Doctoral Thesis for the degree of Doctor of Philosophy, Faculty of Medicine

ON CELLULAR SOURCES FOR INTIMAL HYPERPLASIA AFTER VASCULAR INTERVENTIONS

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Paper I was reprinted from Eur J Vasc Endovasc Surg. 2005 Jul;30(1):63-70. Healing of PTFE grafts in a pig model recruit neointimalcells from different sources and do not endothelialize. S. Mellander, P. Fogelstrand, K. Enocson, B. R. Johansson E. Mattsson
Paper III is accepted in Eur J Vasc Endovasc Surg (April 2007) and not yet published. Photodynamic therapy reduces intimal hyperplasia in prosthetic vascular bypass grafts in a pig model. J. Heckenkamp, S. Mellander, P. Fogelstrand, S.Breuer, J. Brunkwall, E. Mattsson.
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ABSTRACT

Vascular interventions for the treatment of symptomatic atherosclerosis fail in up to 40% of the cases during the first year. One important reason is the development of a narrowing process known as intimal hyperplasia (IH). The cells forming IH resemble smooth muscle cells (SMCs) from the media of the arterial wall. Therefore the media has generally been regarded as the cellular origin for IH. However, there are reports indicating that other cellular sources might be involved.

The aim of this thesis was to investigate which cellular sources participate in the development of intimal hyperplasia after bypass surgery and balloon injury. Furthermore, we wanted to investigate, if the inhibition of one cellular source could reduce initimal hyperplasia. Studies were made in pig and rabbit. Specific aims were: 1/ To evaluate the blood, the adjacent artery, the media, the adventitia, and the surrounding tissue as cellular sources to intimal hyperplasia 2/ To evaluate the contribution of blood-borne mononuclear cells to IH 3/ To evaluate, if depletion of the cells in the media reduces intimal hyperplasia after vascular interventions.

We found that the adjacent artery at the anastomoses and the surrounding tissue contributed cells in a bypass model in pig. Blood-borne mononuclear cells were labeled ex vivo and retransfused after bypass implantation and balloon injury in pig. These cells were later found in the IH. Some of them co-expressed markers for smooth muscle cells suggesting a trandifferation from a blood-borne mononuclear to a tissue forming cell. In a ballon injury model in rabbit we found that the media with its SMCs was the main cellular source, and that adventitial cells did not contribute to the IH.

After depletion of the medial SMCs by a more severe balloon trauma in rabbit and by photodynamic therapy in the bypass model in pig we found less IH compared with controls, suggesting the media and its cells to be an important source after both interventions.

In conclusion, the results presented in this thesis show that cells from the media, the surrounding tissue, the adjacent artery, and blood-borne mononuclear cells can contribute to IH. By depletion of the cells in the media, intimal hyperplasia following both bypass surgery and balloon injury is reduced.

Key words: Intimal hyperplasia, vascular intervention, smooth muscle cell, blood-borne mononuclear cell, balloon injury, prosthetic graft, pig, rabbit

LIST OF PUBLICATIONS

This thesis is based on the following individual papers, which will be referred to in the text by the Roman numerals I-IV.

I Healing of PTFE grafts in a pig model recruit neointimal cells from different sources and do not endothelialize. Mellander S, Fogelstrand P, Enocson K, Johansson BR, Mattsson E. Eur J Vasc Endovasc Surg. 2005 Jul; 30(1):63-70.

II Blood-borne mononuclear cells contribute to intimal hyperplasia after vascular interventions in pig. Mellander S, Fogelstrand P, Åström-Olsson K, Mattsson E. Manuscript.

III Photodynamic therapy reduces intimal hyperplasia in prosthetic vascular bypass grafts in a pig model. J Heckenkamp, S Mellander, P Fogelstrand, S Breuer, J Brunkwall, E Mattsson. Accepted for publication in Eur J Vasc Endovasc Surg (April 2007).

IV Reduced neointima in rabbit following a more severe balloon-injury. Per Fogelstrand, Stefan Mellander, Erney Mattsson. Submitted.

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ABBREVIATIONS

AP	Alkaline phosphatase
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2-deoxyuridine
CCA	Common carotid artery
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
EEL	External elastic lamina
IEL	Internal elastic lamina
IGF-1	Insulin-like growth factor-1
IH	Intimal hyperplasia
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
LDL	Low density lipoprotein
MMP	Matrix metalloproteinase
NO	Nitric oxide
PCI	Percutaneous coronary intervention
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDT	Photodynamic therapy
PTA	Percutaneous transluminal angioplasty
PTFE	Polytetrafluoroethylene
SEM	Scanning electron microscopy
SM	Smooth muscle
SMCs	Smooth muscle cells
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-β

INTRODUCTION

Progressive atherosclerosis with subsequent stenosis and eventually occlusion of blood vessels is a major cause of morbidity and mortality worldwide. Known risk factors are smoking, diabetes, hypertension, and hyperlipidemia. The main interventional treatments consist of balloon angioplasty, with or without stenting, or bypass surgery. Unfortunately, up to 40% of the interventions will fail within the first year. Important causes for the failures are restenosis and graft-stenosis.

The vessel wall

The blood vessel wall consists of three layers: the intima, the media, and the adventitia. The intimal layer is the innermost and consists of a monolayer of endothelial cells. The endothelium has several functions. It produces a number of vasodilator and vasoconstrictor substances, which regulate vasomotor tone. It is involved in the recruitment and activity of inflammatory cells, and it regulates thrombosis and fibrinolysis.[1-3] The endothelial cells are attached to a layer of connective tissue known as the internal elastic lamina (IEL). The IEL is composed of elastin and the subendothelial surface harbors collagen, laminin, and heparin sulphate. The media, the middle layer, is composed of concentrically arranged smooth muscle cells (SMCs) surrounded by their own basement membrane and an extracellular matrix (ECM) containing elastic fibers, collagen fibers, and proteoglycans. It is the muscular and elastic components that are responsible for the vascular tone. The external elastic lamina separates the media from the adventitia, the outer layer. The adventitia is a collagen-rich connective tissue layer, containing fibroblasts, nerves, lymph vessels, and blood capillaries known as vasa vasorum, which supply the adventitia and the outer part of the media with nutrients and oxygen. The nutrition of the inner part of the media depends on diffusion from the lumen.[4] There is no anatomical border separating the adventitia from the perivascular tissue.

Atherosclerosis

Atherosclerosis is an inflammatory and fibroproliferative disease localized in medium-sized and large arteries. The lesions tend to develop at branch points and in major arterial curvatures.[5] These areas show increased permeability to macromolecules such as low-density lipoproteins (LDL).[6] It is assumed that an endothelial dysfunction induced by factors such as elevated and modified low-density lipoproteins, free radicals, infectious microorganisms, shear stress, hypertension, and toxins after smoking progress to an inflammatory response in the vessel wall. Circulating monocytes adhere and penetrate the vessel wall and become macrophages. Oxidized LDL accumulates in the intima and is taken up

by the macrophages, creating lipid-rich so called foam-cells. These cells form a lipid core covered by a fibrous cap mainly created by migrating and proliferating SMCs. The lipid core together with the fibrous cap constitutes a focal intimal thickening known as a plaque. In advanced disease a narrowing of the arterial lumen develops with a high risk for the formation of superimposed thrombosis leading to an acute ischemia of the end organ.[7, 8]

Treatment options of atherosclerotic lesions

Angioplasty

Angioplasty also named PTA (percutaneous transluminal angioplasty) or PCI (percutaneous coronary intervention) is a commonly used treatment option for atherosclerotic stenoses or even occlusions of the arteries. The technique was first described in 1964 by Charles Dotter.[9] The method was modified in the seventies and eighties and reached a widespread use. A balloon catheter is inserted into the arterial system, usually through the common femoral artery, and guided forward under fluoroscopic vision to the lesion in the artery. The balloon is inflated at high pressure and causes rupture and dissection of the plaque and overstretching of the vessel wall with an increased lumen as the result.[10] Because of the tendency of early elastic recoil and the later negative remodeling of the vessel, balloon- or self-expandable stents (metal net) are often used in combination with the PTA/PCI procedure.

Bypass grafting

Bypass grafting is a surgical option for treatment of ischemia caused by the atherosclerotic lesions. Blood is shunted through a graft from one open artery to another distal to the occluded segment. The saphenous vein is the most commonly used conduit for bypass grafting in peripheral surgery. For patients lacking a suitable vein, artificial grafts can be an alternative. The main prosthetic materials developed for bypass grafting are polyethylene terephthalate (Dacron) and expanded polytetrafluoroethylene (ePTFE). Which artificial graft used is decided by the surgeon's personal preferences, since there are no scientific proof of superiority of any specific material.[11, 12] Small diameter prosthetic grafts (<6 mm) are especially prone to occlude and are therefore rarely used clinically.
In the lower extremities the long-term patency rates of prosthetic grafts above the knee are lower compared to vein grafts.[13, 14] Bypasses below the knee with synthetic grafts have poor patency rates so these reconstructions are usually avoided.[15] Promising results have however been shown in ePTFE grafts seeded with endothelial cells [16] and recently also with heparin-bonded ePTFE grafts.[17]
In coronary bypass surgery the internal mammary artery is the graft of choice due to excellent patency. However, the patient often requires more than one bypass and the complementary grafts commonly used are the saphenous vein or the radial artery, although with lower patency rates.

Complications to interventions

All forms of interventions cause injury to the vessel wall. Besides the risk for early thrombosis or technical failures, restenosis or graft-stenosis will affect the long-term outcome of the intervention.

- *After angioplasty*

Angioplasty is as mentioned a well established method to treat symptomatic atherosclerosis. In spite of a primary success rate of 90-95%, late restenosis occurs in 30-45% of patients within 6 months of the procedure.[18-20] Restenosis is a re-narrowing of the treated vessel and can be divided into a number of interrelated processes: elastic recoil, inflammation, thrombus formation, intimal hyperplasia (IH), and negative remodeling. Following balloon angioplasty 60-70% of the late lumen loss is due to constrictive remodeling of the vessel wall and 30-40% to a thickening of the intimal layer, called intimal hyperplasia.[21] Adventitial fibrosis has been suggested as a major factor causing negative remodeling.[22, 23] Placing a stent (metal net) intraluminally during the PTA-procedure prevents early recoil and later negative remodelling but does not inhibit intimal hyperplasia. The formation of intimal hyperplasia is unfortunately increased with stents compared to balloon angioplasty alone. Despite increased intimal hyperplasia, stenting has lowered the rate of restenosis to approximately 20% [24, 25] and today the majority of balloon dilatations are combined with stent implantation.

- *After bypass surgery*

Early graft occlusions during the first month are commonly caused by thrombosis. Whether it is a vein or a synthetic graft, the luminal surface has thrombogenic properties. Even handling of the vein without direct trauma causes injury to the intima and the media.[26, 27] Other causes of early occlusions are poor run-off in the recipient artery, retained vein valves or technical problems with the surgical procedure.[28] Following bypass surgery, approximately 30% of implanted grafts will develop stenosis within a month to a year due to intimal hyperplasia (IH). Bypass grafts in humans develop IH preferentially at the distal but also at the proximal anastomosis.[29] Surgical trauma caused by stiches, vessel clamps, and arteriotomy might induce IH. Moreover, the bypass is usually performed with an end to side connection, thus creating abnormal hemodynamic forces. Forces such as flow (shear stress), stretch of the vessel wall (tensile stress) and turbulence have shown to be important factors for the development of IH.[30-32] The combination of hemodynamic forces and endothelial/medial cell damage at the anastomotic site might in part explain why the anastomoses are prone to develop IH.[33] In order to detect and treat graft-stenosis before graft occlusion, duplex ultrasound surveillance programs have shown to be effective for patients treated for critical limb ischemia, leading to a reduction of major

amputations and consequently to a reduction in costs.[34] Late occlusions after a year are mostly due to progressive atherosclerosis resulting in poorer run-off and subsequently in graft thrombosis. Another cause of occlusion can be progressive atherosclerosis in the bypass graft, described especially in vein grafts used in coronary surgery.[35]

Intimal hyperplasia - A reparative process

Intimal hyperplasia (IH) is a reparative response to vascular injury. It can occur in all arteries as a response to a wide variety of injuries, including radiation, hemodynamic forces, mechanical injuries such as ballon dilatation or placement of a suture, and even after manipulation of the perivascular tissue without damage to the vessel wall.[36] Intimal hyperplasia occurs physiologically in closure of the ductus arteriosus after birth[37] and pathologically without intervention in pulmonary hypertension.[38]
IH is an entity dependent on an increase of the number of cells and deposition of extracellular matrix (ECM) in the intimal layer of the vessel. At a mature state it consists of 20 % cells and 80 % ECM. [39]

Mechanisms of intimal hyperplasia

The formation of intimal hyperplasia involves the activation and phenotypic modulation of medial smooth muscle cells (SMCs). These cells proliferate and subsequently migrate into the subendothelial space. Numerous extracellular stimuli and intracellular signal transduction pathways are known to be involved. The major extracellular factors include growth factors, cytokines, extracellular matrix proteins and interacting cell-surface receptors. They may be derived from adhering platelets, leukocytes, plasma, injured SMCs or endothelial cells. The most studied model for IH is the carotid balloon injury in rat.[40]

After balloon injury
The balloon injury results in a complete destruction of the endothelium as well as an extensive injury to smooth muscle cells in the media.[41] Platelets adhere to exposed collagen fibers in the vessel wall followed by aggregation and degranulation with the release of e.g. platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-ß), ADP, P-Selectin, and fibrinogen.[42, 43] Leukocytes are recruited early to the injury site. Initially, leukocytes attach loosely and roll along adhered platelets.[44] The activated leukocytes subsequently migrate and invade the vessel wall.[44, 45] An early observed adventitial inflammation probably also plays a role in IH development.[46, 47] Inflammatory cells release growth factors, cytokines, oxygen-derived free radicals, and lysosomal proteinases. These factors directly effect proliferation and migration of smooth muscle cells and also modulate endothelial products such as

inactivation of nitric oxide (NO).[48, 49] In addition, activated SMCs themselves produce a number of substances which endogenously can promote proliferation and migration.[50]

Shortly after injury the contractile SMCs in the media undergo a phenotype modulation into a synthetic state and start to proliferate within the media.[41] The expression of matrix metalloproteinases (MMPs) and the degradation of laminin in the basement membrane together with the deposition of fibronectin have been suggested to be important for the activation of SMCs.[51, 52] Functionally, the cells enter the cell cycle and acquire the capacity to respond to mitogens.[53, 54] Basic fibroblast growth factor (bFGF) has shown to be an important mitogen in the early phase of SMC proliferation. The release of bFGF mainly comes from severely injured smooth muscle cells.[55, 56] Systemic injection of a neutralizing antibody against bFGF prior to balloon catheterization of the rat carotid significantly decreases the induced SMC proliferation.[55] Studies have further shown that exogenously added bFGF increases the proliferation in injured vessels but does not affect the proliferation in uninjured vessels, indicating the injury to be of importance for the response to bFGF.[55, 57]

After the phenotypic modulation, SMCs migrate into the intima. The cells cross the IEL and can be detected in the intima 4-7 days later. The cell migration seems to be stimulated by growth factors such as PDGF. The effect of PDGF has been supported by observations following the infusion of PDGF in the rat model for balloon angioplasty[58], and has further been underlined in a separate study in which thrombocytopenia was induced.[59] With the infusion of PDGF, SMC migration increased while the proliferation was unaffected.[58]
Following their migration, SMCs in the neointima continue to proliferate for another 2-4 weeks.[40] Different growth factors such as PDGF, angiotensin II, and insulin-like growth factor (IGF-1) are believed to be secreted endogenously from the SMCs to stimulate the continued proliferation.[36] The intimal thickening increases further after this initial stage, mainly because of the deposition of ECM-proteins until a steady state has been reached after three months.[39]

The re-endothelialization after injury seems to be of importance in different animal models. Studies in rodent models after angioplasty have shown that proliferation of SMCs is inhibited under regrowing endothelium.[41] Re-endothelialization of the injured site causes cessation of proliferation, possibly in part because of renewed activity of endothelial NO, prostaglandins, and heparin, which normally exert an antiproliferative effect.[60, 61]

After bypass grafting in general

It is generally thought that the sequence of events in the development of intimal hyperplasia in vein grafts and prosthetic graft are similar to that in the balloon injury model, but the events inducing the start of the sequence and the speed of development might be different.[36, 62-64]

- *vein bypass grafting*

Injury induced by surgery and the change to arterial hemodynamics cause an early loss of endothelium and SMCs in the vein.[27, 39] Loss of the endothelial monolayer results in the accumulation of fibrin on the luminal surface and the adherence of platelets. Leukocytes are early recruited to the injured vein.[63] The handling of the saphenous vein during its harvest results in impairment of the release of NO which is an important inhibitor of SMC proliferation.[65, 66] Hemodynamic forces such as tensile stress (circumferential stretch) and shear stress (shoving force) are altered. The changes of hemodynamics in the vein graft induce the formation of intimal hyperplasia.[67, 68] The influence of hemodynamic forces is demonstrated by the fact that the IH in vein grafts can regress when the graft is placed back into the venous circulation.[69]

- *prosthetic bypass grafting*

The mechanisms of the development of IH in prosthetic grafts are thought to be similar to vein grafts. The postulated reasons for IH in prosthetic grafts are mainly mechanical. The difference in compliance between the native vessel and the graft, turbulence and oscillatory changes in direction of flow, and luminal diameter differences at the anastomosis are factors of importance.[29, 33] The anastomotic IH generally occurs at the heel, the toe, and the floor of the recipient artery (Fig 1). Interposing an autologous vein cuff makes the compliance mismatch more gradual and redistributes IH away from the most critical areas of the anastomosis, leading to some improvement in patency but keeping the overall extent of IH unchanged. [70, 71]

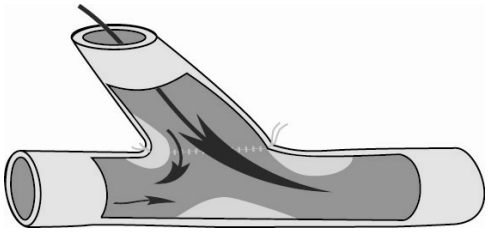

Fig 1. *Intimal hyperplasia in a distal anastomosis. IH preferentially occurs at the heel, the toe, and the recipient artery floor. IH is represented by the brighter areas in the lumen of the vessel.*

The origin of cells in intimal hyperplasia

It has generally been thought that the media with its SMCs is the only cellular source for IH.[49, 72, 73] This has lately been questioned and several studies have shown that other cell types such as adventitial/perivascular cells and blood-borne cells can contribute to the intimal thickening.

The Media

The media consists mostly of SMCs which are contractile in order to maintain the vascular tone. These SMCs have a high content of proteins associated with the contractile apparatus such as smooth muscle (SM) α-actin, SM myosin heavy chain (SM MHC), SM-tropomyosin, calponin, caldesmon, vinculin, desmin, and smoothelin.[74] In response to injury or when placed in cell culture, contractile SMCs can dedifferentiate towards a synthetic phenotype.[75, 76] The synthetic phenotype has a low content of myofilaments and a high amount of synthetic organelles and responds rapidly to proliferative and migratory stimuli.[77]These cells can later be found in the intimal hyperplasi after injury.[49, 55] However, the media contains not only contractile SMCs but also other cells. These cells are called non-SMCs by Holifield et al. [78] They have been found both in the media and in the adventitia and could not be morphologically distinguished from each other in cell culture. Therefore these cells presumably are fibroblasts. The cells have been found as strings in the media of the saphenous vein. Following implantation into the arterial circulation, the proliferation starts among these cells.[79, 80] These observations, combined with the fact that fibroblasts can become SM α-actin positive myofibroblasts[81, 82], suggest that SM α-actin positive cells in the neointima can also be derived from fibroblasts residing in the media or the adventitia.

The Adventitia

The adventitia harbors mainly fibroblast cells. It has been shown to be the first vessel wall layer to respond to ballon injuryby an upregulation of cellular adhesion molecules and chemochines in the capillary bed.[46] Within 24 hours of injury there is a massive invasion of leukocytes into the adventitia and fibroblasts begin to proliferate.[81, 83] This occurs before the early proliferation in the media. Following vascular injury, adventitial fibroblasts have been shown to become myofibroblasts that express SM α-actin.[81, 82] Transforming growth factor β1 seems to play a key role in the transformation of fibroblasts into myofibroblasts.[84, 85] It has been shown that fibroblasts can migrate through the media and contribute to the neointimal mass.[81, 86] This indicates that the adventitia is another cellular source of SM α-actin positive cells found in the intimal hyperplasia.

Blood-borne cells

Blood-borne cells have in different studies been implied to participate in the neointimal formation. Monocytes may be particularly important. As earlier mentioned, they seem to have an important role in atherogenesis.[7] Several experimental studies show that white blood cells, especially monocytes, also play a central role in the restenosis process after balloon angioplasty and stent implantation.[87-90] Replicating monocytes and macrophages have also been found in vein-bypass stenoses months after surgery.[91, 92] It has recently been shown that human hematopoietic mononuclear cells differentiate into SM α-actin positive cells when they are cultured in PDGF BB-enriched medium.[93] Cells double-staining for macrophage markers and α-actin have been found in neointimal lesions after thermal injury in a pig model. Five percent of the neointimal cells co-expressed SM α-actin and a macrophage antigen after one month.[94]

The advance of stem-cell science has revealed that the bone marrow contains multipotent adult stem cells.[95, 96] Both mesenchymal and hematopoietic stem cells from the bone marrow have the ability to differentiate into SM α-actin positive myofibroblasts in vitro.[97, 98] It has also been shown that bone marrow cells contribute to neointimal lesions in vascular injury models in vivo.[99-101] This indicates that SM α-actin positive cells found in IH can be derived from the blood.

The Intima

The intima in an uninjured vessel consists of a monolayer of endothelial cells. After vascular injury it is the site for the formation of intimal hyperplasia. There are observations supporting that endothelial cells themselves are capable of giving rise to SMCs. For example, when bovine aortic endothelial cells are exposed to TGF-β in vitro, they start to express SM α-actin.[102] Since platelets and other cells produce TGF-β among with other mitogens, there is a possibility that endothelial cells can transform into SM α-actin positive cells and participate in the healing process. Since interventions usually cause a local loss of endothelial cells the intima is less probable as an initial cellular source for IH.

Prevention of Intimal Hyperplasia after Angioplasty and Bypass Surgery

Different drugs have been evaluated in the inhibition of IH. Most of them have not shown any effect but a few indicate a potential of favourable outcome. Orally given cilostazol has been effective to prevent thrombotic occlusions and to reduce IH after arterial stenting of iliac arteries in dogs.[103] Cilostazol has also been evaluated in a clinical study. To determine the antiproliferative effect of this agent, cilostazol or aspirin was randomly given for 6 months to 36 patients treated with stent implantation. At follow-up, minimal luminal diameters were significantly greater in the cilostazol group than in the aspirin group ($P < 0.001$).

These results suggest that cilostazol may reduce the incidence of restenosis after stent implantation.[104] Cilostazol-eluting stents have been compared to bare-metal stents in the coronary circulation in a porcine model with favourable results. The demonstrated suppression of IH and reduced late lumen loss indicate that the effects seen with systemic delivery may also be expected with local delivery.[105]

Trapidil (triazolopyrimidine) is an antiplatelet agent that acts as a phosphodiesterase inhibitor and as a competitive inhibitor of a PDGF receptor. Trapidil has been shown to attenuate IH in rat and hamster models of balloon arterial injury and to inhibit restenosis after percutaneous coronary intervention in several small clinical trials. The effect of Trapidil in attenuating IH may in part be attributed to its effects on macrophage accumulation.[106]

In experimental animal models treatment with statins reduced IH [107, 108] but so far no effect is shown in humans.[109] It has been well demonstrated that atherosclerosis develops in saphenous vein bypass grafts after coronary artery bypass surgery. Three trials (CLAS, post-CABG, and CARE) have shown a delayed progression of atherosclerosis in vein grafts and/or a reduction of cardiac deaths, nonfatal MI, and the need for revascularization after lowering of LDL-cholesterol. The recommended target of LDL cholesterol level can safely be reached with diet and monotherapy using one of the statins.[110] So even if statins have not demonstrated any specific effect against IH, their use seems to be beneficial in connection to coronary bypass surgery.

Many locally directed techniques to inhibit IH have been evaluated. Most of the studies have been performed in arterial ballon injurymodels. Since it is thought that the mechanisms for IH are similar in bypass grafts they probably, in part, can be translated between the different interventions.

Gene therapy is one approach with initial promising results. For example, when an antisense oligodeoxynucleotide, antisense c-myc, was applied in a pluronic gel to the adventitia in a rat balloon injury model it demonstrated reduced intimal hyperplasia after 14 days.[111] Cultured human saphenous veins have been transfected successfully with an adenoviral vector encoding bovine endothelial nitric oxide synthase, yielding a marked increase in venous endothelial NO production.[112] Moreover, retrovirus-mediated gene transfer in a rat balloon model resulting in an over-expression of endothelium-specific constitutive NO synthase (ecNOS) inhibited neointimal formation by 37%. [113]

Cryoplasty is a technique involving the use of cryotherapy to locally inhibit intimal hyperplasia. The cryoplasty system simultaneously dilates and cools the plaque and vessel wall by inflating the balloon with cold nitrous oxide.

Cryotherapy induces an acute phase change that triggers apoptosis in the smooth muscle cells.[114] In a clinical study, evaluating cryoplasty for the treatment of femoropopliteal arterial disease, a patency rate of 75% could be reported at a three-year follow-up.[115]

Photodynamic therapy (PDT) is a technique in which laser light activates photosensitizer dyes to produce local free radicals resulting in an eradication of cells in the vascular wall. These reactive species exert their cytotoxic effects by damaging cellular organelles and membranes inducing apoptosis.[116] PDT has also been found to affect extracellular matrix molecules.[117] PDT is a clinically well documented method in the treatment of various neoplasms. It has been used for tumours in the bladder, lung, and oesophagus.[118-120] PDT has also gained interest as a local vascular therapeutical approach to inhibit IH.[121-123] Animal studies have shown promising results to inhibit restenosis after interventions.[123, 124] Clinical safety trials in humans have been performed in which balloon angioplasty has been combined with adjuvant photodynamic therapy to treat peripheral arterial insufficiency.[125, 126] No adverse effects were reported with PDT in these studies.

Brachytherapy has been used to prevent in-stent restenosis in humans with initial promising results, but there are concerns about the late effects.[127, 128] There is an increased risk for thrombosis with brachytherapy. Furthermore the decay of the radiation at the edges of the treated area combined with vessel injury may even locally stimulate neointimal formation.[129]

There has been a focus on drug-eluting stents to reduce restenosis following balloon angioplasty. At the moment, more than 85% of all coronary interventions in the United States are performed with drug-eluting stents.[130] Two pivotal trials have demonstrated striking reductions in angiographic restenosis rates with sirolimus- and paclitaxel-eluting stents.[131, 132] Long-term results are not yet available, and there are indications that the risk for delayed thrombosis has been underestimated.[133, 134] Recent observations have even demonstrated an increased rate of death with the use of drug-eluting stents in the coronary circulation.[135]

In analogy with drug-eluting stents, it has been shown that bypass surgery with prosthetic grafts coated with rapamycin, reduces intimal hyperplasia in pigs.[136] Furthermore, heparin coated grafts have shown to reduce IH in animal studies [137] and promising patency rates have been demonstrated in humans.[17]

Another effort to overcome IH in bypass surgery has been to place an external stent around vein grafts. In a porcine model, placement of a non-restrictive, porous, external Dacron stent around a vein bypass markedly inhibited medial

and intimal thickening.[138, 139] Since the neointimal formation in vein grafts mainly occurs within the first months after implantation, the effect of biodegradable sheaths has also been studied in the short and long-term. These studies demonstrated similar results as with non-degradable stents.[140, 141] The "stented" vein grafts are characterised by a "neoadventitia" in the space between the graft and the stent. The "neoadventitia" has an abundant microvasculature that extends into the media of the veingraft. Microvessels possess the capacity to rapidly regenerate and re-establish an integrated microcirculation. In turn, this will promote oxygenation of the graft and obviate hypoxia. Hypoxia may play a role in mediating vein graft disease. Prolonged hypoxia upregulates the expression of a huge number of proteins including some that promote vein graft disease.[142, 143] Disruption of the vasa vasorum is known to be associated with vascular disease.[144-146] Since the vein graft thickens rapidly, the graft is probably subject to an increase in oxygen demand. Hypoxia as the mechanism by which external stents inhibit vein graft stenosis is plausible but unfortunately not proven. These results taken together are promising and intriguing but the positive effect is not yet shown in humans.

Although several attempts have been made, there is so far no generally accepted treatment modality to clinically reduce the occurrence of restenosis or graft-stenosis. More has to be learned about pathophysiological mechanisms and the participating cellular sources.

AIMS OF THE THESIS

The general aims of the thesis were to investigate which cellular sources participate in the development of intimal hyperplasia after vascular interventions, and if the inhibition of one cellular source could reduce initimal hyperplasia.

The specific aims were:

- to evaluate the adjacent artery, the media, the surronding tissue, and the blood as cellular sources for intimal hyperplasia after prosthetic bypass surgery in pig, and to analyze the contribution of cells from the media, and the adventitia after balloon injury in rabbit.

- to evaluate the contribution of blood-borne mononuclear cells to the formation of intimal hyperplasia after bypass surgery and balloon injury in pig.

- to investigate, if the depletion of cells in the media reduces intimal hyperplasia after bypass surgery with artificial grafts in pig, and balloon injury in rabbit

MATERIALS, METHODS, AND COMMENTS

Detailed descriptions are given in the papers I-IV.
It should be emphasized that the animals used in the studies were young and had no atherosclerotic manifestations. Studies in vascular biology have shown diverging results depending on the species used for the observations. It is generally thought that big animals reflect human biology better than small animals or in vitro studies. The pig was the chosen species in paper I-III. The pig is established as an experimental animal, and their biology is thought to be similar to human biology. To explore the potential diversity between species a rabbit model was also used in the present thesis (paper IV).

Implantation of artificial vascular grafts in pig (Paper I, II, III)
The grafts were implanted through a midline longitudinal incision in the lower abdomen. The iliac arteries were exposed, and PTFE-grafts of 4-5 cm length were implanted bilaterally end-to-side from the common to the external iliac artery. The native arteries were ligated between the anastomoses to shunt the blood through the grafts (Fig 2).

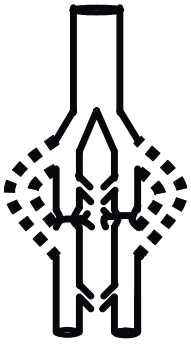

Fig 2. A schematic picture of the pig model. Dotted lines represent the PTFE grafts bilaterally implanted from the proximal to the distal iliac arteries.

Clinically used PTFE-grafts in humans usually have an internodal distance of 20-30 μm (distance between the graft fibers) and they are wrapped with a dense PTFE-layer to avoid aneurysmal development. The wrapping and the short internodal distance inhibit cell migration through the graft fabrics.[147] Thereby, IH in human PTFE-grafts is almost exclusively formed in the anastomoses. In paper I we used unwrapped PTFE-grafts with a wider internodal distance (60μm) to facilitate cellular ingrowth and neointimal formation in the entire graft. This enabled us to study the participation of cells from the surrounding graft tissue and the blood in the mid-graft sections away from the hemodynamic forces at the anastomoses in a time-course model. In paper II, in which the purpose was to study the importance of blood-borne cells, we used a wrapped

graft with an internodal distance of 60μm to inhibit cell migration from the surrounding tissue through the graft. This graft still allowed luminal ingrowth of circulating cells. In paper III we used a wrapped graft with an internodal distance of 30μm, which is identical to the grafts used in humans. The reason was that the PDT-study was performed as a preparation for a future clinical study. From a biological point of view this graft inhibited growth from the surrounding tissue and perhaps reduced ingrowth of circulating cells, leaving the adjacent artery as the major cellular source for the formation of IH. The aim of this study was to see if a reduction of the contribution from the adjacent artery could decrease IH in artificial grafts. In paper II only one graft was implanted in each animal. The reason was to reduce the total time for the interventions since the additional procedure of a coronary ballon injury was included.

Ballon injury to the pig coronary artery (Paper II)

Under fluoroscopic guidance (OEC 9800 Cardiac), an arterial injury was inflicted to the left anterior descending coronary artery (LAD) using an angioplasty catheter. It was inflated three times (6 to 10 atmospheres) for a period of 15 seconds and deflated with 20 seconds intervals between the inflations, thus inducing an over-stretch injury to the vessel wall.[148] The oversizing was 20-30% of the vessel diameter. The occlusion and reperfusion of the LAD were angiographically verified with injections of a contrast agent. The vasculature was accessed through the right common carotid artery. At retrieval of the specimens at four weeks, the injured arteries were easily located by identification of the first marginal branch of the LAD. Rupture of the external elastic lamina was not observed in this study, indicating a relatively mild injury. All but one specimen developed IH. Formed IH was up to 15 cell layers thick but not always evenly distributed around the circumference.

Ballon injury to the rabbit carotid artery (Paper IV)

Balloon-induced intimal hyperplasia in the rabbit common carotid artery (CCA) was studied in paper IV. A 3 French Fogarty embolectomy catheter was inserted and positioned in the right CCA. The balloon was inflated with 0.2 ml physiological saline and was withdrawn smoothly and rotated 90° through the entire CCA. Two withdrawals were considered as a minor injury and four withdrawals as a major injury. The rabbit was chosen because areas of total SMC loss in the media are easy to achieve. We also tested a rat injury model, but medial SMC loss was only seen in the inner half of the media and not in the outer half. Furthermore, the rabbit was chosen to evaluate the medial and adventitial contribution of cells in another species than pig.

PDT-treatment (Paper III)

The effect of PDT-treatment on the vessel wall was evaluated. Before graft-implantation, the iliac artery at the site for the future distal anastomosis was

pressurized at 180 mmHg for 5 minutes with the photosensitizer (methylene blue). The contralateral side was pressurized with saline at 180 mmHg and served as a control. The previously described technique of endovascular PDT application was modified for this model.[121] Homogenous intraluminal light emission was achieved by coupling the laser to a 600 μm optical fiber with a 3 cm diffuser tip, introduced into a transparent balloon angioplasty catheter. The balloon centered the laser diffuser tip in the lumen in order to get an even distribution of the laser energy to the arterial wall.

Since IH in artificial grafts mostly depends on local obstruction, the local cytotoxic effects of PDT seemed to be well suited for the prevention of this side-effect. A depletion of endothelial cells, medial smooth muscle cells, and adventitial fibroblasts can be observed in the targeted segment, when using the correct dosimetry. This occurs without thrombus formation, development of aneurysms, or inflammatory cell infiltration.[149] The reactive components generated by PDT exert their cytotoxic effects by damaging cellular organelles and membranes, but have also been found to affect extracellular matrix molecules, thereby inhibiting cellular migration through the vessel wall.[150] Based on a previous small animal study, [151] the photosensitizer Methylene Blue was tested in a pilot study at different concentrations and laser-light fluencies. Too low dosimetry showed remaining cells in the deeper layers of the media, and too high dosimetry showed an increased inflammatory response. It has been shown in small animals that a PDT overdose can lead to thrombosis of the vessel.[152] This demonstrates the narrow beneficial window with PDT and emphasizes the need for pre-treatment calculations. For the final study, we used the photosensitizer at a concentration of 330μg/ml and a light dose of 150 J/cm^2. Long-term results were not studied in this paper.

Apheresis (Paper II)

Apheresis (Greek: "to take away") is a process that involves removal of specific cells from the whole blood. In this study we used apheresis to separate mononuclear cells from the animals in order to label them ex vivo for later cell tracking. The apheresis apparatus is basically a centrifuge in which the components of the whole blood are separated according to size and weight. The mononuclear cells were withdrawn and collected in a plastic bag and the remaining blood components were continuously returned to the animal. Access to the circulation was gained from the left internal jugular vein using a double-lumen catheter. The cells were separated from other blood components by centrifugation at a spin velocity of 900-1000 rpm (Automated blood cell separator, COBE Spectra, version 7.0, Gambro). A total blood volume of approximately 5000 ml was processed during a time span of two hours in each animal. Thus, the circulating blood volume in the animals was processed several times. Microscopy showed that more than 95% of the collected cells were

mononuclear cells when counted in a Burkner chamber. Cell viability, as determined by trypan blue, demonstrated that over 95% of the cells were viable.

Freezing of cells (Paper II)

In equal amounts of cell suspension (90ml) and plasma (90ml), the collected cells were mixed together with a freeze medium (dimethyl sulfoxide, DMSO, 20ml) to a total volume of 200 ml. The cells were gradually frozen to -130°C for later labeling and re-transfusion to the animal.

Cell labeling (paper II, IV)

PKH 26 (Paper II and IV)

PKH 26 is a fluorescent dye that is incorporated into the lipid bilayer of the cell membrane, thereby marking all cells non-specifically.[153] The dye is not cytotoxic and it has previously been used for in vivo cell tracking.[154, 155] It is a cell marker with a long durability and PKH 26-labeled lymphocytes have been tracked for periods longer than 2 months in vivo.[156] In paper II we labelled the collected cells in a cellsuspension according to the manufacturer's manual (Sigma). After thawing and the PKH 26 labeling, the cell viability was 80-90% as determined by trypan blue staining. With fluorescence microscopy it was estimated that >90% of the cells were PKH 26 positive. In paper IV, both carotid arteries (injured and contralateral uninjured) were dissected free from its surrounding tissue immediately after a minor or a major balloon-injury. A 2 cm segment of each vessel was incubated in 1 mL of 0.1μmol/L PKH 26 for 20 minutes. The arteries were washed with saline solution, and the wound was closed. PKH 26 positive cells could later be detected in retrieved specimens with fluorescence microscopy at a wavelength of 551 nm (red fluorescence). In this study PKH 26 was used to see if labelled cells in the adventitia could later be found in the IH.

Pulse-labeling with BrdU (Paper IV)

BrdU (5-bromo 2-deoxyuridine) is a base analog of thymidine and becomes incorporated into the genome of DNA in replicating cells. BrdU incorporation can be used to trace cell migration from the adventitia to the neointima. Positive cells can be detected by ordinary immunohistochemistry. We found that during the first 24 hours after balloon injury, cells in the adventitia and perivascular tissue were proliferating, but not cells in the media. BrdU-injections at 12 and 24 hours after injury selectively labeled cells in the adventitia/perivascular tissue. The half-time of BrdU in the circulation is only 30 minutes. The rabbits were sacrificed at day 14, and tissue sections were stained for BrdU to investigate, if labeled cells were found in the neointima. We also injected BrdU at 48 and 72 hours after injury to label cells in the media together with cells in the adventitia/the perivascular tissue. The latter was done to show that cells from

the media migrated into the neointima and was used as a positive control for the method. As negative controls we used sections from balloon-injured arteries from rabbits not injected with BrdU. There are some disadvantages with this method. First, cell migration also involves non-replicating cells.[157] Thereby the BrdU method cannot identify all migrating adventitial cells involved in this process. Second, during labeling of proliferating cells in the adventitia and the perivascular tissue, occasional proliferating cells in the media will also become labeled. Third, because of the short half-time of BrdU, cells before, and 30 minutes after the injections will not be labeled although they might participate in the formation of IH. These were some of the reasons why we additionally used PKH 26 as a marker to track adventitial cells.

Morphological analyzes (Paper I, II, III, IV)

Paraffin embedded specimens were deparaffinzed and stained with hematoxylin and eosin for morphological analysis. Areas and intimal thickness were measured in light microscopy with Kontron Electronic image analysing system (KS 400 version 2.0, Carl Zeiss, Germany). In paper II no areas were measured but the thickness of IH was assessed by counting cellular layers.

Immunohistochemistry (Paper I, II, III, IV)

Proliferating cells were detected by help of immunohistochemistry using antibodies against PCNA (proliferating cell nuclear antigen). For morphological studies, formalin-fixated paraffin sections were used, whenever immunohistochemistry allowed, because frozen specimens do not provide an equally clear morphology. The paraffin sections were de-waxed and rehydrated in PBS. The use of formalin to fixate tissues can inhibit antibodies to reach their epitopes. Therefore the specimens were pre-treated 5 minutes with proteinase K or microwaved in a citrate-buffer for 10 minutes to unmask hidden epitopes. The specimens were then incubated 5 minutes with 3% H_2O_2 to block endogenous peroxidases in the tissue. To block unspecific electrostatic interactions between the tissue and the antibodies, the specimen were incubated 15 minutes with milk powder solution (5% in PBS). The specimens were then incubated with the primary antibody followed by incubation with the secondary antibody. The secondary antibodies were either conjugated with horseradish peroxidase (HRP) or with alkaline phosphatase (AP). Visualization was achieved with diaminobenzidine (DAB) as substrate for the HRP (gives a brown color) or fast red as substrate for alkaline phosphatase (gives a red color). For fluorescence microscopy we used secondary antibodies conjugated to fluorescein isothiocyanate (FITC, green fluorescence). DAPI (4',6-diamidino-2-phenylindole) was used to counterstain the nuclei (blue fluorescence). As a negative control for unspecific binding, we replaced the primary antibody with a non-immune antibody. The secondary antibodies were also applied without the primary antibodies to exclude false positive staining.

Double staining (paper II)
We double-stained leukocytes with an antibody against monocyte/macrophages, (clone, MAC 387, recognizing antigens in monocytes, tissue macrophages, and granulocytes) and SM α-actin. These epitopes are all present in the cytoplasm. To circumvent this problem we first stained the leukocytes for MAC 387 and photographed the section. The cover, mounted in saline, was then removed and the MAC 387-staining was washed away with xylene. The same section was then re-stained for α-actin and visualized with DAB. New photos of the same section were taken and laid on top of the first ones in PhotoShop (Adobe).
To assure that the secondary antibody for SM α-actin did not bind to the primary Mac 387 antibody, the same staining procedure was done except that the primary antibody against α-actin was replaced with buffer.

Endothelial cell staining (paper I)
Endothelial cells were identified with antibody against Willebrand Factor (vWF). In pigs it is known that vWF is not universally distributed in various endothelial beds. vWF is proven to be present in the distal abdominal aorta, the vaso vasorum, the thoracic aorta and the pulmonic artery.[158] A variation in distribution could be one possible explanation why the luminal cells in our study did not stain positive. Another possibility is that the observation time was not long enough. However, one graft that was still patent after three month was also negative for vWF staining.

PCNA (paper I)
In pigs, proliferating cells were detected with immunohistochemistry against PCNA. PCNA is essential for DNA replication. It accumulates in the nucleus during the S-phase of the cell cycle. It is also involved in DNA repair. Staining for PCNA has been shown to give an overestimation of the proliferation.[159] However, we were only interested in when and where cells proliferated in the graft and not in the absolute number of proliferating cells.

Confocal microscopy (paper II)
Fluorescence images of the tissue sections were acquired, using an Axiovert 200 inverted microscope equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss, Jena). DAPI was excited with a 405 nm Blue diode laser and detected using a 470-500 band-pass filter. FITC was excited with a 488 nm Argon laser and detected using a 505-530 band-pass filter. PKH 26 was excited with 543 nm laser line of a Green Helium-Neon laser and detected using a 560 long-pass filter. Tissue sections were visualized using the confocal microscope at 1024x1024 pixel resolution through a 63x/1,4 NA Zeiss Plan-Apochromat oil immersion objective. Multi-Tracking mode was used to

eliminate spillover between fluorescence channels. Digital images were prepared in Adobe Photoshop.
A confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. Only the light within the focal plane can be detected, so the image quality is much better than that of wide-field images. Since it detects information from only one point on the specimen, the information needed to make a 2D or 3D image requires scanning of the excited spot over a regular raster in the specimen. The advantage of the method in this study was that we could confine the fluorescence from PHK 26 and SM α-actin to the same cell. In a conventional fluorescence microscope false positive observations can be made because cells might be overlying each other which cannot be excluded since you do not get a 3D view.

Electron microscopy (Paper I)
Electron microscopy was used to analyze the ultrastructure of cells facing the luminal side. Graft specimens (30 day grafts) were analyzed with scanning and transmission electron microscopy (SEM/TEM). The specimens were fixated by immersion in 2% paraformaldehyde + 2.5% glutaraldehyde + 0.1% sodiumazide in cacodylate buffer. Graft pieces measuring 5 x 5 mm that were intended for SEM were postfixated according to the OTOTO method.[160] In short this is a method where specimens are treated with several cycles of osmium tetroxide (OsO_4) and thiocarbohydrazide and then critical point dried. Thiocarbohydrazide enhances the impregnation of osmium in the specimen. Osmium increases the ability of a specimen to conduct electricity and makes a sputter coating unnecessary. Dehydration in graded series of alcohol was followed by infiltration with hexamethyl disilazane, which was allowed to evaporate till dryness. Specimens were mounted on aluminum stubs and were examined in a Zeiss 982 Gemini field emission SEM. TEM samples were postfixated with 1% OsO_4 and 1% potassium-ferrocyanide in cacodylate, followed by uranyl staining en bloc, alcohol dehydration, plastic resin infiltration, and curing. Ultrathin sectioning was performed with a diamond knife and sections were examined in a Zeiss 902.
With SEM the luminal surface resembled the endothelium of an artery. A distinct difference from a normal endothelium was that cells seemed to migrate from a subluminal position and penetrated the luminal cell-layerin a luminal direction. TEM revealed cells in the subluminal space to mostly resemble synthetic SMCs with an abundance of subcellular organelles such as a well-developed endoplasmatic reticulum and golgi apparatus and little contractile proteins. In deeper layers of the IH the cells contained more contractile elements and less developed organelles more resembling a contractile SMC (data not shown).

Statistics and Ethics

All values are expressed as the mean ± SD when not otherwise mentioned. The paired t-test (paper III) and the Mann Whitney U test (paper IV) were used for the statistical evaluations. Differences were considered significant at a p-value less than 0.05.

All animal protocols were approved by the ethical committees at Lund and Göteborg University.

SUMMARY OF RESULTS

Paper I

In this paper, the healing of implanted artificial grafts in a pig model was studied in a time-course. PTFE grafts were implanted end-to-side from the common to the external iliac artery. The grafts were harvested at different time-points from day 1 to day 90. At day 1, the graft fabrics were populated with leukocytes. At day 7, a neoadventitia was formed with α-actin expressing cells. Cells from the neoadventitia started to grow into the graft fabrics and at day 21 they had reached the lumen and contributed to the neointima. The neointima continued to grow and after three months five out of six grafts were occluded.

Additional cellular sources for the neointimal formation seemed to be the luminal blood and the adjacent artery. At day 14, several mid-graft sections contained cellular clusters and small islets of proliferating cells on the luminal side of the graft fabrics. These islets did not have any cellular contact with the neoadventitia or the anastomoses. Some of the cells in the clusters were SM α-actin positive (data not shown).

At day 14, an SM α-actin positive neointima with cellular contact to the adjacent artery was present in the anastomoses. At this time-point the neointima had no cellular contact with the neoadventitia. A connecting bridge between the artery and a focal thickening on the luminal side of the graft was observed.

The cells lining the lumen in the PTFE grafts did not express the endothelial marker vWF. On an overview with SEM the luminal cells resembled endothelial cells, but with TEM they showed characteristics of both endothelial cells and SMCs.

Paper II

In this study we separated mononuclear cells from whole blood with an apheresis technique, and labeled them ex vivo with a cellmarker (PKH 26). The aim of the study was to see if these cells later contributed to the intimal hyperplasia after a coronary artery balloon injury and iliac bypass grafting. The labeled cells were intravenously reinjected at two different time-points after intervention and the marked cells could later be detected within the injury-induced intimal hyperplasia. Specimen examination revealed that some of the initially labeled blood-derived mononuclear cells expressed SM α-actin within the IH, suggesting the in vivo transdifferation to a SM α-actin positive cell. Confocal microscopy confirmed double labeling of PKH 26 dye and SM α-actin confined to the same cell. Additionally, we found that neointimal cells co-expressed a monocyte/macrophage epitope and SM α-actin using immunohistochemichal double-staining techniques. Furthermore, SM α-actin positive cells of both elongated and round shapes were found in a thrombus of one occluded bypass-graft and these cells had no connection to any formed IH, thus indicating a blood-derived origin.

In summary, we observed with different methods that blood-borne mononuclear cells can transdifferentiate into SM α-actin positive cells and contribute to the tissue organization in intimal hyperplasia after vascular interventions. The quantitative importance of this phenomenon was not evaluated.

Paper III

In this paper we investigated the effect of PDT locally given at the site of the distal anastomosis prior to graft implantation in a pig model. Seven pigs were included in the main study. PTFE grafts were bilaterally implanted between the common and external iliac arteries. The recipient artery was PDT treated and the contralateral side served as control. Specimens were analysed 4 weeks later. Graft anastomoses on the PDT-treated side showed significantly less IH compared to the control side (Fig. **3A, B**).

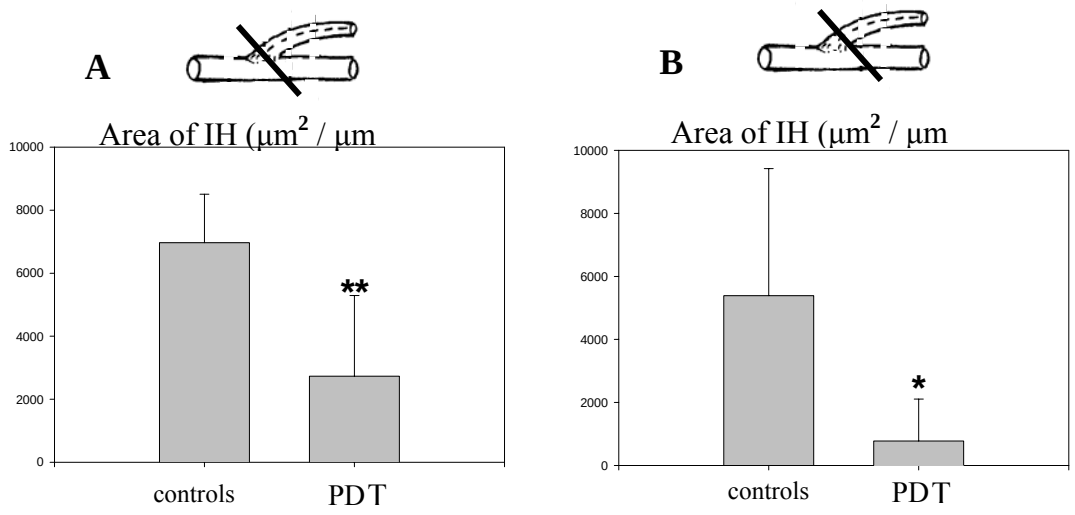

Fig 3. Intimal hyperplasia in the mid-portion of the anastomosis (**A**) and in the graft (**B**). The black lines in the drawings show the level at which the specimens were analysed. PDT-treated anastomoses showed reduced IH in the mid-portions of the anastomosis (**A**) and in the grafts (**B**). **A**, Control: 6970 ±1536, PDT: 2734 ±2560 (Mean ± SD, μm^2 IH /μm graft); **P<0.01. **B**, Control: 5391 ±4031, PDT: 777 ±1331 (Mean ± SD, μm^2 IH /μm graft); *P<0.05. n = 7 in both the control and PDT group.

Paper IV

In this study we examined how balloon injuries of different severity applied to the cells in the media influenced the degree of IH to follow. A balloon catheter was inflated and withdrawn 2 (minor injury) or 4 times (major trauma). At day 4, large areas with total loss of SM α-actin positive staining were seen in the media after the major but not after the minor injury (figure 4 A). The numbers of cells in the media were also significantly reduced with both injuries after 2

hours. The reduction was more prominent with the major compared to the minor trauma. The major injury attracted more leukocytes (CD18-positive) to the media and adventitia. Despite the pronounced SMC loss and inflammatory response, the major injury resulted in less intimal hyperplasia (figure 4 B) at day 14. In the major injury group, intimal hyperplasia first appeared next to SMC-rich parts of the media (day 7) and at day 14 intimal hyperplasia was thicker in those areas. In both groups, BrdU-labeled cells migrated from the media but not from the adventitia into the neointima. To further investigate if cells migrated from the adventitia to the IH, PKH 26-labeling was used. In accordance with the findings with the BrdU-labeling, this demonstrated that cells in the adventitia did not migrate towards the media or the intimal hyperplasia.

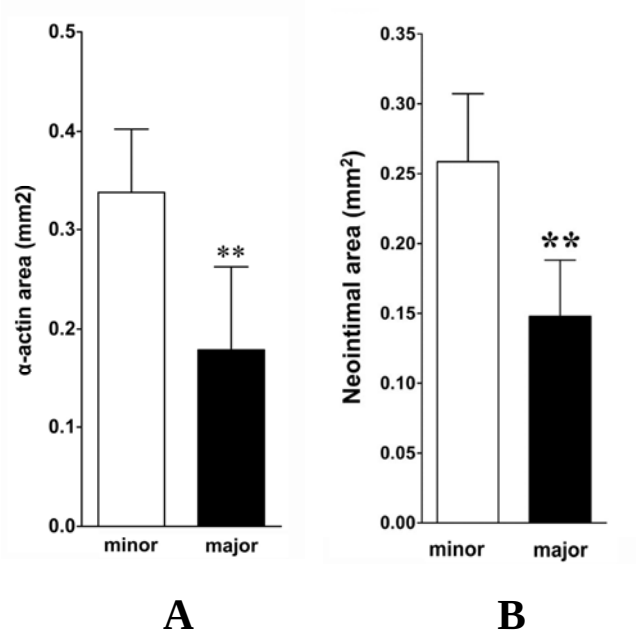

Fig 4. α-actin area (day 4) and neointimal area (day 14) following a minor and a major injury. Mean ± SD, **p<0, 01, $n_{minor} = 6$ and $n_{major} = 8$.

DISCUSSION

Intimal hyperplasia (IH) is a structural change occurring in ballon-dilated arteries, arterialized veins and prosthetic bypass grafts. It results in narrowing of the vessel lumen. Up to 40% of all interventions will be affected within one month to a year with subsequent morbidity and mortality. The first description of intimal hyperplasia has been attributed to Carrel and Guthrie[161], who noted in 1906 that within a few days of a vascular bypass operation the sutures at the anastomoses had become "covered with a glistening substance similar in appearance to normal endothelium". However, it was not until 1971 that the first report appeared citing intimal hyperplasia as the cause of late occlusion in an aortocoronary vein bypass graft.[162] Since then several studies have investigated the pathophysiological mechanisms and studied different modalities to prevent IH but the problem still remains unsolved.

Even if IH is a response which narrows the lumen following all arterial interventions, the impact might be different in different interventions. Following a direct injury to the arterial wall as with balloon angioplasty, a reparative response might be necessary to cover the thrombogenic subendothelial surface. It has been shown that the production of endothelium-specific factors can normalize with the establishment of IH.[163] Furthermore, the clinical studies with sirolimus- and paclitaxel-eluting stents support the general view that IH to a certain extent can be beneficial.[131, 132] The dramatic reduction of restenosis with these agents seems to increase the risk for thrombosis[133, 134] and death.[135] The clinical use of brachytherapy with abolishment of IH also increases the risk for thrombosis. Therefore a reparative response within certain limits following balloon injury may be beneficial. The fact that the majority of patients do not get restenosis following balloon angioplasty shows that these individuals might have a biological regulation of the reparative response that others lack. The delicate balance between cellular sources and the need for external regulation of a biological response in selected cases is obviously a big challenge.

The implantation of vein grafts is a standard procedure for the treatment of atherosclerotic stenoses in coronary and peripheral arteries. The transfer of a vein to the arterial circulation never occurs in nature. A dormant ability of the vein to adapt to the new hemodynamic environment cannot be expected. The surgery and the change to arterial hemodynamics[67, 68] also cause an early loss of the endothelium which further promotes the development of IH.[27, 39] Accordingly, one important impact of IH in connection to vein grafts is to support mechanical adaptation. It is therefore of great interest that external synthetic stents or sheaths induce a complete inhibition of neointimal formation in vein grafts and an overall reduction of graft thickening[138-141]. However, non-degradable stents or sheaths may elicit unpredictable effects in the long term,

such as mechanical and inflammatory effects that may ultimately be counter-productive. Biodegradable sheaths may therefore represent the way forward. The cellular sources that induce IH in connection to the anastomoses and the contribution from blood-borne cells are still to be addressed in vein bypass surgery. The biological response at the anastomosis might preferentially be studied in artificial grafts.

The artificial grafts do have enough mechanical stability and do not harbor any cells to begin with that can respond to any trauma or hemodynamic influences. The grafts in clinical use exclude a number of cellular sources and the impact from the arteries at the anastomoses ought to be of central importance. IH in artificial grafts seems to be a reparative response to the mechanical injury following surgery and to the hemodynamic forces that arise in connection to the anastomoses.
In summary, even if IH is a response which narrows the lumen following all arterial interventions, the inducing factors seem to be different in different interventions. An increased biological knowledge of contributing cellular sources and their interplay in each specific situation is therefore crucial for a better understanding of this side effect, which hopefully can improve the outcome of interventions in the arterial tree.

It has generally been thought that the media with its SMCs is the only cellular source for IH. This has over the last decade been questioned and several studies have indicated that other cell types such as adventitial cells and blood-borne cells might contribute to the neointimal mass. Even if the media has been questioned as the only cellular source for IH, this thesis demonstrates that a depletion of the number of cells in the media reduces in short term the IH to follow after artificial bypass grafting in pig and balloon injury in rabbit.

The adjuvant application of PDT at the site of the recipient artery reduced the amount of intimal hyperplasia inside artificial grafts in our porcine model. PDT exerts its cytotoxic effects by cellular apoptosis. After PDT we observed almost a total removal of cells in the media of the recipient artery and the development of IH was significantly reduced but not completely inhibited. Achieving correct photosensitizer, light and dosimetry in arteries of bigger size is difficult. Furthermore, perivascular cells and blood-borne cells might have contributed to the formed IH. These sources were not inhibited by the present PDT-treatment to the artery. PDT unfortunately has a narrow beneficial window between no effect and an increased risk for thrombosis. Since the principal finding has a potential to be immediately transferred to the clinical setting, an alternative might be to use thermal angioplasty with radiofrequency energy, which causes medial smooth muscle cell necrosis and transform the media into an extracellular matrix barrier.[94] This response is very similar to what we have

experienced with PDT. The thermal injury however seems to lack the side-effects known with PDT. The intriguing reduction of IH by eliminating the cellsin the media of the recipient artery can be questioned since the thesis does not include long term results and the potential of a delayed compensatory response from perivascular and blood-borne cells was not evaluated. In mice a high contribution of bone marrow-derived cells seems to be dependent on a shortage of local mesenchymal cells.[101] Furthermore, there are observations supporting that the origin of neointimal cells does not influence the final neointimal size.[164] Thus, it is likely that the neointimal size is locally regulated and therefore a similar neointimal thickness will be formed as long as there is a sufficient supply of cells. Anyhow, even if questioned, the cells in the media of the recipient artery seem to be very important for the formation of stenosis in artificial grafts in the short term perspective.

In many species, an extensive loss of SMCs in the media can presumably be compensated for by myofibroblasts from the adventitia. In rats, a pronounced neointima is still formed following a balloon-trauma in which 90-100% of the SMCs in the media are lost.[83, 165] In this thesis we demonstrate that the rabbit lacks cellular contribution from the adventitia following balloon injury. We therefore hypothesize that the media is the main source for IH in rabbit and that a massive loss of SMCs causes a shortage of responding cells and thus less intimal hyperplasia. The lesson to be learned is that the responding cellular sources must be known in the species of interest if an effective treatment against intimal hyperplasia is to be found.

In summary, when the number of alternative cellular sources is reduced, depending on either the species treated or the type of intervention used, a precise reduction of the cells in the media might give promising results. The media is therefore still an important cellular source for IH but it has to be defined in its context.

In canine, non-SMCs from the adventitia or the media cannot be morphologically distinguished from each other. These cells have been found in the neointima following balloon injury.[78] These observations, combined with the fact that fibroblasts can become SM α-actin positive myofibroblasts[81, 82], suggest that SM α-actin positive cells in the neointima can be derived from fibroblasts originally residing either in the media, the adventitia or presumably the perivascular tissue. The fibroblasts in the adventitia start to proliferate before the cells in the media following a balloon trauma[81, 83]. It has also been shown that fibroblasts from the adventitia can migrate through the media and contribute to the neointimal mass. The adventitia and perivascular tissue are also locations of entry for leukocytes into the vascular wall. Within the first 24 hours the cellularity increases in the adventitia/the perivascular tissue due to a massive infiltration of neutrophils and macrophages[46, 166]. This demonstrates the quick response from the adventitia/ perivascular tissue following injury. Experimental

observations support the presence of a close relation between the perivascular tissue and the intima. For example perivascular application of interleukins or TNF-α can induce intimal hyperplasia.[167] The importance of the tissue outside the classical vascular wall is supported by this thesis in which a "neoadventitia" was formed around the artificial grafts with α-actin expressing cells. These cells started to grow into the graft fabrics and contributed to the neointima. The principle finding indicates that the vessel wall may not be the limit for cellular responses and cellular contribution to intimal pathology. This suggests that vascular wall biology seems to be too narrow as perspective for the understanding of vascular wall disease.

Peripheral blood carries progenitor cells. Both hematopoietic stem cells and mesenchymal stem cells from the bone marrow have the ability to differentiate into myofibroblasts in vitro.[97, 98] In vivo studies have been done on bone marrow transplanted mice and rats with recognizable bone marrow cells in the intimal hyperplasia formed [100, 101]. There are studies supporting that white blood cells, especially monocytes/macrophages, play a role in the restenosis process.[89, 168, 169] Furthermore it has been shown that human peripheral blood contains pluripotent cells, which are a subset of peripheral monocytes capable of differentiating into various cell lines.[170] In this thesis mononuclear cells labeled ex vivo could later be detected as α-actin expressing cells in the intimal hyperplasia formed after balloon injury to a coronary artery and bypass surgery. This cellular source contributed to the tissue organization in the intimal hyperplasia after vascular interventions. The quantitative importance of this cellular source in different situations is so far unknown. The finding challenges our present definition and naming of cells, since certain cells must be in an intermediate phase. Furthermore it indicates that cells might have a bigger plasticity than previously anticipated. It might be the functional pressure exerted by the biochemical and biophysical microenvironments that induce the differentiation of cells. The concept of functional adaptation would predict that the environment is just as important as the origin of the cells. Such an approach would even challenge the importance of cellular sources for intimal hyperplasia, which is the title of this thesis.

In summary, this thesis has shown that cells from the adjacent artery, the media, the surrounding tissue, and blood-borne mononuclear cells can contribute to intimal hyperplasia after prosthetic bypass surgery. The media and blood-borne mononuclear cells can contribute to the IH after balloon angioplasty but not the adventitia in rabbits. Furthermore, depletion of the cells in the media reduces the intimal hyperplasia after bypass surgery in pig and after balloon injury in rabbit.

CONCLUSIONS

In this thesis we have studied which cellular sources participate in the development of intimal hyperplasia after bypass surgery and ballon injury. Furthermore, we have investigated, if the depletion of one cellular source, the media, could reduce intimal hyperplasia. The studies were perfomed in a bypass and balloon injury model in pig and a balloon injury model in rabbit.

We have found:

- that the adjacent artery, the media, the surrounding tissue, and the blood can contribute cells to the intimal hyperplasia after prosthetic bypass surgery in pig. The media but not the adventitia, contributes cells after balloon injury in rabbits.

- that blood-borne mononuclear cells can contribute to the formation of intimal hyperplasia after bypass surgery and balloon injury in pig.

- that the depletion of the cells in the media reduces the intimal hyperplasia after bypass surgery with artificial grafts in pig and after balloon injury in rabbit

POPULÄRVETENSKAPLIG SAMMANFATTNING

Förträngande åderförkalkning behandlas med ballongvidgning eller bypass operationer. I upp till 40 % av fallen uppkommer en ny förträngning i form av tillväxande ärrvävnad, även kallad intimal hyperplasi, i den behandlade pulsådern. Förträngningen börjar bildas redan någon månad efter ingreppet och leder oftast till återkommande symptom för patienten i form av kärlkramp, fönstertittarsjuka men även amputation och död. Trots mycket forskning inom området finns ännu ingen bra metod att förhindra denna reaktion. Den förträngande vävnaden består i en tillväxt av celler vars ursprung inte är helt klart. Den vanligaste uppfattningen är att dessa kommer från den behandlade pulsåderns mellersta vägglager, muskelcellslagret.

Vi ville ta reda på vilka cellulära källor som bidrar till ärrvävnaden och om manipulation av någon av dessa cellkällor kunde minska förträngningen. Vi använde oss av en bypass- och ballongskade-modell på gris och en ballongskade-modell på kanin.

I grismodellen utfördes en bypass operation med konstgjorda ådror på bäckenartärerna. Samma kärlsubstitut används kliniskt på människa. Efter tre månader kunde vi konstatera att 90 % av ådrorna slutade fungera pga en förträngande vävnadsinväxt. Celler från utsidan av den konstgjorda ådern, från den intilliggande pulsådern vid inkopplingsstället för bypassen samt blodburna celler tycktes bidra. Att blodburna celler bidrar till förträngande vävnad är sedan tidigare kontroversiellt. Vita blodkroppar är en tänkbar blodburen cell som kan bidra till förträngningarna.

I grismodellen avskiljdes cirkulerande vita blodkroppar från blodcirkulationen och märktes in med en detektionsmarkör. Därefter utfördes både en ballongvidgning av en kranspulsåder i hjärtat och en bypass operation på bäckenartärerna. De inmärkta cellerna återfördes till djuret strax efter ingreppet. En månad senare fann vi att vissa av de inmärkta blodcellerna återfanns i ärrvävnaden och antog kännetecken likt kärlväggsceller, efter både bypass och ballongvidgning. Vi kunde därmed visa att cirkulerande blodburna celler kan ändra skepnad och delta i bildandet av ärrvävnaden.

Vidare ville vi se om vi kunde påverka ärrvävnadsbildning genom att slå ut en cellkälla. I grismodellen behandlades den mottagande artären på platsen för inkopplingen av bypassen, med en specifik ljusterapi. I kanin jämfördes en liten och en stor ballongskada på halspulsådern. Båda metoderna resulterade i en utslagning av cellerna i det mellersta lagret i artären. I kanin åsdtadkoms större utslagning av celler efter stor skada jämfört med liten. Vid analys efter

ingreppen kunde vi konstatera att ärrvävnaden var signifikant mindre där vi slagit ut de glatta muskelcellerna jämfört med kontrollerna. Med detta visade vi att påverkan av en cellkälla, i detta fall pulsåderns mellersta cellager, resulterar i mindre förträngande ärrvävnad efter både bypass kirurgi och ballongvidgning.

Sammanfattningsvis har vi visat att celler från omgivande vävnad, den närliggande artären och blodburna celler kan bidra till förträngande ärrvävnad efter bypasskirurgi och ballongvidgning i gris. Blodburna celler kan omvandlas till byggstenar i ärrvävnaden. I kanin så bidrar celler från pulsåderns mellersta lager, men inte celler från kärlets utsida till förträngningen. Förträngningen kan reduceras, efter både bypass kirurgi och ballongvidgning, om man slår ut cellerna i pulsåderns mellersta lager.

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