, placebo-controlled study has been reported, in a small group of patients using percutaneous delivery to ischaemic myocardium of VEGF plasmid. This study showed a reduction in anginal class in the VEGF group[323].

The Kuopio Angiogenesis Trial (KAT), a phase II clinical trial involving 103 patients, used adenovirus gene transfer for VEGF, mainly in stented patients. At six months, no detectable difference was seen in restenosis rates compared to controls, but there was a significant improvement in myocardial perfusion[324].

1.8.13 The VEGF family of proteins.

1.8.13.1 VEGF-B

VEGF-B is also known as VEGF-related factor (VRF) and is co-expressed in tissues with VEGF, especially in heart and skeletal muscle tissue. Structurally similar to VEGF, it forms both homo-dimers and, with VEGF, hetero-dimers and may control the bioavailability of VEGF. In contrast to VEGF-A, VEGF-B is expressed constitutively. No response to hypoxia was seen *in vitro*[325]. VEGF-B deficient mice develop normal cardiovascular systems, but show atrial conduction abnormalities[326].

1.8.13.2 VEGF-C

VEGF-C, or VEGF related protein (VRP), has 32% homology with VEGF[283]. It binds to the VEGFR-2 and –3 receptors. The VEGFR-3 receptor is largely restricted to the lymphatic system[327] and it seems likely that VEGF-C is involved with the development and function of

the lymphatic system. VEGF-C has been shown to have angiogenic effects in hindlimb ischaemia models[328]. There may not be a physiological function for VEGF-C in adult blood vessels, but the protein may be useful for therapeutic angiogenesis.

1.8.13.3 VEGF–D

VEGF-D or c-fos-induced growth factor (FIGF) is structurally related to VEGF-C. It shows mitogenic effects predominantly on fibroblasts and binds to VEGFR-2 and –3[329].

1.8.13.4 VEGF-E/ *Orf* virus.

Two sequences of DNA have been identified in the *orf* virus with some homology to VEGF. This pathogen causes skin lesions in goats and humans characterised by extensive microvascular proliferation. Members of this family of proteins have also been labelled VEGF-E. They appear to bind to the VEGFR-2[330].

1.8.13.5 Platelet Derived Growth factor (PDGF)

VEGF has striking homology with both PDGF A and B chains. All eight cysteine residues are conserved suggesting a similar tertiary structure, along with about 15-20% identical amino acid residues[233]. PDGF has angiogenic effects on the endothelial cell[331].

1.8.13.6 Placenta Growth Factor (PlGF)

PlGF is a protein with 53% homology to VEGF, isolated from placental tissue. PlGF is also thought to be an angiogenic growth factor and binds to the VEGFR-1 receptor[283]. In choriocarcinoma cells both proteins have been isolated as a PlGF/VEGF heterodimer[332].

1.9 Summary

The scale of the clinical problem posed by restenosis has been made clear. With increasing numbers of patients undergoing PCI in the UK, this problem will only become more pressing. Despite the reduction in restenosis derived from the increasing use of stents, the problem still remains significant. Alternative therapies, including brachytherapy, have not yet found widespread acceptance and carry their own associated problems. The importance of understanding the restenotic process at a cellular level has been emphasised. The use of pharmacological agents that interfere with the restenosis process has been discussed. Many of these have seemed promising in initial trials only to disappoint in clinical use and the ideal drug has not yet been identified. Local drug delivery, using a polymer-coated stent as the delivery vehicle, holds promise as a means of reducing restenosis. This method allows the use of expensive or systemically toxic agents as it delivers small amounts of an agent at the site where it is needed most.

In summary, it has been demonstrated that in-stent restenosis is a significant clinical problem with the potential to be prevented by local delivery of drugs using stents themselves as the delivery vehicle. VEGF has been selected to test this hypothesis.

1.10 Original hypothesis

VEGF, acting on the endothelial cell, holds may speed the re-endothelialisation of a stent. By loading it onto a polymer-coated stent, this re-endothelialisation process may reduce the incidence of stent thrombosis and in-stent restenosis. This has not been tested before.

Chapter 2 deals with the pharmacokinetics of the absorption and release of VEGF from polymer-coated stents. Chapter 3 examines the effects of VEGF and VEGF-eluting stents on endothelial cell culture. Chapter 4 deals with the *in vivo* study of the effects of the VEGF-eluting stent in a rabbit model for restenosis/thrombosis.

Chapter 1 References

1 SIGN. Coronary revascularisation in the management of stable angina pectoris. http://www.sign.ac.uk/guidelines/fulltext/51/index.html

2 Anonymous. Coronary angioplasty versus medical therapy for angina: the second Randomised Intervention Treatment of Angina (RITA-2) trial. RITA-2 trial participants. *Lancet* 1997; **350**:461-468.

3 Serruys PW, de Jaegere P, Kiemeneij F, *et al.* A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. *New England Journal of Medicine* 1994;**331**:489-495.

4 Fischman DL, Leon MB, Baim DS, *et al.* A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. *New England Journal of Medicine* 1994;**331**:496-501.

5 Kastrati A, Schomig A, Elezi S, *et al.* Predictive factors of restenosis after coronary stent placement. *Journal of the American College of Cardiology* 1997;**30**:1428-1436.

6 Savage MP, Fischman DL, Rake R, *et al.* Efficacy of coronary stenting versus balloon angioplasty in small coronary arteries. Stent Restenosis Study (STRESS) Investigators. *Journal of the American College of Cardiology* 1998;**31**:307-311.

7 Serruys PW, Emanuelsson H, van der Giessen W, *et al.* Heparin-coated Palmaz-Schatz stents in human coronary arteries. Early outcome of the Benestent-II Pilot Study. *Circulation* 1996;**93**:412-422.

8 Van Belle E, Bauters C, Hubert E, *et al.* Restenosis rates in diabetic patients: a comparison of coronary stenting and balloon angioplasty in native coronary vessels. *Circulation* 1997;**96**:1454-1460.

9 Lau KW, He Q, Ding ZP, Johan A. Safety and efficacy of angiography-guided stent placement in small native coronary arteries of < 3.0 mm in diameter. *Clinical Cardiology* 1997;**20**:711-716.

10 Moliterno DJ, Topol EJ. Restenosis: Epidemiology and treatment. In: *Comprehensive Cardiovascular Medicine*. Topol EJ. ed. 1998;2397-2432.

11 Topol EJ, Leya F, Pinkerton CA, *et al.* A comparison of directional atherectomy with coronary angioplasty in patients with coronary artery disease. The CAVEAT Study Group. *New England Journal of Medicine* 1993;**329**:221-227.

12 Emanuelsson H, Beatt KJ, Bagger JP, *et al.* Long-term effects of angiopeptin treatment in coronary angioplasty. Reduction of clinical events but not angiographic restenosis. European Angiopeptin Study Group. *Circulation* 1995;**91**:1689-1696.

13 Topol EJ, Nissen SE. Our preoccupation with coronary luminology. The dissociation between clinical and angiographic findings in ischemic heart disease. *Circulation* 1995;**92**:2333-2342.

14 Weintraub WS, Kosinski AS, Brown CL, King SB. Can restenosis after coronary angioplasty be predicted from clinical variables? *Journal of the American College of Cardiology* 1993;**21**:6-14.

15 Lee HS, Hutchison IV, Pravica V *et al.* Association between transforming growth factor beta polymorphism and coronary in-stent restenosis. *XXIst Congress of the European Society of Cardiology* 1999;3250(Abstract)

16 Hirshfeld JWJ, Schwartz JS, Jugo R, *et al.* Restenosis after coronary angioplasty: a multivariate statistical model to relate lesion and procedure variables to restenosis. The M-HEART Investigators. *Journal of the American College of Cardiology* 1991;**18**:647-656.

17 Savage MP, Douglas JSJ, Fischman DL, *et al.* Stent placement compared with balloon angioplasty for obstructed coronary bypass grafts. Saphenous Vein De Novo Trial Investigators. *New England Journal of Medicine* 1997;**337**:740-747.

18 Le Feuvre C, Bonan R, Lesperance J *et al.* Predictive factors of restenosis after multivessel percutaneous transluminal coronary angioplasty. *American Journal of Cardiology* 1994;**73**:840-844.

19 Hermans WR, Rensing BJ, Foley DP, *et al.* Therapeutic dissection after successful coronary balloon angioplasty: no influence on restenosis or on clinical outcome in 693 patients. The MERCATOR Study Group (Multicenter European Research Trial with Cilazapril after Angioplasty to prevent Transluminal Coronary Obstruction and Restenosis). *Journal of the American College of Cardiology* 1992;**20**:767-780.

20 Schwartz RS, Huber KC, Murphy JG, *et al.* Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. *Journal of the American College of Cardiology* 1992;**19**:267-274.

21 Rodriguez AE, Santaera O, Larribau M, *et al.* Coronary stenting decreases restenosis in lesions with early loss in luminal diameter 24 hours after successful PTCA. *Circulation* 1995;**91**:1397-1402.

22 Mintz GS, Popma JJ, Pichard AD, *et al.* Arterial remodeling after coronary angioplasty: a serial intravascular ultrasound study. *Circulation* 1996;**94**:35-43.

23 Christen T, Verin V, Bochaton-Piallat M-L, *et al.* Cellular mechanisms in in-stent restenosis after balloon angioplasty. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000;(Abstract)

24 Haudenschild C. Restenosis: Pathophysiologic considerations. In: *Textbook of Interventional Cardiology 2nd Edition.* Topol EJ. ed. WB Saunders, Philadelphia, PA 1994;382-399.

25 Pauly RR, Passaniti A, Bilato C, *et al.* Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. *Circulation Research* 1994;**75**:41-54.

26 Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA. Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circulation Research* 1994;**75**:539-545.

27 Wight T. The role of matrix in in-stent restenosis and restenosis after balloon angioplasty. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000;(Abstract)

28 Strauss BH, Robinson R, Batchelor WB, *et al.* In vivo collagen turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. *Circulation Research* 1996;**79**:541-550.

29 George SJ, Johnson JL, Angelini GD *et al.* Adenovirus-mediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and neointimal formation in human saphenous vein. *Human Gene Therapy* 1998;**9**:867-877.

30 Tada T, Reidy MA. Endothelial regeneration. IX. Arterial injury followed by rapid endothelial repair induces smooth-muscle-cell proliferation but not intimal thickening. *American Journal of Pathology* 1987;**129**:429-433.

31 Fingerle J, Kraft T. The induction of smooth muscle cell proliferation in vitro using an organ culture system. *International Angiology.* 1987;**6**:65-72.

32 Haudenschild CC, Schwartz SM. Endothelial regeneration. II. Restitution of endothelial continuity. *Laboratory Investigation* 1979;**41**:407-418.

33 Ruitenberg M, Pasterkamp G, de Kleijn DPV *et al.* Constrictive arterial remodelling after balloon angioplasty is not related with the absence of macrophages and endothelial cells. *XXIst Congress of the European Society of Cardiology* 1999;2590(Abstract)

34 Minick CR, Stemerman MB, Insull W, Jr. Role of endothelium and hypercholesterolemia in intimal thickening and lipid accumulation. *American Journal of Pathology* 1979;**95**:131-158.

35 van Beusekom HM, Whelan DM, Hofma SH, *et al.* Long-term endothelial dysfunction is more pronounced after stenting than after balloon angioplasty in porcine coronary arteries. *Journal of the American College of Cardiology* 1998;**32**:1109-1117.

36 Castellot JJJ, Addonizio ML, Rosenberg R, Karnovsky MJ. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. *Journal of Cell Biology* 1981;**90**:372-379.

37 Yang Z, Birkenhauer P, Tanner FC *et al.* Effects of HMG-CoA reductase inhibition on human saphenous vein endothelial and smooth muscle cells. *XXIst Congress of the European Society of Cardiology* 1999;760(Abstract)

38 Rubin P, Williams JP, Riggs PN, *et al.* Cellular and molecular mechanisms of radiation inhibition of restenosis. Part I: role of the macrophage and platelet-derived growth factor. *International Journal of Radiation, Oncology, Biology, Physics.* 1998;**40**:929-941.

39 Komatsu R, Ueda M, Takahiko N. Immunohistochemical analysis of the neointimal tissue response at sites of coronary stenting in humans. *Circulation* 1997;(Suppl I):I-403-2260.(Abstract)

40 Feldman L, Aguirre L, Ziol M, *et al.* Interleukin-10 Inhibits Intimal Hyperplasia After Angioplasty or Stent Implantation in Hypercholesterolemic Rabbits. *Circulation* 2000;**101**:908-916.

41 Li G, Chen S-J, Oparil S, Chen Y-F, Thompson JA. Direct In Vivo Evidence Demonstrating Neointimal Migration of Adventitial Fibroblasts After Balloon Injury of Rat Carotid Arteries. *Circulation* 2000;**101**:1362-1365.

42 Powers JB, Connolly TM, Yoon J-H. Prevention of neointimal hyperplasia with recombinant leech antiplatelet protein (RLAPP) via platelet adhesion. *Heart* 1997;70th Scientific Session:I-289(Abstract)

43 Willerson JT. Stent restenosis: can effective antithrombotic therapy be protective? *Circulation* 1997;**96**:383-385.

44 Haunstetter A, Izumo S. Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circulation Research* 1998;**82**:1111-1129.

45 Malik N, Francis SE, Holt CM, *et al.* Apoptosis and cell proliferation after porcine coronary angioplasty. *Circulation* 1998;**98**:1657-1665.

46 Isner JM, Kearney M, Bortman S, Passeri J. Apoptosis in human atherosclerosis and restenosis. *Circulation* 1995;**91**:2703-2711.

47 Steg PG, Tahlil O, Aubailly N, *et al.* Reduction of restenosis after angioplasty in an atheromatous rabbit model by suicide gene therapy. *Circulation* 1997;**96**:408-411.

48 Koster R, Vieluf D, Kiehn M *et al.* Nickel allergy in patients with in-stent restenosis. *XXIst Congress of the European Society of Cardiology* 1999;3264(Abstract)

49 McKenney P, Currier J, Haudenschild C *et al.* Cyclosporine A does not inhibit restenosis in experimental angioplasty. *Circulation* 1991;**84**:II-70(Abstract)

50 Folts JD, Crowell EBJ, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. *Circulation* 1976;**54**:365-370.

51 Kiesz RS, Buszman P, Martin JL, *et al.* Local Delivery of Enoxaparin to Decrease Restenosis After Stenting: Results of Initial Multicenter Trial : Polish-American Local Lovenox NIR Assessment Study (The POLONIA Study). *Circulation* 2001;**103**:26-31.

52 More RS, Rutty G, Underwood MJ *et al.* A time sequence of vessel wall changes in an experimental model of angioplasty. *Journal of Pathology* 1994;**172**:287-292.

53 Fischell TA, Carter AJ, Laird JR. The beta-particle-emitting radioisotope stent (isostent): animal studies and planned clinical trials. *American Journal of Cardiology* 1996;**78**:45-50.

54 Kollum M, Kaiser S, Kinscherf R *et al.* Apoptosis after stent implantation compared with balloon angioplasty in rabbits. Role of macrophages. *Arteriosclerosis, Thrombosis & Vascular Biology* 1997;**17**:2383-2388.

55 Kuiper KK, Robinson KA, Chronos NA *et al.* Phosphorylcholine-coated metallic stents in rabbit iliac and porcine coronary arteries. *Scandinavian Cardiovascular Journal* 1998;**32**:261-268.

56 Rogers C, Edelman ER, Simon DI. A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proceedings of the National Academy of Sciences of the United States of America* 1998;**95**:10134-10139.

57 Van Belle E, Tio FO, Chen D, Maillard L, Kearney M, Isner JM. Passivation of metallic stents after arterial gene transfer of phVEGF165 inhibits thrombus formation and intimal thickening. *Journal of the American College of Cardiology* 1997;**29**:1371-1379.

58 Erickson LA, Bonin PD, Wishka DG, *et al.* In vitro and in vivo inhibition of rat vascular smooth muscle cell migration and proliferation by a 2-aminochromone U-86983. *Journal of Pharmacology & Experimental Therapeutics* 1994;**271**:415-421.

59 Schwartz RS. Animal models of human coronary restenosis. In: *Textbook of Interventional Cardiology 2nd Edition*. Topol EJ. ed. WB Saunders, Philadelphia, PA 1994;365-381.

60 Santoian EC, Foegh M, Gravanis MB. Treatment with angiopeptin inhibits the development of smooth muscle proliferation in a balloon overstretch model of restenosis. *Journal of the American College of Cardiology* 1992;**19**:164A(Abstract)

61 Gallo R, Padurean A, Jayaraman T, *et al.* Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. *Circulation* 1999;**99**:2164-2170.

62 Sousa JE, Costa MA, Abizaid A, *et al.* Lack of Neointimal Proliferation After Implantation of Sirolimus-Coated Stents in Human Coronary Arteries : A Quantitative Coronary Angiography and Three-Dimensional Intravascular Ultrasound Study. *Circulation* 2001;**103**:192-195.

63 Miller JM, Ohman EM, Moliterno DJ. Restenosis: The clinical issues. In: *Textbook of interventional Cardiology 3rd Edition.* Topol EJ. ed. 1998;379-415.

64 Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circulation Research* 1995;**76**:412-417.

65 Serruys PW, Foley DP, Jackson G, *et al.* A randomized placebo-controlled trial of fluvastatin for prevention of restenosis after successful coronary balloon angioplasty; final results of the fluvastatin angiographic restenosis (FLARE) trial. *European Heart Journal* 1999;**20**:58-69.

66 Ishiwata S, Nakanishi S, Nishiyama S, Seki A. Prevention of restenosis by bezafibrate after successful coronary angioplasty. *Coronary Artery Disease* 1995;**6**:883-889.

67 Lablanche JM, Grollier G, Lusson JR, *et al.* Effect of the direct nitric oxide donors linsidomine and molsidomine on angiographic restenosis after coronary balloon angioplasty. The ACCORD Study. Angioplastic Coronaire Corvasal Diltiazem. *Circulation* 1997;**95**:83-89.

68 Shechter M, Agranat O, Har-Zahav Y *et al.* Prostaglandin E1 during angioplasty as preventative therapy for coronary restenosis. *American Journal of Therapeutics* 1997;**4**:395-400.

69 Gershlick AH, Spriggins D, Davies SW, *et al.* Failure of epoprostenol (prostacyclin, PGI2) to inhibit platelet aggregation and to prevent restenosis after coronary angioplasty: results of a randomised placebo controlled trial. *British Heart Journal* 1994;**71**:7-15.

70 Cheng LP, Kuwahara M, Jacobsson J, Foegh ML. Inhibition of myointimal hyperplasia and macrophage infiltration by estradiol in aorta allografts. *Transplantation* 1991;**52**:967-972.

71 Engelse MA, Lardenoye JHP, Neele JM *et al.* Activin, A TGF-Beta-like factor, inhibits neointima formation *in vitro* and *in vivo*. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:465-465.(Abstract)

72 Hong MK, Kent KM, Tio FO, *et al.* Single-dose intramuscular administration of sustained-release angiopeptin reduces neointimal hyperplasia in a porcine coronary in-stent restenosis model. *Coronary Artery Disease* 1997;**8**:101-104.

73 Wilson DP, Saward L, Zahradka P, Cheung PK. Angiotensin II receptor antagonists prevent neointimal proliferation in a porcine coronary artery organ culture model. *Cardiovascular Research* 1999;**42**:761-772.

74 Anonymous. Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? Results of the

MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. Multicenter European Research Trial with Cilazapril after Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MERCATOR) Study Group. *Circulation* 1992; **86**:100-110.

75 Schweizer J, Kirch W, Koch R *et al.* Effect of high dose verapamil on restenosis after peripheral angioplasty. *Journal of the American College of Cardiology* 1998;**31**:1299-1305.

76 Jorgensen B, Simonsen S, Endresen K, *et al.* Restenosis and clinical outcome in patients treated with amlodipine after angioplasty: results from the Coronary AngioPlasty Amlodipine REStenosis Study (CAPARES). *Journal of the American College of Cardiology* 2000;**35**:592-599.

77 Sawada N, Itoh H, Ueyama K, *et al.* Inhibition of Rho-Associated Kinase results in suppression of neointimal formation of balloon-injured arteries. *Circulation* 2000;**101**:2030-2034.

78 Tamai H, Katoh O, Suzuki S, *et al.* Impact of tranilast on restenosis after coronary angioplasty: tranilast restenosis following angioplasty trial (TREAT). *American Heart Journal* 1999;**138**:968-975.

79 Ohsawa H, Noike H, Kanai M, *et al.* Preventive effects of an antiallergic drug, pemirolast potassium, on restenosis after percutaneous transluminal coronary angioplasty. *American Heart Journal* 1998;**136**:1081-1087.

80 Waltenberger J, Uecker A, Kroll J, *et al.* A dual inhibitor of platelet-derived growth factor beta-receptor and Src kinase activity potently interferes with motogenic and mitogenic responses to PDGF in vascular smooth muscle cells. A novel candidate for prevention of vascular remodeling. *Circulation Research* 1999;**85**:12-22.

81 Faxon DP, Spiro TE, Minor S, *et al.* Low molecular weight heparin in prevention of restenosis after angioplasty. Results of Enoxaparin Restenosis (ERA) Trial. *Circulation* 1994;**90**:908-914.

82 Ali MN, Mazur W, Kleiman NS, *et al.* Inhibition of coronary restenosis by antithrombin III in atherosclerotic swine. *Coronary Artery Disease* 1996;**7**:851-861.

83 Buchwald AB, Hammerschmidt S, Stevens J *et al.* Inhibition of neointimal proliferation after coronary angioplasty by low-molecular-weight heparin (clivarine) and polyethyleneglycol-hirudin. *Journal of Cardiovascular Pharmacology* 1996;**28**:481-487.

84 Brack MJ, Ray S, Chauhan A, *et al.* The Subcutaneous Heparin and Angioplasty Restenosis Prevention (SHARP) trial. Results of a multicenter randomized trial investigating the effects of high dose unfractionated heparin on angiographic restenosis and clinical outcome. *Journal of the American College of Cardiology* 1995;**26**:947-954.

85 Serruys PW, Herrman JP, Simon R, *et al.* A comparison of hirudin with heparin in the prevention of restenosis after coronary angioplasty. Helvetica Investigators. *New England Journal of Medicine* 1995;**333**:757-763.

86 Berg JM, Kelder JC, Suttorp MJ, *et al.* Effect of Coumarins Started Before Coronary Angioplasty on Acute Complications and Long-Term Follow-Up : A Randomized Trial. *Circulation* 2000;**102**:386-391.

87 Galassi AR, Tamburino C, Nicosia A, *et al.* A randomized comparison of trapidil (triazolopyrimidine), a platelet-derived growth factor antagonist, versus aspirin in

prevention of angiographic restenosis after coronary artery Palmaz-Schatz stent implantation. *Catheterization & Cardiovascular Interventions* 1999;**46**:162-168.

88 Kunishima T, Musha H, Eto F, *et al.* A randomized trial of aspirin versus cilostazol therapy after successful coronary stent implantation. *Clinical Therapeutics* 1997;**19**:1058-1066.

89 Savage MP, Goldberg S, Bove AA, *et al.* Effect of thromboxane A2 blockade on clinical outcome and restenosis after successful coronary angioplasty. Multi-Hospital Eastern Atlantic Restenosis Trial (M-HEART II). *Circulation* 1995;**92**:3194-3200.

90 Serruys PW, Rutsch W, Heyndrickx GR, *et al.* Prevention of restenosis after percutaneous transluminal coronary angioplasty with thromboxane A2-receptor blockade. A randomized, double-blind, placebo-controlled trial. Coronary Artery Restenosis Prevention on Repeated Thromboxane-Antagonism Study (CARPORT). *Circulation* 1991;**84** :1568-1580.

91 Anonymous. Acute platelet inhibition with abciximab does not reduce in-stent restenosis (ERASER study). The ERASER Investigators. *Circulation* 1999;**100**:799-806.

92 de Smet BJGL, de Kleijn DPV, Hanemaiijer R, *et al.* Metalloproteinase inhibition reduces constrictive arterial remodeling after balloon angioplasty: a study in the atherosclerotic Yucatan micropig. *Circulation* 2000;**101**:2962-2967.

93 Gray TJ, Strauss BH, Hinek A. Inhibitory mechanisms by which suramin may attenuate neointimal formation after balloon angioplasty. *Journal of Cardiovascular Pharmacology* 1999;**33**:960-971.

94 Johansen O, Brekke M, Seljeflot I *et al.* N-3 fatty acids do not prevent restenosis after coronary angioplasty: results from the CART study. Coronary Angioplasty Restenosis Trial. *Journal of the American College of Cardiology* 1999;**33**:1619-1626.

95 McKenna CJ, Burke SE, Opgenorth TJ, *et al.* Selective ET(A) receptor antagonism reduces neointimal hyperplasia in a porcine coronary stent model. *Circulation* 1998;**97** :2551-2556.

96 Tardif JC, Cote G, Lesperance J, *et al.* Probucol and multivitamins in the prevention of restenosis after coronary angioplasty. Multivitamins and Probucol Study Group. *New England Journal of Medicine* 1997;**337**:365-372.

97 Squadrito F, Altavilla D, Saporito F, *et al.* IRFI 042, a novel dual vitamin E-like antioxidant stimulates reendothelialization and attenuates intimal hyperplasia after arterial injury. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:469-469.(Abstract)

98 Chen SJ, Chen YF, Miller DM, Li H, Oparil S. Mithramycin inhibits myointimal proliferation after balloon injury of the rat carotid artery in vivo. *Circulation* 1994;**90**:2468-2473.

99 von Essen R, Ostermaier R, Grube E, *et al.* Effects of octreotide treatment on restenosis after coronary angioplasty: results of the VERAS study. VErringerung der Restenoserate nach Angioplastie durch ein Somatostatin-analogon. *Circulation* 1997;**96**:1482-1487.

100 Serruys PW, Foley DP, Hofling B *et al.* Carvedilol for Prevention of Restenosis After Directional Coronary Atherectomy. Final Results of the European Carvedilol Atherectomy Restenosis (EUROCARE) Trial . *Circulation* 2000;**101**:1512-1518.

101 O'Keefe JHJ, McCallister BD, Bateman TM *et al.* Ineffectiveness of colchicine for the prevention of restenosis after coronary angioplasty. *Journal of the American College of Cardiology* 1992;**19**:1597-1600.

102 Lardenoye JHP, Lamfers MLM, de Vries MR *et al. In vivo* suppression of neointima formation by adenovirus-mediated gene transfer of ATF.BPTI, A cell surface directed plasmin inhibitor. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:467-467.(Abstract)

103 Lincoff AM, Topol EJ, Ellis SG. Local drug delivery for the prevention of restenosis. Fact, fancy, and future. *Circulation* 1994;**90**:2070-2084.

104 Pieper M, Serruys PW. Trapidil for prevention of in-stent restenosis - the TRAPIST trial. *The Journal of Heart Disease* 1997;**1**:114-456.(Abstract)

105 Lincoff AM, Weinberger J. Local drug delivery and endovascular radiation. In: *Comprehensive Cardiovascular Medicine*. Topol EJ. ed. 1998;2433-2451.

106 Kimura T, Miyauchi K, Yamagami S *et al.* Local delivery infusion pressure is a key determinant of vascular damage and intimal thickening. *Japanese Circulation Journal* 1998;**62**:299-304.

107 Gunn J, Holt CM, Francis SE, *et al.* The effect of oligonucleotides to c-myb on vascular smooth muscle cell proliferation and neointima formation after porcine coronary angioplasty. *Circulation Research* 1997;**80**:520-531.

108 Mitchel JF, Azrin MA, Fram DB, *et al.* Inhibition of platelet deposition and lysis of intracoronary thrombus during balloon angioplasty using urokinase-coated hydrogel balloons. *Circulation* 1994;**90**:1979-1988.

109 Ibukiyama C. Angiogenesis. Angiogenic therapy using fibroblast growth factors and vascular endothelial growth factors for ischemic vascular lesions. *Japanese Heart Journal.* 1996;**37**:285-300.

110 Tio RA, Scheuermann TH, Lebherz C, Luo Z, Dvorak AM *et al.* Reduction in in-stent restenosis in pig coronary arteries by adenovirus-mediated GAX gene transfer. *XXIst Congress of the European Society of Cardiology* 1999;3257(Abstract)

111 Wilensky RL, March KL, Hathaway DR. Direct intraarterial wall injection of microparticles via a catheter: a potential drug delivery strategy following angioplasty. *American Heart Journal* 1991;**122**:1136-1140.

112 Yoon Y-S, Jang Y, Chung K-H *et al.* Local intraluminal infusion of oestradiol containing liposome in rat carotid balloon injury model. *XXth Congress of the European Society of Cardiology* 1998;(Abstract)

113 Hogrefe K, Swanson N, Baron J *et al.* In-vivo evaluation of locally delivered bispecific antibody inhibiting platelet GPIIb/IIIa receptor and tissue factor. *European Heart Journal* 2000;**21**:164-164.(Abstract)

114 Slepian MJ. Polymeric endoluminal gel paving: therapeutic hydrogel barriers and sustained drug delivery depots for local arterial wall biomanipulation. *Seminars in Interventional Cardiology* 1996;**1**:103-116.

115 Valassis G, Vogel-Wiens C, Gonschior P. Local photodynamic therapy in stented porcine vessels. *XXIst Congress of the European Society of Cardiology* 1999;3256(Abstract)

116 Baumbach A, Oberhoff M, Bohnet A, *et al.* Efficacy of low-molecular-weight heparin delivery with the Dispatch catheter following balloon angioplasty in the rabbit iliac artery. *Catheterization & Cardiovascular Diagnosis* 1997;**41**:303-307.

117 Kornowski R, Hong MK, Tio FO, Choi SK, Bramwell O, Leon MB. A randomized animal study evaluating the efficacies of locally delivered heparin and urokinase for reducing in-stent restenosis. *Coronary Artery Disease* 1997;**8**:293-298.

118 Bartorelli AL, De Cesare NB, Kaplan AV, *et al.* Local heparin delivery prior to coronary stent implantation: acute and six-month clinical and angiographic results. *Catheterization & Cardiovascular Diagnosis* 1997;**42**:313-320.

119 Franzen D, Schannwell M, Oette K, Hopp HW. A prospective, randomized, and double-blind trial on the effect of fish oil on the incidence of restenosis following PTCA. *Catheterization & Cardiovascular Diagnosis* 1993;**28**:301-310.

120 Imanishi T, Arita M, Tomobuchi Y *et al.* Effects of locally administered argatroban on restenosis after balloon angioplasty: experimental and clinical study. *Clinical & Experimental Pharmacology & Physiology* 1997;**24**:800-806.

121 Liu B, Fisher M, Groves PH. Locally-delivered oligodeoxynucleotides to mitogen activated protein kinase downregulate target protein and inhibit neointima formation following balloon angioplasty. *XXIst Congress of the European Society of Cardiology* 1999;P494(Abstract)

122 Iverson P, Skrinska V, Keane E. Local Delivery of antisense phosphorodiamidate compound - restin NG inhibits myointimal hyperplasia following balloon angioplasty. *The Journal of Heart Disease* 1999;**1**:106-422.(Abstract)

123 Ferguson JJ. Meeting Highlights: Highlights of the 20th Congress of the European Society of Cardiology. *Circulation.* 1999 **99**(9):1127-1131.

124 Bailey SR. Local drug delivery: current applications. *Progress in Cardiovascular Diseases* 1997; **40**:183-204.

125 Cox DA, Anderson PG, Roubin GS *et al.* Effect of local delivery of heparin and methotrexate on neointimal proliferation in stented porcine coronary arteries. *Coronary Artery Disease* 1992;**3**:237-248.

126 Herdeg C, Blattner A, Oberhoff M. Local delivery of paclitaxel in the rabbit carotid artery results in reduced neointima formation and vessel enlargement. *European Heart Journal* 1997;**18**:2695(Abstract)

127 Ahn HY, Cho MC, Kim YG *et al.* Prevention of stenosis by local delivery of green tea catechins in rat carotid artery injury model. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:462-462.(Abstract)

128 Gu JL, Pei H, Thomas L, *et al.* Ribozyme-Mediated Inhibition of Rat Leukocyte-Type 12-Lipoxygenase Prevents Intimal Hyperplasia in Balloon-Injured Rat Carotid Arteries. *Circulation* 2001;**103**:1446-1452.

129 Asahara T, Bauters C, Pastore C, *et al.* Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation* 1995;**91**:2793-2801.

130 Laitinen M, Hartikainen J, Hiltunen MO, *et al.* Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. *Human Gene Therapy* 2000;**11**:263-270.

131 Yasuda S, Noguchi T, Gohda M, *et al.* Single low-dose administration of human recombinant hepatocyte growth factor attenuates intimal hyperplasia in a balloon-injured rabbit iliac artery model. *Circulation* 2000;**101**:2546-2553.

132 Hong MK, Bhatti T, Matthews BJ, *et al.* The effect of porous infusion balloon-delivered angiopeptin on myointimal hyperplasia after balloon injury in the rabbit. *Circulation* 1993;**88**:638-648.

133 Breuss JM, Cejna M, Bergmeister H *et al.* Adenoviral gene transfer of I-kappa-B into ballooned rabbit iliac arteries for prevention of restenosis. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:492-492.(Abstract)

134 Fukumoto Y, Shimokawa H, Kozai T, *et al.* Tyrosine kinase inhibitor suppresses the (re) stenotic changes of the coronary artery after balloon injury in pigs. *Cardiovascular Research* 1996;**32**:1131-1140.

135 Meiners S, Laule M, Rother W, *et al.* Ubiquitin-proteasome pathway as a new target for the prevention of restenosis. *Circulation* 2002;**105**:483-489.

136 Zhu NL, Wu L, Liu PX, *et al.* Downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation. *Circulation* 1997;**96**:628-635.

137 Claudio PP, Fratta L, Farina F, *et al.* Adenoviral RB2/p130 gene transfer inhibits smooth muscle cell proliferation and prevents restenosis after angioplasty. *Circulation Research* 1999;**85**:1032-1039.

138 Sinnaeve P, Chiche JD, Gillijns H, *et al.* Overexpression of a constitutively active protein kinase G mutant reduces neointima formation and in-stent restenosis. *Circulation* 2002;**105**:2911-2916.

139 Hu Y, Zou Y, Dietrich H, Wick G, Xu Q. Inhibition of neointima hyperplasia of mouse vein grafts by locally applied suramin. *Circulation* 1999;**100**:861-868.(Abstract)

140 Sebastian M, Honda H, Robbins J, *et al.* Catheter-based delivery of ribozymes targeting PCNA mRNA after stenting reduces neointimal hyperplasia in porcine model. *XXIst Congress of the European Society of Cardiology* 1999;P2728(Abstract)

141 Liu MW, Anderson PG, Luo JF, Roubin GS. Local delivery of ethanol inhibits intimal hyperplasia in pig coronary arteries after balloon injury. *Circulation* 1997;**96**:2295-2301.

142 Kaul S, Terhakopian A, Cercek B *et al.* Intrapericardial Apolipoprotein A-I MILANO reduces intimal and adventitial hyperplasia in porcine coronary overstretch injury model. *48th Annual Scientific Session of the American College of Cardiology* 1999;(Abstract)

143 Herdeg C, Axel DI, Blattner A, *et al.* Assessment of the biological effects of retinoic acid in cell cultures in vitro and in vivo after local delivery in the rabbit carotid artery. *48th Annual Scientific Session of the American College of Cardiology* 1999;(Abstract)

144 Chen D, Asahara T, Krasinski K, *et al.* Antibody blockade of thrombospondin accelerates reendothelialization and reduces neointima formation in balloon-injured rat carotid artery. *Circulation* 1999;**100**:849-854

145 Gruntzig AR, Senning A, Siegenthaler WE. Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. *New England Journal of Medicine* 1979;**301**:61-68.

146 Sigwart U, Puel J, Mirkovitch V *et al.* Intravascular stents to prevent occlusion and restenosis after transluminal angioplasty. *New England Journal of Medicine* 1987;**316**:701-706.

147 Muramatsu T, Tsukahara R, Ho M, *et al.* Clinical outcome of stent implantation in small coronary arteries using different types of coronary stents. *Journal of Invasive Cardiology* 2001;**13**:634-639.

148 Colombo A, Hall P, Nakamura S, *et al.* Intracoronary stenting without anticoagulation accomplished with intravascular ultrasound guidance. *Circulation* 1995;**91**:1676-1688.

149 Betz E. Cell culture systems to study progression and inhibition of intimal proliferations. *Basic Research in Cardiology* 1991;**86**:79-86.

150 Scott NA, Robinson KA, Nunes GL, *et al.* Comparison of the thrombogenicity of stainless steel and tantalum coronary stents. *American Heart Journal* 1995;**129**:866-872.

151 Garasic JM, Squire JC, Edelman ER. Stent and artery geometry determine intimal thickening independent of deep arterial injury. *Circulation.* **101**(7):812

152 Tominaga R, Harasaki H, Sutton C *et al.* Effects of stent design and serum cholesterol level on the restenosis rate in atherosclerotic rabbits. *American Heart Journal* 1993;**126**:1049-1058.

153 Tominaga R, Kambic HE, Emoto H, Harasaki H, Sutton C, Hollman J. Effects of design geometry of intravascular endoprostheses on stenosis rate in normal rabbits. *American Heart Journal* 1992;**123**:21-28.

154 Tseng DY, Rogers C, Edelman ER. Effcts of stent geometry, balloon compliance an deployment pressure on balloon-artery interactions during stetn placement: a finite element anlysis approach. *Circulation* 1997;(Suppl I):I-402-2257.(Abstract)

155 Rogers C, Tseng DY, Squire JC, Edelman ER. Balloon-artery interactions during stent placement: a finite element analysis approach to pressure, compliance, and stent design as contributors to vascular injury. *Circulation Research* 1999;**84**:378-383.

156 Park SW, Park HK, Hong MK, *et al.* Comparison of slotted tube versus coil stent implantation for ostial left anterior descending coronary artery stenosis: initial and late clinical outcomes. *Journal of Korean Medical Science* 1998;**13**:483-487.

157 DePalma VA, Baier RE, Ford JW *et al.* Investigation of three-surface properties of several metals and their relation to blood compatibility. *Journal of Biomedical Materials Research* 1972;**6**:37-75.

158 Hehrlein C, Zimmermann M, Metz J *et al.* Influence of surface texture and charge on the biocompatibility of endovascular stents. *Coronary Artery Disease.* 1995;**6**:581-586.

159 Schomig A, Kastrati A, Neumann FJ. Impact of gold plating on the outcome after coronary stent placement. *Circulation* (Suppl) 1998;855-4486.

160 Seliger C, Schwennicke K, Schaffar C *et al.* Influence of a rough, ceramic-like stent surface made of iridium oxide on neointimal structureand thickening. *European Heart Journal* 2000;**21**:286-286.(Abstract)

161 Windecker S, Mayer I, De Pasquale G, *et al.* Stent Coating With Titanium-Nitride-Oxide for Reduction of Neointimal Hyperplasia. *Circulation* 2001;**104**:928-933.

162 Dolmatch BL, Tio FO, Li XD, Dong YH. Patency and tissue response related to two types of polytetrafluoroethylene-covered stents in the dog. *Journal of Vascular & Interventional Radiology* 1996;**7**:641-649.

163 Tepe G, Duda SH, Hanke H, *et al.* Covered stents for prevention of restenosis. Experimental and clinical results with different stent designs. *Investigative Radiology* 1996;**31**:223-229.

164 Stefanadis C, Toutouzas K, Tsiamis E, *et al.* Total reconstruction of a diseased saphenous vein graft by means of conventional and autologous tissue-coated stents. *Catheterization & Cardiovascular Diagnosis.* 1998;**43**:318-321.

165 Stefanadis C, Tsiamis E, Vlachopoulos C, *et al.* Arterial autologous graft-stent for treatment of coronary artery disease: a new technique. *Catheterization & Cardiovascular Diagnosis.* 1997;**40**:302-307.

166 Heublein B, Ozbek C, Pethig K. Silicon carbide-coated stents: clinical experience in coronary lesions with increased thrombotic risk. *Journal of Endovascular Surgery* 1998;**5**:32-36.

167 Unverdorben M, Schywalsky M, Labahn D. A new silicon carbide coated stent - experience in the rabbit. *European Heart Journal* 1995;**16**:178(Abstract)

168 de Scheerder I, Szilard M, Yanming H, *et al.* Evaluation of the biocompatibility of two new diamond-like stent coatings (Dylyn) in a porcine coronary stent model. *Journal of Invasive Cardiology* 2000;**12**:389-394.

169 King SB, Williams DO, Chougule P, *et al.* Endovascular beta-radiation to reduce restenosis after coronary balloon angioplasty: results of the beta energy restenosis trial (BERT). *Circulation* 1998;**97**:2025-2030.

170 Teirstein PS, Massullo V, Jani S, *et al.* Catheter-based radiotherapy to inhibit restenosis after coronary stenting. *New England Journal of Medicine* 1997;**336**:1697-1703.

171 Fischell TA, Hehrlein C. The radioisotope stent for the prevention of restenosis. *Herz* 1998;**23**:373-379.

172 Hehrlein C, Kubler W. Advantages and limitations of radioactive stents. *Seminars in Interventional Cardiology* 1997;**2**:109-113.

173 Waksman, R. Total occlusion rates across VBT trials. Tenth Endovascular Therapy Course,(Paris). 5-21-1999. Conference Presentation

174 Zidar JP, Lincoff AM, Stack RS. Biodegradable stents. In: Anonymous1994;787-802.

175 Tamai H, Igaki K, Kyo E, *et al.* Initial and 6-Month Results of Biodegradable Poly-l-Lactic Acid Coronary Stents in Humans. *Circulation* 2000;**102**:399-404.

176 Ye YW, Landau C, Willard JE, *et al.* Bioresorbable microporous stents deliver recombinant adenovirus gene transfer vectors to the arterial wall. *Annals of Biomedical Engineering.* 1998;**26**:398-408.

177 Heublein B, Rohde R, Niemeyer M, Kaese V, Hartung W, Rocken C. Degradation of metallic alloys - a new principle in stent technology? *Journal of the American College of Cardiology* 2000;**35**:14-14.(Abstract)

178 Seifert LM, Greer RT. Evaluation of in vivo adsorption of blood elements onto hydrogel-coated silicone rubber by scanning electron microscopy and fourier transform infrared spectroscopy. *Journal of Biomedical Materials Research* 1985;**19**:1043-1071.

179 Fontaine AB, Koelling K, Clay J, *et al.* Decreased platelet adherence of polymer-coated tantalum stents. *Journal of Vascular & Interventional Radiology* 1994;**5**:567-572.

180 van der Giessen WJ, Lincoff AM, Schwartz RS, *et al.* Marked inflammatory sequelae to implantation of biodegradable and nonbiodegradable polymers in porcine coronary arteries. *Circulation* 1996;**94**:1690-1697.

181 Fischell TA. Polymer coatings for stents. Can we judge a stent by its cover? *Circulation* 1996;**94**:1494-1495.

182 Matsuhashi T, Miyachi H, Ishibashi T *et al.* In vivo evaluation of a fluorine-acryl-stylene-urethane-silicone antithrombogenic coating material copolymer for intravascular stents. *Academic Radiology* 1996;**3**:581-588.

183 Hardhammar PA, van Beusekom HM, Emanuelsson HU, *et al.* Reduction in thrombotic events with heparin-coated Palmaz-Schatz stents in normal porcine coronary arteries. *Circulation* 1996;**93**:423-430.

184 Kim SW. Platelet adhesion and prevention at blood-polymer interface. *Artificial Organs* 1987;**11**:228-236.

185 Lansky AJ, Popma JJ, Hanzel GS. Predictors of Late Clinical Outcome After Gianturco-Roubin II Stent Use. Final Results of the GR II Randomized Clinical Trial. *AHA 71st sessions* 1998;3478

186 McKenna CJ, Camrud AR, Sangiorgi G, *et al.* Fibrin-film stenting in a porcine coronary injury model: efficacy and safety compared with uncoated stents. *Journal of the American College of Cardiology* 1998;**31**:1434-1438.

187 Hwang CW, Wu D, Edelman ER. Physiological transport forces govern drug distribution for stent-based delivery. *Circulation* 2001;**104**:600-605.

188 Golomb G, Dixon M, Smith MS *et al.* Controlled-release drug delivery of diphosphonates to inhibit bioprosthetic heart valve calcification: release rate modulation with silicone matrices via drug solubility and membrane coating. *Journal of Pharmaceutical Sciences* 1987;**76**:271-276.

189 Rogers C, Kjelsberg MA, Seifert P, Edelman ER. Heparin-Coated Stents Eliminate Mural Thrombus Deposition for Days Without Affecting Restenosis. *Circulation* 1997;**96** :710-71I.

190 Strecker EP, Gabelmann A, Boos I, *et al.* Effect on intimal hyperplasia of dexamethasone released from coated metal stents compared with non-coated stents in canine femoral arteries. *Cardiovascular & Interventional Radiology* 1998;**21**:487-496.

191 Lincoff AM, Furst JG, Ellis SG *et al.* Sustained local delivery of dexamethasone by a novel intravascular eluting stent to prevent restenosis in the porcine coronary injury model. *Journal of the American College of Cardiology* 1997;**29**:808-816.

192 de Scheerder I, Wang K, Wilczek K, *et al.* Local methylprednisolone inhibition of foreign body response to coated intracoronary stents. *Coronary Artery Disease.* 1996;**7**:161-166.

193 Klugherz BD, Jones PL, Chen W *et al.* Successful gene transfer to swine coronary arteries using a polymer-coated, DNA-eluting stent. *XXIst Congress of the European Society of Cardiology* 1999;1931(Abstract)

194 Lambert TL, Dev V, Rechavia E *et al.* Localized arterial wall drug delivery from a polymer-coated removable metallic stent. Kinetics, distribution, and bioactivity of forskolin. *Circulation* 1994;**90**:1003-1011.

195 Kruse KR, Crowley JJ, Tanguay JF, *et al.* Local drug delivery of argatroban from a polymeric-metallic composite stent reduces platelet deposition in a swine coronary model. *Catheterization & Cardiovascular Interventions* 1999;**46**:503-507.

196 Folts JD, Maalej N, Keaney JF. Palmaz-Schatz stents coated with a NO donor reduces reocclusion when placed in a pig carotid artery for 28 days. *Journal of the American College of Cardiology* 1996;(Supplement A):904-44.

197 Alt E, Haehnel I, Beilharz C, *et al.* Inhibition of Neointima Formation After Experimental Coronary Artery Stenting A New Biodegradable Stent Coating Releasing Hirudin and the Prostacyclin Analogue Iloprost . *Circulation* 2000;**101**:1453-1458.

198 Foo R, Gershlick AH, Hogrefe K, Baron J, Johnson TW, de Bono DP. Inhibition of platelet thrombosis using an activated protein C loaded stent: in vitro and in vivo results. *Thrombosis & Haemostasis* 2000;**83**:496-502.

199 Mir-Akbari H, Sylven C, Lindvall B *et al.* PhVEGF coated stent reduces restenosis intimal hyperplasia. *XXIst Congress of the European Society of Cardiology* 1999;P1536(Abstract)

200 Yamawaki T, Shimokawa H, Kozai T, *et al.* Intramural delivery of a specific tyrosine kinase inhibitor with biodegradable stent suppresses the restenotic changes of the coronary artery in pigs in vivo. *Journal of the American College of Cardiology* 1998;**32**:780-786.

201 Drachman DE, Edelman ER, Kamath KR, *et al.* Sustained stent-based delivery of paclitaxel arrests neointimal thickening and cell proliferation. *Circulation* 1998;**98**:740I(Abstract)

202 Kornowski R, Hong MK, Ragheb A. Slow-release taxol coated GRII stents reduce neointimal formation in a porcine coronary in-stent restenosis model. *Circulation* 1997;(Suppl I):I-341-1900.(Abstract)

203 Oei F, Pomerantzeva I, Eury R, *et al.* Evaluation of *in vivo* release of ^{3}H-labeled QP-2 in rabbit iliac arteries. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000;(Abstract)

204 Carter AJ. Stent-based rapamycin reduces neointima in a 30-day porcine coronary model. *Journal of the American College of Cardiology* 2000;**35** (Suppl A):13A(Abstract)

205 Aggarwal RK, Ireland DC, Azrin MA *et al.* Antithrombotic potential of polymer-coated stents eluting platelet glycoprotein IIb/IIIa receptor antibody. *Circulation* 1996;**94**:3311-3317.

206 Santos RM, Tanguay JF, Crowley JJ, *et al.* Local administration of L-703,081 Using a Composite Polymeric Stent Reduces Platelet Deposition in Canine Coronary Arteries. *American Journal of Cardiology* 1998;**82**:673-675.

207 de Scheerder I, Huber R. New concepts for eluting stents. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000;(Abstract)

208 Leclerc G, Martel R, Vick T *et al.* Optimization of parameters affecting DNA oligonucleotide loading onto phosphorylcholine (PC) coated BiodivYsio drug delivery stent. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000;(Abstract)

209 Sollott SJ, Cheng L, Pauly RR, *et al.* Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. *Journal of Clinical Investigation* 1995;**95**:1869-1876.

210 Axel DI, Kunert W, Goggelmann C, *et al.* Paclitaxel inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using local drug delivery. *Circulation* 1997;**96**:636-645.

211 Heldman AW, Cheng L, Jenkins M, *et al.* Paclitaxel stent coating inhibits neointimal hyperplasia at 4 weeks in a porcine model of coronary restenosis. *Circulation* 2001;**103**:2289-2295.

212 Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *Journal of Clinical Investigation.* 1989;**83**:1774-1777.

213 Maalej N, Albrecht R, Loscalzo J, Folts JD. The potent platelet inhibitory effects of S-nitrosated albumin coating of artificial surfaces. *Journal of the American College of Cardiology* 1999;**33**:1408-1414.

214 Aggarwal RK, Ireland DC, Ragheb A. Platelet adhesion to polymer coated drug eluting stents. An in vitro study. *Clinical Science* 1995;**89(1)**:96-28P.(Abstract)

215 Wohrle J, Al-Khayer E, Grotzinger U, *et al.* Comparison of the heparin coated vs the uncoated Jostent - no influence on restenosis or clinical outcome. *European Heart Journal* 2001;**22**:1808-1816.

216 Barone GW, Conerly JM, Farley PC *et al.* Endothelial injury and vascular dysfunction associated with the Fogarty balloon catheter. *Journal of Vascular Surgery* 1989;**9**:422-425.

217 Grewe PH, Deneke T, Machraoui A *et al.* Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. *Journal of the American College of Cardiology* 2000;**35**:157-163.

218 Menendez JC, Casanova D, Amado JA, *et al.* Effects of radiation on endothelial function. *International Journal of Radiation, Oncology, Biology, Physics.* 1998;**41**:905-913.

219 Hehrlein C, Gollan C, Donges K, *et al.* Low-dose radioactive endovascular stents prevent smooth muscle cell proliferation and neointimal hyperplasia in rabbits. *Circulation* 1995;**92**:1570-1575.

220 Lazarous DF, Shou M, Scheinowitz M, *et al.* Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. *Circulation* 1996;**94**:1074-1082.

221 Waksman R. Late Thrombosis after radiation. *Circulation* 1999;**100**:780-782.

222 Thompson MM, Budd JS, Eady SL, *et al.* Platelet deposition after angioplasty is abolished by restoration of the endothelial cell monolayer. *Journal of Vascular Surgery* 1994;**19**:478-486.

223 van der Giessen W, Serruys PW, Visser WJ. Endothelialization of intravascular stents. *Journal of Investigative Surgery* 1988;**1**:109-120.

224 Dichek DA, Neville RF, Zwiebel JA *et al.* Seeding of intravascular stents with genetically engineered endothelial cells. *Circulation* 1989;**80**:1347-1353.

225 Flugelman MY, Weisz A, Koren B *et al.* Stent-based gene therapy: from transfection efficiency to biological effect. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000;(Abstract)

226 Harada M, Numaguchi Y, Osanai H, *et al.* Catheter-based prostacyclin synthase gene transfer prevents in-stent restenosis in atheromatous rabbits: a potential strategy for therapeutic angiogeneis via vascular endothelial growth factor. *European Heart Journal* 2000;**21**:394-394.(Abstract)

227 Krasinski K, Widmer MB, Yang J, Losordo DW. In vivo blockade of TNF using a soluble receptor accelerates re-endothelialization after arterial balloon injury. *Circulation* 1997; (Suppl):1603(Abstract)

228 Foegh ML, Zhao Y, Farhat M, Ramwell PW. Oestradiol inhibition of vascular myointimal proliferation following immune, chemical and mechanical injury. *Ciba Foundation Symposium* 1995;**191**:139-145.

229 Krasinski K, Spyridopoulos I, Asahara T *et al.* Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation* 1997;**95**:1768-1772.

230 Ohno N, Itoh H, Ikeda T, *et al.* Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. *Circulation* 2002;**105**:1623-1626.

231 Hiltunen MO, Laitinen M, Turunen MP, *et al.* Intravascular Adenovirus-Mediated VEGF-C Gene Transfer Reduces Neointima Formation in Balloon-Denuded Rabbit Aorta. *Circulation* 2000;**102**:2262-2268.

232 Senger DR, Galli SJ, Dvorak AM *et al.* Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;**219**:983-985.

233 Favard C, Moukadiri H, Dorey C *et al.* Purification and biological properties of vasculotropin, a new angiogenic cytokine. *Biology of the Cell* 1991;**73**:1-6.

234 Belgore FM, Mzimba ZS, Yip GYH *et al.* Localisation of the vascular endothelial growth factor A,B,C and their receptors in human atherosclerotic arteries. *Journal of the American College of Cardiology* 2001;**37**:1A-648A.(Abstract)

235 de Vries C, Escobedo JA, Ueno H *et al.* The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992;**255** :989-991.

236 Millauer B, Wizigmann-Voos S, Schnurch H, *et al.* High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993;**72**:835-846.

237 Takahashi T, Yamaguchi S, Shibuya M. The autophosphorylation of 1175-tyrosine residue on KDR/Flk-1 (VEGF receptor-2) is essential for the activation of PLC-γ and MAP kinase pathway for DNA synthesis in vascular endothelial cells. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:437-437.(Abstract)

238 Joukov V, Pajusola K, Kaipainen A, *et al.* A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO Journal* 1996;**15**:1751

239 Achen MG, Jeltsch M, Kukk E, *et al.* Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4).

Proceedings of the National Academy of Sciences of the United States of America 1998; **95**:548-553.

240 Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;**92**:735-745.

241 Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB Journal* 1999;**13**:9-22.

242 Murohara T, Horowitz JR, Silver M, *et al.* Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation* 1998;**97**:99-107.

243 Tsurumi Y, Murohara T, Krasinski K, *et al.* Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. *Nature Medicine* 1997;**3**:879-886.

244 van der Zee R, Murohara T, Luo Z, *et al.* Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 1997;**95**:1030-1037.

245 Martin JF. VEGF gene transfer reduces intimal thickening via increased production of nitric oxide in carotid arteries. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:490-490.(Abstract)

246 Zachary I, Mathur A, Yla-Herttuala S, Martin J. Vascular protection: A novel nonangiogenic cardiovascular role for vascular endothelial growth factor. *Arteriosclerosis, Thrombosis & Vascular Biology* 2000;**20**:1512-1520.

247 Maloney JP, Silliman CC, Ambruso DR *et al.* In vitro release of vascular endothelial growth factor during platelet aggregation. *American Journal of Physiology* 1998;**275**:H1054-H1061

248 Carmeliet P, Ferreira V, Breier G, *et al.* Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;**380**:435-439.

249 Bikfalvi A, Sauzeau C, Moukadiri H, *et al.* Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: binding, internalization, degradation, and biological effects. *Journal of Cellular Physiology.* 1991;**149**:50-59.

250 Paques M, Massin P, Gaudric A. Growth factors and diabetic retinopathy. *Diabetes & Metabolism* 1997;**23**:125-130.

251 Keyt BA, Nguyen HV, Berleau LT, *et al.* Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *Journal of Biological Chemistry.* 1996;**271**:5638-5646.

252 Sirois MG, Edelman ER. Vascular Endothelial Growth Factor effect on vascular permeability is mediated by platelet-activating factor. *Circulation* 1997;(Suppl I):973(Abstract)

253 Christofori G, Naik P, Hanahan D. Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. *Molecular Endocrinology* 1995;**9**:1760-1770.

254 Matsuura A, Yamochi W, Hirata K *et al.* Stimulatory interaction between vascular endothelial growth factor and endothelin-1 on each gene expression. *Hypertension* 1998;**32**:89-95.

255 Ku DD, Zaleski JK, Liu S, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *American Journal of Physiology.* 1993;**265**:t-92

256 Kroll J, Waltenberger J. A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. *Biochemical & Biophysical Research Communications* 1999;**265**:636-639.

257 Clauss M, Gerlach M, Gerlach H, *et al.* Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *Journal of Experimental Medicine* 1990;**172**:1535-1545.

258 Bikfalvi A, Han ZC. Angiogenic factors are hematopoietic growth factors and vice versa. *Leukemia* 1994;**8**:523-529.

259 Shalaby F, Rossant J, Yamaguchi TP, *et al.* Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;**376**:62-66.

260 Pepper MS, Ferrara N, Orci L, Montesano R. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochemical & Biophysical Research Communications.* 1991;**181**:902-906.

261 Asahara T, Chen D, Tsurumi Y, *et al.* Accelerated restitution of endothelial integrity and endothelium-dependent function after phVEGF165 gene transfer. *Circulation* 1996;**94**:3291-3302.

262 Bauters C, Asahara T, Zheng LP, *et al.* Recovery of disturbed endothelium-dependent flow in the collateral-perfused rabbit ischemic hindlimb after administration of vascular endothelial growth factor. *Circulation* 1995;**91**:2802-2809.

263 Yang W, de Bono DP. A new role for vascular endothelial growth factor and fibroblast growth factors: increasing endothelial resistance to oxidative stress. *FEBS Letters.* 1997; **403**:139-142.

264 Cohen T, Nahari D, Cerem LW *et al.* Interleukin 6 induces the expression of vascular endothelial growth factor. *Journal of Biological Chemistry.* 1996;**271**:736-741.

265 Warren RS, Yuan H, Matli MR *et al.* Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. *Journal of Biological Chemistry* 1996;**271**:29483-29488.

266 Okuda Y, Tsurumaru K, Suzuki S, *et al.* Hypoxia and endothelin-1 induce VEGF production in human vascular smooth muscle cells. *Life Sciences* 1998;**63**:477-484.

267 Deroanne CF, Hajitou A, Calberg-Bacq CM *et al.* Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. *Cancer Research* 1997;**57**:5590-5597.

268 Pertovaara L, Kaipainen A, Mustonen T, *et al.* Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *Journal of Biological Chemistry* 1994;**269**:6271-6274.

269 Kronemann N, Bouloumie A, Bassus S *et al.* Aggregating human platelets stimulate the expression of vascular endothelial growth factor in cultured vascular smooth muscle cells through a synergistic effect of transforming growth factor beta and platelet derived growth factor AB. *Circulation* 1999;**100**:855-860.(Abstract)

270 Pepper MS, Mandriota SJ. Regulation of vascular endothelial growth factor receptor-2 (Flk-1) expression in vascular endothelial cells. *Experimental Cell Research* 1998;**241**:414-425.

271 Giraudo E, Primo L, Audero E, *et al.* Tumor necrosis factor-alpha regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. *Journal of Biological Chemistry.* 1998;**273**:22128-22135.

272 Mandriota SJ, Menoud PA, Pepper MS. Transforming growth factor beta 1 down-regulates vascular endothelial growth factor receptor 2/flk-1 expression in vascular endothelial cells. *Journal of Biological Chemistry.* 1996;**271**:11500-11505.

273 Namiki A, Brogi E, Kearney M, *et al.* Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *Journal of Biological Chemistry.* 1995;**270**:31189-31195.

274 Forsythe JA, Jiang BH, Iyer NV, *et al.* Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Molecular & Cellular Biology* 1996;**16**:4604-4613.

275 Natarajan R, Bai W, Lanting L *et al.* Effects of high glucose on vascular endothelial growth factor expression in vascular smooth muscle cells. *American Journal of Physiology* 1997;**273**:H2224-H2231

276 Levy AP, Levy NS, Loscalzo J, *et al.* Regulation of vascular endothelial growth factor in cardiac myocytes. *Circulation Research* 1995;**76**:758-766.

277 Ruef J, Hu ZY, Yin LY, *et al.* Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circulation Research* 1997;**81**:24-33.

278 Ramos MA, Kuzuya M, Esaki T, *et al.* Induction of macrophage VEGF in response to oxidized LDL and VEGF accumulation in human atherosclerotic lesions. *Arteriosclerosis, Thrombosis & Vascular Biology.* 1998;**18**:1188-1196.

279 Patterson C, Marschall RS. Therapeutic angiogenesis. *Circulation* 1999;**99**:2614-2616.

280 Annex BH, Torgan CE, Lin P, *et al.* Induction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle. *American Journal of Physiology.* 1998;**274**:t-7

281 Williams B. Mechanical influences on vascular smooth muscle cell function. *Journal of Hypertension* 1998;**16**:t-9

282 Li J, Hampton T, Morgan JP, Simons M. Stretch-induced VEGF expression in the heart. *Journal of Clinical Investigation* 1997;**100**:18-24.

283 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocrine Reviews.* 1997;**18**:4-25.

284 Maeda K, Chung YS, Ogawa Y, *et al.* Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996;**77**:858-863.

285 Benjamin LE, Golijanin D, Itin A *et al.* Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *Journal of Clinical Investigation.* 1999;**103**:159-165.

286 Unterberg C, Stevens J, Nebendahl K, Buchwald AB. Temporal and spatial pattern of VEGF and KDR/FLK expression in reendothelialization and neovascularisations after coronary angioplasty. *XXIst Congress of the European Society of Cardiology* 1999;3253(Abstract)

287 Luo Z, Asahara T, Tsurumi Y *et al.* Reduction of vein graft intimal hyperplasia and preservation of endothelium-dependent relaxation by topical vascular endothelial growth factor. *Journal of Vascular Surgery* 1998;**27**:167-173.

288 Iwaguro H, Yamaguchi J, Kalka C, *et al.* Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation* 2002;**105**:732-738.

289 Lindner V, Reidy MA. Expression of VEGF receptors in arteries after endothelial injury and lack of increased endothelial regrowth in response to VEGF. *Arteriosclerosis, Thrombosis & Vascular.Biology.* 1996;**16**:1399-1405.

290 Belli G, Eccleston MC, Horrigan JG. Locally delivered vascular endothelial growth factor (VEGF) does not inibit neointimal proliferation in the porcine restenosis model. *Journal of the American College of Cardiology* 1997;Abstracts:52A

291 Lemstrom KB, Krebs R, Nykanen AI, *et al.* Vascular endothelial growth factor enhances cardiac allograft arteriosclerosis. *Circulation* 2002;**105**:2524-2530.

292 Zhao Q, Egashira K, Inoue S, *et al.* Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation* 2002;**105**:1110-1115.

293 Van Belle E, Tio FO, Couffinhal T, Maillard L, Passeri J, Isner JM. Stent endothelialization. Time course, impact of local catheter delivery, feasibility of recombinant protein administration, and response to cytokine expedition. *Circulation* 1997;**95**:438-448.

294 Unemori EN, Ferrara N, Bauer EA, Amento EP. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *Journal of Cellular Physiology* 1992;**153**:557-562.

295 Senger DR, Claffey KP, Benes JE *et al.* Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. P*roceedings of the National Academy of Sciences of the United States of America.* 1997;**94**:13612-13617.

296 Pels K, Labinaz M, Miller H *et al.* Coronary artery expression of VEGF after balloon angioplasty correlates with vasa vasorum angiogenesis: potential role in arterial remodelling. *XXIst Congress of the European Society of Cardiology* 1999;P535(Abstract)

297 El Gendi H, Violaris AG, Panikker S *et al.* Endogenous, local, vascular endothelial growth factor production in patients with chronic total coronary occlusion: further evidence of its role in angiogenesis. *XXIst Congress of the European Society of Cardiology* 1999;210(Abstract)

298 Inoue M, Itoh H, Ueda M, *et al.* Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation* 1998;**98**:2108-2116.

299 Takeshita S, Zheng LP, Brogi E, *et al.* Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *Journal of Clinical Investigation* 1994;**93**:662-670.

300 Bauters C, Asahara T, Zheng LP, *et al.* Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. *American Journal of Physiology* 1994;**267**:H1263-H1271

301 Hopkins SP, Bulgrin JP, Sims RL *et al.* Controlled delivery of vascular endothelial growth factor promotes neovascularization and maintains limb function in a rabbit model of ischemia. *Journal of Vascular Surgery* 1998;**27**:886-894.

302 Takeshita S, Tsurumi Y, Couffinahl T, *et al.* Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo. *Laboratory Investigation.* 1996;**75**:487-501.

303 Pearlman JD, Hibberd MG, Chuang ML, *et al.* Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nature Medicine* 1995;**1**:1085-1089.

304 Hariawala MD, Horowitz JR, Esakof D, *et al.* VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *Journal of Surgical Research* 1996;**63**:77-82.

305 Sellke FW, Wang SY, Stamler A *et al.* Enhanced microvascular relaxations to VEGF and bFGF in chronically ischemic porcine myocardium. *American Journal of Physiology.* 1996;**271**:t-20

306 Lopez JJ, Laham RJ, Carrozza JP, *et al.* Hemodynamic effects of intracoronary VEGF delivery: evidence of tachyphylaxis and NO dependence of response. *American Journal of Physiology.* 1997;**273**:t-23

307 Banai S, Jaklitsch MT, Shou M, *et al.* Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation* 1994;**89**:2183-2189.

308 Sayeed-Shah U, Mann MJ, Martin J. Combined gene therapy with TMR improves hemodynamic performance in chronically ischemic porcine hearts. *American Heart Association* 1998;1125(Abstract)

309 Pepper MS, Ferrara N, Orci L, Montesano R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. *Biochemical & Biophysical Research Communications.* 1992;**189**:824-831.

310 Asahara T, Bauters C, Zheng LP, *et al.* Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation* 1995;**92**:(Suppl)-71

311 Takeshita S, Pu LQ, Stein LA, *et al.* Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. *Circulation* 1994;**90**:II228-II234

312 Tsurumi Y, Takeshita S, Chen D, *et al.* Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation* 1996;**94**:3281-3290.

313 Glennon PE, Clarke SC, Kume SM, *et al.* Catheter-based endomyocardial injection of viral or plasmid DNA in vivo stimulates local VEGF production irrespective of the gene delivered. *European Heart Journal* 2000;**21**:58-58.(Abstract)

314 Partyka L, Dulak J, Sobhian B *et al.* Intramuscular gene transfer of VEGF165 in rabbit model of hind-limb ischemia. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:435-435.(Abstract)

315 O'Connor DS, Schechner JS, Adida C, *et al.* Control of apoptosis during angiogenesis by survivin expression in endothelial cells. *American Journal of Pathology* 2000;**156**:393-398.

316 Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *Journal of Biological Chemistry* 1998;**273**:13313-13316.

317 Fava RA, Olsen NJ, Spencer-Green G, *et al.* Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *Journal of Experimental Medicine* 1994;**180**:341-346.

318 Baumgartner I, Pieczek A, Manor O, *et al.* Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 1998;**97**:1114-1123.

319 Henry TD, Rocha-Singh K, Isner JM. Results of intracoronary recombinant human vascular endothelial growth factor (rhVEGF) administration trial. *Journal of the American College of Cardiology* 1998;(Supplements):65A

320 Henry TD. VIVA (vascular endothelial growth Factor in ischemia for vascular angiogenesis) *Clinical Cardiology* 1999;**22**:369(Abstract)

321 Rosengart TK, Lee LY, Patel SR. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation* 1999;**100**:468-474.

322 Symes JF, Losordo DW, Vale PR, *et al.* Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Annals of Thoracic Surgery* 1999;**68**:830-836.

323 Losordo DW, Vale PR, Hendel RC, *et al.* Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation* 2002;**105**:2012-2018.

324 Hedman M, Hartikainen J, Syvanne M, *et al.* Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia. *Circulation* 2003;**107**:2677-2683.

325 Enholm B, Paavonen K, Ristimaki A, *et al.* Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* 1997;**14**:2475-2483.

326 Aase K, von Euler G, Li XD, *et al.* Vascular Endothelial growth factor-B- deficient mice display an atrial conduction defect. *Circulation* 2001;**104**:358-359.

327 Lymboussaki A, Partanen TA, Olofsson B, *et al.* Expression of the vascular endothelial growth factor C receptor VEGFR-3 in lymphatic endothelium of the skin and in vascular tumors. *American Journal of Pathology* 1998;**153**:395-403.

328 Witzenbichler B, Asahara T, Murohara T, *et al.* Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *American Journal of Pathology* 1998;**153**:381-394.

329 Joukov V, Kaipainen A, Jeltsch M, *et al.* Vascular endothelial growth factors VEGF-B and VEGF-C. *Journal of Cellular Physiology.* 1997;**173**:211-215.

330 Kiba A, Ogawa S, Yamaguchi S, Shibuya M. Mapping of the residues involved in receptor interaction of VEGF-ENZ7 for VEGF receptor-2/KDR. *Journal of Submicroscopic Cytology & Pathology* 2000;**32**:453-453.(Abstract)

331 Risau W, Drexler H, Mironov V, *et al.* Platelet-derived growth factor is angiogenic in vivo. *Growth Factors* 1992;**7**:261-266.

332 Cao Y, Linden P, Shima D, Browne F, Folkman J. In vivo angiogenic activity and hypoxia induction of heterodimers of placenta growth factor/vascular endothelial growth factor. *Journal of Clinical Investigation.* 1996;**98**:2507-2511.

Chapter 2.

Absorption and elution studies of VEGF.

2.1 Background

VEGF was chosen for study since it has been demonstrated to accelerate the re-endothelialisation process after PCI, when delivered locally. It has not however been investigated as a stent-based therapy. The experiments detailed in this chapter are concerned with the study of how VEGF is absorbed into and released from the polymer of the Cook Supra G stent (West Lafayette, IN 47906). 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.

2.1.1 Passive absorption

The absorption and release of drugs into the polymer coating of a stent depends on the property of passive absorption. Passive absorption is the adhesion 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 longer periods of time, up to many days[1].

2.1.2 Experimental design

2.1.2.1 Stent structure

Since absorption and elution curves were dependent on the polymer present, it was decided to examine the stent/polymer structure more closely using scanning electron microscopy.

2.1.2.2 Absorption and elution experiments.

Preliminary assessment of the optimum conditions of VEGF loading onto polymer-coated stents looked at: optimum concentration of VEGF solution; pH of buffer solution used to dilute VEGF; length of immersion in VEGF. Next, stent sections were absorbed with VEGF and then were immersed in buffer solution for various lengths of time. This system is described as "simple" since stents were not actively perfused. Elution occurred by diffusion. To try and mimic the *in vivo* situation, a perfusion circuit was built, a modification of previously published work[2]. This placed the stents in tubing of internal diameter similar to the coronary arteries and perfused them with a perfusate containing physiological concentrations of protein at normal pH and temperatures. Finally, the elution of VEGF from a polymer-coated stent was determined in an *ex vivo* model. This was to determine whether the VEGF-coated stent would release the drug into the tissue or merely into the perfusion fluid. The tissue, in this case internal mammary artery obtained from patients undergoing a bypass operation, was stented with the VEGF-eluting stent and perfused for 24 hours.

2.2 Methods and materials

2.2.1 Characteristics of the Supra G, polymer-coated stent.

This is a hoop and flex design stent (*see fig. 2.1*) which can easily be divided into hoop and flex sections. The stent is coated with a 10-20 micron thickness polymer coating of a linear chain of aromatic hydrocarbons. (*Personal Communication Dr A Ragheb, Cook Inc, IN, USA*). The process by which this is bonded to the stent remains a commercial secret. The volume of polymer on each hoop section is 0.31-0.62mm^3 or 0.22-0.44mm^3 for the two sizes available (*fig.2.2*).

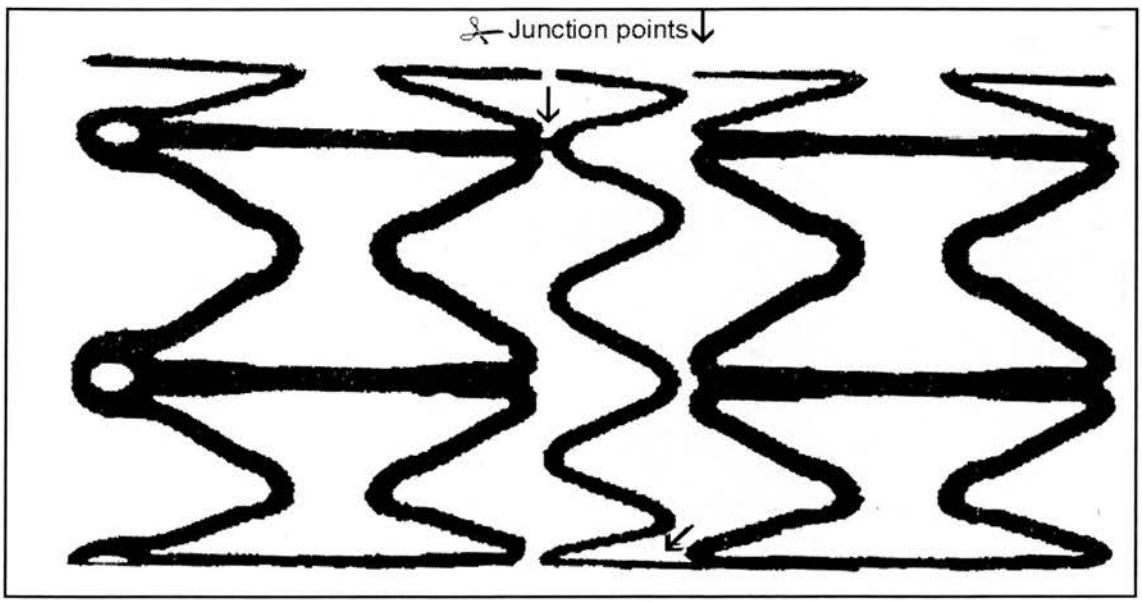


Figure 2.1. Diagram showing hoop and flex design of Supra G stent.

Stent size (mm)	Hoop Sections			Flex Sections		
	n	Lumenal Area ($cm^{2)}$	Total Area (cm^2)	n	Lumenal Area ($cm^{2)}$	Total Area (cm^2)
4.0x20	5	0.10	0.31	4	0.03	0.1
3.0x20	6	0.07	0.22	5	0.02	0.09

Figure 2.2. Table of areas of sections of Supra G stent. (Personal communication from Dr Ragheb, Cook Inc.)

2.2.2 Radiolabelling of VEGF - Iodogen method.

Radiolabelling was chosen as the means to quantify the absorption and subsequent rate of release of the VEGF. The Iodogen method employs a gentle solid-phase oxidising agent and is preferable to chloramine-T for proteins sensitive to chemical damage[3]. 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. The radiolabel used was Iodine-125, a gamma emitting isotope.

VEGF was diluted to 1mg/ml in borate-saline buffer (BBS) pH 8.5, ionic strength 0.1. 30 Eppendorf tubes and disposable test tubes were labelled. Iodogen (1,3,4,6-tetrachloro-3α, 6α-DI-phenylglycouril) was dissolved in chloroform to make a concentration of 0.1mg/ml (e.g. 2mg Iodogen in 20ml chloroform). 0.2ml (20μg Iodogen) was added to ten Eppendorf tubes. Chloroform was evaporated in a stream of nitrogen, rotating the tubes to leave a film of residue on the inside of the tube. Tubes were removed onto ice and stored in fridge. A Sephadex-G PD-10 (Pharmacia) column was equilibrated with PBS. Non-specific binding was blocked with 0.25% gelatin in PBS. The column was washed through again with PBS twice.

The iodination procedures were done in a shielded isotope room with the hood fan on. 200μl of VEGF (i.e. 200μg of VEGF) was aliquotted into an Eppendorf tube on ice. 200μl of VEGF was added to the reaction tube. The reaction was initiated with 4μl (0.25mCi) of $Na^{125}I$ and incubated for 20min. The resulting solution was decanted into a new tube and left for another 10min for reactive iodine to decay. Labelled protein and free iodine were separated by running the reaction mixture through the gel column. PBS was added to the column to produce elution. Eluent drops were collected into the numbered Eppendorf tubes, the first ten drops into the first two tubes then five to seven drops in the rest.

5μl of each fraction from the numbered Eppendorf tubes was placed, with the pipette tip, into the corresponding numbered disposable test tube. These were counted in the automated gamma counter and the five tubes containing the maximum amount of labelled activity selected for storage/use. Labelled VEGF was stored in lead containers in a 4°C fridge. An example of the radiolabelling process is shown (*fig 2.3*).

Later experiments were done with previously labelled VEGF. This allowed the amount of radiation used to be minimised.

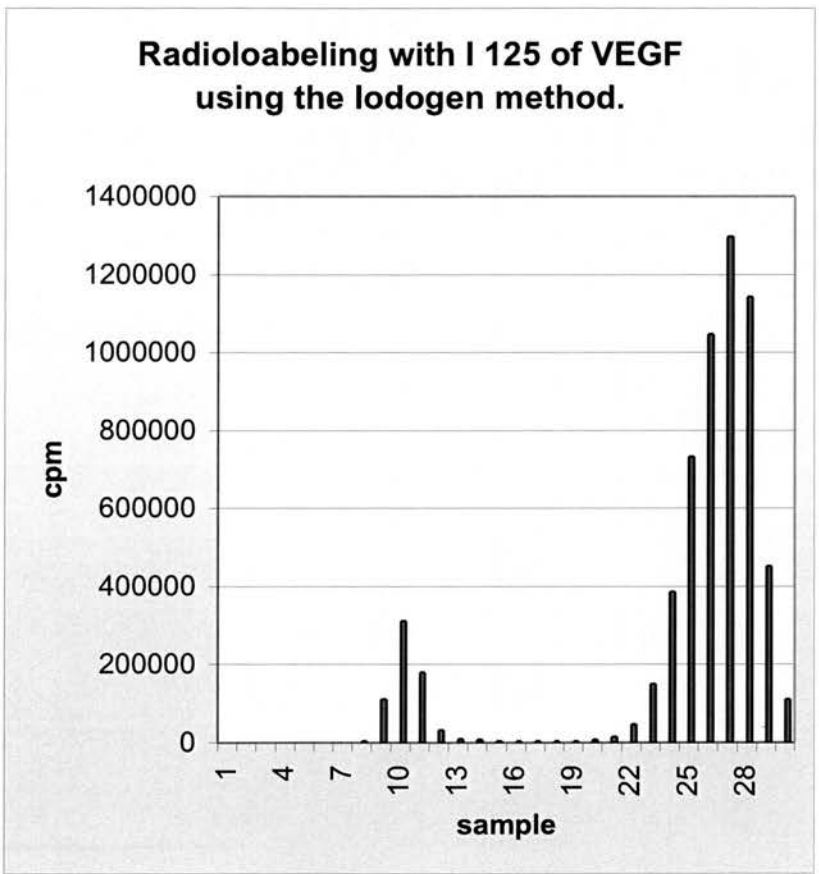


Figure 2.3 "Spiking" of VEGF with radiolabelled Iodine. The first peak represents labelled protein, the second is free Iodine, which is discarded.

2.2.3 Absorption studies.

2.2.3.1 Absorption Kinetics of VEGF (Variation with pH of solution).

Three buffer solutions were used:

"Enzyme Buffer"	pH	4.5
PBS	pH	7.4
BBS	pH	8.5

(For composition of buffers see Appendix)

450µl of each buffer was placed into an Eppendorf. 50µl of 5mg/ml VEGF solution was added to the Eppendorfs. This gave 500µl at a final concentration of 0.5mg/ml of VEGF in each buffer solution. To each 500µl was added 5µl of radiolabelled VEGF as a "spike". Sections of Supra G stents were obtained by cutting them with a scalpel blade at the junction points where the thick hoop and thinner flex sections intersected (*see fig 2.1*). This was partly to conserve limited supplies of stents and also because longer sections of stent had a tendency to retain large droplets of VEGF solution within the lumen of the stent. Each thick hoop section was weighed to allow for differences among them in the way they were cut. Hoop sections (n=6) were placed into Eppendorfs again. To these Eppendorfs 80µl of VEGF/buffer solution was added. A control was used for each buffer to allow for a background radiation estimate to be made in the absence of a "spike". 5µl of the remaining buffer solution were assayed for its radioactivity. This allowed for a correlation to be made between the counts recorded and the amount of VEGF these represented. Each 5µl contained 2.5µg of unlabelled VEGF. All samples were left at room temperature for two hours to allow the VEGF to passively absorb onto the stents. After two hours, each stent section was removed from the radiolabelled solutions and washed in PBS and then placed in a test tube. All 24 test tubes (including controls) were measured in a gamma counter on two occasions. The counts were adjusted to account for the small differences in the weights of the hoops (A typical 3 x 20mm stent as used in clinical practice weighs about 55mg). An average of all the values for each buffer solution was obtained.

2.2.3.2 Absorption Kinetics of VEGF (Variation with concentration).

Concentrations of VEGF were made up in PBS (pH7.4) to give final concentrations of 0.5mg/ml, 1mg/ml and 2mg/ml. These doses were chosen because previous published work with absorbed proteins (e.g. urokinase and abciximab) into similar polymers had showed maximal absorption at a concentration of 1mg/ml[1; 4], (MD thesis of Dr. Rajesh Aggarwal). Each 300µl of diluted VEGF was "spiked" as before with 10µl of radiolabelled VEGF. Nine hoop sections were placed into Eppendorfs. To these Eppendorfs 100µl of VEGF/buffer solution was added to produce three samples for each VEGF concentration. A control was used for each concentration to allow for a background radiation estimate to be made in the absence of a "spike". Two 5µl aliquots of the remaining VEGF solution at each concentration were assayed for their radioactivity. Stent hoops were removed from the radiolabelled solutions and washed in PBS and then placed in test tubes. These were measured in a gamma counter on two occasions. A second hoop was placed into the same "spiked" solution of VEGF for each time point and concentration in each Eppendorf. The counts were adjusted to account for the weights of the hoops and an average of all the corrected values for each concentration/time point was obtained.

2.2.3.3 Absorption kinetics of VEGF (Variation with Time).

The radio-iodinated protein was diluted to 2mg/ml in PBS buffer, pH 7.4, maintained at room temperature. Polymer-coated stent hoops of known weight were immersed in each solution for 72, 48, 24 & 2 hours (n=3). All stent segments were then removed and counted in a gamma well counter to quantify protein absorption. The readings were adjusted for background and weight of stent sections to give a final reading of micrograms of VEGF per stent.

2.2.4 Elution studies.

2.2.4.1 Simple elution of VEGF.

Four stent hoops of known weight were immersed in 2mg/ml VEGF solution in PBS for 24 hours at room temperature. The hoops were removed, excess VEGF solution shaken off and surface VEGF solution removed by running the hoop section over filter paper until dry. The hoops were counted in the gamma counter twice as before to give a baseline reading. The

hoops were immersed in PBS, 2ml per hoop each. After 5 and 15 minutes and 1, 2, 24, 48, 72, 96, 120 and 192 hours, the hoops were removed from the solution, excess solution shaken off and counted twice in the gamma counter. The readings were adjusted for background and for the weight of the hoops.

2.2.4.2 Elution of VEGF in a perfusion circuit.

Stent hoops were immersed in 2mg/ml solutions of radiolabelled VEGF for 24 or 48 hours. Hoops were rinsed with 5ml of PBS and gamma-counted. The hoops (n=3, with each absorption time) were then perfused continuously at 20ml/min in a closed loop circuit (*fig. 2.4*) with PBS containing 4% bovine serum albumin. Pairs of stent hoops were placed into circuits in polypropylene tubes with a total volume of perfusing solution in the circuit of 100ml. Circuits were maintained at 37°C by immersing the reservoirs containing PBS/BSA in a water bath (Grant Instruments). Three separate circuits were set up for a total of six hoops. The PBS was pumped through the circuit using a peristaltic pump (Watson-Marlow 302S, Falmouth, U.K.). Sterile silicone tubing (3mm bore, Fisons, Loughborough, U.K.) was used to carry the perfusate to the stent hoops. After 5 and 15 minutes, and 1, 3, 8, and 24 hours all hoops were removed from the perfusion circuit and counted twice in a gamma well counter to quantify the amount of VEGF remaining bound to each, and then returned to the perfusion circuit. This was repeated for nine days. On each day the standards containing a known amount of VEGF were also counted so that all the results could be adjusted to account for radioactive decay of the Iodine label.

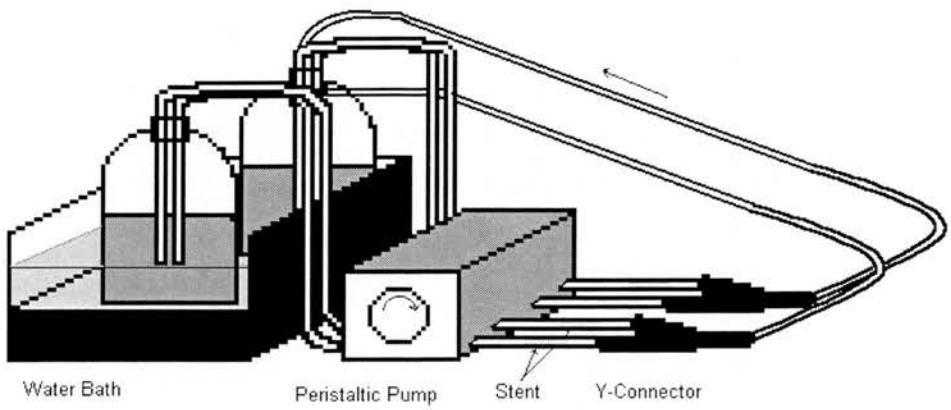


Figure 2.4. Schematic of perfusion circuit used in elution experiments.

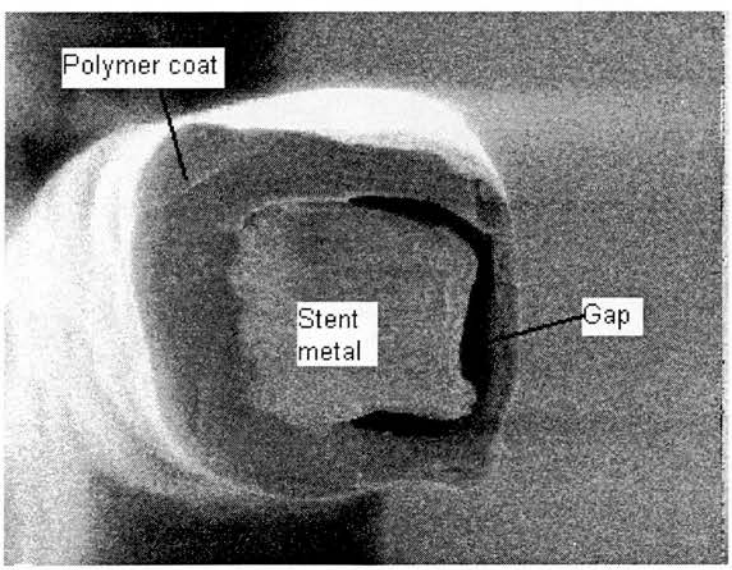


Figure 2.5. Scanning electron microscopy at 15kV of Supra G structure in cross section. Note variability in polymer thickness and large gap.

2.2.4.3 *Ex vivo* assessment of VEGF-elution in perfusion circuit.

Sections of polymer-coated stents were immersed in "spiked" 2mg/ml VEGF solution. These were then left for 24-72 hours to absorb VEGF passively. Sections of stent were then air-dried and gamma-counted. Sections of stent were then mounted onto an angioplasty balloon catheter (Cook). Separately, sections of internal mammary artery were obtained, with verbal prior consent, from patients undergoing coronary bypass grafting. The samples were the free ends of the dissected artery, which is normally discarded as waste. These arterial sections were typically 1-2cm in length and 2-3mm in internal diameter. Internal size was estimated visually. Samples were transported in DMEM and excess adventitia or adherent clots were removed carefully. All clips used during the operation to seal side-branches of the vessel were removed. Each arterial section was cut to a length equal to the stent segments. Using a (Cook) angioplasty catheter, a single inflation to eight atmospheres for one minute was performed in the tissue. This tissue then had the radiolabelled VEGF-soaked stent deployed within it, inside a length of polypropylene tubing of appropriate internal diameter. The stent was thus apposed to the tissue of the vessel wall. The short length of tubing, containing a stented piece of vessel, was then placed in the perfusion circuit for 24hr. Sections were removed and the tissue dissected free of the stent section. The tissue and the stent section were then counted in the gamma-counter. Controls for background, and samples of known VEGF content from the initial solution were also counted. Finally, 1ml aliquots of the residual PBS/BSA solution were counted to assess how much VEGF had been washed off into the total circulating volume. Proportions of radioactivity remaining in stent and vessel were then calculated relative to the initial radioactivity on the stent.

2.2.4.4 Data analysis

Absorption data were summarised as means ± standard deviation. Significance was established using Student's t-test. Elution kinetics were ascertained by nonlinear regression (curve fitting) using a computerised scientific data analysis package (Prism, GraphPad Software Inc., CA, USA). R^2 values were determined for each fitted curve to assess the overall goodness-of-fit and analysis of residuals performed to assess whether observed data were consistent with the chosen regression equation. A p value of >0.05 for analysis of residuals was considered to be consistent with non-significant deviation from the model.

2.3 Results

2.3.1 Results of passive absorption of VEGF to polymer-coated stent sections.

2.3.1.1 pH studies.

No significant differences in the absorption rates of VEGF were seen amongst the three pH conditions used (*see fig. 2.6*). Further work was all done on VEGF diluted with PBS. No clear advantage to using this buffer was demonstrated over the others. It was used largely as it has the same pH as blood and might therefore be less likely to denature or damage the VEGF protein.

2.3.1.2 Concentration of VEGF.

This experiment established that an increase in the concentration did result in an increased absorption of the drug. Higher concentrations of drug were not used in order to reduce wastage. 2mg/ml was used in further experiments (*see fig. 2.7*).

2.3.1.3 Absorption of VEGF with variation of time of immersion.

Increasing the length of time that the stent sections were immersed in VEGF solution showed an initial rapid rise in the amount of VEGF absorbed. After two hours, however, no further significant immobilisation of protein to the stents was seen. Further work was done with at least two hours of immersion time. A maximum of 21.7±1.1μg of VEGF was absorbed onto a whole stent (55mg) according to these studies over time (*see fig. 2.8*).

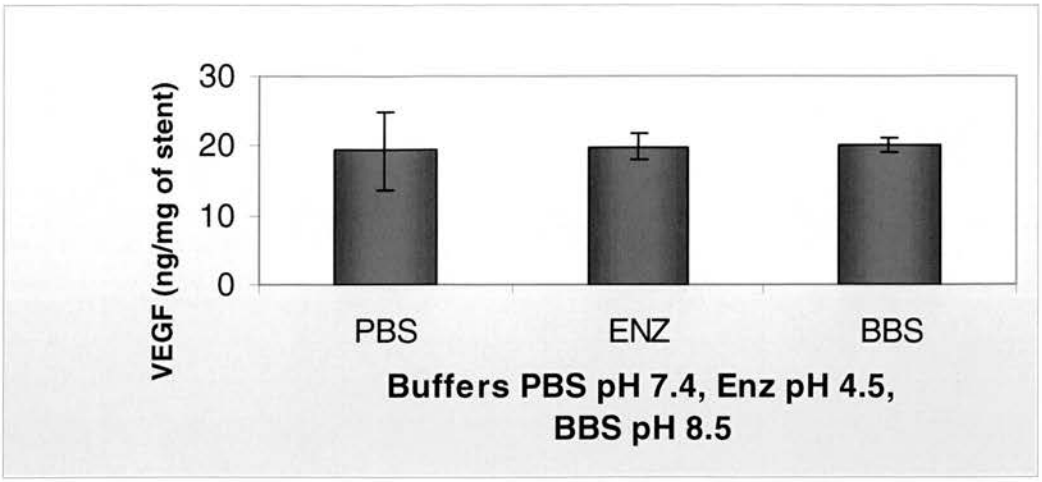


Figure 2.6 – Absorption of VEGF to Cook stents in differing pH conditions.

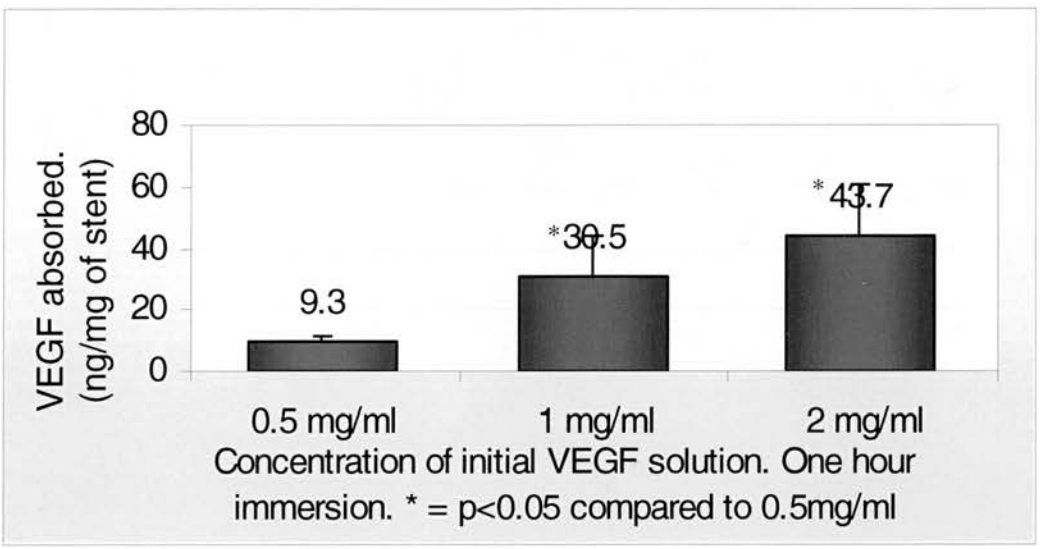


Figure 2. 7 – VEGF absorption to stent sections in differing concentrations.

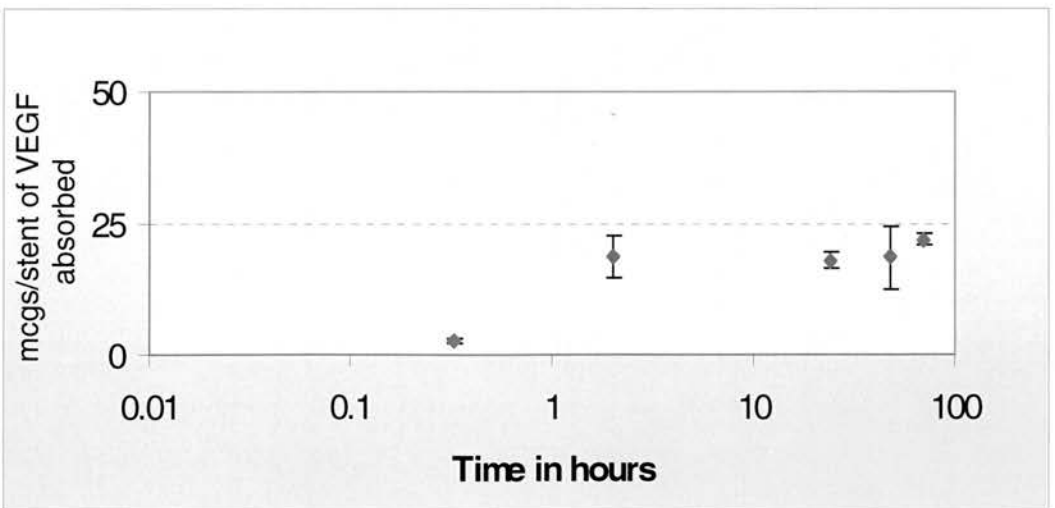


Figure 2. 8 – VEGF absorption after differing immersion times.

2.3.2 Results of VEGF elution experiments

2.3.2.1 Simple elution

This experiment showed that VEGF absorbed onto the Supra G stent was initially released very quickly, with a second, slower elution phase (*fig. 2.9*).

2.3.2.2 Elution in the perfusion circuit

The results obtained in this experiment were very similar to those obtained in the simple elution experiment. Again, an initial rapid decay was seen with an overall bi-exponential decay. At five and nine days, 28% and 20% respectively of the initial dose still remained on the stent sections (*fig.2.10 & 2.11*).

2.3.2.3 Elution in the internal mammary artery.

The results of this experiment are shown below (*fig. 2.12*). At 24 hours, 11±6.8% of the initial VEGF loaded onto the stents was seen in the tissue. 12.3±1.7% remained on the stents. A large proportion of the initial VEGF-associated radioactivity was not detected in the stent, the tissue or the perfusate. This was assumed to have become adherent to the plastic tubing of the circuit or to small losses incurred when the stent or tissues were manipulated. The length of the tubing was such that the relatively small amounts of radioactivity involved would be very thinly dispersed.

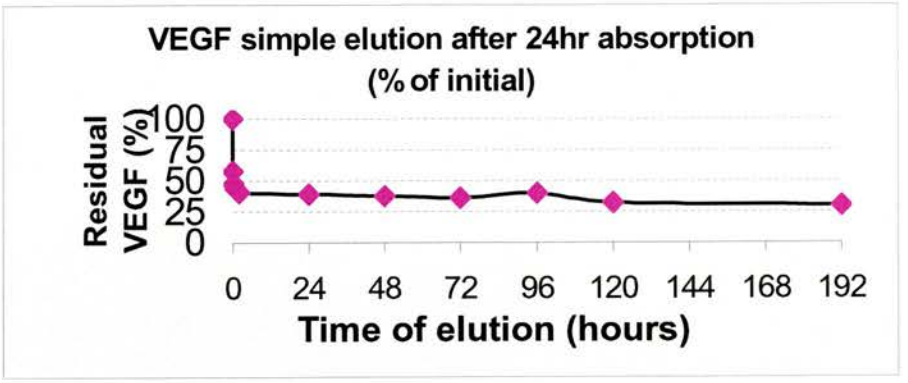


Figure 2.9. Simple VEGF elution experiment results over eight days.

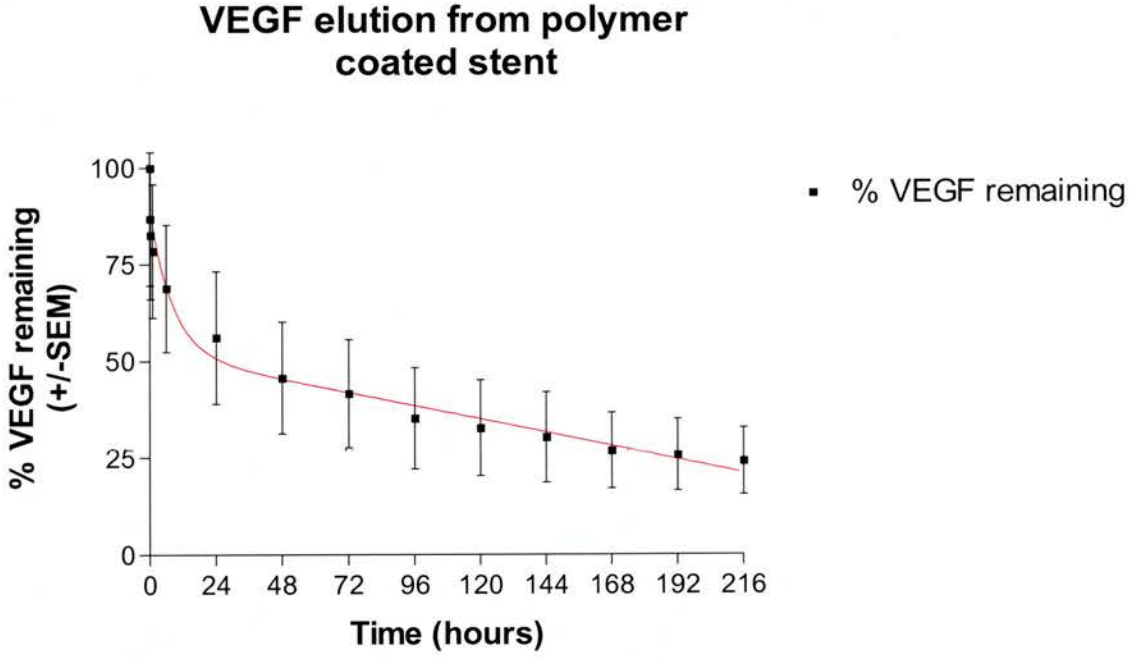


Figure 2.10. VEGF elution in perfusion circuit 1. These stents had been dipped for 48 hours. R^2=0.99. Analysis of residuals p= 0.11, not significant[5].

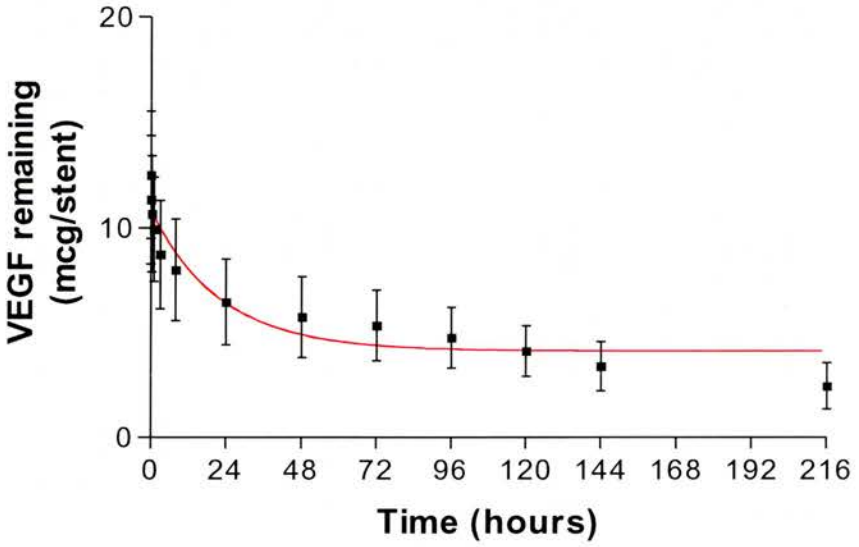


Figure 2.11. VEGF elution in perfusion circuit 2. These stents had been dipped for 24 hours. Line fitted to bi-exponential curve, R^2 =0.98, but p<0.05 for analysis of residuals.

Location	% of initial value (mean)	Standard deviation (%)
Stent section	12.3	1.7
Tissue specimen	11.0	6.8
"systemic" delivery	76.7	2.9

Figure 2.12. VEGF release in arterial tissue. Distribution of VEGF after 24 hours perfusion (initial loading 7.9+/-2.54mcgs of VEGF on a half-length, i.e.10mm, stent). "Systemic" delivery was the remaining VEGF deposited in the glassware, tubing or in the perfusate[5].

2.4 Discussion

The radiolabelling experiments showed that about 20μg of VEGF could be absorbed per stent. This is within the same range that had been delivered with beneficial effects in similar experiments. It did so in a manner independent of pH but influenced by both the initial concentration of the solution and, to a lesser extent, the length of time that the polymer was exposed to the solution. Absorption of VEGF was shown to reach a plateau over time, with a lower absorption within the first 20min. This suggested that the drug was actually being incorporated into the structure of the polymer (absorption), a process that would take some time, rather than merely coating the exterior surface of the polymer (adsorption), which would be expected to occur as soon as the stent section was immersed in the drug solution. A large excess of protein was present in all the solutions compared to the small quantities absorbed on the stent. However, at the lower concentration of VEGF solution a reduced absorption of the protein was seen, despite long immersion times. This suggests that the protein competes to be absorbed onto the stent with surrounding molecules, i.e. the constituents of the buffer solution. Around 10% of the released protein was present in arterial tissue, in an *ex vivo* circuit.

More importantly, the radiolabelling experiments showed that although a large quantity of VEGF 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 a week. In a simple perfusion circuit, retention of drug within the vessel was significantly higher than that seen commonly after local drug delivery balloon use. The elution kinetics of VEGF from polymer-coated stent sections were biphasic as evidenced by the R^2 values (all >0.98) obtained by non-linear regression to fit a two-phase exponential decay model. This pattern of elution may be explained by an initial high rate of loss of VEGF from the stent, due to VEGF that was only very superficially *adsorbed* to the surface of the polymer. The slower release of VEGF seen over the later part of the curve would then be explained by a gradual release of VEGF that had been fully *absorbed* within the polymer hydration space. The retention 19.7% of the initial dose of the drug after nine days perfusion was an encouraging finding since re-endothelialisation begins within this time frame. The release of protein was similar to results seen in previous work[1; 2] with agents that then showed physiological benefit when tested *in vivo*. It also favoured the concept of the stent as a local delivery device over local instillation of solutions by balloon. These latter tend to deliver very small quantities of active drug to the targeted tissue relative to the total amount infused, typically around 1%.

2.5 Study limitations

Great variability was seen in the total amount of VEGF that was shown to be absorbed in the different experiments. Despite replicating the same absorption conditions, very variable amounts of VEGF were detected in the gamma counter. These differences can be attributed to relatively small amounts of VEGF-containing fluid adherent to the polymer surface. These are difficult to remove, partly because the stent sections are complex three-dimensional structures that hold droplets in the interstices of the structure. Various techniques were tried to overcome this variability. Initially, stent sections were counted after having been merely agitated to lose any residual droplets of VEGF solution. A second method was to rinse the stent section after their absorption period in plain buffer. However, this resulted in dramatic falls in the counted protein and it was felt that this step was really part of the elution process and therefore did not accurately reflect the amount of VEGF absorbed. It also failed to produce results with little variability. The final method settled on was to agitate the stents and then to remove any residual droplets by rolling the wet hoops on filter paper. This method produced reasonable reproducibility within each experiment.
Another explanation for the great variability in the amount of VEGF absorbed to any one stent lies in the structure of the polymer coating itself. As can be seen from the electron micrograph (*fig. 2.5*) of cross sections of the stent, large variability is seen in the thickness of the polymer coating. Furthermore, gaps appear to be present between the metal of the stent and the polymer coat. It is possible that these abnormalities are artefactual, occurring as a result of a tangential cut through the stent strut, or as a result of the EM processing. However, the findings are constant in several cross sections. These gaps will retain variable quantities of dissolved drug above that absorbed into the substance of the polymer. The release of drug from these gaps may be different from the release kinetics of the polymer.

Some assumptions have been made in conducting these experiments. It was assumed that radiolabelled VEGF would absorb to, and elute from, a coated stent in the same way as unlabelled VEGF. The size of the protein is, however, considerably larger than the added iodine group and the process has been well established as a marker of proteins. Only three concentrations of VEGF were tested and a plateau of VEGF absorption was not demonstrated. It might be postulated that higher still concentrations of VEGF would result in even higher absorption. Limited quantities of VEGF proved a practical constraint on

exploring this possibility. Furthermore, higher concentrations of protein would tend to form protein aggregates that would not behave in a comparable method to the isolated protein. The favourable *in vitro* elution kinetics of the protein evaluated may be significantly different *in vivo* when perfused by blood in physiological conditions, particularly given the presence of plasma proteins and proteases. This limitation was overcome in part by using a pH7.4 buffer containing 4% serum albumin to mimic the presence of plasma proteins. Attempts to reproduce the cellular milieu into which stents are placed were made in the final part of the experiment using IMA tissue. Plasma proteins *in vivo* would be absorbed onto any artificial surface in the circulation potentially displacing any drugs already absorbed to that surface[6]. Perfusion of the stent sections with blood would have some additional advantages, most notably the potential to allow cellular and protein interactions with the immobilised proteins. However, this option was impractical for the prolonged perfusion periods used in the study. The *ex vivo* viability of cellular blood components is limited and unlikely to reflect the *in vivo* situation. Some reassurance that the *in vitro* results may well be reproduced *in vivo* can be found in the finding that the results seen in the simple elution experiment are not altered by the introduction of the slightly more realistic conditions in the perfusion circuit.

Chapter 2 references

1 Aggarwal RK, Ireland DC, Azrin MA *et al.* Antithrombotic potential of polymer-coated stents eluting platelet glycoprotein IIb/IIIa receptor antibody. *Circulation* 1996;**94**:3311-3317.

2 Foo R, Gershlick AH, Hogrefe K *et al.* Inhibition of platelet thrombosis using an activated protein C loaded stent: in vitro and in vivo results. *Thrombosis & Haemostasis* 2000;**83**:496-502.

3 Woltanski KP, Besch W, Keilacker H *et al.* Radioiodination of peptide hormones and immunoglobulin preparations: comparison of the chloramine T and iodogen method. *Experimental & Clinical Endocrinology* 1990;**95**:39-46.

4 Baron JH, Aggarwal RK, de Bono DP. Adsorption and elution of c7e3 Fab from polymer-coated stents in vitro. *Heart* 1999;503-P2902(Abstract)

5 Swanson N, Hogrefe K, Javed Q, Gershlick AH. *In vitro* evaluation of Vascular Endothelial Growth Factor (VEGF) eluting stents. *International Journal of Cardiology* 2003;In Press:

6 Andrade JD, Hlady V. Plasma protein adsorption: the big twelve. *Annals of the New York Academy of Sciences.* 1987;**516**:158-172.

Chapter 3.

VEGF-eluting stents - assessment in cell cultures.

3.1 Introduction

The experiments described in the previous chapter deal with the physical properties of VEGF-eluting stents and demonstrated that it was technically possible to use polymer-coated stents to absorb and then gradually elute VEGF. The following experiments were designed to demonstrate whether or not the VEGF eluted from the stents was sufficient to significantly affect the target cells, i.e. the endothelium. The tissue culture work done was performed on two endothelial cell types, Bovine Aortic Endothelial Cells (BAECs) and Human Umbilical Vein Endothelial Cells (HUVECs). BAECs are a cell type known to express VEGF receptors[1]. BAECs are known to possess receptors for VEGF and stimulated growth of such cells has been shown under the influence of VEGF[1; 2]. However, such growth is less than that seen with other cell types. HUVECs were used in further experiments to demonstrate the efficacy of eluted VEGF in human, rather than bovine, tissue.

3.1.1 Experimental design

To establish that the cells used were endothelial cells rather than fibroblast overgrowths or other cell types, they were stained for von Willebrand Factor using immunofluorescence techniques. All endothelial cells express von Willebrand's Factor, which binds to the platelet glycoprotein Ib receptor on platelets, regulating the adherence of the platelets to the endothelium. vWF is not expressed on any other cell types.

The growth of endothelial cells was determined in order to show the stimulatory effects of VEGF either as an additive to the growth medium, or eluting from the polymer coated stent. This was to demonstrate that the VEGF that had been obtained from Genentech was physiologically active in endothelial cells. It also was to demonstrate that the principle of a VEGF-eluting stent was capable of delivering a concentration of VEGF to nearby cells that was sufficient to stimulate their growth.

As part of the varying of the conditions under which the experiment was performed, some BAECs were grown under low serum conditions. This was initially done to minimise the effects of any growth factors active within the growth medium so the effects of VEGF would not be obscured..

VEGF was then added to cell cultures using the polymer-coated stents as a vehicle. Cell growth was determined using the PMS/MTS assay and cell counting with radiolabelled thymidine.

3.1.1.1 Background to the PMS/MTS assay

MTS (3H,4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium), or Owen's reagent, is bioreduced in the mitochondria of viable cells into a formazan product that, when dissolved in culture medium, can be measured colorimetrically at a wavelength of 490nm. This has been validated elsewhere[3]. The quantity of formazan produced is directly proportional to the number of viable cells in each well. The stability of MTS is enhanced in solution by the addition of the electron-coupling agent phenazine methosulphate (PMS).

3.1.1.2 Background to thymidine labelling as a measure of cell growth.

Thymidine, one of the four bases essential for DNA synthesis, is incorporated into DNA as cells undergo mitosis. Labelled thymidine can be a marker for cell growth. In a rapidly dividing population of cells, such as the endothelial cells under study *in vitro* in this series of experiments, thymidine incorporation will approximate to total cell numbers as all the cells will undergo mitosis during the course of twenty four hours. Thymidine is typically labelled with tritium in place of a normal hydrogen atom. This is a beta-emitting isotope that can readily be detected in the laboratory.

3.1.1.3 Functional assay for the VEGF-eluting stent.

VEGF is known to stimulate the production of NO from cells at nanomolar concentrations. In previous cell culture work this has been demonstrated, with maximal NO production following exposure to NO after eight minutes[4]. Detection of Nitric Oxide production by endothelial cells can be effected by the use of the Griess Reaction. This is a colorimetric assay method where a azo-derivative is produced by the reaction of diazonium ions with N-(1-naphthyl) ehtylendiamine. The diazonium ion is formed when acidified NO_2^- ions react with sulfanilic acid. To examine whether VEGF maintained these functional effects after delivery using the VEGF-eluting stent, a further experiment was performed using HUVEC cell culture.

3.2 Methods and materials.

3.2.1 Obtaining cells

BAECs were cultured from aortae of cows, freshly slaughtered at the local abattoir. Cells were kept in liquid nitrogen until required. At this time they were thawed out and then grown in sterile flasks to obtain enough cells for experimental use. Cells were divided when they reached confluence using trypsin. These methods are set out in Appendix 2. HUVECs were obtained from fresh human umbilical cords (Appendix 2). They were used directly, rather than being frozen.

3.2.2 Positive staining for endothelial cells with vWF antibody.

Endothelial cells at a concentration of 2.5 x 10^4 cells/ml were plated out into eight well chamber slides (Life Technologies Ltd.), 400µl in each well, i.e. 10^4cells per well. Cells were left overnight in an incubator. Cells were washed three times with HBBS containing 1% BSA. Wells were then incubated with 200µl of 2% paraformaldehyde in PBS and incubated at 4°C for 16 hours, to fix the cells. The wells were washed three times with PBS/ 2%FCS/ 0.1% saponin.

3.2.2.1 Primary antibody

A mouse monoclonal antibody was used. A 1:500 dilution in PBS/saponin/FCS was used, because this proved to be the optimal concentration in previous titration experiments, and 100µl of this was added to the wells. Control wells had no primary antibody. Wells were left on a rocker for half an hour to allow labelling of the antigen. The antibody solution was washed off with a further three cycles of PBS/ 2%FCS/ 0.1% saponin. Saponin is a detergent that permeates the cell membrane, allowing access to the nucleus.

3.2.2.2 Secondary antibody and nuclear stain.

The secondary antibody used was a FITC (Fluorescein isothiocyanate) labelled rabbit anti-mouse antibody. DNA in cell nuclei was stained with the fluorescent fluorochrome Hoescht 33258 stain at low concentration (100nmol). This nuclear stain allowed all cells to be identified. Solutions of Hoescht and secondary antibody were made up in PBS/ 2%FCS/ 0.1%

saponin and 300µl added to the wells. Again they were left for half an hour during which they were gently agitated to allow labelling to take place. After half an hour, slides were washed with PBS/ 2%FCS/ 0.1% saponin three times to remove the secondary antibody. The chambers were removed carefully and the slides had coverslips fitted with an anti-fade fluorescent mounting agent (DAKO). This was to reduce quenching of the fluorochrome. Slides were left overnight in the fridge to allow the mountant to dry.

3.2.2.3 Imaging

Slides were visualised using oil immersion on a fluorescent microscope (Zeiss) fitted with a digital video camera (Hamamatsu Photonics). This was connected to a computer with "Motion Picture" software (ATM Ltd). Images were taken using separate filters for the Hoechst and the FITC stains. These images were recorded separately for both positive and control slides. The recorded images were then superimposed and processed using Adobe Photoshop software. False colour was used.

3.2.3 Replication of ECs

Cells were seeded at an initial concentration of 10^6 cells per $75cm^3$ flask and grown in DMEM with 20% FCS. Flasks were observed daily until the cells appeared to be nearing confluence (at which time normal growth might be expected to slow due to contact inhibition). The cells were then trypsinised as before and then counted with the haemocytometer. 10^6 cells per $75cm^3$ flask were then seeded once more and the cell numbers counted again. Numbers of cell doublings were calculated using the formula $N_c/N_s = 2x$ where N_s is the number of cells seeded, N_c is the number of cells counted after tryspinisation and x is the number of population doublings.

3.2.3.1 Cell counting – the PMS/MTS EC proliferation assay

Endothelial cells were cultured in a 96-well plate with 0.1ml of DMEM+20% FCS culture medium at 37°C in an incubator under sterile conditions for 1 hour. 20µl of "One solution" was then added to each well, taking care not to produce any bubbles. A final well containing only culture medium and "One Solution" was used as a control or blank. The plate was incubated for a further hour before being read twice in a spectrophotometer (Titertek Multiskan Plus (ICN) Mk11). Any reading from a well containing a bubble gave a very high absorbance and so any well with a visible bubble was eliminated from the analysis.

3.2.4 Replication of ECs with VEGF

3.2.4.1 BAEC growth curves in six well plates.

ECs were grown until they reached confluence in a culture flask. The cells were then trypsinised and re-suspended in fresh DMEM culture medium. The cells were well mixed and then counted twice using an automated cell counter (Coulter 4C Plus 51). 100ml of culture medium containing VEGF at 1ng/ml were prepared under sterile conditions. This concentration was used as it was three times higher than the dose required to half maximally stimulate foetal BAECs in previously published work[5]. To 50ml of this solution were added a small quantity of the endothelial cell stock, with a final concentration of 1 x 10^4 cells/ml and 1ng/ml VEGF.

An identical quantity of medium containing cells (1 x 10^4 cells/ml) was prepared. Sterile six well plates were seeded with 3ml of cell-containing culture medium. Two plates were seeded with medium containing VEGF and two without. At the same time every day after the cells were seeded, one well from each plate was trypsinised by adding 1ml of warmed trypsin for three minutes. The suspended cells were spun down to a pellet and re-suspended in 2ml of fresh medium. These samples were counted in the cell counter and the results recorded. The PMS/MTS assay and direct observation of cell numbers with a haemocytometer slide under a light microscope were also used to quantify cell proliferation.

The results of the initial growth experiment proved difficult to replicate, due to fungal contamination in the cell culture wells. This recurred despite using freshly made medium and trypsin solutions. The high risk of contamination was felt to be a consequence of the equipment used in the experiment. The plates used to grow the cells were not sealed with an air filter. Furthermore, they were opened many times as each well in a plate was processed on a different day.

Further experiments were done with a modified protocol. The main change was to use small ($25cm^3$) culture flasks with an air filter. Each day of the experiment led to the use of its own specific flasks only. Flasks containing cells to be processed on other days were not affected. A 10ng/ml solution of VEGF was used, to see if higher concentrations continued to have the same or any adverse effects effects on growth of cells. 5x10^4 cells were seeded per flask, because each flask was larger than the wells used in previous work. Similarly, 5ml of medium was used per flask rather than 3ml. The automated cell counter was not used due to the inaccuracy of its readings. Cell counting was performed as before.

3.2.5 BAEC growth with VEGF after prolonged culture in low serum medium.

Cloned BAECs, which were at or around their 20th population doubling since primary culture, were cultured for over two weeks in either 2% or 10% culture medium. After this time, changes in the cell morphology (large, multinucleate or amorphous) microscopically began to be seen and this was taken as an indication that the cells were approaching senescence (*see examples in fig. 3.1*). Cells at this point were trypsinised and seeded in 25cm^2 sterile flasks at a density of 50000 cells per flask. To these flasks was added medium with either 2 or 10% serum as before with or without 10ng/ml VEGF. The flasks were cultured for up to four days and then trypsinised, the cells were removed and then seeded into a 96 well plate, 100µl per well. To these wells, the PMS/MTS assay solution was added as before. The experiment was repeated and the numbers of cells in each flask were also calculated by counting them using a haemocytometer on day four. Examples of these flasks were formalin-fixed and photographed.

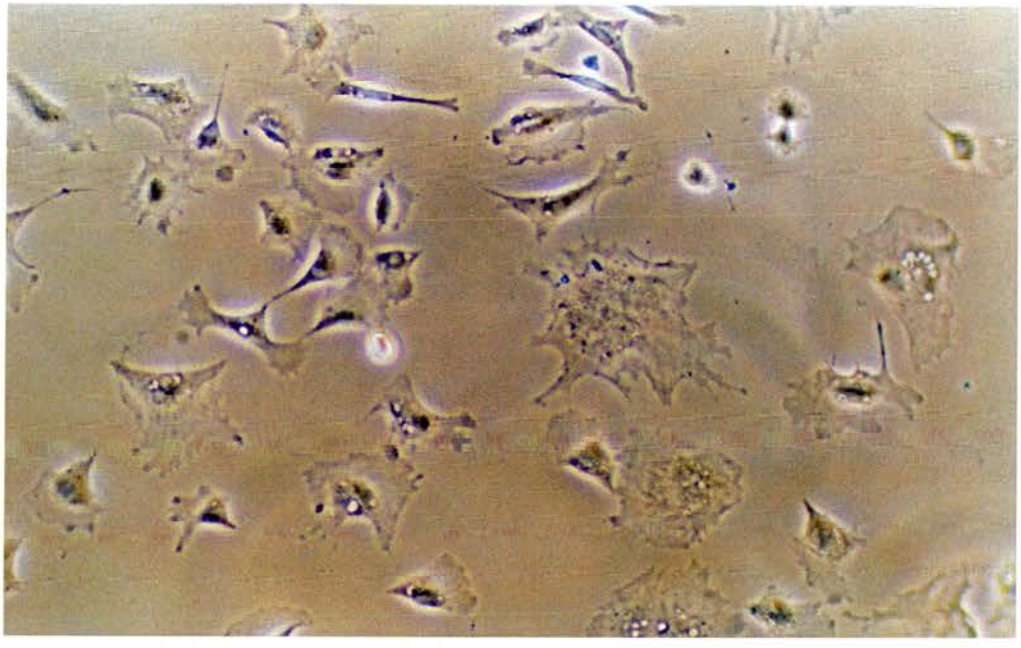


Figure 3.1. BAECs approaching senescence. Cells are large, with poorly defined nuclei and have lost their typical morphology.

3.2.6 HUVEC growth with and without VEGF.

HUVEC cells were grown in 24 well plates. They were seeded at an initial density of 10^4 or 2 x 10^4 cells/well, in 1ml of medium. To this was added either a further 1ml of medium or 1ml of medium containing VEGF (10ng/ml). 10 wells were used for each group and the growth of cells was studied at three and four days. On the third day, the medium in the wells to be studied was removed and 500µl of fresh medium was added. A further 100µl of PMS/MTS assay reagent was added to each well. This preserved the ratio used in previous 96 well plates to ensure the reagent would react normally. The plates were then replaced in the incubator for a further hour. After one hour, 120µl of the solution in each well was removed and added to a 96 well plate, taking care not to produce any bubbles in the wells. Any bubbles seen were punctured with a needle to avoid artefact. The plate was counted twice in a spectrophotometer at 492nm as before. The results were automatically adjusted with a control blank containing 120µl of culture medium incubated alongside the other wells with PMS/MTS reagent (i.e. no cells).

The results were recorded and the ratio between the cells treated with and without VEGF calculated to determine the % increase in growth due to the addition of VEGF. On the third day the medium was changed for the wells to be studied in the same fashion at four days and a further 2ml of medium was added to the wells again either with or without 10ng.ml of VEGF.

3.2.7 VEGF-eluting stents in BAEC culture – thymidine labelling.

BAECs were prepared at a concentration of 125000/ml in growth medium (DMEM containing 20% FCS and gentamicin). The cells were then plated out onto 24-well plates under sterile conditions, 1ml of cell-containing medium per well. Eight wells were left empty to act as controls in the absence of cells. The cells were then incubated for 24 hours adhere to the base of the wells. Sections of stents were cut and sterilised with ethylene oxide. 250µl of 2mg/ml VEGF solution in PBS was placed into two separate Eppendorfs. Into each Eppendorf four hoop sections were placed. The containers were agitated to ensure the hoops were fully covered by the VEGF solution. The hoops were left in this solution for 24 hours. Eight control hoops were placed in two identical Eppendorfs containing 250µl of PBS only. At 24 hours, the hoops were taken out of the VEGF solution, excess solution shaken off them and they were then left to dry in air. Control hoops were treated identically. The 24-well plate was removed from the incubator and the remaining medium was removed, taking care not to

touch the base of the plate where the cells were adhering. All hoops were placed into the 24-well plate, one to a well. The hoops were then covered with 2ml of fresh medium. After 24 hours of incubation the plate was removed from the incubator and the medium removed, together with any VEGF that had eluted from the stents into the medium. This corresponds to the loss of VEGF that would occur *in vivo* as blood washed some VEGF away from the target site into the general circulation. The medium was replaced with fresh medium. Every two to three days thereafter, the medium was replaced. At five days the medium was removed and replaced with medium containing tritiated thymidine. The cells were then cultured for a final 24 hours to allow for all growing cells to incorporate the thymidine. After this last 24 hours, the radioactive medium was removed and discarded appropriately. The hoops were all taken out of the wells and kept separately. The wells were then processed to assess thymidine incorporation.

3.2.7.1 Protocol for radiolabelled thymidine use with endothelial cells.

ECs were incubated with culture medium containing 0.037MBq/ml ^{3}H thymidine (adding 1µl of 37MBq/ml stock into 1ml of medium) for 24 - 72 hours, dependent on experimental need, at 37°C. The amount of tritiated thymidine containing culture medium was 1ml/well in 24 well plate. The isotope-containing medium was removed, cells washed with fresh medium twice, and chased with fresh isotope-free medium for 60min at 37°C to reduce the unincorporated tritiated thymidine in cytoplasm. Samples were then washed again with PBS, and treated with 5% ice-cold trichloroacetic acid for 60min to precipitate DNA and incorporated tritiated thymidine (1ml/well in 24 well plate). After removal of trichloroacetic acid the cells were washed with ethanol once, then a small amount of fresh ethanol added and left to dry overnight to remove any remaining free isotope. The washed precipitate was re-dissolved in 2N NaOH for 60min, 100µl/well in 24 well plate. The cell lysate was transferred to a counting vial containing 5ml of "Hisafe" (Wallac) scintillant. After good mixing the samples were left still for at least four hours to eliminate chemiluminance. The radioactivity in the samples was counted by Tri-Carb 1500 liquid scintillation analyser (Packard) with counting window set at tritium and a counting efficiency of about 65%. Each sample was counted for 4min and all the samples were counted for two rounds to check variation. Control samples containing 2N NaOH only were used to allow for background radiation.

3.2.8 HUVEC growth under the influence of VEGF from stents

Fresh HUVEC cultures were prepared from umbilical cords. These were cultured in Endothelial Growth Medium (see Appendix). The cells were confirmed to be endothelial cells using vWF staining. Stent sections were immersed in VEGF 2mg/ml in PBS or BSA 2mg/ml in PBS. These were left for 24hr. Stent sections were then removed and air-dried. All sections were sterilised using ethylene oxide gas. Stent sections were placed in a 24 well plate with 1ml of EGM for 24 hours. This was to allow the elution process to commence. A refinement of the experiment was to investigate whether stents loaded with VEGF retained their stimulatory effect on HUVECs even after a 24-hour period of passive elution into culture medium. This medium, containing the VEGF which had already eluted from the stent in that time, was removed, and fresh medium including HUVECs, was added to the stents. These stents were then cultured for 72 hours and the growth of HUVECs measured using the same assay techniques. The eluate was added to a separate group of cells and their growth measured. A stock solution was prepared in EGM of HUVECs with a final concentration of 1.6×10^4 cells/ml. 1ml of this stock solution was added to each of the wells containing a stent and to the control wells.

Eight groups of wells were studied including several control groups,

1 Negative control	Cells cultured without VEGF.
2 BSA group	Cells cultured with stent sections dipped in BSA.
3 VEGF group	Cells cultured with stent sections dipped in VEGF, medium unchanged.
4 VEGF group	Cells cultured with stent sections dipped in VEGF, medium changed after first 24 hours passive elution.
5 Positive control	Cells cultured with 10ng/ml VEGF, as before.
6 Positive control	Cells cultured with 500ng/ml VEGF – This was to exclude any adverse effect of the relatively high dose of VEGF delivered by stent.
7 Eluate	Cells cultured with the medium containing eluate from the group 4 stents.
8 Blank	Culture medium only, no cells.

The medium in each well was made up to 2ml by adding medium either with or without VEGF. All wells were then cultured for 72 hours. The medium was not changed during this time. After 72hr the medium in each well was removed and replaced in all cases with 500µl of fresh EGM. To this was added 100µl of PMS/MTS reagent. 120µl aliquots of each well

were drawn off and their absorption counted at 492nm. The results were adjusted for the blank well. Two counts were made and averaged. The stents from each well were also placed into fresh medium with PMS/MTS and this was cultured from up to four hours. Two positive control groups were also studied. These cell cultures were those to which VEGF had been added without stents. Two concentrations were used. The first was the same as in previous cell growth experiments, i.e. 10ng/ml. The second was a concentration of 500ng/ml, to test the effects on cell growth of very large doses of VEGF and was calculated from the absorption of VEGF seen in the radiolabelling experiments. It was assumed that the maximum concentration the HUVECs might be exposed to would be the entire absorbed dose of VEGF diluted in the 2ml medium in each well. As both these doses are at least an order of magnitude above the ED50 from previously published work[5], no further stimulation of EC growth was expected. However, it remained possible that VEGF delivery by stent might have adverse effects on EC growth.

3.2.9 NO production from HUVECs under the influence of VEGF.

Detection of NO was performed using the commercially available Total Nitric Oxide Assay (R&D DE1600) according to the manufacturers recommended protocol.
$2x10^5$ HUVECs were cultured to confluence, trypsinised and plated out in a 24 well plate. The wells were left overnight to reach confluence, thus each well had an equal number of confluent HUVECs. Stent sections were prepared with either BSA or VEGF.
Stents were added to each well of the 24 well plate. Negative control wells containing either cells alone or just culture medium were also included in the study. After eight minutes 100µl of cell culture supernate was removed and stored at 4°C. The cells were exposed to the stent section for a further 22 minutes, i.e. up to half an hour total exposure. At this point a further 100µl of cell culture supernate was removed and also stored. The cell supernate samples were centrifuged for 10mins at 1500rpm to remove particulates. Each sample was diluted in an equal volume of reaction buffer. The samples were then filtered through a 10000MW cutoff filter using a Heraeus centrifuge at 13,000rpm for 15min (11000g) to remove proteins, as these may interfere with the assay.
NADH was reconstituted in distilled water and kept on ice throughout the assay. It was further diluted immediately prior to use. Nitrate reductase was reconstituted in buffer, vortexed and kept on ice during the assay. It also was diluted immediately prior to use in Reaction Buffer. To make the nitrate standard, doubling dilutions were made with reaction buffer using the stock solution included in the kit,. A range of standard concentrations between 3.12µmol/l and 100µmol/l were prepared.

50µl of sample, standard solution or plain buffer (negative control) were added to wells in a 96 well plate. 25µl of NADH and 25µl were added to each well and the plate was incubated at 37°C for 30mins to allow the conversion of nitrate to nitrite. 50µl of the Griess I & II reagents were added and the plate incubated for a further 10 minutes to allow the production of the final coloured azo-derivative. The plate was then read in a microplate reader set at 540nm. All samples were assayed in duplicate. Results were adjusted to account for background counts. The whole assay was repeated with the samples obtained at 30min in an identical fashion. A standard curve was derived from the known concentrations (*fig. 3.2*).

3.2.10 Statistical analysis used.

The results of the experiments were analysed to show that the data were compatible with a normal distribution. The data were then subjected to a two-tailed t-test. Equality of variance was calculated in each case and the results factored into the t-test. In almost all cases, equality of variance was seen. Results are calculated as p values and displayed as means ± standard deviation.

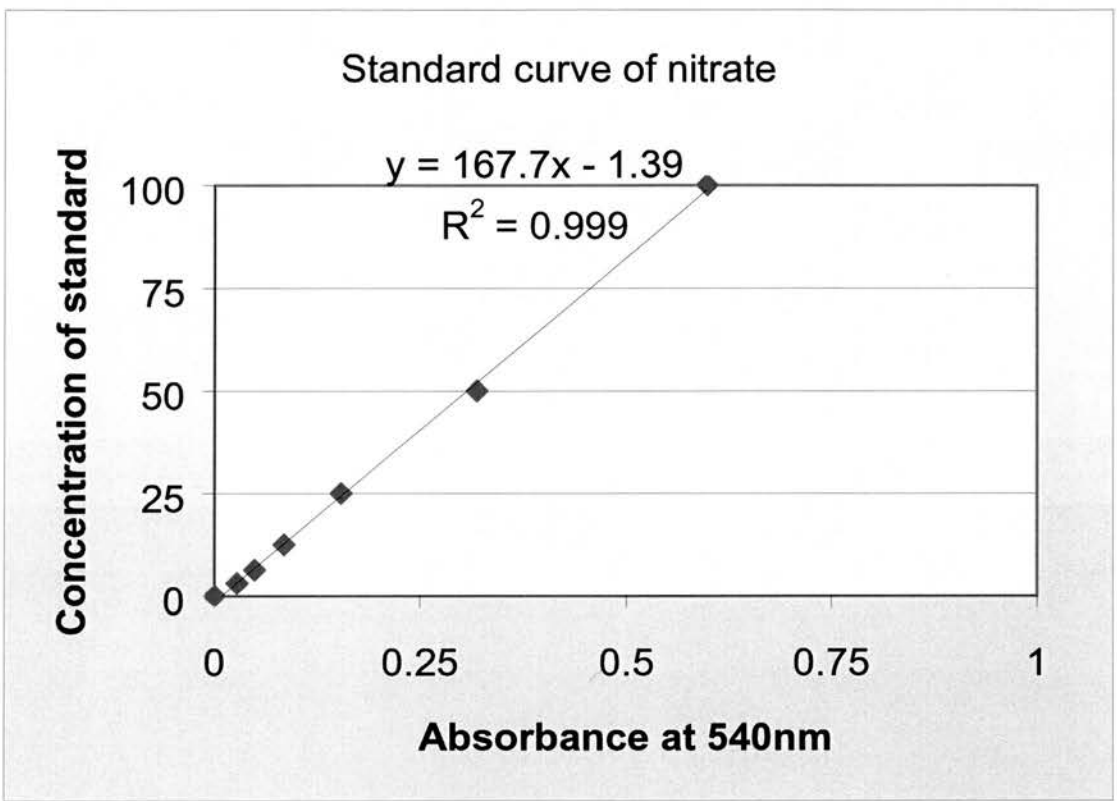


Figure 3.2. Standard curve obtained with Nitric Oxide solutions of known concentration. This was used to detect the amounts of NO produced from HUVEC cultures with and without exposure to VEGF.

3.3 Results

3.3.1 von Willebrand staining of cells and cell morphology

The cells used stained uniformly with the anti-vWF antibody. No cells were seen that did not express this antibody. The cells were therefore a pure culture of ECs. Furthermore, under normal light microscopy, the cells demonstrated typical "cobblestone" morphology when they reached confluence. An example of these findings is demonstrated below *(fig. 3.3&4).*

3.3.2 Endothelial cell growth curves

Steady exponential growth was seen in the cell cultures *(fig. 3.5).*

3.3.3 Effects of VEGF on BAECs and HUVECs

Two cell types were used. BAECs were used initially. However, the results obtained with them proved variable and for this reason, the experiments were repeated with HUVECs. More reproducible results were obtained with these *(fig 3.7.a-d).*
Slower growth was seen in low serum culture *(fig. 3.8)*. This variation to the experimental design gave an unexpected result in cells grown in these conditions for over two weeks. These cells seemed to be actually *reduced* in number compared to controls. As this was surprising, the experiment was repeated twice under pre-set conditions VEGF stimulated the rate of growth of normal endothelial cells in favourable growth conditions. However, senescent cells cultured in adverse (low serum) conditions showed a significant decline in numbers compared to similar cells grown in normal medium or without VEGF.

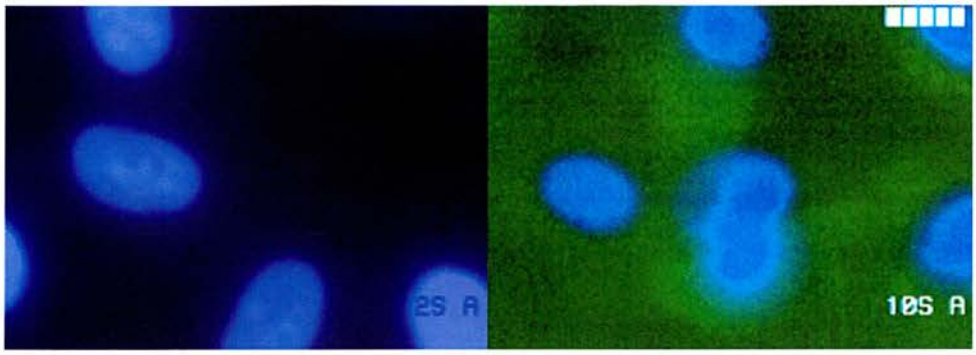


Figure 3.3a *Figure 3.3b*

Figure 3.3&b. vWF staining of endothelial cells.
In figure 3.3a, cell nuclei are stained dark blue with Hoechst stain. In figure 3.3b, cells are also stained for vWF factor, which is stained fluorescent green. All cells have green staining, so all cells are ECs.

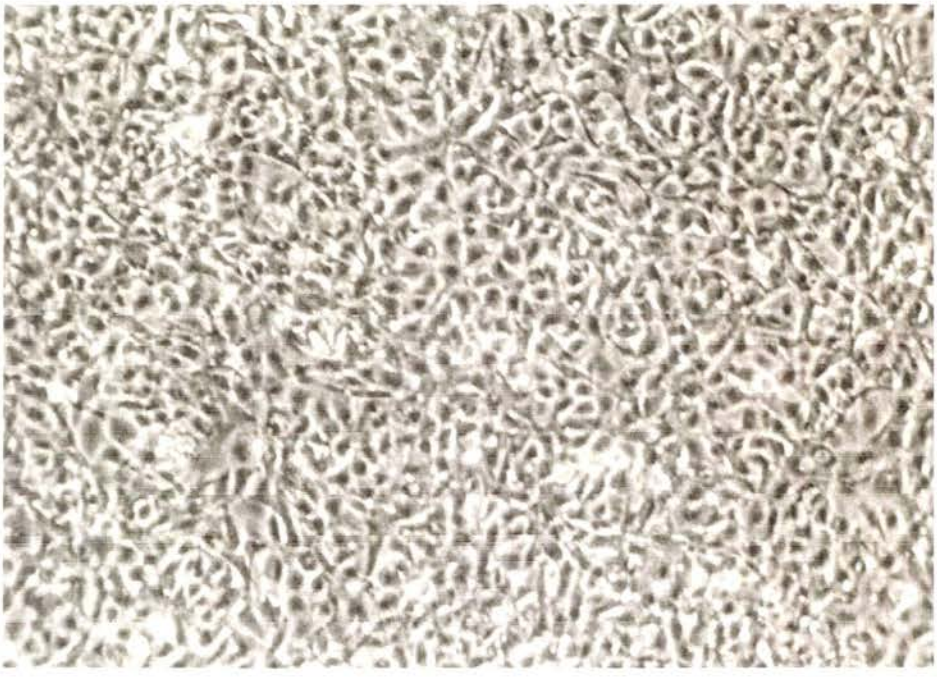


Figure 3.4 Light microscopy of endothelial cells. Photo of confluent BAECs showing typical cobblestone morphology, confirming that the cells are endothelial (x10 magnification).

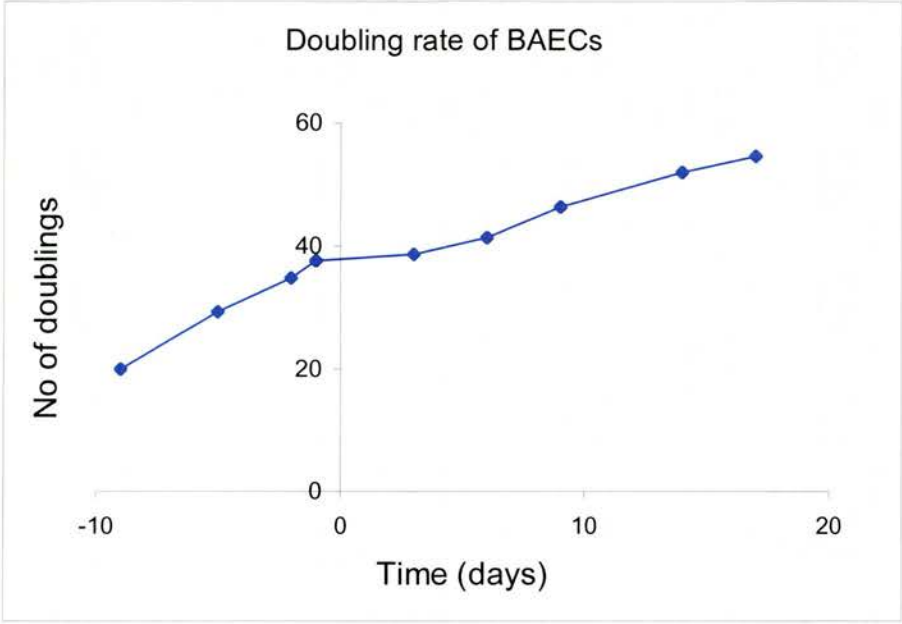


Figure 3.5. Growth curve of BAECs. This used DMEM/FCS medium and showed continuing cell doublings, i.e. cells had not reached senescence.

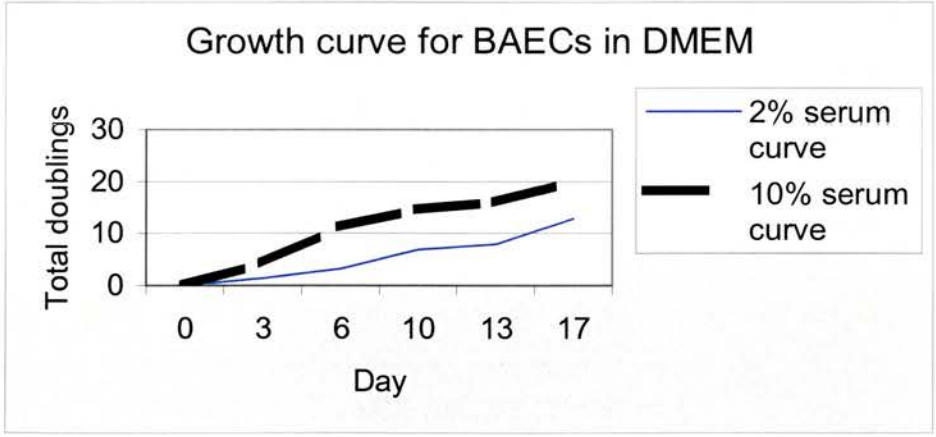


Figure 3.6. BAEC growth in low serum culture medium. Slower growth is seen.

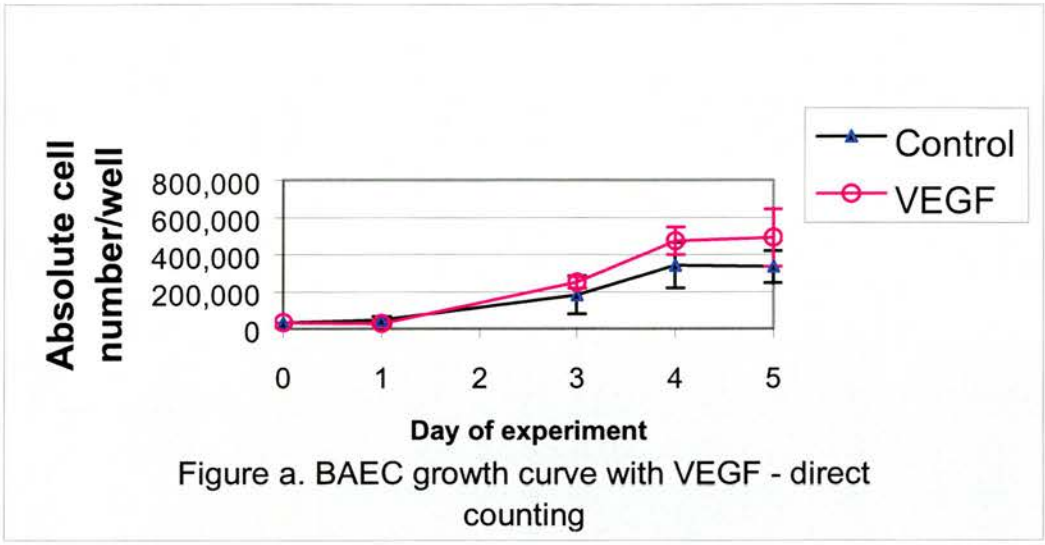


Figure a. BAEC growth curve with VEGF - direct counting

Figure 3.7a

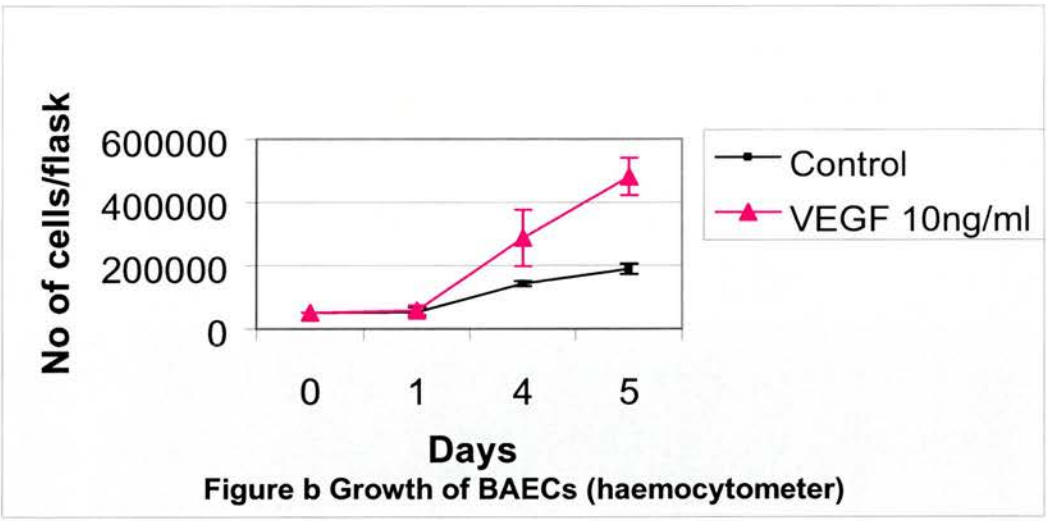


Figure b Growth of BAECs (haemocytometer)

Figure 3.7b

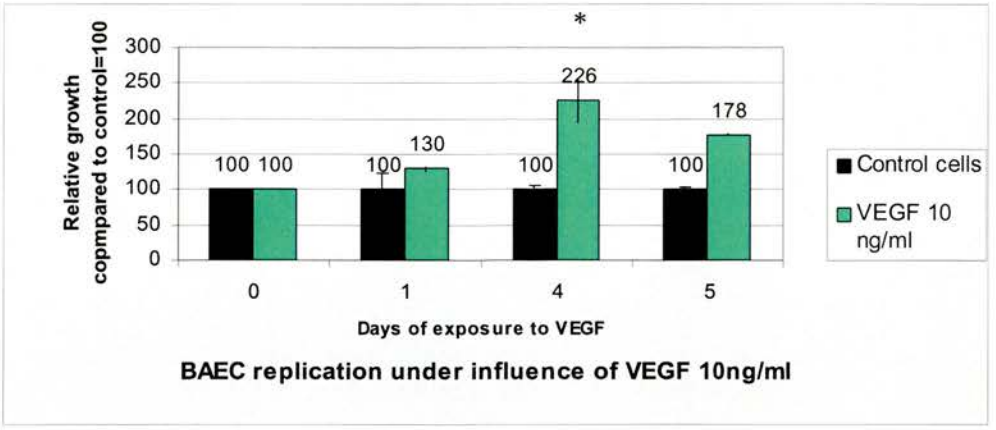


Figure 3.7c

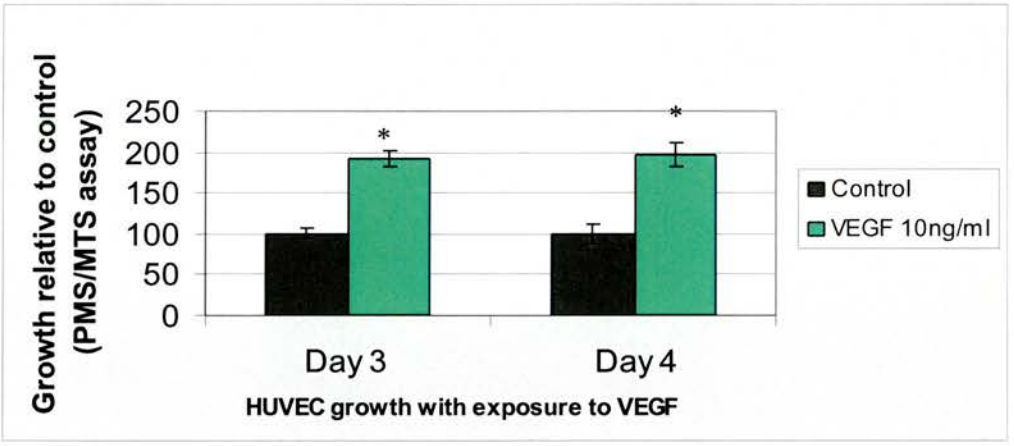


Figure 3.7d

Figures 3.7a,b,c &d. Effects of VEGF on the replication of endothelial cells. 3.7a shows the results obtained when counted directly by light microscope. 3.7b shows the results obtained with a haemocytometer. 50000 cells were initially seeded per flask. 3.7c shows the use of the PMS/MTS assay that was used most commonly, because of its reproducibility (= p<0.01). In each case "control" was identical medium, lacking only the addition of VEGF.*
3.7d. Shows the results for HUVEC replication, measured using the PMS/MTS assay (= p<0.001).*

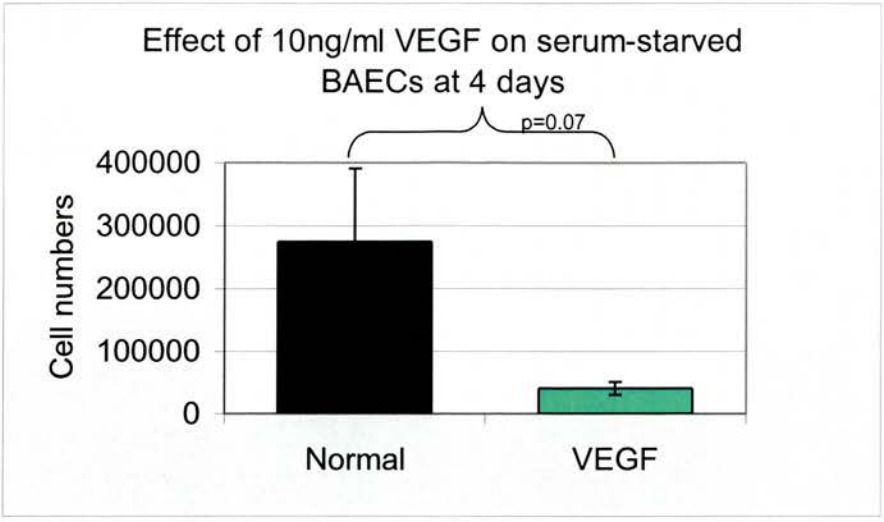


Figure 3.8a

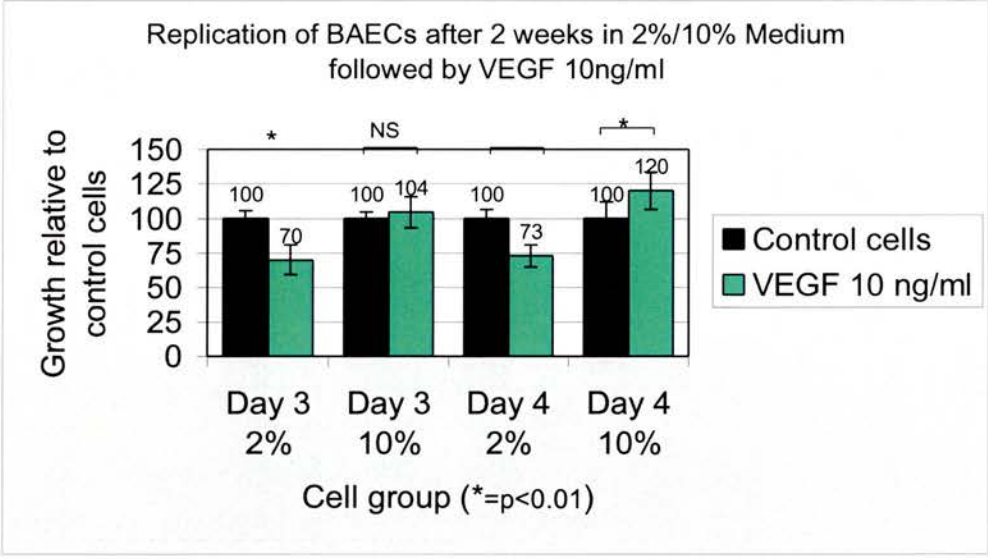


Figure 3.8b

Figure 3.8&b. Replication of BAECs after prolonged culture followed by exposure to VEGF-containing or normal medium. Direct counting used microscope (3.8a) and the PMS/MTS assay (3.8b). In low serum conditions a reduction in cell numbers was seen with VEGF exposure (Statistical significance tested using 2-tailed t-test, variance not assumed to be equal).

3.3.4 BAEC growth with VEGF-eluting stent sections.

An increase in endothelial growth in the groups treated with VEGF-eluting stents was seen, but this was not reproducible. There was an absolute difference in cell numbers between groups treated with bare stents and VEGF-eluting stents but this did not reach significance. The results are described below (*figs. 3.9a&b*). The experiments with thymidine labelling were repeated on three occasions. There was some variability in the radioactivity detected in all cell groups. BAEC numbers in this experiment were significantly greater in the populations exposed to the VEGF-eluting stent than to control groups of cells (22901 counts +/- 5501 *vs.* 6576 +/- 2480 in controls ($p<0.01$)).

3.3.5 HUVEC growth with VEGF-eluting stents.

The results obtained showed an 11.3±4.9% ($p<0.05$) increase in cell growth over controls. This was not significantly different to the effects seen when VEGF was added to the cell cultures without a stent (*figs3.10a,b&c).*

3.3.6 Functional assay for the VEGF-eluting stent

Using a standard curve, the amounts of NO produced over either 8 or 30min. were calculated for each group of wells and plotted (*fig3.11*).

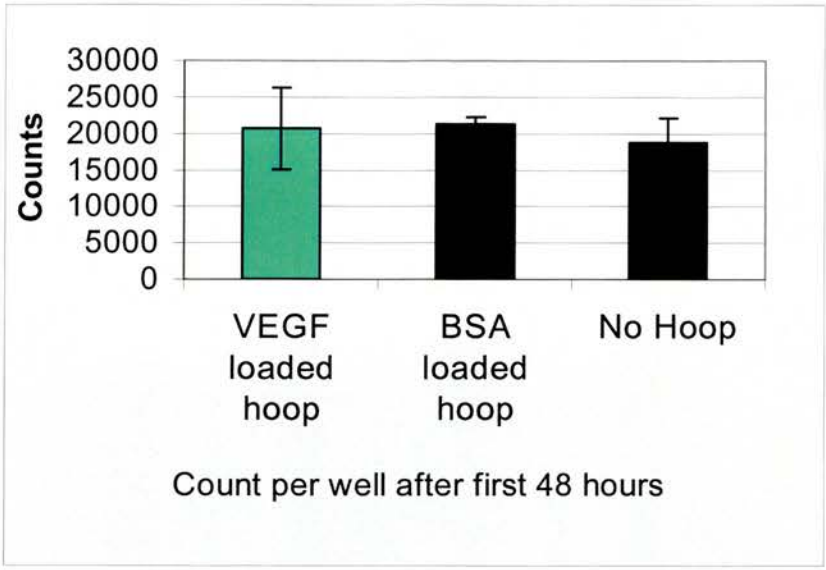


Fig 3.9a

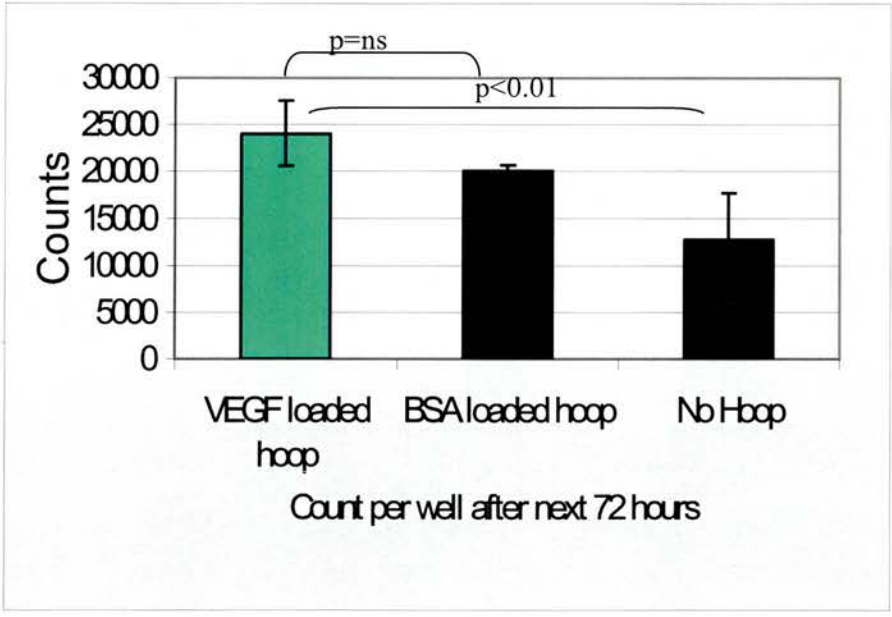


Figure 3.9a&b. The growth of BAECs when cultured with or without VEGF-absorbed stent sections (hoops). After 48 hours, no differences were seen in the different groups. However, by five days, a significant difference was seen between VEGF hoops and the cells with no stent hoop. Although an absolute difference was seen between the groups with VEGF hoops and bare hoops, this was not significant.

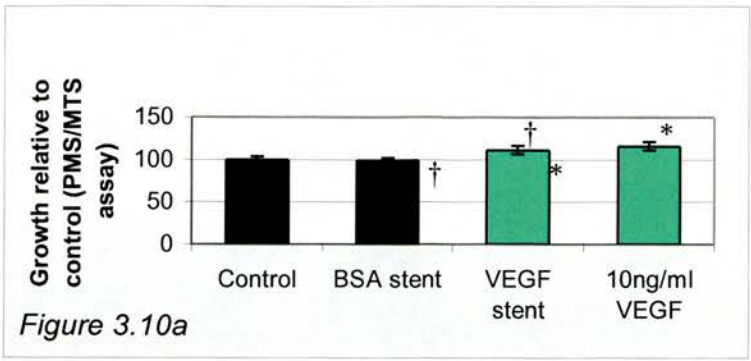


Figure 3.10a

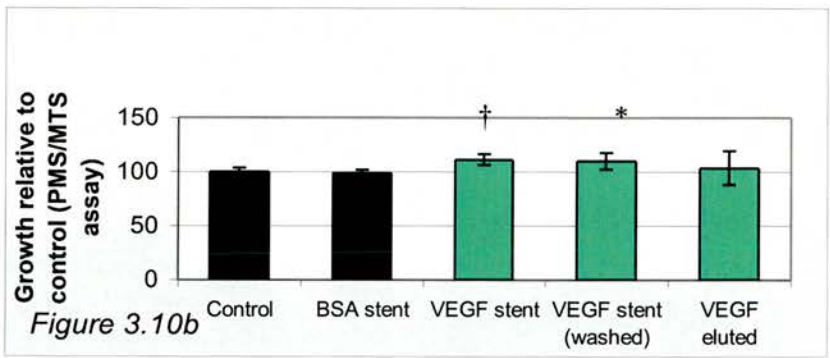


Figure 3.10b

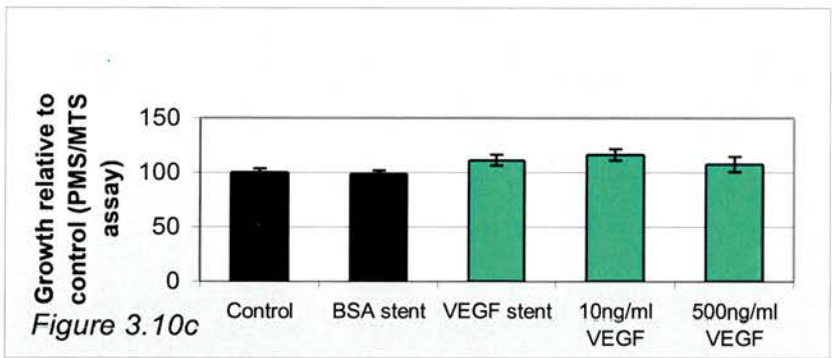


Figure 3.10c

Figures 3.10a,b&c. Replication of HUVECs after VEGF exposure, bound to a stent section (= $p<0.05$, † = $p<0.001$ compared to control group).*

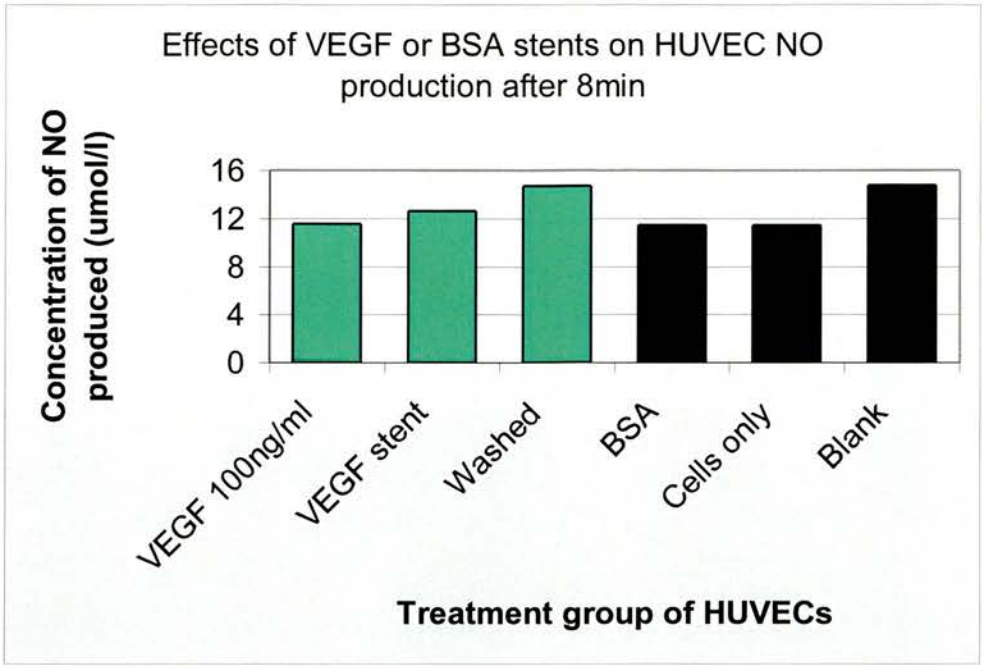


Figure 3.11. NO production from cells treated with or without VEGF. Results were similar at the 30min endpoint. No difference was seen between the different groups.

3.4 Discussion

3.4.1 Growth of ECs with VEGF

A variable response was seen to VEGF with the BAECs. This may be due to errors in reproducibly plating out small numbers of endothelial cells at the outset. As these cells will normally undergo exponential growth over the course of an experiment, any error in numbers of cells seeded initially will be greatly magnified. Secondly, BAECs produce FGF, a non-specific endothelial cell mitogen[6]. This growth factor may exert a proliferative response in the cells irrespective of the presence or absence of VEGF and thus obscure the effects of VEGF.

3.4.2 Serum-starved cells.

Prolonged serum starvation of cells adversely affects their growth and morphology, by stimulating apoptosis[7]. Adding VEGF to such cells led to a *decrease* in the growth of BAECs. A failure of VEGF to promote growth has been seen in BAECs treated with oxidised LDL, which impairs the growth and function of ECs[6]. Oxidative stress can adversely affect the growth of BAECs. This process was accelerated by the addition of VEGF[8]. It is postulated that damaged cells exposed to VEGF will be driven into apoptosis.
The angioplasty site is an area covered normally by an endothelial layer of cells damaged by, amongst others, oxidative stress and hyperlipidaemia. These cells are largely stripped away during PCI. VEGF might complete this process by driving any remaining dysfunctional cells to apoptose. Later, VEGF may promote the overgrowth of endothelium from nearby, less atherosclerotic, parts of the artery. Such a hypothesis would be compatible with the results seen in animal models, but cannot be substantiated without study in patients undergoing PCI, where the endothelium is a mix of healthy and unhealthy subtypes.

3.4.3 Growth of HUVECs with VEGF – eluting stents.

VEGF-loaded stents were effective in promoting endothelial cell growth, albeit by only 11% more than the control groups in the HUVEC study. The effect seen was comparable to the stimulus seen when VEGF was added directly to the culture medium, so the delivered dose of VEGF was higher than that necessary to produce maximal growth stimulation. The relative increase in cell numbers was not large in the VEGF group. This may be because the cells in the control group were already proliferating at a near maximal rate. Other studies have shown

previously that the dose of VEGF needed to produce half-maximal growth stimulation was less than 1ng/ml[5]. The stents used in this work will release significantly more than this into the surrounding medium. No toxic effect on cell growth of this dose was seen, nor did the polymer of the stents themselves have any adverse effects on endothelial cell growth.

3.4.4 Functional effects of VEGF-eluting stents

Positive NO production was seen in all the wells equally, despite the absence of any ECs in the negative control wells. This implies contamination at an early stage in the experiment with a nitrate or nitrite-containing substance. It is possible that the growth medium used contained substances, possibly peptides, which were small enough to pass through the 10000MW cut-off filter and contaminate all the readings.
No comment can be made about the effectiveness of VEGF released from stents in producing any *functional* response in HUVECs.

In summary, VEGF has been shown to promote EC growth. Stent sections maximally absorbed with VEGF released VEGF into the surrounding fluid, in this case cell culture medium. Increased cell growth was seen in cells exposed to VEGF-coated stents. The effect on growth was preserved beyond any very rapid loss of VEGF from the stent, since stent sections that had been washed for 24 hours with medium and then added to the cell cultures still significantly increased cell growth. VEGF-coated stents retained their effectiveness after sterilisation of the stents.

Chapter 3 References

1 Thieme H, Aiello LP, Takagi H *et al.* Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes* 1995;**44**:98-103.

2 Visweswaran P, Johnson AD, Tock CL, *et al.* Stimulation of growth and migration of vascular endothelial cells by vascular endothelial growth factor transduced smooth muscle cells in co-culture. *ASAIO Journal* 1997;**43**:M753-M758

3 Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Communications* 1991;**3**:207-212.

4 van der Zee R, Murohara T, Luo Z, et al. Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 1997;**95**:1030-1037.

5 Favard C, Moukadiri H, Dorey C *et al.* Purification and biological properties of vasculotropin, a new angiogenic cytokine. *Biology of the Cell* 1991;**73** :1-6.

6 Chen CH, Jiang W, Via DP, *et al.* Oxidized low-density lipoproteins inhibit endothelial cell proliferation by suppressing basic fibroblast growth factor expression. *Circulation* 2000;**101**:171-177.

7 D'Arcangelo D, Facchiano F, Barlucchi LM, *et al.* Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor and vascular endothelial growth factor expression. *Circulation Research* 2000;**86**:312-318.

8 Yang W, de Bono DP. A new role for vascular endothelial growth factor and fibroblast growth factors: increasing endothelial resistance to oxidative stress. *FEBS Letters.* 1997;**403**:139-142.

Chapter 4

VEGF-eluting stents - *In vivo* assessment.

4.1 Introduction.

To test the apparent beneficial effects of VEGF-eluting stents on endothelium and neointima, they were tested *in vivo*. A rabbit iliac artery model was chosen, after consideration of alternative models available (*see section 1.4.1*).
Rabbits used underwent one of two different procedures. These were the acute and chronic groups.

4.1.1 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 VEGF eluted from a stent might have some effect on the thrombosis process early on. This effect would be independent of any mitogenic effects on the endothelium. Rather, this would be due to the NO-stimulation known to occur with VEGF (*see section 1.8.3*). NO-donor coated stents have been shown to be anti-thrombotic *in vivo*[1]. VEGF released early after implantation might protect a stent against thrombosis through its stimulation of NO. Such an anti-thrombotic effect might also be related to the anti-restenotic effects of VEGF, since thrombus formation is itself a stimulus for intimal hyperplasia (*see section 1.3.3.4*).

4.1.2 The chronic model.

The chronic model was used to examine several reactions to the VEGF-eluting stent. Firstly, endothelialisation at 7 days over the stented area of vessel was assessed. Secondly, stent thrombosis at 7 days was observed. The animals studied at seven days were examined for the amount of endothelialisation that had occurred over the stented vessels – either with or without VEGF. This time point was chosen as it is known that in the rabbit endothelialisation occurs quickly and is complete at 28 days[2]. Partial endothelialisation is of more interest, as it is here that any differences between the groups might be seen. Seven days was used as the

time point of studies by Van Belle and colleagues[3] when administering VEGF through a perfusion balloon to stented rabbit iliac vessels.
The extent of in-stent neointima formation at 28 days was measured using digital morphometry. Digital morphometry was also used to ascertain whether the different stent types had had any discernible effect on either the media, where the proliferating vascular smooth muscle cells originate, or on the luminal area. This latter corresponds to the measure most commonly used clinically to determine the success or otherwise of any new treatment, i.e. quantitative coronary angiography.

4.1.3 Background to techniques used.

4.1.3.1 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[4]. ^{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[5; 6].

4.1.3.2 Cyclic Flow Variations.

Cyclic flow variation (CFV) is the observation that flow down a damaged vessel varies. Typically, flow gradually reduces and then is abruptly restored to normal, due to the gradual accumulation of thrombus and its sudden dislodgement[7; 8]. The stimuli to CFVs occurring include adrenaline[9]. Clopidogrel can prevent adrenaline-enhanced cyclic flow variation in canine coronary arteries[9]. In a canine study[10], CFVs were produced in endothelial-injured coronary arteries. Transient coronary occlusion during CFVs induced electrocardiographic ST segment changes which returned to baseline after reflow. In those dogs which developed persistent coronary occlusion, histologic examination showed thrombus formation at the stenotic site and evidence of myocardial infarction. Eichhorn and colleagues[11] found

spontaneous variations in coronary blood flow velocity in 3 of 13 patients undergoing angioplasty for severe angina. The authors concluded that flow variations were related to platelet aggregation, vasoconstriction, or both at the site of angioplasty induced-injury. Sunamara *et al*[12] showed that although CFV was rare in clinical practice (~5% of cases), it was an important predictor of imminent, clinically significant thrombus formation or even acute occlusion.

4.1.3.3 Evans' Blue dye.

Evans' Blue is an azo dye that binds to circulating albumin. This dye/protein complex cannot cross a viable endothelium. Sections of vessel with an intact endothelium do not stain with the dye, whilst segments with dysfunctional or absent endothelium will allow the dye to stain the underlying surface of the vessel[13]. This dye has no active effects on the vessel, but serves as a marker of areas that are denuded of endothelium, such as occurs immediately after angioplasty. The recovery of this endothelium has been traced using this dye in a rabbit model[14].

4.1.3.4 Silver staining.

Silver nitrate solution can be infused into vessels segments. The stain is taken up at the cell boundary of ECs. Under scanning electron microscopy, this allows the cells to be more easily differentiated from underlying tissues[13].

4.1.3.5 Critical point drying.

Water has a relatively high surface tension at the liquid-gas phase and when wet tissues are exposed to a vacuum excessive distortion and shrinkage of the tissues occur. Other substances, including acetone, lose the liquid-vapour stage at certain temperatures and pressures (the critical point). Tissue integrity may be better preserved by replacing the water in them with acetone and then subjecting the samples to critical point drying. The process of critical point drying replaces the dehydrating fluid, in this case acetone, with a transition fluid such as liquid CO_2 at the critical point for the fluids. This transition fluid is then removed at the critical point, leaving a dehydrated tissue sample. Despite the improvement in tissue integrity that is seen by the use of these dehydrating and intermediate stages, it is

recognised that some tissue distortion or shrinkage is inevitable. Nevertheless, critical point drying is at least as good as other methods of drying animal specimens[15].

4.1.3.6 Sputter coating.

Sputter coating is a method used to coat samples for SEM 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 then 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.

4.2 Methods and materials.

4.2.1 Loading of stents with VEGF for *in vivo* work.

3x10mm Supra G stents polymer-coated stents were gently removed from the delivery balloon and immersed, unexpanded, in 2mg/ml VEGF in PBS solution. After twenty-four hours, they were removed from the solution and allowed to dry. The stents were then replaced on the delivery balloon with minimal handling of the stent itself. Stents were removed from balloons to coat all surfaces of the stent and to prevent superficial VEGF adhesion to the surface of the balloon. 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 VEGF were treated identically. Stents were stored, in sterile packs, at 4°C, for up to two months.

4.2.2 Angioplasty and stent model.

Male New Zealand White rabbits aged 4-8 months and weighing 3.0 - 4.5kg were used. 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.
Clopidogrel and Aspirin (1mg/kg/day) were administered in drinking water for five days before procedures and until sacrifice. General anaesthesia was induced 30 minutes after premedication with Hypnorm 0.3ml/ kg (a combination of fentanyl citrate 0.315 mg/ml and fluanisone 10mg/ml) (Janssen-Cilag, High Wycombe, Bucks). 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 intra-operative monitoring of heart rate, respiratory rate and rectal temperature. All animals were given 1000IU unfractionated heparin before angioplasty.

4.2.3 Acute Model.

The ventral aspects 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. The

right and left neurovascular bundles, composed of femoral artery, nerve and vein, were exposed by blunt dissection and the superficial femoral arteries carefully freed of surrounding adventitial connective tissue. Next, a midline abdominal incision was used to open the peritoneum; the abdominal contents were deflected to the right lateral position and the iliac arteries exposed and dissected free of surrounding tissues to facilitate placement of a perivascular flow-probe on each side.

An arteriotomy was fashioned between two ligatures placed loosely around the superficial femoral artery distal to its lateral circumflex branch (*fig.4.1*); arterial bleeding was controlled by tension on the proximal ligature. A 3x15mm diameter, non-compliant, coronary angioplasty balloon catheter 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 3x10mm Cook Supra G stent was placed using a further delivery balloon into the iliac artery. These were either a control or a VEGF-loaded stent, also mounted on a 3x10mm diameter non-compliant balloon. Choice of stent was non-blinded, but determined before starting the body of work. VEGF and control stents were spread evenly throughout the course of work to avoid one group being influenced by any effects of a learning curve in performing the procedures. The stent was introduced into the femoral artery, advanced to the proximal common iliac artery under direct vision 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.

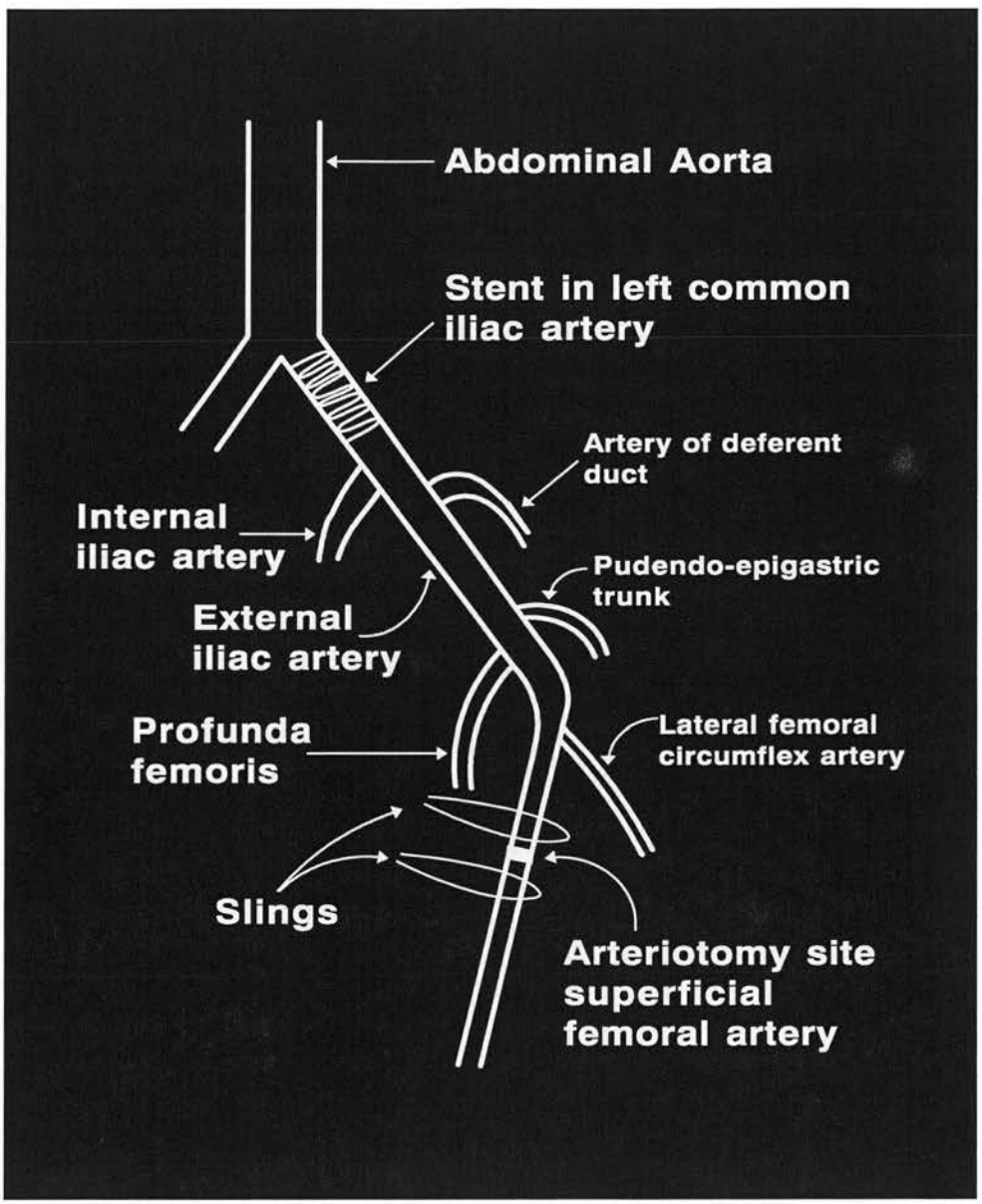


Figure 4.1. Schematic illustration of operative anatomy. (illustration from MD thesis by Rajesh Aggarwal).

4.2.3.1.1 Cyclical flow measurements.

Baseline blood flow before any angioplasty or stent placement was recorded for 2-3 minutes through each common iliac artery using perivascular transit time flow-probes (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 two hours thereafter with the flow-probe placed immediately distal to the stent.

4.2.3.1.2 Indium labelling of autologous platelets.

A minor modification of the method of Hawker *et al* was used to label rabbit platelets with indium-111[16], as detailed below.

17ml of rabbit blood were obtained, by cannulating an ear artery, in 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 was used to minimise 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 how much volume remained in the tube that was not PRP. 10µl of PRP was removed, to be used to count the total platelet concentration involved (see below). The blood from the other syringe was centrifuged at 640g (2100rpm, Wifug centrifuge) for 10min. 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 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 added dropwise 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 re-suspended 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 re-injected 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.

To calculate the number of platelets represented by a single radioactive count, the platelets per ml were counted. This used 10µl of PRP taken before the labelling process. 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.

4.2.3.1.3 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.

4.2.4 Chronic model.

Animals that were allowed to recover after stent deployment for 7 or for 28 days were anaesthetised and prepared as above. Operative dissection was restricted to left-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 7 or 28 days and vessels dissected free as before.

4.2.4.1 Method for study of endothelialisation and thrombus formation at 7 days.

The rabbits were sedated and anaesthetised under G.A. as before. Under G.A., 40mg (i.e.approx. 10mg/kg) in 2ml normal saline Evans Blue dye was injected intravenously. This was allowed to circulate freely for at least half an hour to stain any endothelium-denuded vessels. An abdominal incision was made and the abdominal aorta and vena cava visualised. Using blunt dissection, the aorta was dissected free of surrounding structures as far down as 1cm below the iliac artery stent site. The iliac artery on the contralateral side was also dissected free. When these structures had all been identified, a small gauge catheter was placed into the abdominal aorta. 10ml of normal saline was flushed through. This was followed by 10ml of 0.25% silver nitrate solution over 20 seconds. Simultaneously an overdose of sodium pentobarbitone was given intravenously. The silver nitrate was followed by a further 10ml of normal saline to flush the vessels. The animal was exsanguinated post-mortem. This allowed more ease at dissecting out the iliac arteries and distal aorta. The arterial block of tissue was placed in 10% formalin for fixation prior to further examination for endothelialisation.

The extent of endothelialisation and thrombosis were assessed by:

a) Dividing the stents lengthwise and partly flattening them. The flattened stents, stained with Evans Blue, were examined under light microscopy and scanned photographs were examined by a blinded observer to evaluate the extent of endothelialisation.
b) Examining the tissues under scanning electron microscopy.
c) Wet and dry weights of any adherent thrombus in the stented section.

A macroscopic assessment of the stented arterial segment was made. 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

Any thrombus visible was carefully removed from the stent structure and weighed. This thrombus was re-weighed at a later date when completely dried. The semi-quantitative

measure of stent thrombus formation in this model has been used in previously published work (MD thesis, Dr. R. Aggarwal).

4.2.4.1.1 Endothelialisation – Light microscopy.

Stented vessels were cut open longitudinally, leaving the stent *in situ*. The stent struts were cut carefully longitudinally and gently prised apart to reveal the luminal aspect of the stent, still in contact with the vessel. It was not possible to completely flatten the stent without detaching it from the vessel wall and so all studies were done on the portion of the stent opposite the cut struts. The opened vessel and stent were pinned to cork and photographed under a binocular light microscope (Olympus SZ). This was set at a magnification of x40 for the maximum close-ups of the stented vessel. The vessels were lit from above with a cold-light source (Schott KL1500). 800ASA colour film was used. Multiple photographs were taken of each section, in particular of the stent struts. Each photo was scanned into digital format, focusing on the parts of the photo where the stented vessel was uninjured by the process of opening the stent longitudinally, i.e. the part of the vessel opposite the incision point. A blinded observer was asked to assess the amount of blue staining in each section relative to undamaged tissue in the same samples. The staining was graded 1 to 5 in increasing subjective impression of the amount or intensity of Evans' Blue staining.

4.2.4.1.2 Endothelialisation – scanning microscopy.

Samples of tissue were initially preserved in 4% formaldehyde in PBS. This was replaced after three or four days with pure PBS. The tissue was dehydrated gradually in increasing concentrations of alcohol and acetone (appendix 2). The dried tissues were then carefully removed from their cork backing and the pins removed. The tissue was mounted on a pedestal and sputter coated with a 15nm layer of gold in a Polaron sputter coating unit E5150.

4.2.4.2 Method for study of restenosis at 28 days.

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 N. Malik. The technique used to resin-embed the sections is described fully in published work[17]. The author of this work supervised the processing of the tissues used in this work.

4.2.4.2.1 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 hydroxyethyl methacrylate) for 24 hours at 4°C. Blocks were then transferred into an embedding solution (infiltrating solution with tetramethyl aniline) and orientated in polythene 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-attak adhesive.

4.2.4.2.2 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 (see appendix 2). 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.

4.2.4.2.3 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 32 separate slides were examined (8 animals per group, two slides

from each, control and VEGF coated stents). Day zero slides were examined to ascertain whether there were baseline differences in the deployment of VEGF *vs.* bare stents. This was determined by measuring the luminal area in both groups as a guide to whether the stents had been deployed in an equal fashion.

The 28-day slides were examined and intimal growth quantified, using two different techniques. At a low power magnification of the vessel cross-section the area of the lumen was calculated. Then the total area of the neointima, the stent sections and lumen was measured and the luminal area subtracted. At a higher magnification, further measurements were made of each quarter of the vessel. This allowed more precise tracing of the boundary between media and intima and gave a measure of the average intimal thickness within each quarter. The results for each quarter were averaged to give the mean intimal depth in that cross-section of the vessel. All measurements were performed by a single, blinded observer. To ensure the results were reproducible, three randomly selected vessels were re-measured and the various measurements plotted against the first reading. Intima:media ratios were also calculated. Luminal area was measured in all the 28-day specimens and the results compared both between the two treatment groups and against the day 0 (baseline) average luminal area (i.e. an indication of late luminal loss).

4.2.4.2.4 Tissue fixation and staining.

H&E staining of tissue specimens was performed as described in Appendix 2.

4.2.4.2.5 Injury scoring.

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*[18]. 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 thicknesses 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.

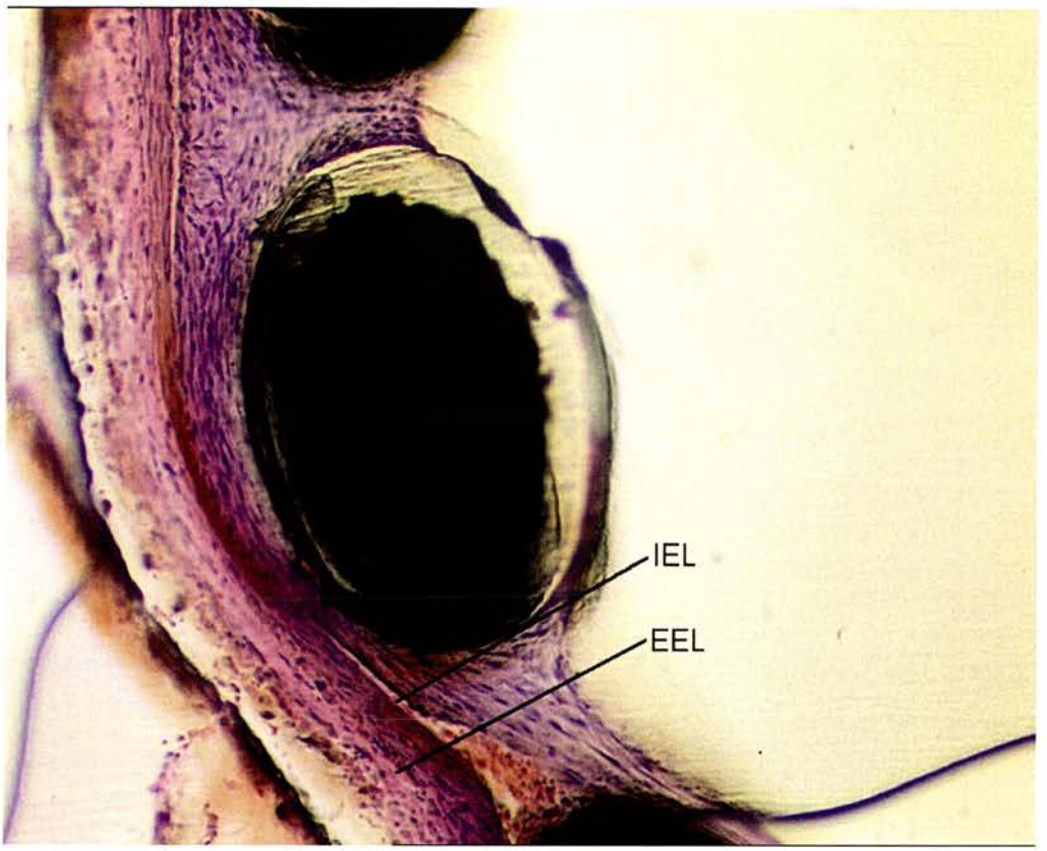
The scoring system that was described in this paper and was used here is as follows. Two slides (out of the 20+ from each specimen) from each stented vessel were examined. Each vessel was examined microscopically and the number of stent/vessel contacts was counted.

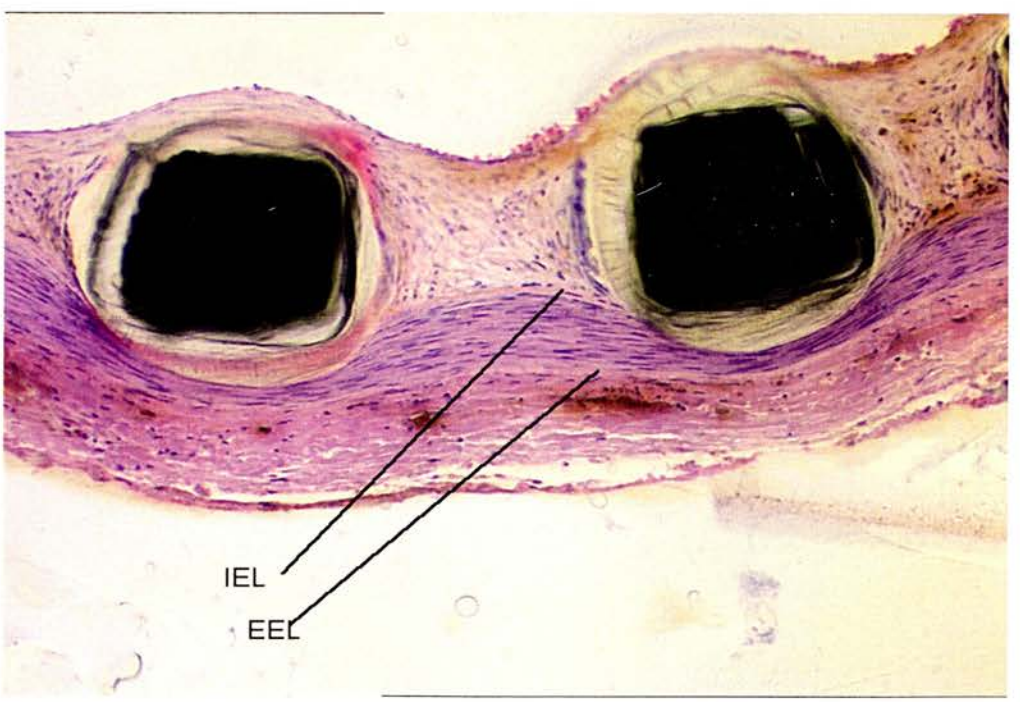
Each contact point was then scored as follows to indicate how much injury that stent section had caused to the adjacent vessel wall:

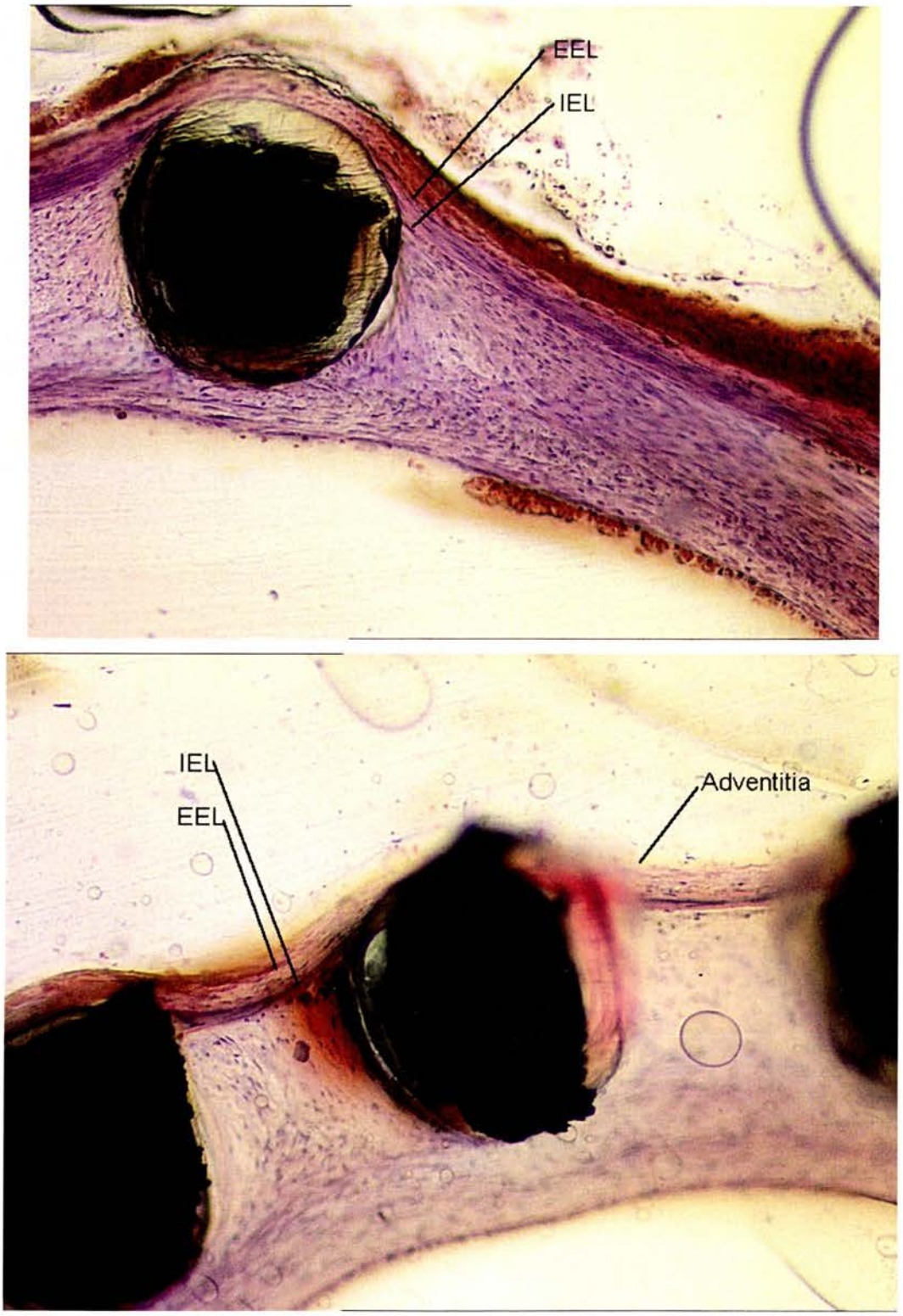
Score	Description of injury
0	IEL intact, media compressed slightly
1	IEL lacerated, media compressed
2	IEL lacerated, media compressed >50%, EEL compressed but intact
3	IEL and EEL lacerated, stent section visible in adventitia.

(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 VEGF-stent and plain-stent groups.


IEL
EEL


IEL
EEL



Figures 4.2. Varying injuries to vessel wall. These pictures demonstrate the different injuries seen in the rabbit iliac vessel after stent insertion. Variable severity of injury is seen with damage progressively to the internal elastic lamina (IEL), the media and the external elastic lamina (EEL). Rarely, the injury can be so severe that the stent section breaks right through into the adventitia.

4.2.4.3 Method for study of systemic distribution of VEGF from *in vivo* studies.

Since the main reason for using stent-based delivery is to ensure a local delivery of VEGF at the target site, it was decided that the extent, if any, of systemic delivery should be evaluated. During the termination procedures of the various parts of the *in vivo* studies, samples were taken of liver and kidney tissue, the most likely organs to eliminate any excessive amounts of VEGF. The presence of above normal concentrations of VEGF in either of these tissues would demonstrate the extent to which VEGF delivered locally was being distributed systemically. The technique used to detect VEGF in these tissues was an immunoassay (Quantikine DVE00) using techniques suggested by the manufacturers (R&D Systems Europe Ltd).

Liver and kidney tissue was stored at 4°C in 4% formalin solution. Tissues were then vacuum-dried for at least eight hours using a FTS systems Flexi-dry MP machine. This gave a desiccated sample. The dried samples were then powderised with a mortar and pestle in liquid nitrogen, which aided the fragmentation of the samples. The powdered tissue was added to 4ml HEPES buffer (see appendix). The resulting solution was then homogenised and centrifuged at 3000g for 10min at 2°C. The supernatant from this was collected and centrifuged at 100,000g for 45min at 2°C for 45min. The resultant supernatant was collected and labelled as the cytosol fraction. The pellet was washed with HEPES buffer and re-suspended in TBS buffer using a small glass-glass homogeniser to yield the membrane sample. Total protein content of each preparation was determined using the Pierce-Bradford reagent (see below). The amount of VEGF was detected using the VEGF Quantikine kit (DVE00) from R&D systems. This involved diluting 25µg of the sample solution in RD5K (buffered protein base and preservatives) diluent from the kit to a final volume of 200µl. Where insufficient protein was in the samples, 200µl was used directly and the amount of protein this represented recorded. A VEGF dilution series was made using the stock solution of 2µg/ml VEGF provided in the kit. Doubling dilutions were performed using RD5K as before.

A micro-plate pre-coated with monoclonal antibody specific to VEGF was used. 50µl of assay diluent was added to each well, followed by 200µl of either standard solution (including zero blank) or sample. The samples were incubated at room temperature for two hours. Each well was then aspirated dry and washed three times with wash buffer, taking care to remove all fluid after each wash, to wash off any unbound substances. To each well was then added 200µl of VEGF conjugate. This polyclonal antibody for VEGF had been

conjugated with horseradish peroxidase (HRP). The conjugate was also left for two hours at room temperature to adhere to all the VEGF present. Unbound conjugate was removed by again washing each well three times. 200µl of substrate solution (hydrogen peroxide and tetramethylbenzidine) was added to each well. This was metabolised by the HRP to form a coloured product. The reaction was halted after 20min by the addition of an acidic stop solution. The plate was then counted for the absorbance of each well at 450nm. Readings were adjusted for optical imperfection in the plate by also reading the plate at 540nm and subtracting these readings. All readings were also corrected by the subtraction of the reading obtained in the zero standard well.

A standard curve was drawn of the absorption and a formula calculated that allowed the absorption measurements to be correlated to a concentration of VEGF in each of the samples examined. The readings were finally expressed as nanograms of VEGF per gram of total protein.

4.2.4.3.1 Protein estimation.

Total protein in each sample was calculated using the BCA reaction[19]. The reagent is made up using a 1:50 ratio of BCA Protein Assay reagent B: reagent A. (Pierce). 200µl of this mix was put into each well of a 96-well plate with 50µl of diluted sample or diluted standard. The BSA standard was serially diluted to provide data for a standard curve (*see fig. 4.3*). The combined solution was gently agitated to mix the reagents and then incubated at 37°C for 30min. The plate was then counted in a colorimeter at 540nm.

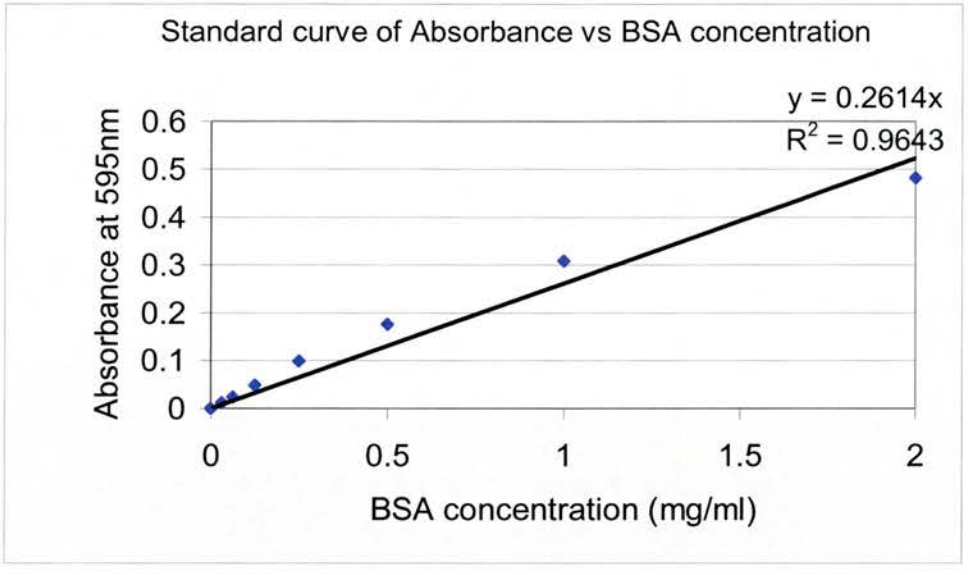


Figure 4.3. Standard curve obtained using the Pierce Bradford reagents. This allowed the calculation of protein content of tissue samples.

4.2.5 Statistical Analyses and power calculations.

Power calculations for sample sizes were calculated using MINITAB software as follows. 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. The paper by Van Belle *et al* that had used similar methods with plasmid-derived VEGF was used as a guide to the likely observed differences expected in work with a VEGF-eluting stent. 2-sample t-Tests were used with a predicted power of 90% and a significance value of $p<0.05$.

a) Stent endothelialisation

In Van Belle's paper the actual difference in means of percentage was 54%, (SEM 7.4%, n=7 SD=0.196). To reproduce these results, the sample size needed would be 5. To calculate sample sizes in this work, a smaller reduction in endothelialisation was assumed. Alpha = 0.05, sigma = 0.196, mean difference of 0.35, target power of 0.9. This resulted in a sample size required of 8 (actual power of 0.9127)

b) Restenosis

In Van Belle's work the actual difference in means of intimal hyperplasia areas was $0.83mm^2$, (SEM 0.11, n=8 SD=0.31). To reproduce these results sample size needed would be 5. To calculate sample sizes in this work, a smaller reduction in restenosis was assumed. Alpha = 0.05, sigma = 0.31, mean difference of 0.55, target power of 0.9. This resulted in a sample size required of 8 (actual power of 0.9093)

The sample size for both endothelialisation and restenosis groups was eight animals. This gave a highly powered probability of detecting substantial differences between plain and VEGF coated stents. This number of animals was furthermore a practical proposition that took into account ethical considerations without compromising scientific rigour.
Data are presented as means ± standard deviation unless otherwise stated. Differences between means were compared using the two-tailed Student's T-Test, assuming unequal variance. Differences between plain polymer and VEGF-coated stents were analysed using the t test unless the data were non-normally distributed, in which case a non-parametric test (Mann-Whitney U Test) was used. Significance was defined as $P<0.05$.

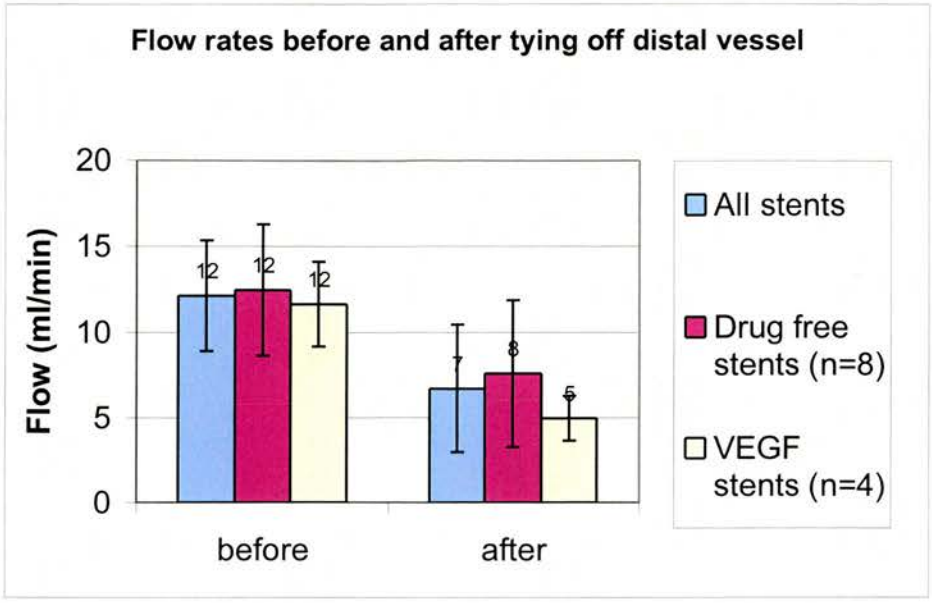
4.3 Results.

4.3.1 Acute model.

4.3.1.1 Flow following stent implantation/cyclic flow variations.

Flow measurements were obtained from six animals (i.e. 12 vessels), four control and two given VEGF-coated stents. Two of the animals assigned to the VEGF-coated stent 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 (*fig. 4.4.*), together with an example of the flow seen during this procedure (*fig.4.5).*

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. Changes suggestive of CFV was only seen in one vessel, one of the animals receiving a normal, drug-free stent. The changes in flow that indicate CFV are shown below (*fig. 4.6*).



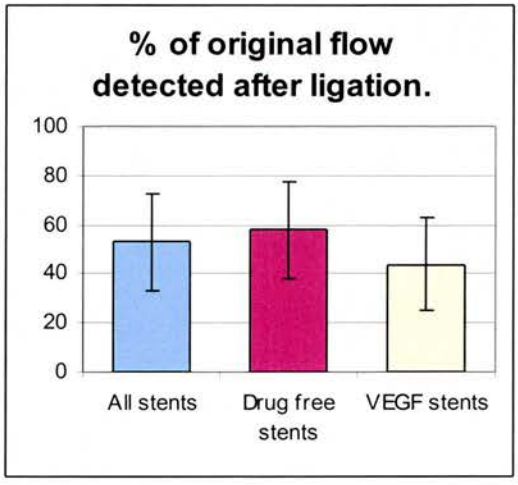


Figure 4.4. Flow through iliac arteries prior to stent insertion. No significant differences existed between the two groups prior to stenting. After ligation, flow was still about 53% of that prior to tying the vessel (53.1±19.7 %).
n.b. Although results are given for both "VEGF" and "control" stents, this differentiation is arbitrary because, at the time of flow recording, stents had not yet been implanted.

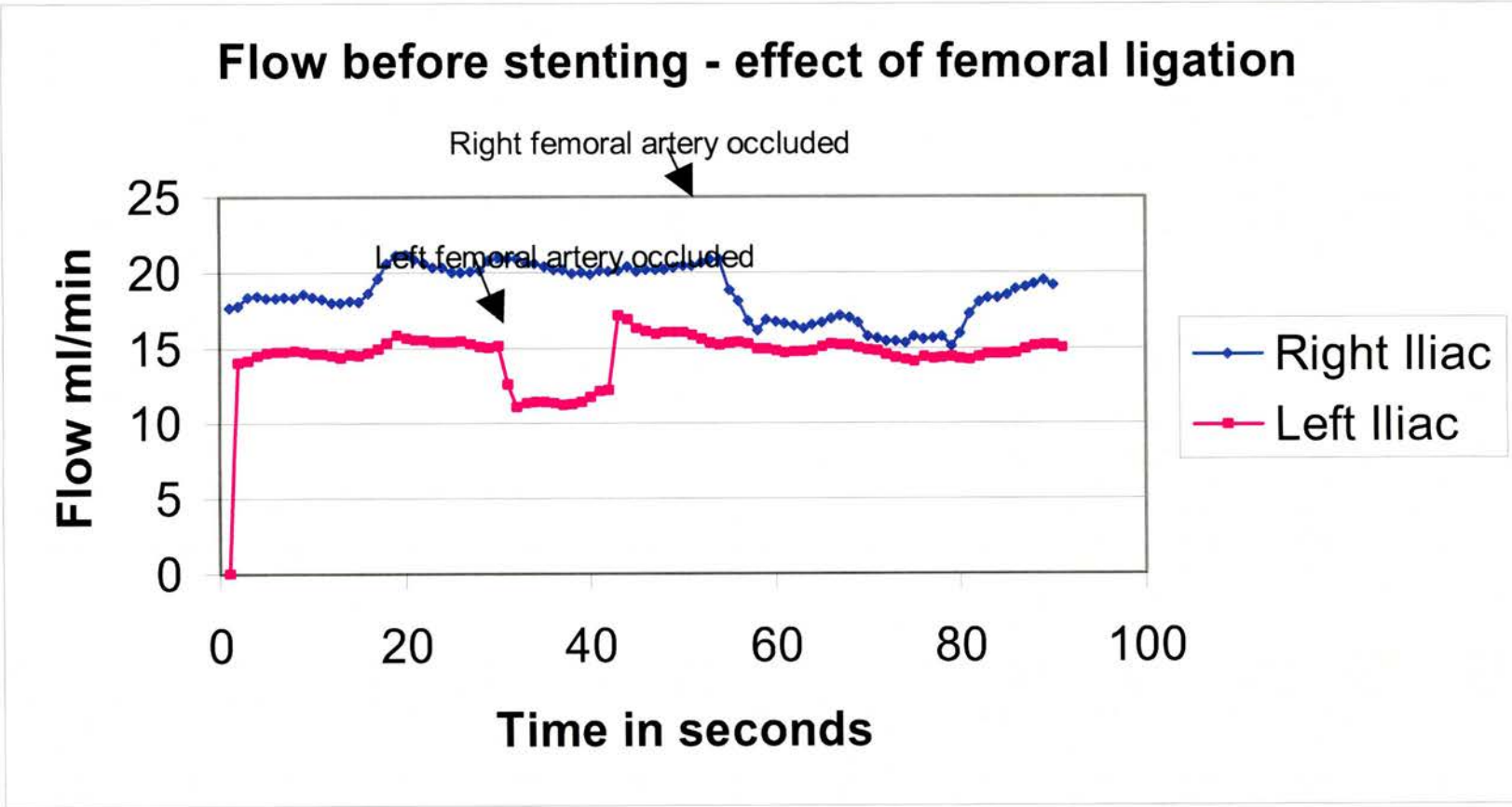


Figure 4.5. Example of flow rates through left and right iliac vessels – effects of temporary ligation of the femoral artery. Note the drop in average flow after ligation to approximately half of original flow. When the ligature is removed, flow returns to the original rate in both vessels.

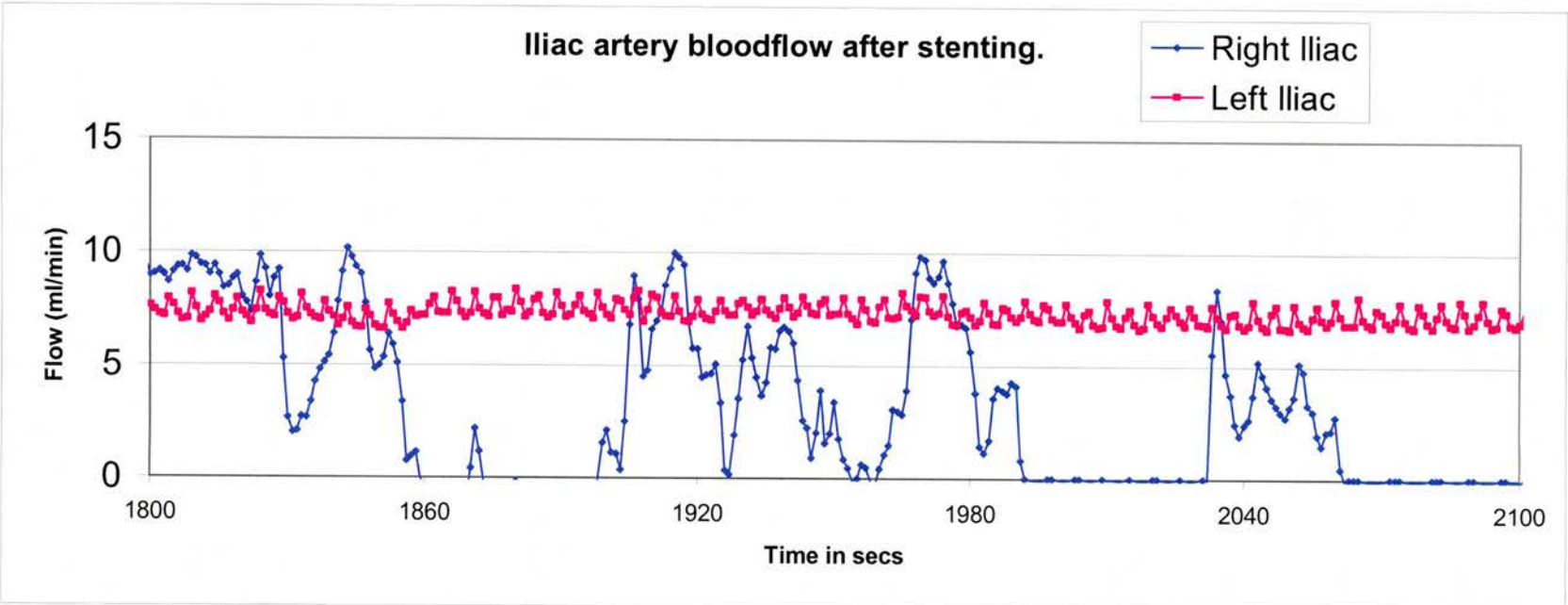


Figure 4.6. Average flow through both iliac vessels, with CFV. For a period of five minutes, 30 minutes into the recording, recurrent falls and abrupt recovery of flow is seen in the right iliac recording. Simultaneous recording of the left is normal throughout. This is typical of cyclical flow variation due to thrombus buildup that then dislodges to allow flow to recover. This occurred in one of the control group stents (CO4). n.b. Flow cannot conclusively be said to be due to CFV alone as thrombus formation was not directly measured. Spasm of the artery may also contribute.

4.3.1.2 Deposition of indium-labelled thrombus.

After the two-hour flow observation period had been completed, stented vessels were dissected free. They were flushed gently with saline to remove blood, but not adherent, formed thrombus and then fixed in formaldehyde. Any adherent fat or adventitia was removed. The vessels were then counted in a gamma counter along with samples of the labelled platelet solutions for each rabbit. Together with the known platelet counts 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. These results are shown below (*fig. 4.7*).

Labeling efficiency was calculated each time the platelets were labeled. This was 86.2±6.6%, in keeping with published efficiency in similar work and demonstrated that the labeling technique itself was done effectively.

Stent	Animal	Stent counts	Count per platelet	Platelets per stent	Mean platelets per stent in each group	Standard deviation
Control	1	57829	24.4	2369.7	19464.0	24497.2
		60703		2487.5		
	2	43971	2.42	18163.7		
		47113		19461.6		
	3	84398	17.9	4709.5		
		97292		5429.0		
	4	48040	1.74	27613.0		
		131314		75478.3		
VEGF	1	38605	6.50	5934.0	3377.9	2950.1
		38586		5931.1		
	2	34037	39.5	861.7		
		30992		784.6		

Figure 4.7. Results of Indium-labelled platelet deposition on stented iliac vessels. Variability was seen, particularly in the platelet count per animal. Two animals died perioperatively in the VEGF group, but before the stent had been inserted. No data were obtained therefore from these animals. No statistically significant difference in platelet adhesion was seen (p= 0.20; 95% CI -3444,69544).

4.3.2 Chronic model – day 7 results.

4.3.2.1 Thrombus formation.

2/8 stents in the control group were deployed too distally and were not deployed fully open. They both were filled with occlusive thrombus. These stents were not included in the analyses. 2/8 in the control group and 1/8 in the VEGF were deployed in the lower aorta rather than the iliac artery as originally planned. These stents were deployed otherwise normally and they were included in the analyses. Removing these results from the analysis gave an even greater difference between the stent groups, so any bias due to the different artery used if anything decreased the observed differences. The observed amounts of thrombus are reproduced below (*fig. 4.8*).

Treatment	**Stent number**	Extent of thrombosis	Comments	Thrombus (wet)	Thrombus (dry)
Control	1	Occlusive		92mg	17mg
	2	Minor		10mg	1mg
	3	Minor	Stent detached from vessel	9mg	2mg
	4	Occlusive	Stent maldeployed		
	5	Luminal	Stent in aorta	15mg	2mg
	6	None	Stent in aorta	0	0
	7	Occlusive		56mg	7mg
	8	Occlusive	Stent maldeployed (Under inguinal lig.)		
VEGF	1	None		0	0
	2	None		0	0
	3	None	Stent in aorta	0	0
	4	None		0	0
	5	None		0	0
	6	None		0	0
	7	None		0	0
	8	Minor	Stent detached from vessel	<1mg	<1mg

Figure 4.8. Table of results of stent thrombosis. Median clot demonstrated was 12.5mg in control stents vs. 0mg (p=0.0142; 95% CI 8.49,55.99) in VEGF stents. The stented vessels were examined after seven days. Visual inspection was made as to the extent of any adherent thrombus. This was carefully removed from the vessel and weighed, both wet and dry. Where the stent was deployed other than in the iliac artery this was also noted.

4.3.2.2 Endothelialisation.

4.3.2.2.1 Light microscopic appearances.

Almost all the sections showed uniform Evans' Blue staining, suggesting that no significant endothelialisation had occurred, or that any endothelial cells that were present were completely dysfunctional (*fig. 4.9*). 2/7 control sections and 3/8 VEGF sections were not stained at all, mainly due to blood clot adhering to the section preventing any staining.
In four of the control vessels endothelial recovery was absent because these were completely occluded with thrombus. Of the remaining four vessels, three had macroscopically visible thrombus that would have prevented some endothelialisation. In the remaining stent (CE6), which had had no visible thrombus, the Evans' Blue staining was homogenous, suggesting no significant endothelial recovery. Examples of all the light microscopy findings are shown below (*fig 4.10).* The results of the Evans' Blue staining were inconclusive. No clear demarcation was seen between newly endothelialised sections (or areas that had never had any endothelial damage) and de-endothelialised areas. In both groups a very similar, diffuse staining for Evans' Blue was seen in most of the stents, suggesting little or no endothelialisation had occurred. The lack of clearly demarcated boundaries between stained and unstained vessel wall meant computer morphometry measurements to quantify more precisely the degree of staining were not possible. In two of the vessels (VE6 and VE7) Evans' Blue staining appeared patchy, suggesting some re-endothelialisation had occurred. These sections were studied in particular detail under scanning electron microscopy.

Stent group	Number	Extent of re-endothelialisation	Other comments
Control	1	Unable to determine	Yellow
	2	3	
	3	3	
	4	1	
	5	2	
	6	1	
	7	Unable to determine	Dark Brown, clotted
VEGF	1	3	
	2	Unable to determine	Yellow
	3	1	
	4	Unable to determine	Dark brown, clotted
	5	Unable to determine	Brown, clotted
	6	3	Brown
	7	2	
	8	2	

Figure 4.9. Table showing the blinded assessment of Evans' Blue staining. A score of 1 to 5 (increasing endothelialisation) was used to produce a semi-quantitative result.

Average count in control = 2.0

Average count in VEGF = 2.2 (p=0.74).

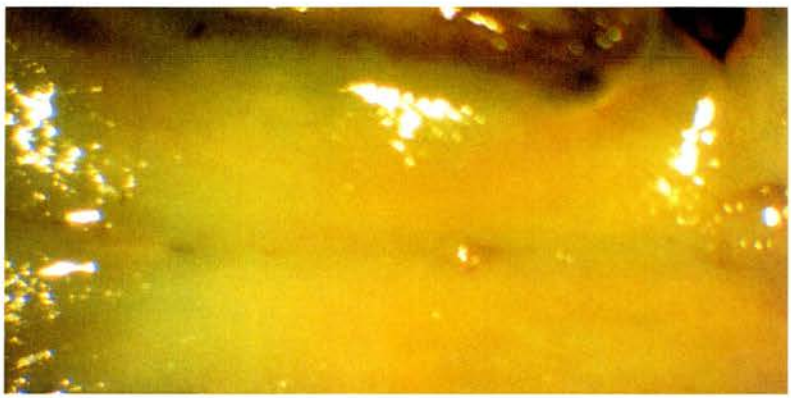


Figure 4.10a Undamaged aorta (CE7) (x20 magnification).

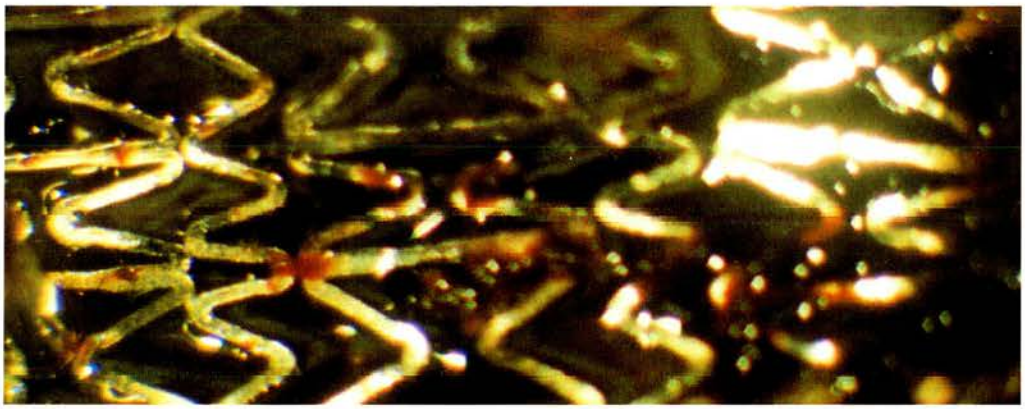


Figure 4.10b. Control stent (CE7). Note superficial adherent thrombus (x20 magnification).

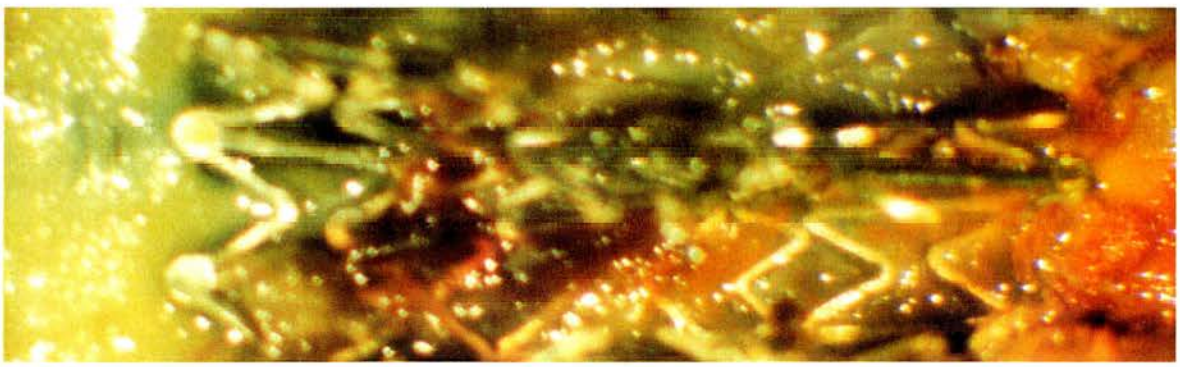


Figure 4.10c Control stent (CE5). Note transition from white (undamaged endothelium) to blue (de-endothelialised tissue). Adherent thrombus visible as orange (x10 magnification).

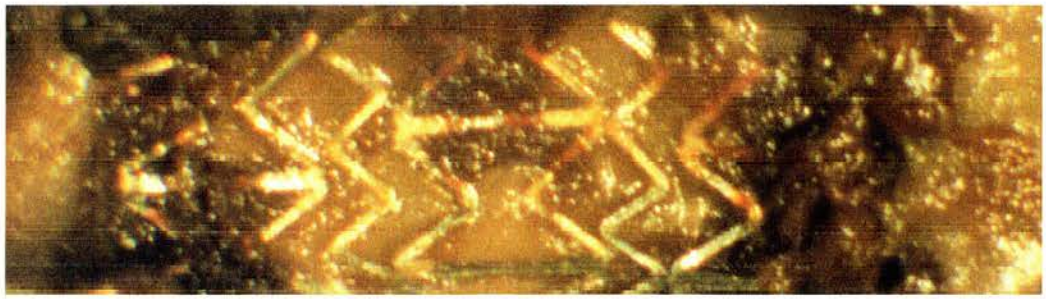


Figure 4.10d. Control stent CE1. This stent was totally occluded and so took up no Evans Blue dye at the stent site. Much adherent thrombus is visible (x10 magnification).

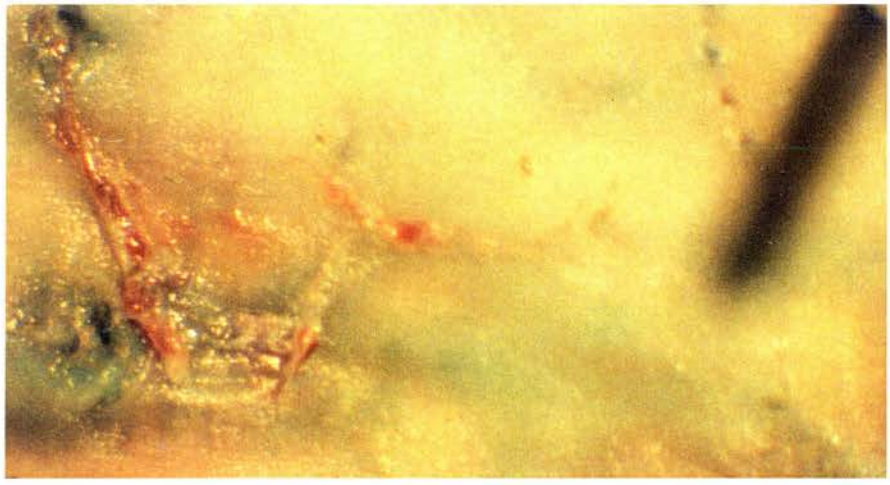


Figure 4.10e. VEGF aorta (VE6). The black shadow is artefact from a pin in the tissue (x20).

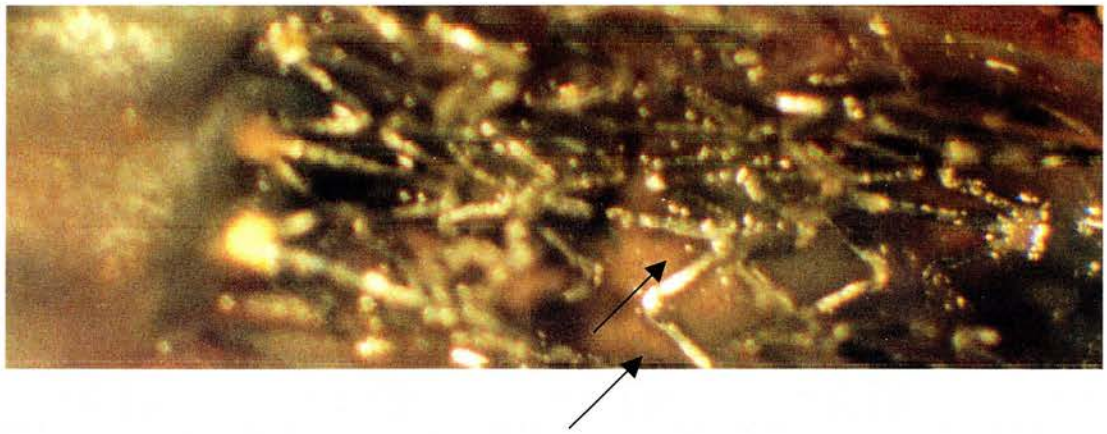


Figure 4.10f. VEGF stent (VE6). Although much of the specimen is blue, there are patches of white vessel (arrowed), within the stent that suggest re-endothelialisation (x10).

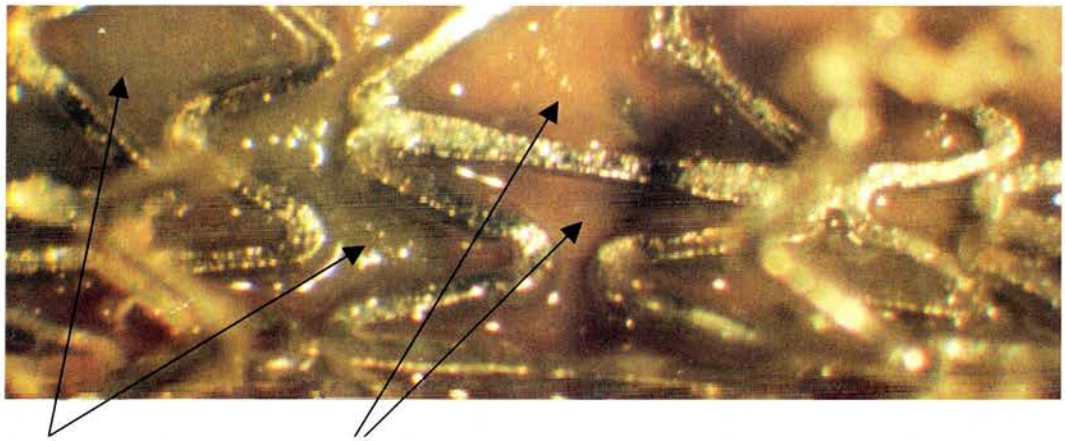


Figure 4.10g.VEGF stent (VE6). Higher power picture of same vessel as in 4.10f, showing partial re-endothelialisation (x40).

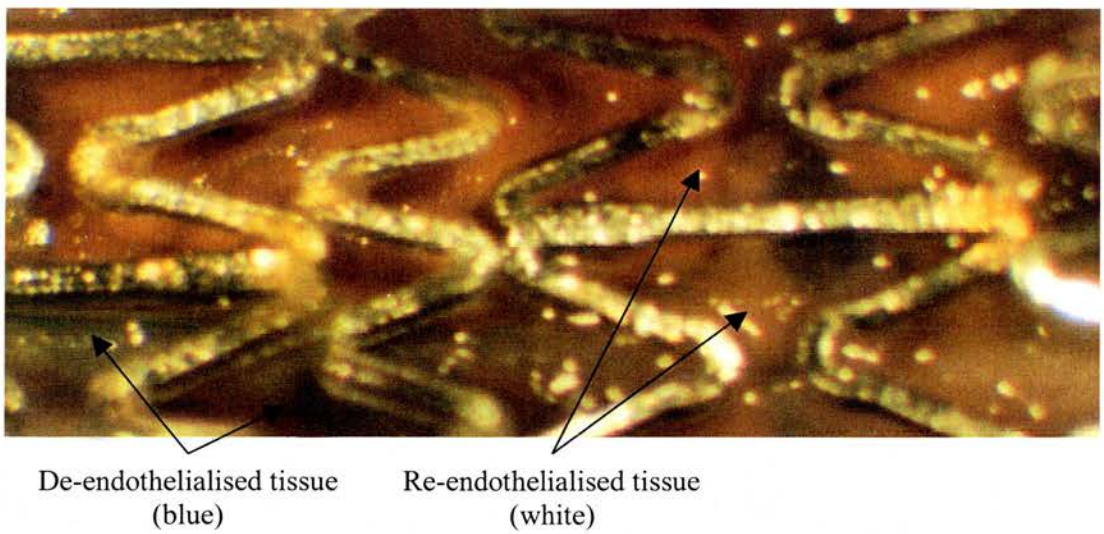


Figure 4.10h. VEGF stent (VE7). This section also showed patches of white vessel, suggestive of re-endothelialisation (x40).

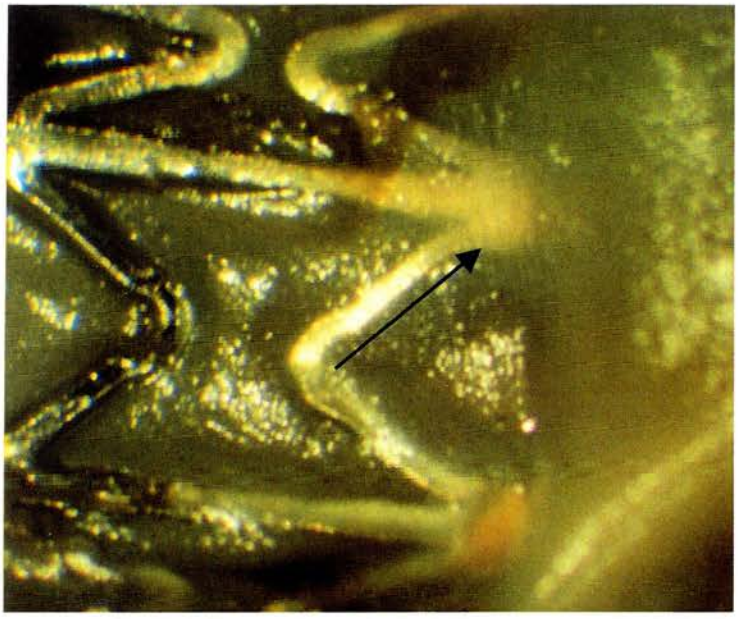


Figure 4.10i. VEGF stent (VE1) with little endothelialisation evident other than at the extreme end of the stent. In the figure, a sheet of endothelium is seen encroaching over the end of the stent struts (arrowed) (x40).

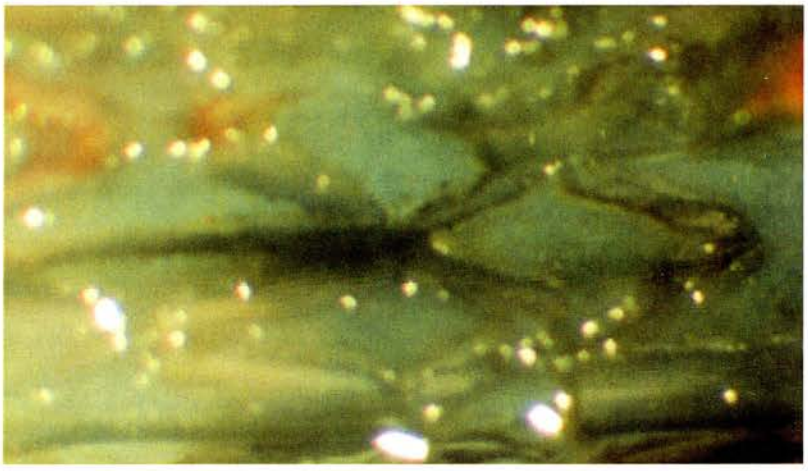


Figure 4.10j. VEGF stent (VE2). In this section the stent has been removed following staining to show the vessel wall underlying the stent struts (x40).

Figure 4.10a-j. Light micrographs of stented, Evans' Blue stained tissue. All sections were taken from animals terminated seven days after stent insertion. Evans Blue dye was administered systemically 30min before termination and tissue retrieval. All photographs were taken using a cold light source from above (giving occasional bright, white reflection artefacts). The film used was 800 ASA.

4.3.2.2.2 Scanning electron microscopy.

Undamaged areas of perfusion-fixed aortae were examined under scanning electron microscopy to provide negative controls where the endothelium had not been damaged. An example of such a section is shown below (*fig. 4.11*). In many of the vessels with control stents, extensive sheets of erythrocyte-rich thrombus had formed (*fig. 4.12*). Vessels with VEGF stents had a similar picture. No significant re-endothelialisation was seen in the stented segments. Less than 10% of the surface of the vessel wall was re-endothelialised. Stents VE6 and VE7 appeared to show patches of endothelial recovery in between stent struts, although the struts themselves remained completely devoid of endothelial coverage. Examples of these endothelialised areas are shown below (*fig. 4.13.a&b*).

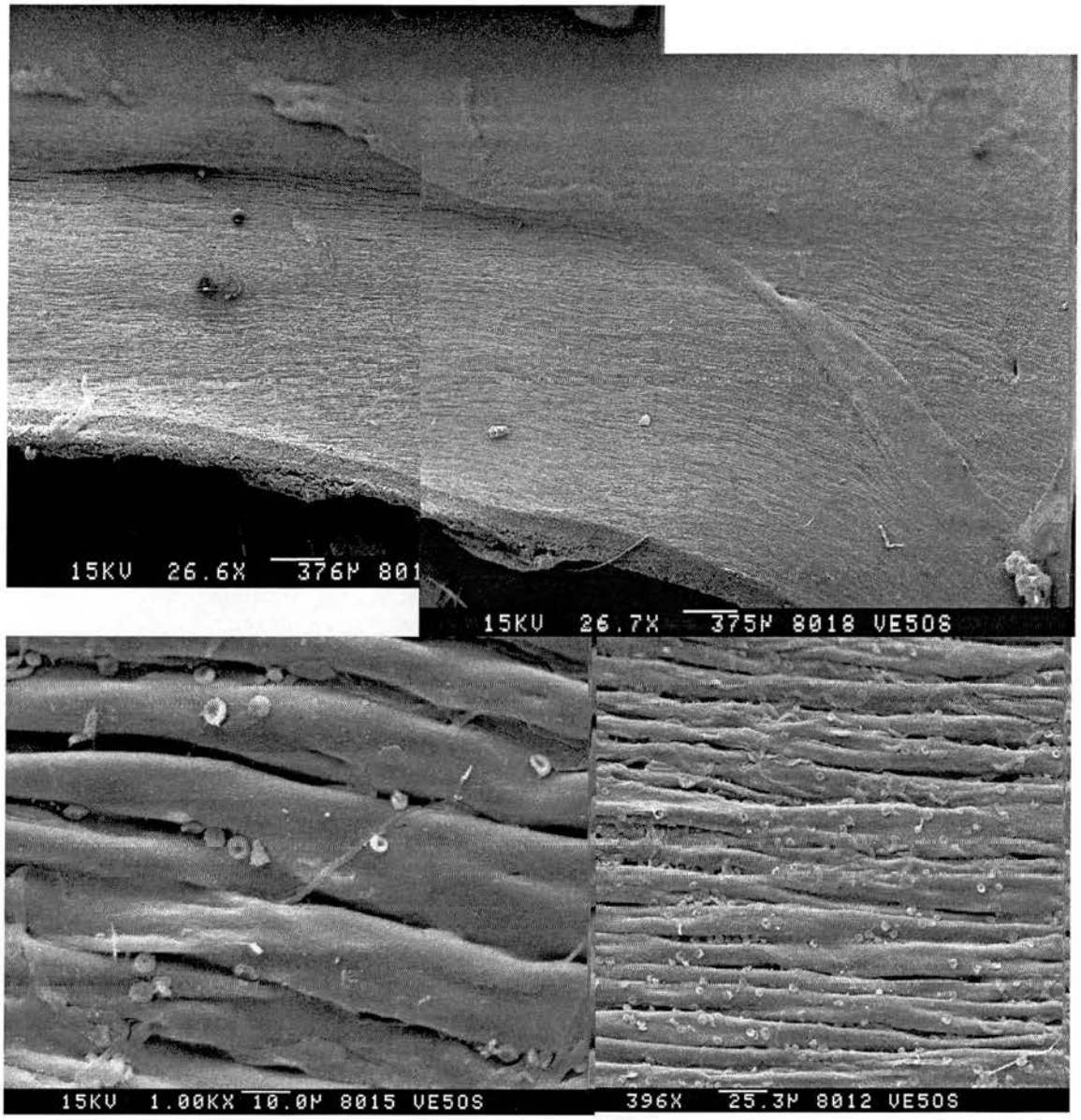


Figure 4.11. SEM pictures of undamaged, perfusion-fixed, rabbit aorta. The luminal aspect shows typical parallel ridges of endothelial cells. Top x26.6, Bottom left x396, bottom right x1000.

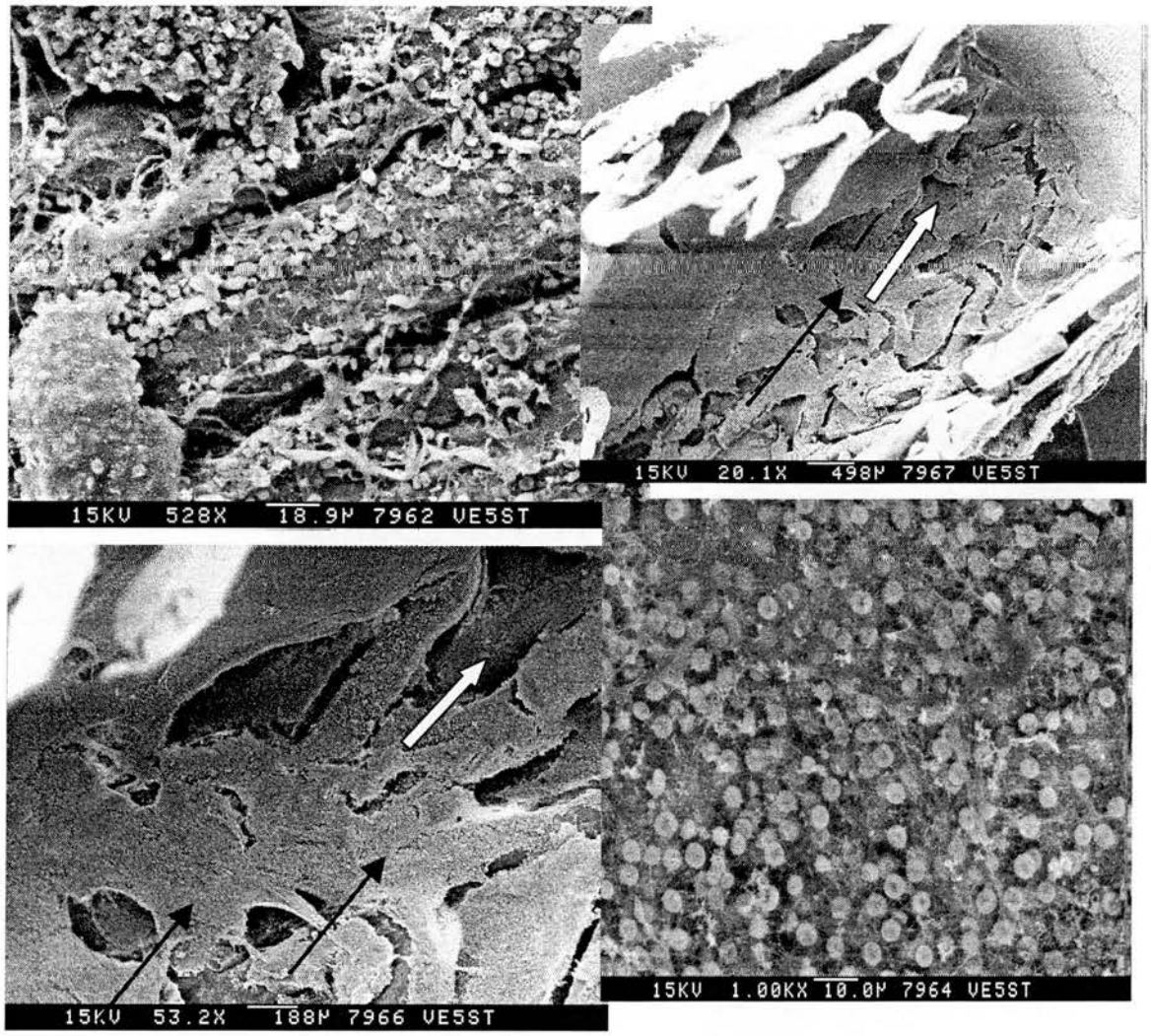


Figure 4.12 Scanning electron microscopy of stents in situ *showing extensive thrombus formation on the stent (dark arrows). Underlying these thrombus sheets, de-endothelialised tissue is seen (white arrows). Increasing magnifications up to 1000x.*

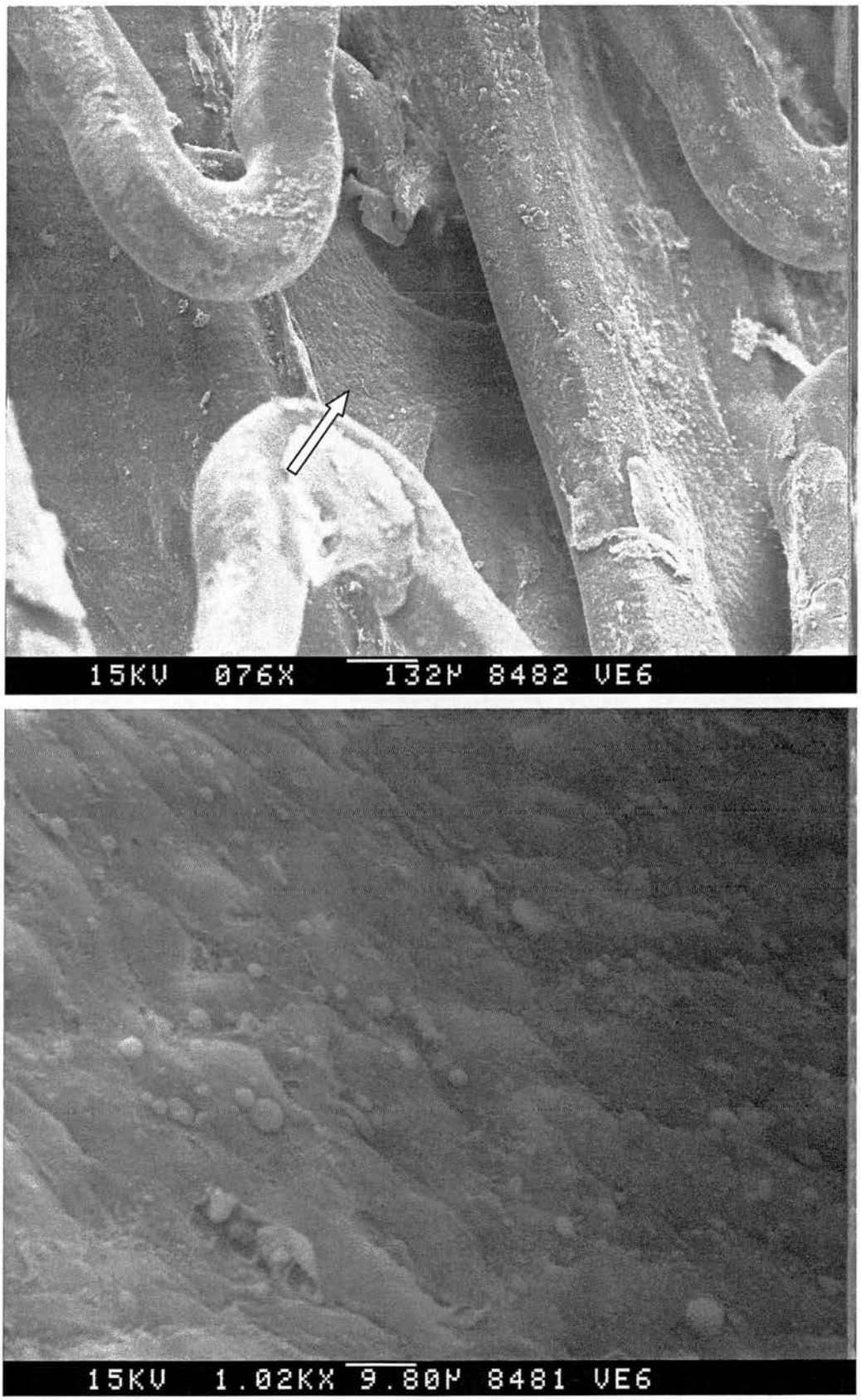


Figure 4.13a Stent VE6 at low and high SEM magnification. This shows endothelialisation between stent struts (arrowed).

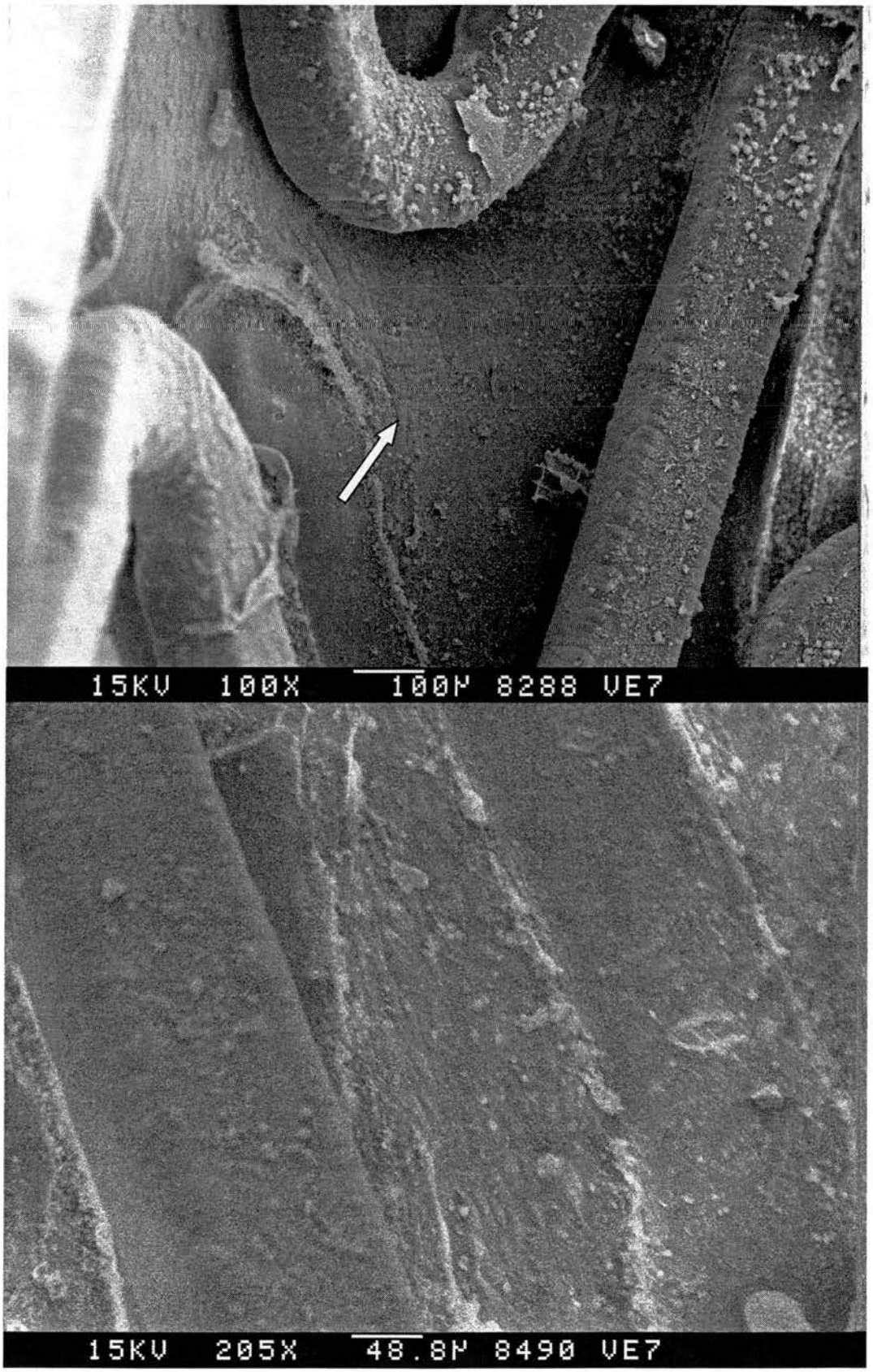


Figure 4.13b Stent VE7 at low and high SEM magnification. This shows endothelialisation between stent struts (arrowed).

4.3.3 Chronic model - day 28 results.

4.3.3.1 Injury scores.

No differences were seen in the average injury scores for the restenosis specimens at 28 days (Control 1.0±0.28 *vs.* VEGF 1.0±0.16, p=0.91).

4.3.3.2 Digital morphometry of media, intima and lumen.

Lumen measurements in the acute model samples obtained at day 0 are shown below (*fig.4.14*). The internal control results are shown in graph form (*fig.4.15*). The results of the measurements of neointimal and media areas and thicknesses are summarised below, (*fig.4.16*). No significant difference was seen in neointimal thickness or area between the two groups. Media area and media thickness also showed no statistically significant differences between the two stent groups. A slight trend (p=0.07) towards a smaller media in the VEGF group was detected. The reduction was 24% and 21% for media thickness and area respectively. Comparison of luminal size at 28 days, including comparison with luminal area in the day 0 (acute model) stents was made. No significant difference was seen in luminal area between the control and VEGF-coated stents. A trend towards decreased luminal area was, as might be expected, seen between baseline measurements and those obtained at 28 days (p=0.08 for both VEGF and control compared to day 0).
In the control group an average intima:media ratio of 1.99 ±1.05 was seen *vs.* 2.19 ± 1.06 (p=0.73). No evidence of positive remodelling was seen.

4.3.4 Chronic model - systemic delivery of VEGF.

The amount of VEGF detected in liver and kidney tissues is shown below (*fig. 4.17*).

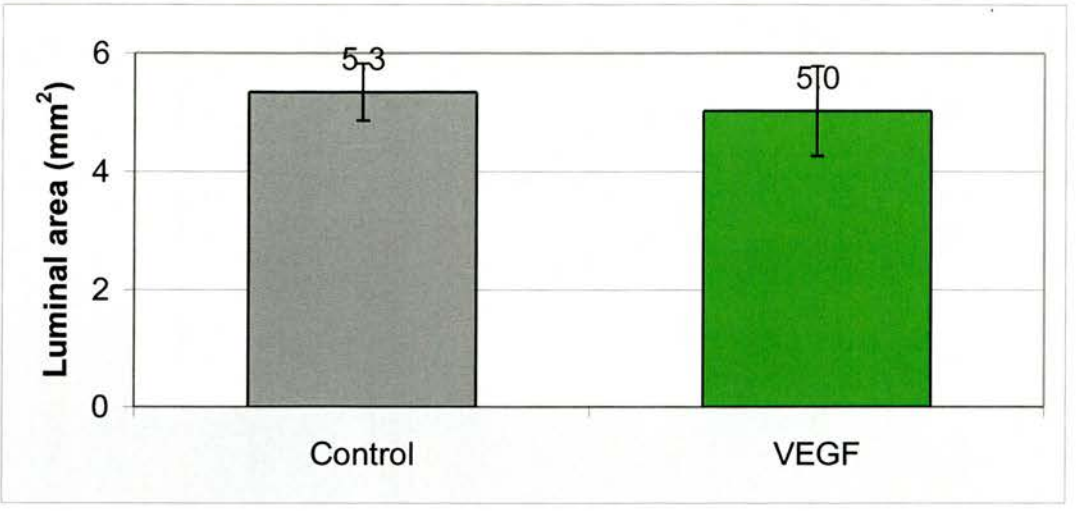


Figure 4.14. Comparison between day 0 (baseline) vessel lumens. No significant difference was seen (p=0.32) between the two groups at day 0, suggesting that the stents were equally deployed in the vessels. Values quoted are means±SD.

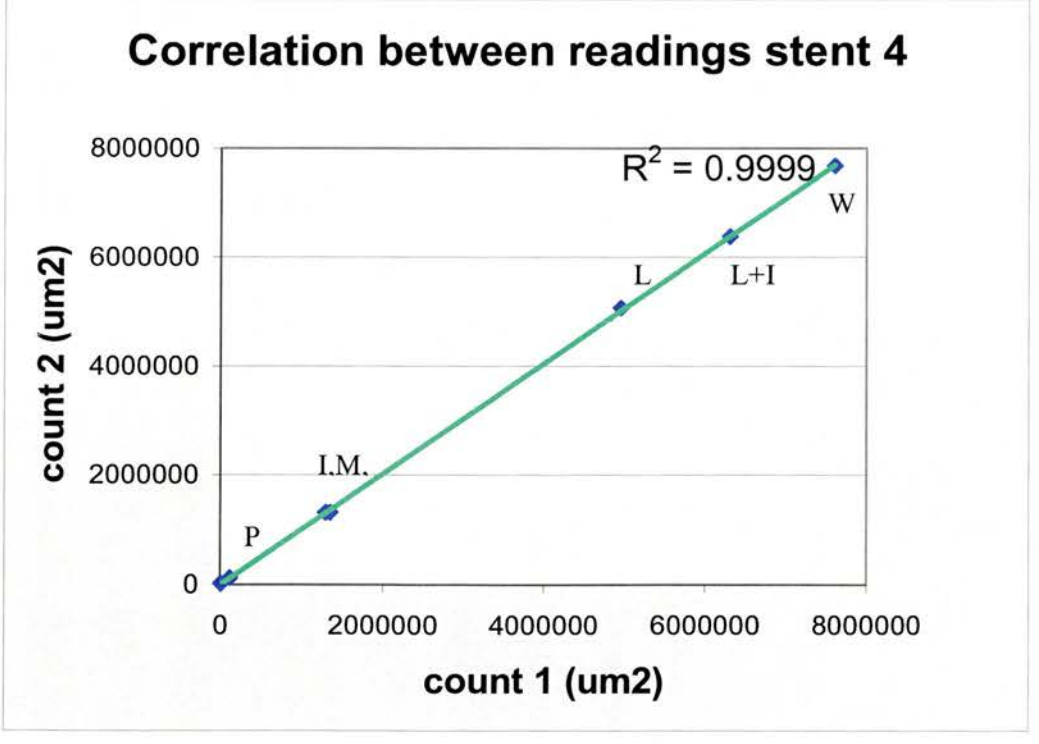


Figure 4.15a

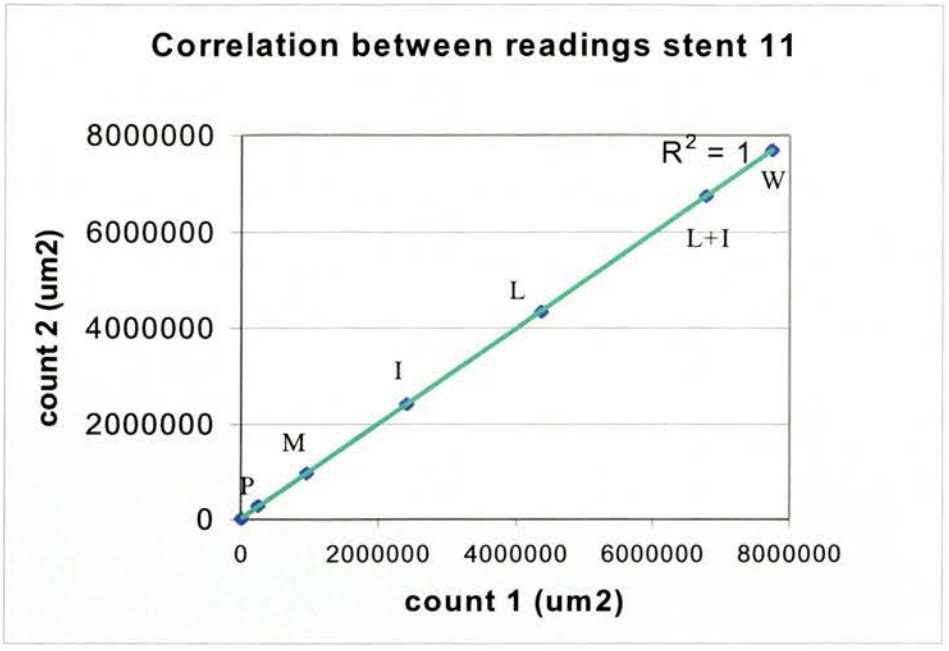


Figure 4.15b

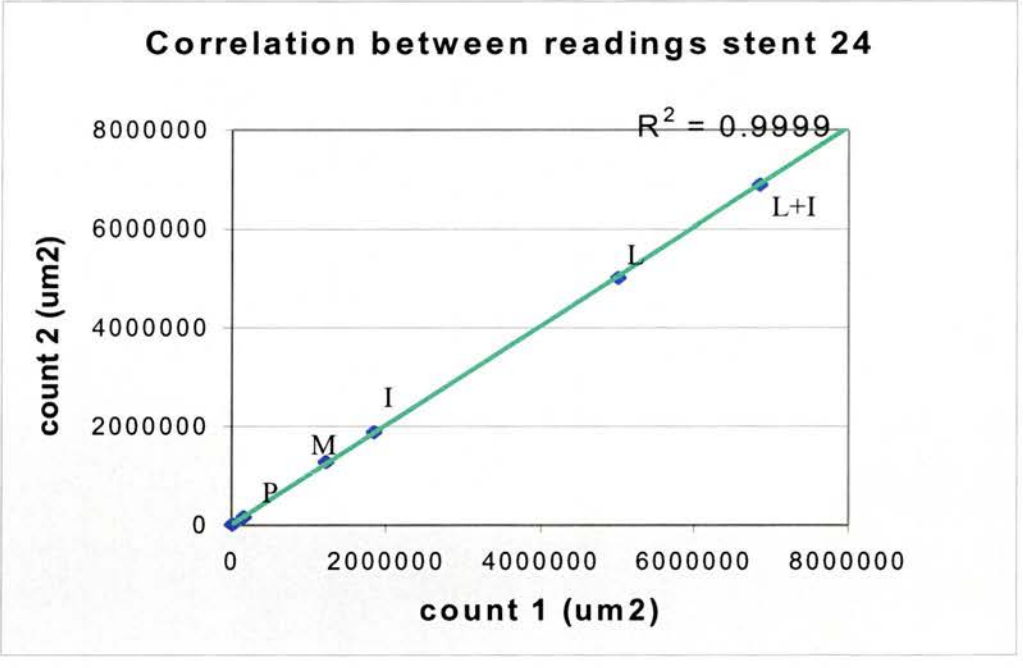


Figure 4.15c

Figures 4.15a-c. Internal controls of cross-sectional digital morphometry. A high degree of correlation in measurements of areas was seen. Thus, the readings can be confidently said to be reproducible (P=Perimeter (μm), M= media, I= intima, L=lumen, L+I=lumen plus intima, W=Whole vessel).

	Area (mm^2)			Thickness (μm)	
Intima-VEGF	2.4±1.8	P=0.81		163±50	P=0.26
-control	2.2±0.9			189±39	
Media-VEGF	0.9±0.3	P=0.07		93±28	P=0.1
-control	1.2±0.3			117±27	
Lumen-Day 0	5.2±0.6	N/A	P=0.08	NA	
-VEGF	4.7±0.5	P=0.44			
-control	4.4±1.0				

Figure 4.16. Results of digital morphometry of stented cross-sections at 28 days. Results are mean±SD.

Treatment	VEGF in ng/g total protein
Control stent day 7 kidney	807
Control stent day 7 liver	2455
Control stent day 28 kidney	5990
Control stent day 28 liver	255
VEGF stent day 7 kidney	312
VEGF stent day 7 liver	1572
VEGF stent day 28 kidney	1190
VEGF stent day 28 liver	255
Tissue with 10μg exogenous VEGF (positive control)	260974

Figure 4.17. Table showing the detected quantities of VEGF per gram of total protein in various tissue samples from the experimental animals. The average detected in the control animals was 2575±2390ng/g VEGF. Samples from VEGF treated animals were 832±653ng/g VEGF, p=0.32 suggesting that no detectable increase in VEGF was present as a consequence of the VEGF stent treatment in distal tissues.

4.4 Discussion.

4.4.1 Study Limitations.

The stent model used in these studies is a model known to have a high thrombosis rate. In this work in the control groups three out of six vessels showed partial or complete thrombotic occlusion. This was due to a combination of flow reduction and deep vessel injury. Although deep vessel injury does occur after stent implantation in clinical practice, use of flow reduction represents a divergence from the clinical situation.

The model allows for an evaluation of any effects of the therapy on thrombosis rates. In clinical practice, with normal flow rates, this is a rare event. To study it in an animal model without prohibitively large numbers of animals being sacrificed necessitates a model like this one where thrombosis rates are higher. Flow reduction may allow longer persistence of VEGF thereby exaggerating its benefit, although the *in vitro* elution studies discussed in section 2.3.2. showed no significant change in elution rate of VEGF with a flow model compared to a "simple elution" model. Some effects on restenosis may result from this low flow model compared to a normal flow model, but this effect will not be more or less in one group, so should not introduce bias into the results.

A bolus dose of 1000IU of heparin was given to all animals. Practical considerations limited the ability to monitor coagulation parameters intra-operatively. It was impossible to accurately size stents for implant as angiography was not available. Stents were all the same size, but vessel diameter will have varied to some extent.

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[20].

No direct measurement of VEGF delivery into the stented tissue (apart from the *in vitro* work with IMA specimens).

4.4.2 Flow changes.

CFVs are due to platelet adhesion within the vessel and occur more rarely in the presence of antiplatelet medications. In the model used for this work two separate antiplatelet agents, aspirin and clopidogrel, were used as well as the anti-thrombotic heparin and, in half the cases, VEGF. High doses of aspirin given intravenously have been shown to abolish CFVs in a canine model[21]. Clopidogrel, at a much higher dose than in this work, has also been shown to abolish CFVs in a primate model of thrombosis[22]. The antiplatelet drugs given to the animals in this work may have greatly reduced the chances of vessel thrombosis and therefore CFVs. The flow recordings performed in the acute model animals show a large drop in flow of around 50%. Despite this, 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. One vessel displayed the changes suggestive of CFV, but there are not enough data to conclude any reduction in thrombosis due to the presence of VEGF on the stents used.

4.4.3 Endothelialisation.

No evidence was obtained of accelerated endothelialisation using the VEGF-eluting stent in this set of experiments. Light microscopy using Evans' Blue dye gave very dense staining of the specimens with the dye. This suggests little or no functional endothelium. No discernible differences were seen between the two groups. Under scanning electron microscopy, this finding was confirmed. Stent struts remained either completely exposed or were covered with sheets of organised, erythrocyte-rich thrombus. In isolated areas, re-endothelialisation had commenced, but with no convincing evidence that this was occurring faster or more comprehensively in the VEGF-treated group than the controls.
Therefore, at the time point chosen (seven days), in this particular rabbit model and using this stent/polymer combination, re-endothelialisation was significantly less than had been the case in similar studies at the same time point. In contrast to the published work of Van Belle *et al,* where VEGF had produced 90% endothelial coverage by this time, less than 10% of the stents structure was endothelialised in this work. There was, subjectively, more evidence for re-endothelialisation between stents struts in two of the specimens studied that had had VEGF-eluting stents implanted.

Two main issues arise from these negative results – the slower endothelialisation than expected in the control vessel and the lack of benefit in VEGF coated stents compared to controls.

The slower endothelialisation may reflect differences between the model used in this work and that used in the similar work by Van Belle *et al.* A different stent was used. The stent's physical effects on the vessel may have been responsible for the delayed endothelialisation. It is possible that it presents a more thrombogenic stimulus than the stent used in other work or that the vessel wall injury is more severe, although this was not detected using the injury scoring system. Apart from the structure of the stent, the polymer coating itself may have had an adverse effect on the endothelialisation process. This polymer has not been tested clinically and although designed to have little or no negative effects on inflammation, thrombosis or restenosis, may have done so in this model. It is perhaps noteworthy that the polymer coating was not completely smooth and even. Under SEM examination (*see fig 2.5*), it was evident that the polymer was of varying thickness. SEM showed gaps between the metal of the stent and the overlying polymer, i.e. faults in the coating process. The surface of the polymer did not present an absolutely smooth surface and numerous minor imperfections and irregularities were seen. The polymer may have had adverse effects on the vessel – through thrombosis, inflammation or turbulent bloodflow – either from its physical structure or from interactions with the blood and tissue. In this work, NZW rabbits were used weighing between 3.0 and 4.4 kg, mean 3.6kg. In Van Belle's work, the rabbits were larger (5-5.5kg). This difference may be important because a relatively greater injury, due to smaller average iliac artery sizes, may have been induced in the smaller animals. This greater injury from a relatively larger stent may have impeded endothelial recovery. As well as the stent injury, the arteries were also injured by repeated (over)inflation of the angioplasty catheter. This catheter was longer than the subsequent stent. At each end of the stent there would have been, therefore, a balloon-injured, de-endothelialised area. If much of the re-endothelialisation process occurred from the two ends of the stent, as appeared to be the case in the stents examined for endothelialisation, then the balloon-injured area outside the stent may have slowed down the ingrowth of endothelial cells, delaying stent re-endothelialisation beyond the seven day period under study. The balloon was deliberately longer than the stents used because of the technical difficulties in ensuring that the stent was implanted exactly over the angioplastied site. With the larger balloon this could be done with some confidence, as there was some margin for error. In clinical practice this is not necessary as the stent is placed in the correct site under direct visualisation.

Other small differences exist between the model described in this work and those previously published. Most of these seem unlikely to have exerted any effects on endothelialisation. One significant difference in this work compared to previous groups was the use of clopidogrel as part of the standard treatment before and after stent implantation. This was done partly to minimise the incidence of acute stent occlusion in a relatively pro-thrombotic (reduced-flow) model. As importantly, it was used throughout since this is what is done in clinical practice and brings the model closer to reproducing the effects seen in patients after PCI. No obvious explanation exists for why clopidogrel might slow endothelialisation and so this difference in technique is probably not a contributor to the delayed endothelialisation seen. It is assumed that the lack of endothelialisation in the samples seen reflects accurately what occurred in the animals during life. It is possible that the removal of the stented vessel and subsequent processing may have dislodged any lightly adherent endothelium around the stent struts. Great care was taken during handling of the specimens to prevent this eventuality.

VEGF seemed to have no beneficial effects on the endothelialisation process, Several possible explanations for this may be postulated. VEGF may have been washed off the stent quicker than expected from the *in vitro* results. This may have occurred before the implantation process, as the stent was passed through the femoral vessels. During this passage, the stent was exposed to blood, which may have washed off some of the most superficial VEGF. During implantation, great stress is placed on the structure of the stent and polymer as the stent is expanded using high pressure. This distortion of the delivery vehicle may have released VEGF into the bloodstream to be washed away ineffectually. Calculated elution was that in an *in vitro* circuit. This may be different from the elution *in vivo* in important ways. The stents *in vivo* are exposed to all the components of the blood, including transport proteins that may absorb VEGF, enzymes that may degrade it and the coagulation cascade that may have sealed VEGF within the polymer and prevented its release. VEGF may have been released, but not where its effects could be exerted. No endothelialisation was seen under stents. Here, where the VEGF-soaked polymer had been pressed into the vessel wall, there was no room for endothelial cells to grow in from the sides. Most of the absorbed VEGF from the stent may have been released into the vessel wall, where there are no target cells for VEGF. This may be compared with balloon delivery where VEGF is delivered to tissues (including any residual endothelium in the main vessel or that in side branches with endothelial cells) where endothelial cells are still present.

VEGF may have been washed off downstream and lost. Only that amount which was released between the struts would potentially be exposed to the target endothelial cells. It may be that not enough VEGF was delivered laterally along the vessel wall to have any benefits – although enough did appear to have been released to reduce thrombosis. Thrombosis occurs earlier than endothelialisation and so relatively small amounts of VEGF released quickly after implantation may be enough to prevent thrombosis but not be sufficient or sufficiently prolonged to stimulate re-endothelialisation. Insufficient VEGF may have been delivered with this stent. In Van Belle's work 100μg was delivered locally. The VEGF stents in this work carried approximately 10μg. Local delivery by balloon has been shown to be a very inefficient means of delivering drug and the apparently low amount of VEGF delivered by stent is likely to be delivering a higher amount of VEGF locally in practice, once the inefficicency of balloon delivery is accounted for.

Endothelial cell proliferation may have been occurring at a maximal rate. VEGF may have not been able to increase the rate of endothelial cell growth above that produced by the vessel injury in the first place. VEGF may have been denatured or inactivated by the storage/sterilisation process. Stents were kept, refrigerated for up to two months after initial absorption with VEGF. In addition, VEGF-coated stents were sterilised using a cold ethylene oxide sterilisation process. This treatment reflects the minimum level of sterilisation required for products to be used in humans and the storage time is a realistic reflection of the storage time of interventional products before their use. In order for the VEGF-coated stent to be anything other than of research interest it must retain effectiveness through such treatment. No loss of VEGF's effects was seen in the cell culture work where the VEGF-coated stent sections were ethylene oxide sterilised, so it seems unlikely that this had any adverse effects on the stents used *in vivo*. There seems to be no obvious reason for VEGF to degrade spontaneously during a two-month storage period. VEGF is normally refrigerated for storage and remains active for at least a year (according to manufacturer's literature). Finally, it may be that VEGF remained active throughout the procedure and in adequate amounts but that it is simply not a powerful enough mitogen to produce the desired effect. Despite its name, VEGF's most pronounced effects are on vascular permeability and not cell proliferation. In the cell culture experiments, only a modest increase in endothelial cell growth was seen; about 10% higher than that seen with controls. The extent of endothelial loss in this model was very great and any minor improvement in endothelial recovery may have been undetectable. Such a small amount of accelerated re-endothelialisation would be clinically insignificant. It interesting to note the poor results obtained in clinical trials of VEGF where it has not led to any clinically significant proliferation of blood vessels (neoangiogenesis),

despite its anticipated stimulation of endothelial (and so blood vessel) growth. Such a poor proliferative effect of VEGF may explain its failure to stimulate re-endothelialisation in this model, but would leave unexplained the strikingly positive results obtained by Van Belle *et al.*

4.4.4 Thrombosis.

Previously published work where VEGF has been delivered has focused largely on the effects of this agent as a promoter of endothelialisation, thus reducing intimal hyperplasia. VEGF is responsible for the production, by endothelial cells, of a number of factors that act on the coagulation and platelet activation systems (see 1.8.4).

In the rabbit iliac artery model, an apparent reduction in macroscopic thrombus accumulation was seen at the seven-day time point, with a significant difference between the VEGF-coated and the uncoated stents. At the earlier time point, no significant differences were seen in the amounts of radio-labelled platelets measured as adhering to the stents, although this may be due to the variations in the readings, which gave wide error bars.

The mechanism behind this apparent finding is unclear. VEGF may act as an inhibitor of thrombus formation, through its stimulation of NO, PGI_2, tPA and urokinase. To exert such effects however, VEGF must act through the medium of functioning endothelial cells, since VEGF has no direct antiplatelet or anticoagulant effects. It has already been discussed that in these studies, little or no endothelium was to be seen over or amongst the struts of the stents, even those treated with VEGF. How then can VEGF be said to be responsible for the apparent reduction in thrombus formation in the VEGF treated stents?

VEGF is released from the polymer of these stents quickly, and in a sustained fashion, as reported in chapter 2. Inevitably, some of this VEGF is washed downstream from the site of stent implantation, or down small side branches from the main artery near the stent site. These downstream or side vessels remain endothelialised, since they were not injured by the percutaneous intervention. These cells may be stimulated to produce significant quantities of anticoagulant/antiplatelet factors that exert local effects, including around the nearby stent. Some VEGF may permeate, through the vessel wall, to the endothelial cells that immediately border the stent upstream.

As well as an effect on thrombus formation through platelets, VEGF may have other indirect but equally important actions on clotting. In this reduced flow model, thrombus formation is promoted. VEGF, acting through endothelium-derived NO, may produce vascular relaxation,

increasing flow and inhibiting thrombus formation. No increase in flow was seen in the arteries treated with VEGF-coated stents.

Any effects of stent-delivered VEGF on thrombosis must occur at low delivered doses, or over much shorter time point, since no re-endothelialisation was seen over the same time points. Further work would need to be performed, directed at the effects of VEGF on stent thrombosis, for these results to be validated. Without a credible mechanism for a VEGF-mediated reduction of thrombus independent of effects on the endothelium, the results in this study have to be treated with caution, unless confirmed in any subsequent studies.

4.4.5 Restenosis.

No benefit in reducing restenosis was seen in these results. Any possible beneficial effects on reduced thrombosis were not accompanied by a corresponding reduction in intimal hyperplasia. The explanation for the lack of any reduction in intimal hyperplasia lies with the observed failure of VEGF-eluting stents to promote re-endothelialisation. VEGF has no direct effects on smooth muscle cell proliferation, requiring a functional endothelium to passivate the underlying vessel wall. Since no endothelium was seen coating the VEGF-eluting stents, intimal hyperplasia was likely to continue unchecked until much later than hoped for.

4.4.6 Systemic delivery.

No increase in VEGF was seen in the distal tissue studied at the two study endpoints. Although sustained VEGF release had been demonstrated from this stent polymer in the earlier experimental work, this released VEGF was not seen in appreciable amounts distant from the stent in the tissues where it was most likely to be metabolised or excreted from the animal's body. The lack of VEGF detection in distant tissues suggests that significant systemic effects are unlikely using this delivery method.

Chapter 4 references

1 Maalej N, Albrecht R, Loscalzo J, Folts JD. The potent platelet inhibitory effects of S-nitrosated albumin coating of artificial surfaces. *Journal of the American College of Cardiology* 1999;**33**:1408-1414.

2 Van Belle E, Tio FO, Couffinhal T *et al.* Stent endothelialization. Time course, impact of local catheter delivery, feasibility of recombinant protein administration, and response to cytokine expedition. *Circulation* 1997;**95**:438-448.

3 Van Belle E, Tio FO, Chen D *et al.* Passivation of metallic stents after arterial gene transfer of phVEGF165 inhibits thrombus formation and intimal thickening. *Journal of the American College of Cardiology* 1997;**29**:1371-1379.

4 Malhotra S, Teirstein PS. The SCRIPPS trial--catheter-based radiotherapy to inhibit coronary restenosis. *Journal of Invasive Cardiology* 2000;**12**:330-332.

5 Kay IP, Wardeh AJ, Kozuma K, et al. Radioactive Stents Delay but Do Not Prevent In-Stent Neointimal Hyperplasia. *Circulation* 2001;**103**:14-17.

6 Javed Q, Swanson N, Warner E *et al.* Plasminogen Activator and Plasminogen Activator Inhibitor Gene Expression in Human Saphenous Vein Organ Culture. *Experimental and molecular Biology* 2001;**70**:146-153.

7 Bush LR, Campbell WB, Buja LM *et al.* Effects of the selective thromboxane synthetase inhibitor dazoxiben on variations in cyclic blood flow in stenosed canine coronary arteries. *Circulation* 1984;**69**:1161-1170.

8 Folts JD, Gallagher K, Rowe GG. Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation? *Circulation* 1982;**65**:248-255.

9 Fraker PJ, Speck JC, Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril. *Biochemical & Biophysical Research Communications* 1978;**80**:849-857.

10 Woltanski KP, Besch W, Keilacker H *et al.* Radioiodination of peptide hormones and immunoglobulin preparations: comparison of the chloramine T and iodogen method. *Experimental & Clinical Endocrinology* 1990;**95**:39-46.

11 Eichhorn EJ, Grayburn PA, Willard JE, *et al.* Spontaneous alterations in coronary blood flow velocity before and after coronary angioplasty in patients with severe angina. *Journal of the American College of Cardiology* 1991;**17**:43-52.

12 Sunamura M, di Mario C, Piek JJ, *et al.* Cyclic flow variations after angioplasty: a rare phenomenon predictive of immediate complications. DEBATE Investigator's Group. *American Heart Journal.* 1996;**131**:843-848.

13 Barone GW, Farley PC, Conerly JM *et al.* Morphological and functional techniques for assessing endothelial integrity: the use of Evans blue dye, silver stains, and endothelial derived relaxing factor. *Journal of Cardiac Surgery* 1989;**4**:140-148.

14 Barone GW, Conerly JM, Farley PC *et al.* Endothelial injury and vascular dysfunction associated with the Fogarty balloon catheter. *Journal of Vascular Surgery* 1989;**9**:422-425.

15 Bray DF, Bagu J, Koegler P. Comparison of hexamethyldisilazane (HMDS), Pedri II, and critical-point drying methods for scanning electron microscopy of biological specimens. *Microscopy research and technique* 1993;**26**:489-495.

16 Hawker RJ, Hawker LM, Wilkinson AR. Indium (111In)-labelled human platelets: optimal method. *Clinical.Science* 1980;**58**:243-248.

17 Malik N, Gunn J, Holt CM, *et al.* Intravascular stents: a new technique for tissue processing for histology, immunohistochemistry, and transmission electron microscopy. *Heart* 1998;**80**:509-516.

18 Schwartz RS, Huber KC, Murphy JG, *et al.* Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model. *Journal of the American College of Cardiology* 1992;**19**:267-274.

19 Guerlava P, Izac V, Tholozan JL. Comparison of different methods of cell lysis and protein measurements in Clostridium perfringens: application to the cell volume determination. *Current Microbiology* 1998;**36**:131-135.

20 Wilensky RL, March KL, Hathaway DR. Direct intraarterial wall injection of microparticles via a catheter: a potential drug delivery strategy following angioplasty. *American Heart Journal* 1991;**122**:1136-1140.

21 Folts JD, Crowell EBJ, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. *Circulation* 1976;**54**:365-370.

22 Yao SK, McNatt J, Cui K, *et al.* Combined ADP and thromboxane A2 antagonism prevents cyclic flow variations in stenosed and endothelium-injured arteries in nonhuman primates. *Circulation* 1993;**88**:2888-2893.

Chapter 5.

Discussion and Conclusions

5.1 Introduction

In this chapter the results of the work with locally delivered VEGF will be summarised. The scope for further study of VEGF-eluting will be discussed. The place of VEGF-eluting stents in humans will be explored in particular, including the limitations on their use. Finally, conclusions will be drawn from the whole body of experiments described thus far.

5.2 Radiolabelling experiments

The absorption of approximately 20μg VEGF per stent into the polymer is within the range of delivered VEG that had been shown to exert a significant effect on the recovery of endothelium over a stent in previous studies. In the elution experiments attempts were made to recreate the physiological conditions the drug-coated stents would be exposed to *in vivo* – flow, pH, temperature and plasma proteins. The sustained VEGF release over a week with VEGF-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. Furthermore, the internal mammary artery tissue experiment showed that a much higher proportion of the VEGF was retained in the tissue than is the case with delivery balloons.

5.3 Cell culture work.

5.3.1 VEGF-eluting stents in cell culture.

VEGF was released in sufficient quantities to exert a stimulatory effect on nearby endothelial cells, in the same way as was hypothesised would need to be the case *in vivo*, when the VEGF released from the stent would have to diffuse around the stent struts sufficiently to stimulate the growth of residual endothelial cells within or near to the angioplastied area of artery. The increased HUVEC proliferation was also seen in cell cultures, exposed to a VEGF-absorbed stent that had been washed for 24 hours with culture medium beforehand. This meant that the VEGF elution from the stent was sustained well beyond the initial

immediate wash off of VEGF, and that this sustained release of VEGF was sufficient to exert a beneficial effect.

The beneficial effect seen in all groups was modest, at around 10% more than that seen in control groups of cell culture. In the light of the subsequent failure of such VEGF-eluting stents to significantly improve the re-endothelialisation process, it may be that such a slight improvement in endothelial cell growth is insignificant *in vivo*.

5.4 *In vivo* experiments.

The in vivo experiments did not show the expected improvement in endothelialisation or reduction in intimal growth. There are many possible explanations for this finding, including deficiencies in the techniques or model used. It seems likely however that the lack of efficacy may well be a true finding as there did appear to be a detectable effect of the VEGF-eluting stents, i.e. the reduction in measured thrombus formation on the stents. Such an effect might make these stents less thrombogenic than current clinically used stents. This effect requires ongoing *in vivo* evaluation. VEGF may be a suitable agent for local delivery by stent in patients in whom a high risk of stent thrombosis may be anticipated, including patients with small vessel stenoses.

5.5 Future studies of a VEGF-eluting stent.

The *in vivo* experiments leave doubt as to whether there is any place for a VEGF-delivering stent. Three possible roles are now considered for the stent. These are:

- as an anti-restenosis device,
- to promote neoangiogenesis in ischaemic tissue,
- to inhibit stent thrombosis in high-risk situations.

5.5.1 VEGF-eluting stents to reduce restenosis.

VEGF has not been shown to speed re-endothelialisation or 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 VEGF-eluting stent in a coronary model would require the use of a larger animal, e.g. the porcine model. VEGF has already been studied in a porcine model (*see 1.8 and 1.9*). However, no studies have been performed in the pig that have given the VEGF in a sustained fashion.

5.5.2 VEGF-eluting stents to promote neoangiogenesis

The VEGF-eluting stent could be tested for a neoangiogenic response in ischaemic tissues. VEGF–eluting stents could be implanted in patients with critical limb ischaemia. Work discussed in chapter 1 (1.8.9) used VEGF in patients with critical ischaemia to delay amputation. A clinical trial would gather data on the safety/side-effect profile of the stent/VEGF system and establish the efficacy of local VEGF delivery in humans.
Stents pre-absorbed with sterile VEGF could be placed percutaneously into the arterial tree of the affected leg. The positioning of the stent would be immediately proximal to the occluded section(s) of artery. The endpoints of such a study would be:

- Progression of disease sufficient to warrant amputation.
- Withdrawal due to side-effects/complications of VEGF or of stent implantation.
- At three months, angiographic reassessment of collateral density.
- Symptoms, ABPI, exercise tolerance and serum levels of VEGF would be assessed at 7, 14 and 28 days and at three months.

If successful and safe, then a similar trial could be attempted in patients with severely ischaemic myocardium who were judged to be unsuitable for traditional revascularisation techniques.

5.5.3 VEGF-eluting stents to reduce stent thrombosis.

Stent thrombosis is, in general, a rare occurrence since the introduction of antiplatelet and anticoagulant agents post-stenting. However, in certain situations, stent thrombosis remains a relatively common, and potentially very serious, complication. One such high-risk group would be patients undergoing vascular brachytherapy for restenosis (*see 1.6.3.3*). Late thrombosis occurred in 9.1% of patients have suffered acute occlusion of the treated area, 40% of these with resultant myocardial infarction over a time period as long as nine months post-treatment (*see fig.5.1*)[1; 2].

Trial	Radiation	Late thrombosis %
SCRIPPS	γ	7.7.
WRIST	γ	9.0
PREVENT	β	11.0
GAMMA	γ	6.1
BERT	β	7.6
BETA-WRIST	β	10.0

Figure 5.1. The incidence of late thrombosis in various brachytherapy trials.

These adverse findings may be due to the inhibitory effect that VBT has on the re-endothelialisation process. Previous work has demonstrated that radiation slows re-endothelialisation and degrades the morphology and function of endothelial cells *in vivo*[3-7]. A VEGF-eluting stent may provide a reduced incidence of stent thrombosis in irradiated vessels, either through a direct anti-thrombotic effect or by promoting re-endothelialisation in a situation where it is particularly retarded by the effects of radiation on the endothelium. To study the effects of the VEGF stent in such a situation the rabbit iliac artery model could be adapted to include the use of a radioactive source (such as the BETACATH β-emitting train that is in current UK use) with either a plain stent or a VEGF-eluting stent inserted after. Thrombosis would be assessed at various time points as has been done in the existing animal work using the VEGF-eluting stent.

5.6 Long-term future of locally-delivered VEGF.

VEGF may yet find a place as a locally delivered agent in human cardiological problems. Three areas of research following on from the work of this MD thesis have been discussed that would explore the potential of the VEGF-eluting stent. If these studies showed encouraging results there are further obstacles to be overcome before the VEGF-eluting stent could be used in mainstream clinical practice. These obstacles include concerns over the safety of VEGF administration in humans.

- The aspect of most concern to any future use of VEGF in clinical trials is the risk of death consequent to the therapy. Use of VEGF in clinical trials was temporarily halted in 2000 after the death of a young man in an angiogenesis trial. In this case large amounts of the adenoviral vector used to deliver DNA coding for VEGF was accidentally injected into the patient's hepatic artery with fatal consequences[8]. Ultimately it was felt that this case was not directly due to the use of VEGF, but rather to the viral vector. No other deaths have yet been associated with VEGF use in humans, but the total number of patient that have been treated with VEGF in any form still is significantly less than 1000.
- VEGF is a potential carcinogen. Use of VEGF may have unwanted neoplastic effects either in the heart or at distal sites, although this has not yet been demonstrated in human subjects. Myoblasts genetically engineered to synthesise VEGF have been implanted into the heart in a mouse model. Most animals treated with VEGF either died or developed large tumours, usually of vascular origin[9]. In cell culture, VEGF receptors have been demonstrated in uterine smooth muscle cells, which proliferate in response to VEGF[10]. VEGF may therefore cause unwanted growth of uterine tissue, e.g. fibroids. VEGF receptors have also been demonstrated in human cell tumour lines including glioma, melanoma and squamous cell carcinoma of neck[11].
- Species differences in response to VEGF may be significant. VEGF has been shown to reduce restenosis in a rabbit model (although not when delivered by stent). However, in a porcine model, workers have not shown a reduction in restenosis. Results in rats have been variable (*see section 1.8.8*). If interspecies differences can be so great in the animal models, there is therefore no guarantee that VEGF will exert an inhibitory effect on the in-stent restenosis process in humans, regardless of any encouraging initial studies in animals. It is striking that, despite various positive

studies in animals of locally delivered VEGF, there was no effect in the **VIVA** trial (**V**ascular Endothelial Growth Factor in **I**schaemia for **V**ascular **A**ngiogenesis). In this study, VEGF165 was given to patients with untreatable severe ischaemia, most of it by systemic delivery, with an intracoronary bolus. Despite some signs of improved perfusion, no increase of exercise time or reduction of angina was seen with VEGF compared to placebo. A strong, sustained placebo effect was seen[12]. In a separate study, no ill-effects were seen in a series of ten patients who received VEGF plasmid infusion into the coronaries after angioplasty[13]. This very small study found no reduction in restenosis in the VEGF group. Finally, the KAT study failed to demonstrate any reduction in restenosis using VEGF gene transfer (*see section 1.8.12*).

- VEGF is not purely an endothelial cell growth factor. One group has reported the detection of KDR (VEGR-2) receptors on human smooth muscle cells in specimens of atherosclerotic arteries[14]. The authors suggest that the atherosclerotic process itself is the stimulus for the production of VEGF receptors in the smooth muscle cells. It is not known whether these receptors have a significant effect *in vivo*. Nevertheless, it is a disquieting finding that casts doubt over the appropriateness of using VEGF as an agent to reduce restenosis in atherosclerotic vessels like the diseased coronary. It is possible that in the presence of atherosclerosis, VEGF promotes smooth muscle cell replication and might in fact exacerbate the growth of neointima.
- The differences between healthy and atherosclerotic arteries may be of further significance. VEGF may have the potential to accelerate atherosclerosis/destabilise plaques. VEGF mRNA and VEGF receptors have been demonstrated in atherosclerotic, but not normal, human coronary arteries[15]. VEGF may therefore be an important promoter of the atherosclerotic process and exogenous delivery of VEGF to diseased coronary vessels may accelerate the growth of these plaques. The additional effects of VEGF (vascular permeability, chemoattraction for macrophages) may destabilise existing plaques leading to increased risk of plaque rupture and resulting acute coronary syndromes. This has not, as yet, been proven to occur in the existing, small-scale, human trials of VEGF in ischaemic heart disease.

5.7 Conclusions.

The work described in this MD thesis covers several new areas of investigation.
The potential for successful drug absorption and elution has been demonstrated for VEGF. This was important, as one of the key advantages to the use of stents to deliver drug is that the release of the drug is sustained. This has been demonstrated clearly and reproducibly using radiolabelled markers. The effects of VEGF as a stent delivered agent have been looked at in cell culture. To demonstrate the direct effect of a drug-coated stent on endothelial cells is an original finding. Previous work records the effect of different drugs on endothelial cell culture, but this work extends this by introducing the stent itself into the culture dish. This may be important since *in vivo* the drug is released by passive diffusion into nearby tissues, producing a gradient of concentration of drug that reduces the further away the tissue is from the stent struts. Adding a drug in a solution to a cell culture dish produces a uniform concentration across the dish. This model also allows for any direct effect on cell growth of the stent or the polymer coating on the stent to be measured. In this case there did not appear to be any such effect. The *in vivo* work is completely original since VEGF protein 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 endothelialisation or 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.

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. VEGF appeared to inhibit endothelial cell growth in a particular subset of cells, i.e. older passage cells in adverse culture conditions. Secondly, VEGF-eluting stents appeared to reduce thrombus formation on the stents when assessed at seven days. These results provide a starting point for further research into VEGF in the future. The possible future usage of the VEGF-eluting stent have been discussed together with caveats regarding the widespread use of VEGF in humans.

The importance of the field of restenosis prevention has been emphasised, not least because of the ever-increasing numbers of patients undergoing stenting. The work reported here is part of a very dynamic research field. Ongoing and recently completed trials of various drug-eluting stents have begun to produce very favourable looking results with substantial reductions in restenosis and MACE, at least in the short to medium term after stent implantation. It is not yet clear whether one agent will be superior to others. It seems more likely that there will be a range of agents competing on the market. Some may have particular benefits in different patient populations. VEGF-eluting stents may have a role in preventing restenosis or thrombosis in particular situations where re-endothelialisation is particularly likely to be poor.

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.

Chapter 5 references

1 Waksman, R. Total occlusion rates across VBT trials. Tenth Endovascular Therapy Course,(Paris). 5-21-1999. Conference Presentation.

2 Costa MA, Sabate M, van der Giessen W *et al.* Late coronary occlusion after intracoronary brachytherapy. *Circulation* 1999;**100**:789-792.

3 Menendez JC, Casanova D, Amado JA, *et al.* Effects of radiation on endothelial function. *International Journal of Radiation Oncology, Biology, Physics.* 1998;**41**:905-913.

4 Qi F, Sugihara T, Hattori Y *et al.* Functional and morphological damage of endothelium in rabbit ear artery following irradiation with cobalt60. *British Journal of Pharmacology* 1998;**123**:653-660.

5 Robinson KA. Reendothelialization following radiotherapy. *6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book.* 2000. 32

6 Thorin E, Meerkin D, Bertrand ME *et al.* Influence of Postangioplasty ß-Irradiation on Endothelial Function in Porcine Coronary Arteries. *Circulation* 2000;**101**:1430-1435.

7 Hehrlein C, Gollan C, Donges K, *et al.* Low-dose radioactive endovascular stents prevent smooth muscle cell proliferation and neointimal hyperplasia in rabbits. *Circulation* 1995; **92**:1570-1575.

8 Epstein SE, Kornowski R, Fuchs S, Dvorak HF. Angiogenesis therapy. *Circulation* 2001;**104**:115-119.

9 Harada K, Friedman M, Lopez JJ, *et al.* Vascular endothelial growth factor administration in chronic myocardial ischemia. *American Journal of Physiology* 1996;**270**:H1791-H1802

10 Herold-Mende C, Steiner HH, Andl T, *et al.* Expression and functional significance of vascular endothelial growth factor receptors in human tumor cells. *Laboratory Investigation* 1999;**79**:1573-1582.

11 Brown LF, Detmar M, Tognazzi K *et al.* Uterine smooth muscle cells express functional receptors (flt-1 and KDR) for vascular permeability factor/vascular endothelial growth factor. *Laboratory Investigation* 1997;**76**:245-255.

12 Henry TD. VIVA (vascular endothelial growth Factor in ischemia for vascular angiogenesis). *Clinical Cardiology* 1999;**22**:369(Abstract)

13 Laitinen M, Hartikainen J, Hiltunen MO, *et al.* Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. *Human Gene Therapy* 2000;**11**:263-270.

14 Belgore FM, Mzimba ZS, Yip GYH *et al.* Localisation of the vascular endothelial growth factor A,B,C and their receptors in human atherosclerotic arteries. *Journal of the American College of Cardiology* 2001;**37**:1A-648A.(Abstract)

15 Inoue M, Itoh H, Ueda M, *et al.* Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation* 1998;**98**:2108-2116.

Acknowledgements

I would like to thank the British Heart Foundation, Genentech, Cook Incorporated and Biocompatibles UK for their support for the work of this MD.

I would like to thank the following people for their scientific advice and assistance: Kai Hogrefe, Elena Moiseeva, Qamar Javed, Weidong Yang, Rachel Boultby, David de Bono, Angie Shone, Liz Warner and the staff of the Biomedical Sciences Department of the University of Leicester for all their technical assistance with animal procedures.

I would also like to thank my supervisors Neal Uren and, particularly, Anthony Gershlick for their support and advice in the design of this programme of research and their editorial help in writing the thesis.

I acknowledge the support of Tom Spyt, who encouraged my development of an IMA organ culture model using specimens obtained from his patients undergoing bypass surgery.

I would like to thank Amanda Stephens Lloyd for her advice regarding the study design, statistical analysis, thesis production and support throughout the work.

Finally I would like to thank my wife Beth for her patience and support during the period of study and thesis production.

Abbreviations

ABPI	Ankle-Brachial pressure index
ADP	Adenosine Diphosphate
BAEC	Bovine aortic endothelial cells
BBS	Borate Buffered Saline
BENESTENT	Belgium Netherlands Stent study
BSA	Bovine Serum Albumin
CFV	Cyclic flow variation
DMEM	Dulbecco's modified Eagle's medium
DMSO	Di-methyl sulphoxide
EEL	External elastic lamina
EGM	Endothelial cell growth medium
EPIC	Evaluation of 7E3 for the Prevention of Ischemic Complications
FCS	Foetal Calf Serum
FGF	Fibroblast growth factor
GAX	growth-arrest specific homeobox
GRII	Gianturco Roubin mark II stent.
H&E	Haematoxylin and Eosin
HBSS	Hanks Balance Saline Solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HUVEC	Human umbilical Vein endothelial cell
IEL	Internal elastic lamina
IHD	Ischaemic Heart Disease.
IMA	Internal mammary artery
IMS	Industrial Methylated spirits.
IRIS	Isostent Restenosis Intervention Study
ISR	In-stent restenosis
IVUS	Intravascular ultrasound
KAT	Kuopio Angiogenesis Trial
LAD	Left anterior descending artery
LDD	Local drug delivery
LDL	Low density lipoprotein
MACE	Major adverse cardiac events.
MLD	Mean luminal diameter
MMP	Matrix metalloproteinase
NADH	Nicotinamide adenine dinucleotide
NAME	N(omega)-nitro-L-arginine-methylester
NO	Nitric Oxide
NZW	New Zealand White
PBS	Phosphate Buffered Saline
PC	Phosphorylcholine
PCI	Percutaneous Coronary Intervention
PCNA	Proliferating Cell Nuclear Antigen.
PCS	Prostacyclin Synthase
PDGF	Platelet Derived Growth factor
PDT	Photodynamic therapy
PGI_2	Prostaglandin I_2
PlGF	Placenta Growth Factor
PLLA	Poly-l-Lactic Acid
PPP	Platelet poor plasma
PRP	Platelet rich plasma

PTCA	Percutaneous Transluminal Coronary Angioplasty
RITA 2	Second randomised intervention treatment of angina trial.
RPB	Randomised, Placebo-controlled, blinded study
SEM	Scanning electron microscopy.
SMC	Smooth muscle cell
STRESS	Stent Restenosis Study
TGFβ	Transforming growth factor beta
TMR	Trans myocardial revascularisation
TNFα	Tumour necrosis factor alpha.
TVF	Target vessel failure
VBT	Vascular Brachytherapy Treatment
VEGF	Vascular Endothelial Growth Factor
VIVA	Vascular endothelial growth Factor in ischemia for vascular angiogenesis trial
VSMC	Vascular Smooth Muscle Cell

Appendix 1.

Composition of buffers, and reagents.

1. 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

2. 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

3 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

4 Borate Buffered Saline 0.1 mol/l – pH8.3 (BBS)

Boric Acid	(0.1mol/l)	6.18g
Sodium tetraborate decahydrate (borax)	0.025mol/l	9.54g
Sodium Chloride (0.075 mol/l)		4.38g

Dilute to 1l with deionised water – check pH 8.4 +/- 0.1

5 Phosphate-buffered saline with 0.25% Gelatin

Phosphate-buffered saline pH 7.4		100ml
Gelatin	250mg	

6 20mM HEPES (200ml)

NaCl	150mM	=1.754g
KCl	5mM	=0.074g
$MgSO_4.7H_2O$	1mM	=0.05g
HEPES (free salt)	10mM	=0.952g

Made up to 200ml with water and pH corrected to 7.4 with NaOH or HCl.

7 HEPES buffer

20mM HEPES, pH 7.4
1.5mM EDTA
0.5mM PMSF
0.5mM Benzamidine
10µg/ml ovomucoid trypsin inhibitor (Sigma)

8 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.

9 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.

10 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 and organ culture media

11 BAEC Cell culture Medium – 20% FCS in DMEM

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

12 HUVEC culture medium

500ml Gibco M199 with Earl Salts with Glutamine (Ref 31150-022)
20% FCS
1% Penicillin/streptomycin
Heparin 2500iu (Monoparin)
Endothelial Cell Growth Supplement (ECGS) 5ml of 5mg/ml solution

Or

13 Endothelial Growth Medium (EGM)

500ml Endothelial Basal Medium (Clonetics)
hEGF 0.5ml
hydrocortisone 0.5ml
Gentamicin 0.5ml
Bovine brain extract 2.0ml
12% FCS

14 Composition of Organ Culture Medium (50ml)

RPMI 1640 Medium	33.5ml
Foetal Calf Serum	15ml
Penicillin.Streptomycin	0.5ml
Glutamic Acid	1.0ml

15 1% Acid/Alcohol (1l)

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

Appendix 2
Endothelial cell culture techniques.

A.1 Primary culture of Bovine Aortic Endothelium.

BAECs were obtained using the methods described by Yang *et al*[1].
Bovine aortas were obtained from the local abattoir as soon as possible after slaughter. Segments of descending thoracic aorta approximately 30cm in length were transferred to the laboratory on ice and processed immediately under sterile conditions. The proximal end of the aorta was ligated and the distal end clamped. Fat or connective tissue was carefully dissected without damaging the root of any intercostals arteries near the aorta. All intercostals arteries were ligated with suture. The clamped end of the vessel was cut off and a kwill filling tube inserted in the distal end and the vessel tied off around it. The vessel was washed thoroughly with saline and filled with 10ml of 0.1% collagenase in DMEM or Hank's solution using a syringe. The syringe was left in place. The vessel was wrapped in cling film and incubated at 37°C for 15 to 20 minutes. The vessel was gently massaged to aid the release of loosened cells. The collagenase/cell solution was removed using the syringe and the solution placed into a sterile universal tube. The lumen was rinsed with 10ml of culture medium. The cell suspension was centrifuged at 1000rpm for ten minutes and the cell pellet rinsed once with culture medium. Finally the cells were resuspended in culture medium and plated in an $80cm^2$ tissue culture flask.
Collagenase treatment removes the cells from the aorta wall in large sheets which make an accurate cell count impossible, therefore the cells obtained from one such length of aorta are initially cultured in 20ml of culture medium and plated in one $80cm^2$ tissue culture flask or resuspended in 40ml of medium and plated in two flasks, depending on the yield, estimated by eye under the microscope. Most of the endothelial cells should adhere to the culture flask within two hours. Thereafter the cells are gently washed and replaced with fresh medium after cell attachment. Generally the cells reach confluence after two to three days at a density of about $8 \times 10^6/80cm^2$ or $10^5/cm^2$.

A.2 Cloning of Bovine Aortic Endothelial Cells.

Cloned endothelial cells were used to avoid non-endothelial contamination.
Primary cultures of bovine aorta endothelial cells which had reached approximately 90% confluence. Batches of cells with high quality and purity (i.e. typical endothelial cell

morphology rather than the "troughs and valleys" appearance of fibroblast overgrowth) were isolated (*see fig.4.2*). These batches were resuspended with 5ml of trypsin solution after washing. This cell suspension was aspirated through a 25 gauge needle two or three times to break up any cell clumps. The cells were diluted with 15ml of serum-containing growth medium to inactivate the trypsin. The cells were recovered by centrifugation at 1000rpm for five minutes and washed with growth medium before finally resuspending in 20ml of medium. The cell suspension was counted with a haemocytometer.

The cell suspension was diluted appropriately so as to achieve a final cell density of 10 cells/ml with growth medium. These cells were plated in a 96 well plate at 100µl/well, equivalent to one cell/well. After 24 hours, each well was visually scored for cell number and individual wells examined for the following three days. Those wells containing a single cell at 24 hours after plating were identified to be a possible clone.

After four days in culture, any wells containing a single colony consisting of 8 to 12 cells were considered to be derived from one single progenitor cell. The culture medium was changed every four to five days using a 50:50, fresh: conditioned medium mixture, until the cells formed a monolayer. It takes about 12 to 18 days for the cells to reach confluence at a density of about 4 x 10^4 cells/well. During this time the cells were estimated to have undergone 13 to 15 cell doublings. The cells were transferred on a well-to-well basis to a 24 x 16mm well plate in normal growth medium. It takes about four to six days for the cells to reach confluence at a density of about 2 x 10^5 cells/well (about $10^5/cm^2$), during which time the cells can be estimated to have undergone another three to four cell doublings. Those cells, which have retained the cobblestone morphology of normal endothelium on reaching confluence and are free of contamination of other cells were expanded to 25cm^2 flasks and grown up for experiments or stored in liquid nitrogen for future use.

A.3 HUVEC preparation.

Human umbilical cords were collected in sealed plastic containers in HBSS and transported in sealed heavy-duty polythene containers. All subsequent procedures were carried out in a class II microbiological Safety Cabinet. Cords from patients known to be infected with HIV, HepB, HSV2 or from patients suspected to be IV drug abusers were not used.

Cords were transferred to a foil-covered tray and cleaned. As much blood as possible was removed from the vein. The ends of the cord were cut off and a plastic cannula inserted. A syringe containing PBS/Albumin was attached at one end. Residual blood was flushed out with this syringe. Once the other end of the cord had been cannulated, collagenase solution

(Gibco) was pushed into the vein until it was fully distended. The cord was then placed into a pre-warmed, cling film-covered tray and then incubated at 37°C for 15min. The cords were then massaged for two minutes gently to dislodge cells.
The collagenase solution was removed into the attached syringe. The vein was flushed again with PBS/Albumin solution and this wash was added to the collagenase solution in a Falcon 50ml tube. This was centrifuged at 1000rpm for five minutes. The resulting supernatant was removed and the pellet of cells resuspended in 4ml HUVEC medium. The suspension was added to a culture flask. This flask was coated with a 1% gelatin solution (made from 2% gelatin mixed 1:1 with PBS). The remaining tissue was placed in disinfectant solution and then disposed of safely.

A.4 Liquid Nitrogen Storage.

Confluent cloned cells in their second passage (in a 24 well plate) were trypsinised as explained previously, washed thoroughly and resuspended in PBS. Cells were then resuspended in 90% FCS: 10% DMSO (Dimethylsuphoxide) at a density of about 2 x 10^5 cells/ml (approximately 1ml/well). The DMSO prevents cell membrane rupture. 1ml aliquots were dispensed into sterile cryotubes. The cells were stored at –70°C for 12 to 24 hours in a well-insulated container to allow a slow drop in temperature, before transferring the cells to a vapour phase in liquid nitrogen.

A.5 Removing cells from Liquid nitrogen storage.

Cells were frozen with liquid nitrogen in a 90% FCS/10 DMSO solution. The DMSO was used to prevent ice crystals forming around the cellular membrane and thus prevents damage to the membrane. The DMSO must be neutralised immediately after thawing.
Under sterile conditions, cell culture medium was made up as described in the Appendix. For each tube of cells which is going to be thawed, 15ml of culture medium was aliquotted into a universal container. The frozen cells were held in a 37°C water bath. Before the cells thawed fully, they were removed from the water bath and the thawing process completed by pipetting a small volume of warmed culture medium into the cell container. This was to allow the cells to thaw surrounded by cell medium. The cells were spun down into a pellet by centrifuging at 1000rpm for five minutes and resuspended in 20ml of culture medium. This cell suspension was pipetted into a tissue culture flask. After one hour's incubation, the

cells were examined microscopically to confirm that they had adhered to the bottom surface of the flask.

A.6 Procedure for trypsinising cells.

Cells used were trypsinised at several points. This is the process where cells adherent to the plastic of the culture flasks and adherent to each other were treated so that they separated into single cells that could be suspended freely. This allowed counting of the cells and also dilution of the cells for further culture. Trypsin is a proteolytic enzyme that cleaves the connections between cells and their surroundings. EDTA is a calcium-chelating agent. Calcium is essential for the cells to adhere successfully. BAECs were cultured in 75ml flasks with 5-10ml of trypsin/EDTA in HBSS (Life technologies) at a temperature of 37°C for five minutes. The flasks were then examined under the light microscope to confirm that the cells had dislodged from the wall of the flask and that the cells were not clumped together. If this process was not completed, the cells were vigorously shaken. Finally, the fluid in the flasks was aspirated up and down with a pipette. The cell-containing trypsin was drawn off from the flask and 10ml of fresh, warmed culture medium was added. This was to inactivate the trypsin to prevent damage to the endothelial cells. The mixture was then centrifuged at 1000rpm for three minutes. A small pellet of cells was seen at the bottom of the container. All remaining fluid was drawn off and discarded. A further 20ml of culture medium was added to the cells. These were then well mixed through the solution.

A.7 Cell counting – haemocytometer.

The number of cells in a well–mixed sample was calculated using a cell-counter slide. Briefly, a few drops of the solution were dripped onto the slide, under a glass coverslip. The slide was then examined under the light microscope. It is marked with counting squares. The number of cells in four of these squares was counted, the result divided by four, to give the number of cells in 0.1µl. This figure was multiplied by 10^4 to give an approximation of the number of cells in every ml of medium.

Appendix 3.

Publications derived from experimental work

Papers

Drug-eluting stents: From lab bench to bedside
N. Swanson, A. Stephens-Lloyd & A.H. Gershlick
Cardiology News, Vol 4,5 June/July 2001

The stent as a local delivery device
N. Swanson, A.H. Gershlick
STENT Vol 2, 3 66-73 *Jan* 2000

The continuing challenge of coronary restenosis – is there a role for Vascular Endothelial Growth Factor?
N. Swanson & A. Gershlick
(*British Journal of Cardiology* 2001; 8 (issue 5): 278-80)

Vascular endothelial growth factor (VEGF) eluting stents. In vivo effects on thrombosis, endothelialisation and intimal hyperplasia.
N. Swanson, K. Hogrefe, Q. Javed, N. Malik & A.H. Gershlick
(*Journal of Invasive Cardiology*, 15 (12), 688-92)

In vitro evaluation of Vascular Endothelial Growth Factor (VEGF) – eluting stents.
N. Swanson, K. Hogrefe, Q. Javed, A.H. Gershlick
(*International Journal of Cardiology*, 2003 Dec, VOL: 92 (2-3)

Book Chapters

Coronary artery stenting: A case-orientated approach
Chapter 15 Local drug delivery from coated stents (pp179-189)
Gershlick J. Baron, J. Armstrong, **N. Swanson**, C. Newman & C. Holt
1st Edition Martin Dunitz Ltd. ISBN 1-85317-718-0
Editors N Curzen, MT Rothman (2001)

Handbook of local drug delivery
Chapter 65 VEGF-eluting stents.
N. Swanson & A.H. Gershlick.
Martin Dunitz Ltd
Editors Camenzind and De Scheerder (2001).

Abstracts, posters and presentations

Novel delivery of vascular endothelial growth factor using polymer-coated stents: Loading and elution characteristics.
Oral presentation and moderated poster British Cardiac Society 2000, Glasgow.
N. Swanson, J. Baron, K. Hogrefe, Q. Javed, A.H. Gershlick

Heart 83 (Suppl 1 P28).

VEGF-eluting stents to reduce stent complications – pharmacokinetics of adsorption and elution.
N. Swanson, K. Hogrefe, J. Baron, Q. Javed, A.H. Gershlick
6th International LDD&R Local Drug delivery meeting and cardiovascular course on Radiation & molecular strategies - Abstract book (2000)

VEGF-eluting coronary stents stimulate endothelial growth *in vitro*
N. Swanson, K. Hogrefe, Q. Javed, A.H.Gershlick
Journal of Submicroscopic Cytology & Pathology 32 (3) 419 B087.

VEGF-Eluting Stents Stimulate Endothelial Cell Growth *In Vitro*
N. Swanson, K. Hogrefe, Q. Javed, M.A. Azrin, A.H. Gershlick.
Journal of the American College of Cardiology March 2001,Suppl 1038-12.

N. Swanson, K. Hogrefe, N. Malik, Q. Javed, AH.Gershlick Vascular endothelial growth factor (VEGF) - eluting stents. In vivo assessment of effects on thrombosis, endothleialisation and restenosis. *Heart* 2002;**87**:P38

VEGF-eluting stents reduce stent thrombosis, but not restenosis, *in vivo*
N. Swanson, K. Hogrefe, Q. Javed, N. Malik, A.H. Gershlick.
European Heart Journal abstract Suppl 2002.

Copies of these works are appended in the following pages.

In vitro evaluation of Vascular Endothelial Growth Factor (VEGF) – eluting stents.

Authors
Neil Swanson MRCP* Kai Hogrefe MRCP Qamar Javed PhD Anthony H. Gershlick FRCP
University of Leicester, Cardiology Clinical Sciences, Leicester UK

International Journal of Cardiology, Volume 92, Issues 2-3, December 2003, Pages 247-251

Structured abstract

Background

Vascular Endothelial Growth Factor (VEGF) is a specific endothelial cell mitogen. It promotes re-endothelialisation of the damaged vessel surface seen after stenting. Stent thrombosis and in-stent restenosis are partly related to endothelial denudation caused by stent implantation. We propose using hydrocarbon polymer-coated stents immersed in VEGF to speed re-endothelialisation and reduce the risk of stent thrombosis and restenosis.

Methods

Stents (3x20mm) were immersed in VEGF solutions and maximal VEGF absorption calculated. VEGF release from these stents was measured in a perfusion circuit. Delivery of VEGF to arterial wall was measured. Sterile VEGF-loaded stents were cultured with Human Umbilical Vein Endothelial Cells (HUVECs).

Results

18.5±4.1μg VEGF was absorbed, 80% of which was released over nine days. 11+/-6.8% of initial VEGF loaded was delivered to the vessel wall. Cells exposed to VEGF-eluting stents showed an 11% increase in growth relative to controls.

Conclusion

VEGF may be a candidate for stent-based delivery and may increase the rate of endothelialisation *in vivo*.

Keywords

Local drug delivery Cell culture
Re-endothelialisation stent
VEGF

Introduction

VEGF is a cytokine originally described in 1983 (1). It is involved in processes essential to the growth, maintenance and repair of vascular structures.
At the time of percutaneous coronary intervention (PCI), the endothelium covering the stenotic area of artery is severely disrupted or even destroyed. In animal models of stent placement, re-endothelialisation begins to occur in the first 2-7 days post placement. Full endothelialisation can take up to three or four weeks (2). It is thought that a similar or longer time-course occurs in humans, although this has been difficult to quantify from the rarity of available autopsy specimens from patients post stent insertion (3). In animals, endothelial dysfunction is seen up to three months post stenting (4). Over time the endothelium grows back over the PCI site and stent. This process has been called stent passivation.
Endothelial dysfunction may contribute to intimal hyperplasia, especially after stent use (4). "Normal" endothelial cells produce heparin sulphate and Nitric Oxide which inhibit cell proliferation (5-6).
Exogenous VEGF has been reported to show accelerated re-endothelialisation of damaged arteries in the rat carotid artery (7). Not only did the group report superior re-endothelialisation in the treated group of rats, but also intimal hyperplasia at the site had been attenuated. Direct transfer of recombinant VEGF or VEGF plasmid in a rabbit hind-limb model of endothelial damage and stenting have been performed with delivery balloons (8,9). A clear increase in the rate of re-endothelialisation was shown in both with reduced intimal hyperplasia and of thrombus formation on the stents.
A human study has been done using a local delivery of VEGF plasmid into angioplastied arteries. At six months, no improvement in restenosis was seen (10). This study was not in stented patients, where the benefit of VEGF might be expected to be most noticeable. It also used a local delivery balloon, which is itself the cause of some intimal hyperplasia that might obscure any beneficial response from the drug.
We suggest that a polymer-coated stent, in this research the Cook Supra G stent, will deliver VEGF locally and for a sustained period, without the local trauma of a delivery balloon. To test this, the absorption and elution characteristics of VEGF on this stent was studied, as were the effects that the eluted VEGF have on endothelial cells, the target cell *in vivo*.

Materials and Methods

The Cook Supra G is a stainless steel slotted tube design stent coated with an aromatic hydrocarbon polymer surface (*personal communication, Dr Ragheb, Medinst, IN, USA*). These stents were divided into sections and sterilized using Ethylene Oxide gas treatment. This was done at low temperatures (<30°C) to avoid denaturing the protein or damage to the polymer.
Human recombinant VEGF-165 was provided by Genentech California in a stock solution of 5mg/ml. Iodine^{125}-radiolabelled VEGF was obtained from Amersham. Small amounts of radiolabelled VEGF were added to each solution of unlabelled VEGF used. The solutions were now termed as "spiked solutions".

Radiolabeling experiments – stent absorption and elution

Stents were immersed for two hours in three buffer solutions of varying pH- 4.5, 7.4 and 8.5 with I^{125}-labelled VEGF (2mg/ml at 25°C). A second batch of stents were immersed in VEGF in different concentrations of I^{125}-labelled VEGF (at 25°C, pH7.4), 0.5,1 and 2mg/ml for one hour. Further stents were immersed in VEGF (2mg/ml at 25°C) for 20min, 2, 24, 48 or 72hr and were gamma-counted at these times to calculate absorption.

Stent segments that had been absorbed with VEGF at the optimal conditions were then perfused in a peristaltic-flow circuit, as in previous work (11), at 37°C with a phosphate buffered saline (PBS)/Bovine serum albumin (BSA) solution representing plasma, for nine days. This had the same protein content as blood (40g/l), was pH7.4 and was perfused at a rate of 20ml/min, approximately the rate at which the stents are perfused in a coronary artery. At pre-determined times during perfusion the stent sections were gamma-counted and replaced in the circuit.

Segments of internal mammary artery were obtained with consent from patients undergoing coronary artery bypass grafting. Sections of artery were cut to be slightly longer than the stents to be deployed. *Ex vivo* retention of radiolabelled VEGF was determined by deploying dipped balloon-mounted stents, at eight atmospheres pressure, into the arterial segments, which were placed within the lumen of the tubing constituting the perfusion circuit previously described. The stented vessels were perfused in 100ml of perfusate in the circuit for 24 hours. Longer perfusion times were not felt to be reliable sources of data regarding retention in the tissue wall as the circuit was not sterile and the tissue not viable in this circuit. After 24 hours, stent sections were separated from the arterial segments. These were trimmed so only the stented sections of vessel were examined further. Stents and vessel sections were gamma counted to estimate absorption of VEGF.

HUVEC cultures exposed to VEGF stents.

Cell culture was performed using Gibco M199 with Earl Salts, glutamine (Ref 31150-022), 1% Penicillin/streptomycin, heparin 2500iu/500ml (Monoparin), Endothelial Cell Growth Supplement (ECGS) and 20% fetal calf serum. Cells were cultured at 37°C in 5% CO_2.

Non-radiolabelled VEGF in PBS was used to coat sterile stents in the optimum fashion determined previously. These stents were then sterilized using ethylene oxide gas treatment. Stents were then co-cultured with 1ml of 1.6x 10^4 cells/ml Human Umbilical Vein Endothelial Cells (HUVECs) solution. The HUVECs had been prepared from early passage cultures obtained as described previously (11). The cells were confirmed to be endothelial both by their typical cobblestone microscopic appearance and by positive staining for von Willebrand Factor. One group of stents was washed with culture medium for 24 hours prior to HUVEC culture (washed stents). This was to determine whether sufficient VEGF remained within the polymer to have continued physiological benefit after any very superficially adherent VEGF had been washed off. The effects of these stents were compared to control stents. These were immersed in 2mg/ml bovine serum albumin (BSA) for 24 hours, dried and sterilized as the VEGF stents. HUVEC growth was determined with a PMS/MTS assay depending on mitochondrial activity in the cells (12). Alongside the wells containing HUVECs and loaded stents were cultured further wells that contained culture medium containing either 10 or 500ng/ml solutions of VEGF to compare the effects of the VEGF delivered by stent to VEGF solution added directly to the medium.

Statistical analysis.

Results are expressed as means +/- standard deviations. Elution kinetics were ascertained by nonlinear regression (curve fitting) using a computerised scientific data analysis package (Prism, GraphPad Software Inc., CA, USA); R^2 values were determined for each fitted curve to assess the overall goodness-of-fit and analysis of residuals performed to assess whether observed data were consistent with the chosen regression equation. A p value of >0.05 for analysis of residuals was considered to be consistent with non-significant deviation from the model.

The results of the proliferation studies were analysed to determine that they were compatible with a normal distribution (Kolmogorov-Smirnov). The data were subjected to a two-tailed t-test. Equality of variance was calculated (Levene's Test) and factored into the t-test. Results were calculated as p values, where significance was defined as $p<0.05$.

Results

Absorption studies

Buffer pH did not affect absorption to the stents (*data not shown*). All further work was done using PBS, as this was of physiological pH7.4. An increase in the concentration did result in an increased absorption of the drug at the concentrations used (*see Figure 1*). 2mg/ml was used in further experiments.
Increasing the length of time that the stent sections were immersed in VEGF solution showed an initial rapid rise in the amount of VEGF absorbed (*see Figure 2*). 18.5±4.1μg of VEGF was absorbed after two hours immersion onto a 3x20mm stent (total stent weight = 55mg). After two hours, however, no further significant immobilisation of protein to the stents was seen. Further work was done with at least two hours of immersion time.

Elution studies – *in vitro, ex vivo* and cell culture

In conditions replicating physiological conditions, the stents (n=8) in the perfusion circuit retained significant amounts of VEGF for nine days. An initial rapid decay was seen with an overall biexponential (r^2=0.99, analysis of residuals p= 0.11) decay. At five and nine days, 28% and 20% respectively of the initial dose still remained on the stent sections (see *Figure 3*). *Ex vivo* distribution of VEGF eluted from a stent placed in a section of internal mammary artery is demonstrated in Table 1. At 24 hours, 11+/-6.8% of the initial VEGF loaded onto the stents was seen in the tissue and 12.3+/-1.7% remained bound to the stents.
HUVECs cultured with VEGF-coated stents showed an 11% increase in growth compared to controls (p<0.001). Stents where the culture medium surrounding them was removed after 24 hours and replaced afresh (washed stents) showed a 10% increase in growth over controls (p=0.004)(*see Figure 4*). Thus, the effect of the stents persisted, even after washing.

Discussion

This work shows that this polymer-coated stent can be used to carry significant quantities of VEGF protein. This stent will then release the drug in a controlled fashion obeying predictable kinetics into the surrounding tissue, as well as the local microcirculation. The release is slow, over many days. The regrowth of endothelial cells over the angioplasty site takes many days or even weeks to occur normally. This prolonged release of VEGF is in contrast to the bolus approach that is seen with balloon catheter delivery systems. Slow release of VEGF has been seen where VEGF plasmid DNA has been delivered locally. The transfection efficiency of this technique is 0.12% of cells (9) and the amount of VEGF released into the local tissue is difficult to quantify and seems likely to be different from case to case. Stent based delivery of the protein itself will release a known amount of VEGF at a predetermined rate.

This work has shown that 11±6.8% of the initial dose of VEGF on the stent is to be found in the vessel wall. This compares favourably with balloon delivery, where lower retention rates are the normal finding. Delivery of 1% or less of the delivered drug is typical (13). The cellular work performed showed that VEGF loaded stents were effective in promoting endothelial cell growth, by 11% more than the control groups. This modest increase in growth was significant. The effect seen was comparable to the stimulus seen when VEGF was added directly to the culture medium so the delivered dose of VEGF was higher than that necessary to produce maximal growth stimulation.

Stent based delivery has been shown to be feasible in our laboratory with favourable *in vivo* effects using the drugs abciximab (ReoPro) (15) and Activated Protein C (16).

Human studies of drug-eluting stents have now been performed. Sirolimus has been shown to have a significant effect in reducing intimal hyperplasia in the RAVEL study [17], which reported with a restenosis rate of 0% incidence of restenosis in the sirolimus group *vs.* a 26% restenosis rate in the control arm ($p<0.001$). Three studies have reported benefits of paclitaxel-coated stents. ASPECT showed a reduction in binary restenosis (4% *vs.* 27%, $p<0.001$). Taxus-I has reported a six month binary restenosis rate of 0% *vs.* 11%, $p=0.011$. ELUTES showed a binary restenosis of 3% *vs.* 21%, $p=0.055$. All these studies have demonstrated the potential of stent-based therapies to reduce intimal proliferation in humans. Although using different drugs, they share a common target cell, the vascular smooth muscle cell.

VEGF-eluting stents, in contrast, target the endothelial cell selectively, since the receptors for VEGF (Flt-1 and KDR) are found almost exclusively on endothelial cells. Inhibition of thrombosis and restenosis would therefore be mediated by a selective proliferation of endothelial cells. Rapid re-endothelialisation of the denuded vessel wall at the stented site would reduce the time that the thrombogenic foreign body would presents a surface to the coagulation system.

Furthermore, since an intact endothelium inhibits smooth muscle cell proliferation, VEGF-eluting stents may indirectly reduce the extent of neointima formation and resultant restenosis

VEGF may be a candidate for stent-based delivery and may increase the rate of endothelialisation *in vivo*.

Acknowledgements: The British Heart Foundation and Cook Inc. have supported this line of research. I gratefully acknowledge the assistance in completing this work of Professor M. Azrin, University of Connecticut.

References.

[1] Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;219(4587):983-5.

[2] Barone GW, Conerly JM, Farley PC, Flanagan TL, Kron IL. Endothelial injury and vascular dysfunction associated with the Fogarty balloon catheter. Journal of Vascular.Surgery 1989;9(3):422-5.

[3] Grewe PH, Deneke T, Machraoui A, Barmeyer J, Muller KM. Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. Journal of the American College of Cardiology 2000;35(1):157-63.

[4] van Beusekom HM, Whelan DM, Hofma SH, Krabbendam SC, van Hinsbergh VW, Verdouw PD, van der Giessen WJ. Long-term endothelial dysfunction is more pronounced after stenting than after balloon angioplasty in porcine coronary arteries. Journal of the.American.College.of Cardiology 1998;32(4):1109-17.

[5] Castellot JJJ, Addonizio ML, Rosenberg R, Karnovsky MJ. Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. Journal of Cell Biology 1981;90(2):372-9.

[6] Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. Journal of.Clinical.Investigation. 1989;83(5):1774-7.

[7] Asahara T, Bauters C, Pastore C, Kearney M, Rossow S, Bunting S, Ferrara N, Symes JF, Isner JM. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. Circulation 1995;91(11):2793-801.

[8] Van Belle E, Tio FO, Couffinhal T, Maillard L, Passeri J, Isner JM. Stent endothelialization. Time course, impact of local catheter delivery, feasibility of recombinant protein administration, and response to cytokine expedition. Circulation 1997;95(2):438-48.

[9] Van Belle E, Tio FO, Chen D, Maillard L, Kearney M, Isner JM. Passivation of metallic stents after arterial gene transfer of phVEGF165 inhibits thrombus formation and intimal thickening. Journal of the.American.College.of Cardiology 1997;29(6):1371-9.

[10] Laitinen M, Hartikainen J, Hiltunen MO, Eranen J, Kiviniemi M, Narvanen O, Makinen K, Manninen H, Syvanne M, Martin JF, et al. Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. Human Gene Therapy 2000;11(2):263-70.

[11] Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. Journal of Clinical Investigation 1973;52(11):2745-56.

[12]Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Communications 1991;3(7):207-12.

[13] Lincoff AM, Weinberger J. Topol EJ, editors.Comprehensive Cardiovascular Medicine. 1998; 90, Local drug delivery and endovascular radiation. p. 2433-51.

[14]Favard C, Moukadiri H, Dorey C, Praloran V, Plouet J. Purification and biological properties of vasculotropin, a new angiogenic cytokine. Biology.of the.Cell 1991;73(1):1-6.

[15] Aggarwal RK, Ireland DC, Azrin MA, Ezekowitz MD, de Bono DP, Gershlick AH. Antithrombotic potential of polymer-coated stents eluting platelet glycoprotein IIb/IIIa receptor antibody. Circulation 1996;94(12):3311-7.

[16] Foo R, Gershlick AH, Hogrefe K, Baron J, Johnson TW, de Bono DP. Inhibition of platelet thrombosis using an activated protein C loaded stent: in vitro and in vivo results. Thrombosis & Haemostasis 2000;83(3):496-502.

[17]Morice MC, Serruys PW, Sousa JE, et al. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. New England Journal of Medicine 2002;346:1773-1780

Figure Legends

*Figure 1 – Absorption of VEGF to Cook stent segments in differing concentrations of VEGF in PBS (Phosphate buffered saline) for one hour. * = $p<0.05$ compared to 0.5mg/ml value.*

Figure 2 – Absorption of VEGF to Cook stent segments after differing immersion times.

Figure 3 VEGF elution in perfusion circuit. These data fit a two-phase exponential decay model (r^2 =0.99, analysis of residuals p= 0.11).

Figure 4 HUVEC growth with the 10 or 500ng/ml VEGF did not differ significantly from the response seen with the VEGF eluting stents (data not shown). BSA stents are those coated with albumin as a control, washed stents were those VEGF loaded stents that had been immersed in culture medium for 24 hours before the addition of HUVECs in fresh medium. This was to show that the effect is not just due to VEGF eluting from the stent very early after immersion in culture medium.

Tables

Location	% of initial value (mean)	Standard deviation (%)
Stent section	12.3	1.7
Tissue specimen	11.0	6.8
"systemic" delivery	76.7	2.9

Table 1 Distribution of VEGF after 24 hours perfusion (initial loading 7.9+/-2.54mcgs of VEGF on a half-length, i.e.10mm, stent, n=4). "Systemic" delivery was the remaining VEGF deposited in the glassware, tubing or in the perfusate.

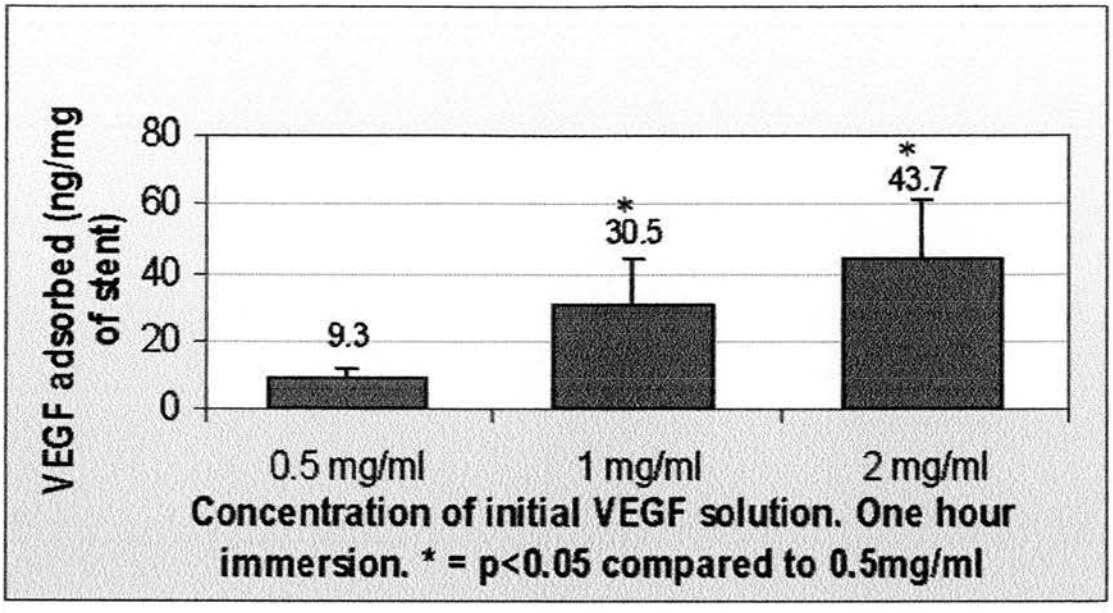

VEGF adsorbed (ng/mg of stent)
80
60
40
20
0
9.3
*
30.5
*
43.7
0.5 mg/ml
1 mg/ml
2 mg/ml
Concentration of initial VEGF solution. One hour immersion. * = p<0.05 compared to 0.5mg/ml

Fig 1

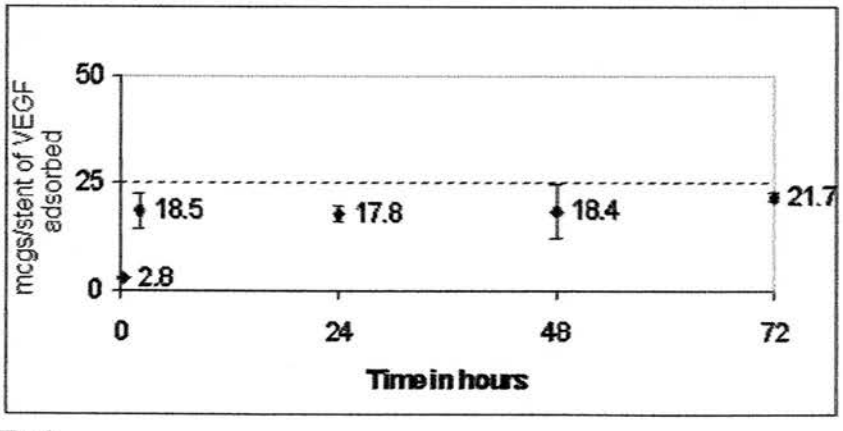

mcgs/stent of VEGF adsorbed
50
25
0
18.5
17.8
18.4
21.7
2.8
0
24
48
72
Time in hours

Fig2

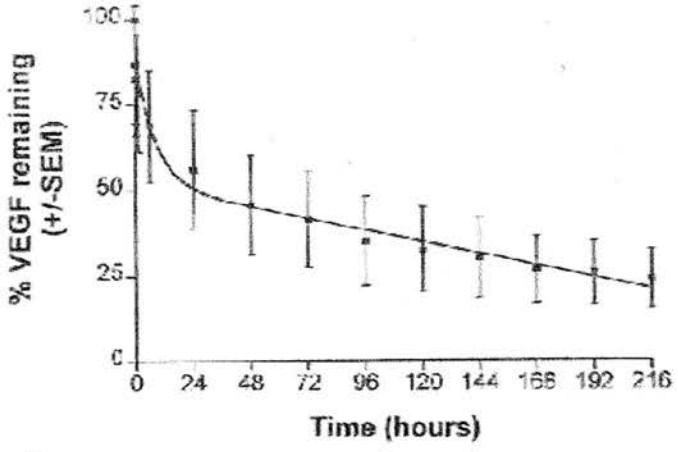

% VEGF remaining (+/-SEM)
100
75
50
25
0
0
24
48
72
96
120
144
168
192
216
Time (hours)

Fig 3

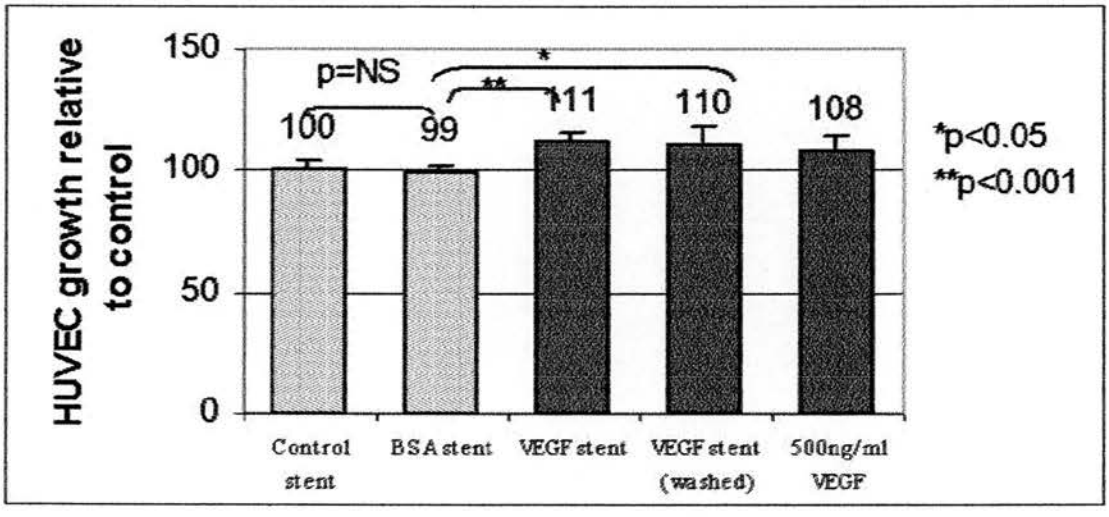

HUVEC growth relative to control
150
100
50
0
p=NS
*
**
100
99
111
110
108
Control stent
BSA stent
VEGF stent
VEGF stent (washed)
500ng/ml VEGF
*p<0.05
**p<0.001

Fig 4

Vascular Endothelial Growth Factor (VEGF)-Eluting Stents: In Vivo Effects on Thrombosis, Endothelialization and Intimal Hyperplasia

Neil Swanson, Kai Hogrefe, Qamar Javed, *Nadim Malik, Anthony H. Gershlick

ABSTRACT: Local drug delivery by stent can reduce in-stent restenosis. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen. After stenting, the arterial wall is almost denuded of endothelium. This loss of endothelium contributes to the smooth muscle cell (SMC) proliferation seen in restenosis, since the endothelium actively inhibits SMC hyperplasia. Over time, the endothelium recovers and SMC hyperplasia is arrested. The capacity of VEGF-coated stents to accelerate re-endothelialization, and to therefore reduce restenosis and thrombosis, was tested in this study. Radiolabeled VEGF was absorbed onto stents and released over nine days in an *in vitro* perfusion circuit. VEGF-coated stents were deployed in arterial segments to study local tissue release. A New Zealand White rabbit iliac artery model for stent implantation was used. Re-endothelialization and thrombosis were assessed after seven days. Further animals were examined 28 days post-procedure for in-stent restenosis. Stented vessels were resin-embedded, sectioned and stained. Intimal thickening was calculated using computerized morphometry. *In vitro*, the stents released 80% of the initial load over nine days. At seven days, thrombus was significantly reduced (12.5 mg for controls versus 0 mg for VEGF; $p = 0.014$). No beneficial effect was seen on endothelialization, nor on intimal hyperplasia. Neointimal area was 2.2 ± 0.9 mm^2 for controls versus 2.4 ± 1.8 mm^2 for VEGF ($p = 0.8$). These VEGF-eluting stents do not accelerate re-endothelialization or inhibit restenosis. Stent thrombosis appears to be reduced, which may make these stents less thrombogenic and be valuable in higher-risk cases.
J INVAS CARDIOL 2003;15:688–692

Key words: endothelial function, growth factors, restenosis, stents

Several recent studies have reported favorable results in preventing restenosis using stent-delivered drugs. The antibiotic and antiproliferative agent sirolimus has been shown to have a significant effect in reducing intimal hyperplasia in the RAVEL study, with a 0% incidence of restenosis.[1] Three other studies (ASPECT, TAXUS-I and ELUTES) have all reported benefits of paclitaxel-coated stents. The STRIDE dexamethasone registry reported a 3.3% occurrence of major adverse cardiac events (MACE) at six months. However, other agents have proven less effective. In the BRILLIANT trial, the MMP inhibitor batimastat was tested but was abandoned because of poor restenosis rates. Similarly, the ACTION trial of actinomycin-D was abandoned due to poor results. All these drugs work by inhibiting the cellular overgrowth, mainly aimed at the smooth muscle cells, which is part of the healing process after percutaneous coronary intervention (PCI). An adverse side effect of such drugs may be to also inhibit endothelial cell function and recovery. "Normal" endothelial cells (EC) produce heparin sulphate and nitric oxide (NO), which inhibit cell proliferation.[2–4] Endothelial dysfunction contributes to intimal hyperplasia, and this dysfunction is prolonged after stent use.[5] In addition, the lack of an endothelium is felt to increase the risk of thrombosis at the PCI site. This often catastrophic condition is rare in most clinical situations, but has been reported in 2–3% in small vessels[6] and in 9.1% of patients in brachytherapy trials.

VEGF is a cytokine involved in processes essential to the growth, maintenance and repair of vascular structures. At the time of PCI, the endothelium covering the stenotic area of artery is severely disrupted. In animal models of stent placement, re-endothelialization begins to occur in the first 2–7 days post-placement and can take up to 3–4 weeks to complete.[7] It is thought that a similar or longer time course occurs in humans.[8]

Exogenous balloon-delivered VEGF or VEGF plasmid have been reported to accelerate re-endothelialization, reduce thrombus formation and reduce intimal hyperplasia in damaged rat carotid arteries[9] and rabbit iliac.[10,11] Previous work has delivered the VEGF with a variety of local delivery balloons. This study assesses these apparent benefits using a polymer-coated stent as the delivery device. Such a stent should release (elute) VEGF gradually, thus prolonging the time during which it may act upon the injured vessel. The effects were studied in a rabbit iliac artery stent model. Elution characteristics of VEGF from the polymer-coated stent were modeled *in vitro*, including the amount retained in the wall of the stented mammary artery.

Methods

Stents and drug. Supra G, stainless-steel, slotted-tube stents (Cook Cardiology, Miami, Florida) coated with an aromatic hydrocarbon polymer surface were used. A previous generation of these stents and polymer was successfully used to deliver the protein abciximab in the animal model used in this work. Human recombinant VEGF-165 was provided by Genentech California in a stock solution of 5 mg/ml.

In vitro elution experiments. VEGF was radiolabeled with the gamma-emitter Iodine 125 using the Iodogen method. Stent segments that had absorbed VEGF at optimal conditions (2 mg/ml stock solution in pH 7.4 PBS buffer for 24 hours at room temperature) were perfused in a peristaltic-flow circuit at 37 °C with a PBS/BSA solution for nine days. This solution was prepared such that it contained the same protein content as blood (40 g/L), was pH 7.4 and was perfused in a pulsatile manner at a rate of 20 ml/minute. After 5 and 20 minutes, and at 1, 2, 4 and 24 hours and every 24 hours of perfusion thereafter, stent sections were gamma-counted and replaced in the circuit.

From the University of Leicester, Cardiology Clinical Sciences, Leicester, United Kingdom and *the University of Manchester, Cardiology Department, Manchester, United Kingdom.

Grants facilitating this work are acknowledged from the British Heart Foundation and Cook Inc., Miami, Florida.

Manuscript received March 7, 2003, provisional acceptance given August 23, 2003, revised manuscript accepted September 9, 2003.

Address reprint requests to: Neil Swanson, Cardiology Clinical Sciences, Glenfield Hospital, Groby Road, Leicester, LE3 9QP, United Kingdom. E-mail: b.n.swanson@talk21.com

Radiolabeling experiments — Stent elution. VEGF solutions were prepared containing Iodine 125 radiolabeled VEGF. Stent segments that had absorbed VEGF at optimal conditions were perfused in a peristaltic-flow circuit. After 5 and 20 minutes, and at 1, 2, 4 and 24 hours and every 24 hours of perfusion thereafter, stent sections were gamma-counted and replaced in the circuit. A diagram of the flow circuit is shown in Figure 1.

In vitro retention of radiolabeled VEGF was determined by deploying dipped stents in segments of internal mammary artery, obtained with consent from patients undergoing coronary artery bypass grafting. Artery sections were cut to the same length as the stents, i.e., 10 mm. The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3). These sections of artery were again perfused in the circuit previously described for 24 hours. As the circuit was not sterile and the tissue was not likely to remain viable in this circuit, longer perfusion times were not felt to be reliable sources of data. After 24 hours, stent sections and arterial segments were separated and gamma counted to estimate absorption of VEGF.

Animal studies. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal studies used male New Zealand White rabbits (3.0–4.6 kg). Animals were prepared for stent implantation with the addition of 1 mg/kg aspirin and 1 mg/kg clopidogrel in their daily drinking water for five days. These drugs were continued daily from the time the animals recovered from initial anaesthesia throughout the study until termination.

Operative procedures for acute studies. General anaesthesia was induced 30 minutes after premedication with Hypnorm 0.3 ml/kg (a combination of fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml; Janssen-Cilag, High Wycombe, Bucks). Inhaled halothane (2–3%) was used for induction and a combination of halothane (0.5–3%) and oxygen (0.5–3 L/minute) was used for maintenance of anaesthesia. Under general anaesthesia, a midline abdominal incision was made and the iliac arteries were exposed to facilitate placement of a perivascular flowprobe on either side. The femoral arteries were also exposed on both sides through separate incisions. An arteriotomy was fashioned between two ligatures placed loosely around the superficial femoral artery distal to its lateral circumflex branch. One-thousand IU of unfractionated heparin were administered. A 3 x 15 mm angioplasty balloon catheter was inflated to 8 atmospheres three times in the iliac artery. A 3 x 10 mm stent (either a control or a VEGF-loaded stent) was deployed at the site of arterial injury. Following stent deployment, the superficial femoral artery was ligated. Blood flow through stented vessels was recorded immediately after superficial femoral artery ligation (T206 small animal blood flow meter with 2.5SB probes, Transonics Inc., Ithaca, New York) and continuously for two hours thereafter with the flowprobe placed immediately distal to the stent. The flow was measured to record the incidence of cyclical flow variations in the stented vessels. These are indicative of transient thrombus adhesion, causing variations in flow rates. After termination, the vessels were dissected free and the stented arteries were fixed and then resin-embedded as described below.

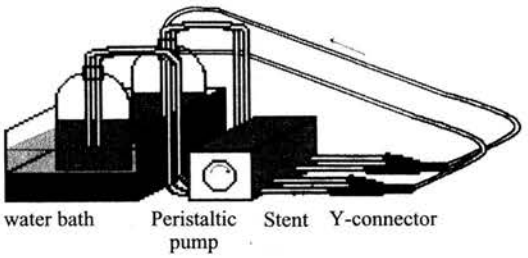


Figure 1. *Diagram of the perfusion circuit used. Each circuit contained 2 stents in parallel, perfused with 4% albumen/buffer solution at 37 °C. The pump generated peristaltic flow.*

Operative procedures for endothelialization, thrombosis and restenosis studies. Animals that were allowed to recover after stent deployment for elective sacrifice after 7 or 28 days had a single, left-sided groin incision for superficial femoral exposure. No abdominal incision was made. Angioplasty and stenting of the left iliac artery was performed as in the acute studies, again with heparin as adjunctive therapy. The animals were recovered, and aspirin and clopidogrel were continued (administered in drinking water) until sacrifice at 7 or 28 days. Animals to be examined for endothelialization (7 days) had 40 mg Evans Blue dye[12] injected intravenously. This was allowed to circulate freely for at least half an hour to stain any endothelium-denuded vessels. At the time of sacrifice, a catheter was placed into the abdominal aorta. Ten milliliters of 0.25% silver nitrate solution were then instilled to improve the delineation of endothelial cells under scanning electron microscopy.[12] The animal was exsanguinated post-mortem. The iliac arteries and distal aorta were dissected free and fixed in 10% formalin.

The extent of endothelialization and thrombosis was assessed as follows: 1) Stents were divided lengthwise and partly flattened. The flattened stents, stained with Evans Blue, were analyzed by computerized morphometry as previously described.[13] A blinded observer was asked to assess the amount of blue staining in each section relative to undamaged tissue in the same samples. The staining was graded 1–5 in increasing subjective impression of the amount or intensity of Evans Blue staining; 2) Tissues were examined under scanning electron microscopy; 3) Thrombus was estimated by removing adherent thrombus from the interior aspect of the stented vessel and weighing it (wet and dry weights). The extent to which the thrombus had occluded the vessel lumen was also noted.

Restenosis studies. The 28-day rabbits were studied for restenosis. Stented vessels were fixed and resin-embedded. Stented vessels were also taken from the animals in the acute studies for baseline measures to be made.

Resin embedding of tissue. The technique allows the preservation of the arterial architecture *with the stent in situ*, while allowing staining to identify the proliferative response of the tissue.[14] Stented tissue was dehydrated in 100% acetone and immersed in infiltrating solution (50% benzoylperoxide with hydroxyethyl methacrylate) for 24 hours at 4 °C. Blocks were then transferred into an embedding solution (infiltrating solution

with tetramethyl aniline) and orientated in polythene tubes. The tubes were hermetically sealed and left at 4 °C for 24 hours. 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 100 µm 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 10–20 mm in thickness. These sections were then affixed to Perspex slides with Super-attak adhesive. Stent cross-sections were stained with hematoxylin and eosin.

Slides from the sections from the 28-day and day 0 animals were examined and intimal growth quantified, using two different techniques. The area of the lumen was calculated at a low power magnification of the vessel cross-section. Then the total area of the neointima, the stent sections and lumen were measured and the luminal area subtracted. At a higher magnification, further measurements were made of each quarter of the vessel. This allowed more precise tracing of the boundary between media and intima and gave a measure of the average intimal thickness within each quarter. The results for each quarter were averaged to give the mean intimal depth in that cross-section of the vessel. All measurements were performed by a single, blinded observer. To ensure the results were reproducible, three randomly selected vessels were remeasured and the various measurements were plotted against the first reading to demonstrate that they were essentially the same.

Injury scoring. The results at 28 days were observed to determine whether the original injuries suffered by the stented vessels were similar in the two groups. Injury scoring was done using the previously published and validated scoring system of Schwartz et al [15]

Systemic delivery samples. In addition to the above assays, samples of liver and kidney were taken from randomly selected animals that received the VEGF-loaded stents. These were freeze-dried and powderized. Resulting solutions were then assayed using a VEGF ELISA kit to detect any systemic delivery of VEGF from the stents. Samples of tissue injected with VEGF solution directly formed the positive controls.

Statistical analysis. Elution kinetics (Figure 2) were ascertained by nonlinear regression (curve fitting) using a computerized scientific data analysis package (Prism, GraphPad Software Inc., California); R^2 values were determined for each fitted curve to assess the overall goodness-of-fit and analysis of residuals performed. Power calculations were based on the findings in similar work.[11] The study was powered to detect a difference of 35% in mean area of endothelialized stent and a 0.55 mm^2 difference in mean area of intimal hyperplasia, both at a power of greater than 90% at a significance level where $p < 0.05$. Differences in thrombus weight between control and VEGF stents were tested with a Mann-Whitney test. Differences in intimal thickness/area were calculated with a two-tailed Student's t-test, assuming unequal variance.

Results

Elution studies — *In vitro*. Radiolabeling data showed that 18.5 ± 4.1 mg of VEGF could be absorbed after two hours of immersion. The stents in the perfusion circuit retained significant amounts of VEGF for many days, with an initial rapid decay and an overall biexponential decay pattern. At five and nine days, 28% and 20% of the initial dose still remained on the stent sections, respectively (Figure 2). *In vitro* distribution of VEGF eluted from a stent showed that at 24 hours, 11 ± 6.8% of the initial VEGF loaded onto the stent was seen in the tissue and 12.3 ± 1.7% remained on the stent.

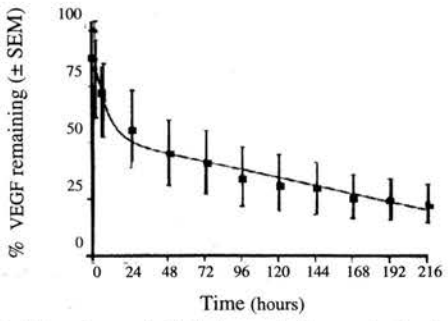


Figure 2. *Vascular endothelial growth factor elution in a perfusion circuit. These data fit a two-phase exponential decay model (r^2=0.99; analysis of residuals $p = 0.11$; $n = 6$).*

Acute studies. Two animals died perioperatively in the VEGF group before the stent had been inserted. No data were obtained from these animals. Cyclical flow variation was seen in one animal, in the control group.

Endothelialization and thrombosis studies. Less thrombus (measured by wet and dry weights of macroscopic thrombus) was seen at seven days in the stents coated with VEGF than in the controls. This was observed macroscopically and confirmed by weighing the adherent thrombus, both when wet and after several days of air-drying to remove water and formalin (Figure 3).

Treatment	Stent number	Extent of thrombosis	Thrombus (wet)	Thrombus (dry)
Control	1	Occlusive	92mg	17mg
	2	Minor	10mg	1mg
	3	Minor	9mg	2mg
	4	Luminal	15mg	2mg
	5	None	0	0
	6	Occlusive	56mg	7mg
VEGF	1	None	0	0
	2	None	0	0
	3	None	0	0
	4	None	0	0
	5	None	0	0
	6	None	0	0
	7	None	0	0
	8	Minor	<1mg	<1mg

Figure 3. *Results of thrombosis and endothelialization 7 days after stent implantation. Thrombosis results are median results of wet weight of thrombus ($p = 0.0142$; 95% confidence interval, 8.49–55.99). Dry weight results were 2 mg vs. 0 mg. The stented vessels were examined after 7 days. Visual inspection was made as to the extent of any adherent thrombus. This was carefully removed from the vessel and weighed, both wet and dry. Two stents in the control group were maldeployed and as a consequence entirely occluded with thrombus. These stents were not included in the final analysis. For the endothelialization at 7 days, a score of 1–5 (increasing endothelialization) was used to produce a semi-quantitative result.*

Almost all sections showed uniform Evans Blue staining, suggesting that no significant endothelialization occurred. No difference was seen between the groups with the Evans Blue staining. Four of the control vessels were completely occluded with thrombus. Three had at least some macroscopically visible thrombus that would have prevented some endothelialization. No significant differences were seen in endothelialization scores between control and VEGF-coated stents (Figure 3). Scanning electron microscopy examination confirmed restricted re-endothelialization in both groups. Less than 10% of the surface of the vessel wall was re-endothelialized.

Restenosis studies. Intimal hyperplasia was seen in all the stents examined at 28 days to a variable degree (Figure 4). No differences were seen in the average injury scores (1.00 versus 1.01; $p = 0.91$; n = 8), suggesting that equal vessel injuries had been made at the outset. Average depth of neointima showed no difference between the two groups (Figure 5). Neointimal area at 28 days again showed no statistically significant difference between the control and VEGF-treated stents (2.2 mm^2 versus 2.4 mm^2, $p = 0.8$). Intima (including stent struts) to media ratios were 1.99 ± 1.05 for control versus 2.19 ± 1.06 for VEGF ($p = 0.73$; n = 8).

The detected quantities of VEGF per gram of total protein in liver or kidney samples from the experimental animals was 2,575 ± 2,390 ng/g VEGF (control group) and 832 ± 653 ng/g VEGF (VEGF group) ($p = 0.32$), i.e., no detectable increase in VEGF systemically as a consequence of the VEGF stent treatment in distal tissues.

Discussion

In vitro results

The initial *in vitro* experiments demonstrated that 18 mg of VEGF protein could be absorbed into the polymer coating of the stents and that this VEGF was gradually released over a period of at least nine days. It was also shown that significant quantities of the released VEGF were found in the vessel wall, where it is likely to be effective. Ten percent of the initial dose of VEGF loaded on the stent was detected in the tissue, which compares favorably with the approximately 1% delivery efficiency in the vessel wall of a local delivery balloon.[16]

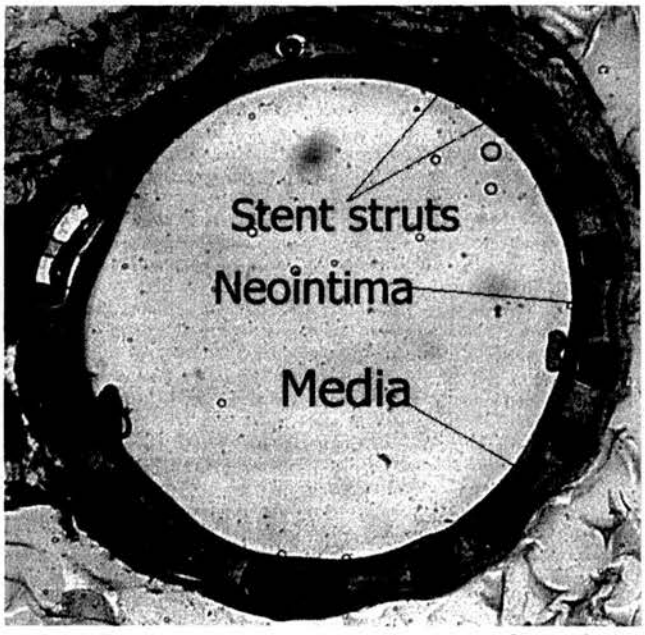


Figure 4. *Representative cross-section of stented vessel stained after 28 days in vivo with H&E to show extent of intimal hyperplasia. No significant differences were seen between the vascular endothelial growth factor and control treated specimens.*

In vivo results

Systemic delivery, endothelialization and restenosis. No increase in VEGF was seen in the distal tissue studied at the two study endpoints. No evidence was obtained of accelerated endothelialization using the VEGF-eluting stent. Stent struts remained either completely exposed or were covered with sheets of organized, erythrocyte-rich thrombus. In contrast to the published work of Van Belle et al., where VEGF had produced 90% endothelial coverage by this time, less than 10% of the stent structure was endothelialized. This reduced re-endothelialization may reflect differences between models used. Different stents were used and they (or the polymer coating) may have contributed to the delayed endothelialization. Smaller animals were used (mean weight, 3.6 kg versus 5–5.5 kg in the Van Belle study). A relatively greater injury may therefore have been induced in the smaller iliac vessels.

There are various possible explanations for the failure of the VEGF-eluting stent to improve re-endothelialization. VEGF may have been inactivated by the storage and sterilization process. VEGF may have been washed off the stent as the stent was passed through the femoral vessels, or downstream after implantation. The VEGF-soaked polymer was pressed into the vessel wall where there was no room for endothelial cells to grow in from the sides. VEGF from the stent will largely have been released into the vessel wall where there were no target cells for the VEGF. Finally, VEGF's most pronounced effects are on vascular permeability and not cell proliferation. In cell culture experiments, only a modest increase in endothelial cell growth was seen, about 10% higher than that seen with controls.[17] In clinical trials, VEGF has not led to any clinically significant proliferation of blood vessels (neoangiogenesis), despite anticipated stimulation of endothelial growth.[18]

No benefit in reducing restenosis was seen in these results, presumably because of the lack of effect on the re-

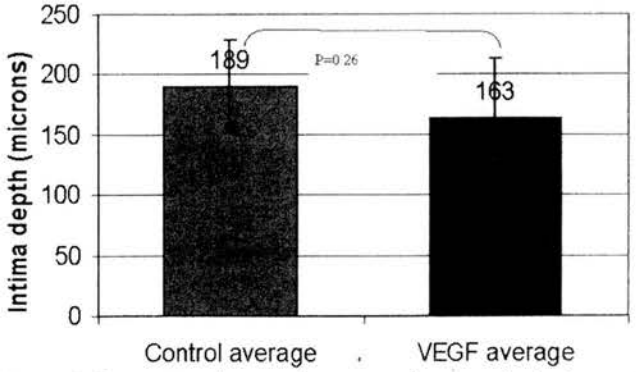


Figure 5. *Comparison between the measured average depth of neointima in the 2 groups. No significant reduction in neointimal depth was seen (n = 8).*

endothelialization process. It would appear that any possible beneficial effects on reduced thrombosis were not borne out by a corresponding reduction in intimal hyperplasia. Notably, a human study using local delivery of VEGF plasmid into angioplastied arteries showed no improvement in restenosis at six months.[19]

Stent endothelialization to reduce restenosis has been tested in several other studies. Some have examined the potential of stents directly coated with endothelial cells (ECs).[20] Several agents other than VEGF have been delivered to the PCI site to speed re-endothelialization. Prostacyclin synthase gene transfer,[21] hepatocyte growth factor,[22] blockade of TNF,[23] systemic estradiol administration[24] and locally-delivered antibody to thrombospondin[25] have all increased re-endothelialization in animal studies. None of these studies used the stent itself as the delivery vehicle. Agents to promote endothelialization, such as VEGF, may not be effective when delivered by stent, because the drug is not delivered to the target cells at either end of the stent, whereas delivery balloons release agent around the PCI site.

Thrombosis. A reduction in thrombus accumulation was seen at seven days using VEGF-eluting stents. In other work, reduced clot formation has been seen on stents implanted in rabbit iliac arteries when washed with VEGF.[11] The mechanism for the observed reduction in thrombus formation in these experiments is not known. VEGF increases the expression of NO, PGI_2, urokinase and tissue-type plasminogen activator,[26] all of which antagonize thrombus formation. VEGF released from the stents may have stimulated endothelial cells adjacent to the PCI site, or in small sidebranches of the main vessel, where the endothelium remained undamaged, to produce some of these factors. Finally, VEGF may produce NO-mediated vascular relaxation upstream, increasing flow and inhibiting thrombus formation.

Study limitations. This study has tested VEGF in a small group of animals with only limited evidence of any benefit. The study is limited by the model used, which is not a true representation of the scenario in man. The vessel used, although arterial, is not a coronary and is free of the mature atherosclerotic plaques seen clinically. The mechanism underlying the apparent anti-thrombotic effect of the VEGF-eluting stent is not clear.

Conclusion. VEGF delivery by stent in this model does not alter re-endothelialization. No beneficial results were seen on restenosis. An apparent beneficial effect on thrombus formation early after stent implantation was seen. Such an effect would make these stents less thrombogenic than current clinically used stents. This effect requires ongoing *in vivo* evaluation. VEGF may be a suitable agent for local delivery by stent in patients in whom a high risk of stent thrombosis may be anticipated, including patients with small vessel stenoses.

Acknowledgments. We acknowledge Professor Michael Azrin and Amanda Stephens-Lloyd for their comments on the manuscript, and Mr T. Spyt for allowing us to approach his patients for arterial specimens.

References

1. Morice MC, Serruys PW, Sousa JE, et al. Randomized study with the sirolimus: A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. *N Engl J Med* 2002;346:1773–1780.
2. Luscher TF. Endothelium in the control of vascular tone and growth: Role of local mediators and mechanical forces. *Blood Pressure* 1994;(Suppl 1):18–22.
3. Castellot JJJ, Addonizio ML, Rosenberg R, Karnovsky MJ. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. *J Cell Biol* 1981;90:372–379.
4. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 1989;83:1774–1777.
5. van Beusekom HM, Whelan DM, Hofma SH, et al. Long-term endothelial dysfunction is more pronounced after stenting than after balloon angioplasty in porcine coronary arteries. *J Am Coll Cardiol* 1998;32:1109–1117.
6. Muramatsu T, Tsukahara R, Ho M, et al. Clinical outcome of stent implantation in small coronary arteries using different types of coronary stents. *J Invas Cardiol* 2001;13:634–639.
7. Barone GW, Conerly JM, Farley PC, et al. Endothelial injury and vascular dysfunction associated with the Fogarty balloon catheter. *J Vasc Surg* 1989;9:422–425.
8. Grewe PH, Deneke T, Machraoui A, et al. Acute and chronic tissue response to coronary stent implantation: Pathologic findings in human specimen. *J Am Coll Cardiol* 2000;35:157–163.
9. Asahara T, Bauters C, Pastore C, et al. Local delivery of vascular endothelial growth factor accelerates re-endothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation* 1995;91:2793–2801.
10. Van Belle E, Tio FO, Couffinhal T, et al. Stent endothelialization. Time course, impact of local catheter delivery, feasibility of recombinant protein administration, and response to cytokine expedition. *Circulation* 1997;95:438–448.
11. Van Belle E, Tio FO, Chen D, et al. Passivation of metallic stents after arterial gene transfer of phVEGF165 inhibits thrombus formation and intimal thickening. *J Am Coll Cardiol* 1997;29:1371–1379.
12. Barone GW, Farley PC, Conerly JM, et al. Morphological and functional techniques for assessing endothelial integrity: The use of Evans blue dye, silver stains, and endothelial derived relaxing factor. *J Cardiac Surg* 1989;4:140–148.
13. Mason RA, Hui JC, Campbell R, Giron F. The effects of endothelial injury on smooth muscle cell proliferation. *J Vasc Surg* 1987;5:389–392.
14. Malik N, Gunn J, Holt CM, et al. Intravascular stents: A new technique for tissue processing for histology, immunohistochemistry, and transmission electron microscopy. *Heart* 1998;80:509–516.
15. Schwartz RS, Huber KC, Murphy JG, et al. Restenosis and the proportional neointimal response to coronary artery injury: Results in a porcine model. *J Am Coll Cardiol* 1992;19:267–274.
16. Lincoff AM, Weinberger J. Local drug delivery and endovascular radiation. In: Topol EJ (ed). *Comprehensive Cardiovascular Medicine.* 1998: pp. 2433–2451.
17. Swanson N, Hogrefe K, Javed Q, Gershlick AH. VEGF-eluting coronary stents stimulate endothelial growth in vitro (Abstr). *J Submicroscopic Cytology Pathology* 2000;32:419–B087.
18. Henry TD. VIVA: Vascular endothelial growth factor in ischemia for vascular angiogenesis (Abstr). *Clin Cardiol* 1999;22:369.
19. Laitinen M, Hartikainen J, Hiltunen MO, et al. Catheter-mediated vascular endothelial growth factor gene transfer to human coronary arteries after angioplasty. *Human Gene Therapy* 2000;11:263–270.
20. van der Giessen W, Serruys PW, Visser WJ. Endothelialization of intravascular stents. *J Invest Surg* 1988;1:109–120.
21. Harada M, Numaguchi Y, Osanai H, et al. Catheter-based prostacyclin synthase gene transfer prevents in-stent restenosis in atheromatous rabbits: A potential strategy for therapeutic angiogenesis via vascular endothelial growth factor (Abstr). *Eur Heart J* 2000;21:394–394.
22. Yasuda S, Noguchi T, Gohda M, et al. Single low-dose administration of human recombinant hepatocyte growth factor attenuates intimal hyperplasia in a balloon-injured rabbit iliac artery model. *Circulation* 2000;101:2546–2553.
23. Krasinski K, Widmer MB, Yang J, Losordo DW. In vivo blockade of TNF using a soluble receptor accelerates re-endothelialization after arterial balloon injury (Abstr). *Circulation* 1997;(Suppl):1603.
24. Krasinski K, Spyridopoulos I, Asahara T, et al. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation* 1997;95:1768–1772.
25. Chen D, Asahara T, Krasinski K, et al. Antibody blockade of thrombospondin accelerates re-endothelialization and reduces neointima formation in balloon-injured rat carotid artery (Abstr). *Circulation* 1999;100:849–854.
26. Zachary I, Mathur A, Yla-Herttuala S, Martin J. Vascular protection: A novel nonangiogenic cardiovascular role for vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* 2000;20:1512–1520.