Vascular Wall Responses to Bypass Grafting

- Studies in Mice

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ABSTRACT

Vein grafts are frequently used in bypass surgery for treatment of coronary artery disease and lower limb ischemia. Unfortunately the long-term patency is impaired by vein graft stenoses due to intimal hyperplasia (IH). It is assumed that exaggerated intimal thickening is initiated by vessel wall injuries or local flow disturbances. Accumulation and proliferation of smooth muscle cells (SMCs) are key events in the formation of IH. It has been an established opinion that these cells have their only origin in the underlying media. This theory has been challenged by recent research, which has demonstrated recruitment of SMCs from other sources than the local vessel wall. Mice models including genetically modified strains have had a major impact on the reformed view upon vascular repair.

In this thesis, recruitment pathways of intimal SMCs and the impact of blood flow on vein graft intimal thickening were investigated.
A new mouse model, which enables studies of different blood flow through vascular grafts was established. The area of IH in vein grafts was measured in two groups, which had a 2.7 times difference in blood flow. The area was 70% larger in the low flow group compared to high flow, which shows that vein graft IH is regulated by the magnitude of blood flow.

Recruitment pathways of SMCs to IH in vein grafts were studied in genetically modified mice, expressing the enzyme LacZ. Thirty percent of the cells originated from sources apart from the grafts. External shielding of the vein grafts resulted in decreased contribution of cells from recipient mice, which point at transadventitial migration as an important recruitment pathway. SMC migration from the adjacent artery could not be detected, which indicate that the connected artery does not play any role for formation of vein graft stenoses.
The ability of externally recruited SMCs to regenerate the vessel wall was investigated by implantation of grafts in which cells had been abolished. Acellular vein grafts developed similar degree of IH as cellular vein grafts, which demonstrate that externally recruited SMCs by themselves can form intimal thickening.
Acellular arteries were also implanted. The externally recruited SMCs to the arteries had limited ability to regenerate the medial SMC population and no vasomotor function was observed. This demonstrates that recipient derived SMC progenitor cells can contribute to pathological cellular formations, but lack ability to re-establish the normal morphology and function of the arterial wall.

In conclusion, the results from this thesis show that the vein wall in response to bypass grafting develops IH, which is regulated by the magnitude of blood flow. The intimal SMCs can be derived from sources outside the vessel wall and may partly be recruited by transadventitial migration but not from the adjacent artery. The SMCs with external origin have ability to contribute to IH but not to the functional population of medial SMCs.

Key words: Intimal hyperplasia, graft stenosis, flow, shear stress, smooth muscle cells, cellular recruitment, mice

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I. Intimal hyperplasia in mouse vein grafts is regulated by flow.
Klas Österberg, Erney Mattsson
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II. Reduced neointima in vein grafts following a blockage of cell recruitment from the vein and the surrounding tissue.
Per Fogelstrand, Klas Österberg, Erney Mattsson
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III. Smooth muscle cells in mouse vein grafts are not recruited from the adjacent artery.
Klas Österberg, Erney Mattsson
In manuscript

IV. Progenitor smooth muscle cells have limited ability to regenerate arterial function and morphology.
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ABBREVATIONS

ACH	Acetylcholine
bFGF	basic Fibroblastic Growth Factor
BrdU	Bromodeoxyuridine
CABG	Coronary Artery Bypass Grafting
CCA	Common Carotid Artery
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EEL	External Elastic Lamina
IEL	Internal Elastic Lamina
H&E	Hematoxylin-Eosin
HF	High Flow
HRP	Horseradish Peroxidase
IH	Intimal Hyperplasia
LF	Low flow
MAC-1	Membrane-activated complex 1
MCP-1	Monocyte Chemoattractant Protein-1
NA	Noradrenaline
PBS	Phosphate-Buffered Saline
PSS	Physiological Salt Solution
PDGF	Platelet-Derived Growth Factor
SMCs	Smooth Muscle Cells
WT	Wild Type

INTRODUCTION

Cardiovascular diseases due to atherosclerosis are the main reasons for deaths in the western world. Atherosclerotic stenoses result in acute or chronic reduction of blood flow to different organs. For many patients with severe symptoms in the heart and lower extremities, bypass-surgery is required. Veins are frequently used and have far better results compared to synthetic conduits. Unfortunately the long-term patency of vein grafts is deteriorated due to flow restricting stenoses. These stenoses are caused by intimal hyperplasia (IH), which is a thickening of the innermost vessel wall layer. There is no effective treatment today against vein graft stenosis. The aim of this thesis was to elucidate some of the mechanisms behind intimal hyperplasia in vein grafts.

The vascular system

Basic structure

A rudimentary vascular system is present by the end of the third week in the human embryo to support rapid growth.[1] The heart and blood vessels develop during the fetal time and the process is not complete until the last modifications just after birth. The cardiovascular system is well adapted to maintain adequate oxygen delivery and metabolic exchange to organs and tissues under conditions ranging from rest to physical exertion.

The pumping heart generates the arterial blood pressure, which force the blood to circulate throughout the body. The blood is distributed via the large elastic arteries, which through elastic recoil maintains a continuous blood pressure during the pulse cycle. From the heart and distally the vessels gradually decrease their content of elastic fibers and acquire prominent circular arrangement of smooth muscle cells (SMCs). The muscular arteries regulate the distribution of the regional blood flow. The tonus of the small arterioles maintains the blood pressure and the control of blood flow into capillary beds. This is due to the action of nervous and vasoactive agents. The exchange of gas and metabolites is carried out in the capillaries. After the capillaries most of the pressure generated by the heart is lost and the blood is transported back to the heart through the veins, a low-pressure system with minor resistance. The hemodynamic forces vary along the circulatory system, which is reflected in the thickness and structure of the vessel wall in different parts of the system. However, there is a basic morphological architecture in all blood vessels, capillaries not included.

The vessel wall

The vessel wall is arranged in three separate layers: the intima, the media, and the adventitia. The intima is composed of a layer of endothelial cells attached to the underlying subendothelial connective tissue. The endothelium lines the inside of all blood vessels and forms the interface between the flowing blood and the vessel wall. At their location the endothelial cells have a critical function to register chemical and mechanical signals, which are crucial for the regulation of vasomotor function, blood cell adherence and blood coagulation.[2-4] The subendothelium consists of a highly organized matrix of molecules that includes collagen, elastin, fibrinonectin, glygosaminoglycans and von Willenbrand factor.
A lamina of elastic fibers separates the tunica intima from the tunica media. This membrane, rich in elastin, is termed internal elastic lamina (IEL) and is normally present in arteries and larger veins while it might be vague or absent in medium sized and small veins.

The media is the muscular layer of the vessel wall and its principal cellular population is SMCs. SMCs are concentrically arranged and embedded in a matrix of connective tissue, composed mainly of elastic fibers, collagen fibers, and proteoglycans. Elastic fibers are condensed to several circumferential elastic lamellae in the arterial media. These are prominent to their structure and numbers in the elastic arteries while they diminish in the smaller muscular arteries, where the SMCs are dominant. SMCs and the network of connective tissue are responsible for the intrinsic vascular tone, which is crucial for maintenance of adequate blood pressure and for the distribution of blood flow to different organs.

The outer limit of the media is demarcated by the external elastic lamina (EEL).
The adventitia is the external coat of the blood vessel and is composed of bundles of collagen-rich connective tissue together with fibroblasts. The adventitia provides the vessel with strong external mechanical support. A network of blood vessels, the vasa vasorum, which penetrate the adventitia supplies larger arteries and veins with nutrients. There are also nerve branches in the adventitia, which connect to the medial layer for regulation of the vessel tonus.
Most of the large arteries and veins are embedded in a thin and loose perivascular network.

Hemodynamic forces

The vessel wall is subjected to two main types of physical forces. The blood pressure exerts a distending force against the vessel wall, which it has to withstand to avoid rupture. As long as the vessel wall is preserved the

transmural pressure is transformed to a tangential stress within the wall, the wall tension (T). The wall tension is directly proportional to the transmural pressure (P) and to the radius(r) of the vessel and is given by LaPlace's law:

$$T = P \quad r$$

Consequently, the wall tension increases with increased blood pressure and increased vessel wall dimension. These characteristics are reflected by the vessel wall structure. Vessels with big radii and vessels subjected to high blood pressure have thicker vessel walls and more elastic fibers.

The other major force exerted on the vessel wall is, shear stress, which is the frictional force along the inner vessel wall surface mediated by the flowing blood. The shear stress (τ) is dependent on the blood viscosity (η), the mean volume blood flow (Q) and the radius of the vessel (r) as given by the following equation:

$$\tau = 4 \eta Q / \pi r^3$$

Accordingly, there will be an increase of shear stress by elevated blood flow, while shear stress will be reduced by an increase of the vessel radius. Shear stress is an important regulator of vessel wall biology under physiological as well as pathophysiological conditions. The vessel tonus is directly influenced by changes in shear stress. Arteries constrict with decreasing shear stress [5] and dilate with increasing shear stress. [6, 7] Through an unknown mechanism endothelial cells sense the shear stress and the signal is transferred to the SMC population of the vessel wall by several mediators, of which nitric oxide has been a topic for extensive research.[8]

Laminar flow is a requirement to generate high levels of shear stress. Turbulent flow arises if the flow profile is disturbed and as a consequence the signal pattern from the endothelium will alter.[9] Areas with a disrupted flow profile are found at vessel bifurcations and graft anastomoses and these sites are associated with pathological vessel wall reactions such as intimal hyperplasia.[10]

Cardiovascular disease and interventions

Atherosclerosis

The etiology behind atherosclerosis is not fully clarified but several risk factors are known, such as hypertension, dyslipidemia, smoking and low levels of exercise.[11]
Atherosclerosis is a generalized vascular disease, which starts with deposition of lipids into the innermost layer of the artery wall, the intima. The disease develops over decades and a chronic inflammatory reaction leads to intimal accumulation of inflammatory cells and SMCs.[12] The vessel wall gains in thickness, which over time diminishes the luminal diameter. Advanced atherosclerotic lesions become calcified, which frequently lead to stenoses and occlusions of arteries, impairing the blood flow.

Even if atherosclerosis is a systemic vascular disease, the advanced lesions are not evenly distributed. Advanced calcified plaques or occlusions are predominately found in the coronary arteries, the carotid arteries, the intracranial arteries, the aorta and the arteries to the lower limbs.[13]

Interventions

Patients with advanced atherosclerotic disease and severe symptoms often require surgical treatment. There are two principal therapeutic options for vascular interventions; endovascular treatment or open surgery. Endovascular techniques, with balloon dilatation with or without stent placement are mostly used for stenoses and shorter occlusions in a wide range of different arteries. Open surgery includes methods where the atherosclerotic plaques are removed as well as bypass surgery. Bypass surgery is predominantly used for coronary artery disease and lower limb ischemia.

Bypass surgery

In bypass surgery blood flow is lead through a vessel conduit, which is sewn onto an artery proximal to the occlusion and attached to an arterial segment beyond the blockage. The method has constantly evolved since the middle of the 20th century when the bypass procedure was developed for treatment of arterial occlusive disease of the superficial femoral artery.[14] In the late sixties, the bypass technique was extended to treatment of atherosclerotic coronary artery disease.[15]

Different materials have been used and evaluated as vascular conduits. Long-term results of bypass surgery show that autologous vessels are superior to

synthetic conduits.[16-18] Autologous arteries are considered to be the best substitute, but have limited availability. In clinical practice it is almost exclusively utilized in coronary artery bypass grafting (CABG), where the internal mammary arteries is used to revascularize one or two coronary arteries.[19-21] Veins are the second best material for conduits and it is the golden standard to achieve good long-term patency in infra-inguinal bypass-surgery. The great saphenous vein is the conduit of choice in the vast majority of vein graft procedures.

There are two principle procedures for how vein graft bypasses are performed. The vein could be completely dissected and harvested followed by implantation in a reversed mode, which overcomes the obstruction to flow from the vein valves. This method is used for CABG and can also be used for infra-inguinal bypasses. The other procedure, in-situ non-reversed vein bypass, has been used increasingly since a modification of the technique was presented 1979.[22] In-situ vein grafts are used for infra-inguinal bypasses and with this method, the saphenous vein is dissected free only in the groin and at the site of the distal anastomosis. The vein valves are cut and side branches ligated to allow blood flow in reversed direction before the distal end is anastomosed to the recipient artery.

Vein graft arterialization

Veins implanted into the arterial circulation face major changes to their environment. First, when the veins are dissected they loose their nutrient supply through disconnection of the vasa vasorum. The vein wall is dependent on these supportive vessels for its oxygen supply since the venous luminal blood has reduced oxygen content. These conditions are switched when arterial blood starts to flow through the vein.[23]

The most extensive environmental change is probably the shift in hemodynamic forces subjected to the vessel wall. The vessel wall which is adapted to low pressure in the venous circulation is suddenly exposed to the arterial pressure, which is several times higher. Consequently the tensile stress in the vessel wall will increase with the same degree. Rapid changes of the wall tension will deform the vessel wall structures which can damage the cells and the network of connective tissue. Furthermore the bypass procedure will also affect the flow patterns in the graft, which may generate altered shearing forces against the endothelium. In addition, surgical handling of the graft may cause injuries, which can influence the vessel wall biology.

A study of morphological changes in human vein grafts, explanted within ten days after bypass surgery, revealed that there was an extensive desquamation of

the endothelium together with a massive infiltration of leukocytes to the vein wall. The media was also severely damaged, resulting in a loss of cells.[24]
All together, there is a wide range of mechanical and humoral stimuli, which can initiate vessel wall remodeling in vein grafts.

With time the vessel wall adapts to the new environment. Several studies have shown that there will be a thickening of the vessel wall. The media will gain in thickness, but there will also be a thickening of the intima, due to IH.[25] These changes are probably to a certain extent necessary for long-term resistance against increased pressure and to avoid aneurysmal formation. It should be emphasized that the majority of vein grafts do not develop stenoses even though IH is formed.

Vein graft stenosis

Veins are not ideal as bypass conduits which is reflected by the incidence of vein graft failure. Within the first year, there is a failure rate of 20-40 per cent, depending on the location and the extension of the bypass.[26-32]

Early postoperatively vein graft failures can usually be attributed to thrombosis, due to low blood flow through the graft, but may also be caused by an unrecognized hypercoaguable state. Impaired flow rates, are in conjunction to the operation, caused by technical errors or poor run-off due to extensive atherosclerosis in the recipient arteries.[33]

Following the postoperative period graft failure is usually caused by flow-restricting stenoses and subsequent thrombosis. Approximately 20 per cent of grafts develop stenoses and most of these occur during the first year.[34-36] Half of the stenoses seem to be intragraft lesions while the remaining ones are localized at the anastomoses.[34] Most of the stenoses consist of localized extensive formation of IH (figure 1).[37, 38]

Late failures, some years after the bypass procedure, are secondary to atherosclerosis in the grafts themselves or progress of the disease in the inflow or the outflow arteries.[39]

For some patients the restrained blood flow, caused by the stenoses, results in relapse of ischemic symptoms giving an opportunity for the condition to be diagnosed and treated. Unfortunately the stenoses are asymptomatic for many patients until the grafts finally occlude. To prevent graft occlusion, patients with peripheral vein graft bypasses often follow surveillance programs with duplex

scanning during the first two years.[27] Detected stenoses can be treated with either balloon dilatation or surgical revision.

Although extensive research has been conducted, the etiology behind vein graft stenoses is still unclear. Mechanical injuries to the vessel wall, caused by the dissection and the suturing of the anastomose, have been suggested as one explantion.[40, 41] In a prospective, randomized trial, it was demonstrated that veins harvested with great care had significant better patency compared to grafts which were harvested with a standard technique.[42] Influence on vessel wall biology by altered flow patterns is another potential pathophysiological mechanism for formation of stenoses. Flow disturbances are present at end-to-side anastomoses, which also is a predilection site for graft stenoses.[43] In a study of infra-inguinal vein grafts, duplex scanning revealed that there was a correlation between early flow disturbances and development of graft stenosis.[44]

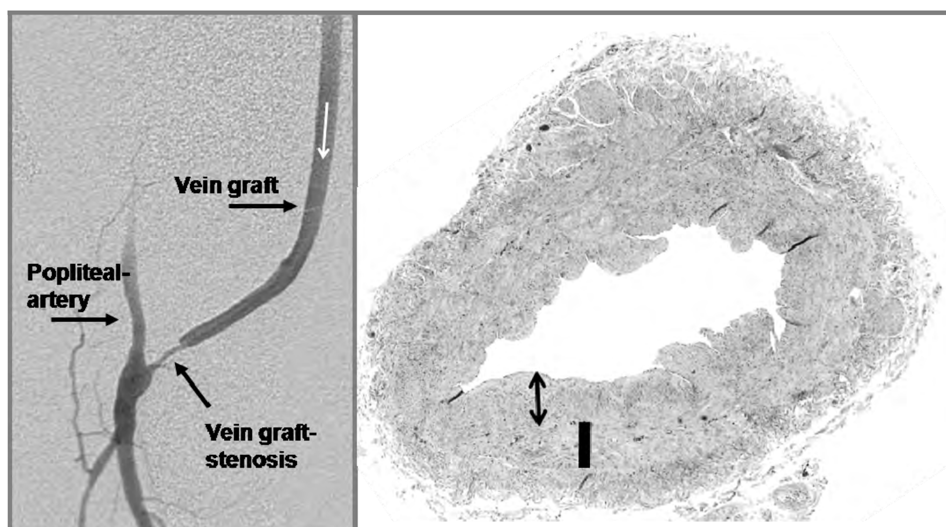

Figure 1. Angiogram and histology of human vein graft stenosis. Left panel demonstrates a vein graft stenosis at the distal anastomosis of a femoro-popliteal bypass. Right panel demonstrates intimal hyperplasia in a vein graft stenosis (double arrow). The bar represents the media of the vessel wall.

Intimal hyperplasia

IH, also termed neointima, is a thickening of the innermost layer of the vessel wall, which occurs as a uniform vessel wall response to different stimuli or injuries.[45] IH is found in a wide range of different conditions, both physiological and pathological. For example, closure of ductus arteriosus in the transformation process of the fetal circulation at partus, is accomplished through IH.[46] In clinical practice neointimal thickening is often associated with unfavorable vessel wall reactions such as restenosis after balloon angioplasty or graft stenoses after bypass surgery. The morphological changes of IH are well characterized and there is strong in vivo evidence implicating involvement of several molecular mediators.[47]

Histology

IH is defined as an abnormal formation of cells and extra cellular matrix (ECM) in the intima of the vessel wall. It is composed of about 20% of SMCs that have migrated to the intima, where they have proliferated and deposited ECM which constitute approximately 80% of the final volume.[48] Macrophages and lymphocytes may also be present. The extension of IH can vary from a few cell layers to extensive formations, which totally fill the lumen and occlude the vessel.

Initation of intimal hyperplasia

- *Mechanical injury*

The ability to survive following vascular injuries has been an advantage during evolution. Intimal hyperplasia has probably, together with the coagulation system, developed as a reparative mechanism, in response to such injuries. Vascular interventions activate the same defense systems and some degree of IH is probably required for healing after these procedures. Unfortunately, the vessel wall reactions may be exaggerated, which can cause stenoses and occlusions.

A mechanical injury can be a direct result of the intervention such as with balloon dilatation, applied vascular clamps, surgical removal of plaques (endarterectomy) or the suturing of the anastomose.[49-51] Vessel wall damage may also arise secondary to increased wall tension.[52]

In experimental studies injury to all components of the vessel wall has been shown to stimulate neointimal formation. Injury restricted to the intima due to

selective endothelial denudation in rat result in neointimal formation.[53, 54] Stretch injury to the media has also been shown to cause IH.[55] In models where the adventitia is injuried it has been demonstrated that this layer is also involved in the formation of IH.[56]

- Inflammation

In human vein grafts there is an early infiltration of leucocytes into the vessel wall.[24] IH is generally preceded by a major inflammatory reaction, which include adherence to the luminal surface and infiltration into the vessel wall of neutrophils and monocytes. [57, 58] The interaction between inflammation and IH is complex. Animal studies indicate that the inflammatory system can influence the degree of IH. Blockade of the membrane-activated complex 1 (MAC-1) receptor with antibodies reduce the leucocyte recruitment and the extent of intimal thickening in a rabbit balloon injury model.[59] Intercellular adhesion molecule-1 (ICAM-1) knockout mice respond with less inflammatory cells adhering to the vessel wall surface following vein graft implantation. Consequently there was a 30%-50% reduction in the neointimal hyperplasia compared to wild type animals.[60] Monocyte chemoattractant protein-1 (MCP-1) is secreted by activated platelets and is a regulator of monocyte/macrophage chemotaxis. Blockage of the MCP-1 receptor, in a vein graft mouse model, resulted in a 51% reduction of the IH. [61]
Inflammation alone seems to be the major stimulus for vessel wall thickening in transplantation vasculopathy. These lesions in arteries transplanted together with the organ of interest are due to IH. The response is caused by a host versus graft immune reaction.[62]

- Platelet activation and coagulation

Endothelial denudation exposes the subendothelial matrix, which cause platelet adhesion and subsequent secretion of neointimal stimulating factors.[63] Soon after the injury, platelets will totally cover the subendothelial matrix.[64] Platelets interact with collagen and other subendothelial components, leading to activation and subsequent release of numerous bioactive substances.[65] These substances, which include growth-factors, cytokines and thrombin, do not only contribute to the hemostasic process, but also have an impact on SMC migration and proliferation. One of these mediators is patelet derived growth factor (PDGF), which has received attention as a chemoattractant for vascular SMCs.[66, 67]

- Hemodynamic forces

Increased wall tension is most commonly associated with medial thickening and not with IH, which was demonstrated in a canine study by Dobrin et al. The same investigation provides evidence for an inverse correlation between graft flow and IH.[68] The influence on neointimal formation has been shown to be

dependent on the degree of shear stress.[69] Low shear stress induces IH at sites with disturbed flow patterns.[70] Spots with pronounced intimal thickening, within the distal anastomoses of coronary bypass vein grafts, show high degree of disrupted flow patterns supporting the above correlation between hemodynamics and IH.[71]

SMC proliferation and migration

SMCs are not to any significant extent present in the intimal layer, which means that these cells need to migrate to this location. The most proximate smooth muscle cell population is located in the underlying media and for decades there has been a focus on investigating migration and proliferation patterns of these cells. Numerous animal studies, mostly before the era of genetically modified mice models, have connected medial cellular processes with IH.

In a canine vein graft model it was demonstrated that the SMCs were transformed to a secretory phenotype in response to vessel wall injury.[40] By the switch of phenotypes the SMCs enter a state enabling proliferation, migration, and synthetic capacity, which is a pre-requisite for neointimal formation.[72]

In experimental models including vein grafts, SMC proliferation starts in the media, and is concentrated to this location during the first seven days after graft implantation, while intimal proliferation is absent. From day seven up to three weeks, the proliferation rate is at its highest within the intima, followed by a later decline.[52, 73] Associated with the onset of proliferation, but with a short delay, development of IH occurs in vein grafts.[74] It has been a well-supported opinion, that the source for the intimal cell population is SMCs from the media. According to this theory, these cells are stimulated to proliferate and migrate to the intima by cellular mediators. [75, 76] This paradigm has been challenged by recent research, which has shown that intimal SMCs can be recruited from other sources, which will be further discussed below.

Regardless of the recruitment pathway, the future neointimal cells respond to intercellular signaling which result in further migration of cells towards the intima. SMCs could be influenced either from paracrin or from an autocrin signaling from mediators in the intima, such as platelets or endothelial cells.

Several molecular mediators, such as basic fibroblastic growth factor (bFGF), interleukin-1β, PDGF, and tumor necrosis factor-α, have been shown to be upregulated in experimental vein grafts during the development of IH.[77] Many of these mediators have a stimulatory effect on SMC migration and

proliferation.[78, 79] Treatments against these mechanisms have been effective in preventing IH in vein grafts in animal studies.[80, 81]

Cellular recruitment

In recent years several reports have provided results which contradict that all neointimal smooth muscle cells originate in the underlying media. Mice models including genetically modified strains have had a major impact on the current view. Accumulated evidence indicates that progenitor cells, not originating from the local vessel wall, contribute to the neointimal formation. Models of transplant vasculpathy have provided results which demonstrate that graft SMCs arise from the donor tissue.[82-85] Mouse studies of IH after arterial injury, demonstrate that the neointimal SMC population partly originate outside the vessel wall.[86, 87] Concerning vein graft IH, mouse studies have shown that a considerable amount of the neointimal SMCs have a graft extrinsic origin.[88, 89]

The origin of the intimal SMCs in the different models is controversial. Recruitment of stem cells from bone marrow has been reported in studies of arterial injury and allograft transplantations while others rejected hematopoietic origin of intimal SMCs.[83, 85-87, 90, 91] Regarding vein grafts, hematopietic origin of neointimal cells has not been demonstrated.[88, 89] This leaves either, some other pool of circulating progenitor cells or SMCs from the adjacent artery to be the sources.
The strongest evidences for recruitment of cells outside the vessel wall have been provided by studies in which vessels have been harvested from one animal, and grafted to another, which harbor a reporter gene.[83, 84, 88, 89] This enables identification of recipient derived cells contributing to IH, allowing the authors to consider the presence of circulating progenitors. These studies all have the alternative explanation that the graft external SMCs, instead of being circulating progenitors, could be migrating cells from the artery to which the graft is anastomosed.

AIMS OF THE THESIS

The aims of the present thesis were:

- To determine if the degree of intimal hyperplasia in mouse vein grafts is regulated by the magnitude of the blood flow.

- To investigate if neointimal smooth muscle cells to vascular grafts in mice are recruited from adjacent arteries and/or through transadventitial migration.

- To explore if smooth muscle cells recruited exclusively from sources outside the vascular graft can form intimal hyperplasia. Furthermore, if externally recruited cells have the ability to regenerate normal morphology and function of the vessel wall.

METHODOLOGICAL CONSIDERATIONS

Surgical procedures

Animals

The studies were all performed on C57BL/6 mice. This means that the animals were inborn and tissue from one animal could be implanted into another without risk of rejection due to immunological reactions. Wild type (WT) animals were used in all studies, either as primary experimental animals (paper I and IV) or as control animals. In addition, two types of genetically modified mice were used. Both types had reporter genes and were designed for cell tracking studies. In paper II, the LacZ transgenic mouse type, ROSA26, which express the enzyme, β-galactosidase in all cells, were used. [92] This enzyme can be detected in histological analyses, and cells with origin from ROSA26 mice can be identified. The other type used (paper III), was SM22α deficient mice with the reporter gene LacZ in the SM22α locus (SM22α-LacZ mice).[93] This construct enables identification of SMCs with origin from SM22α-LacZ animals.

It is important to remark that studies in mice have limitations due to the small size of the animals. Surgical procedures are challenging because of the small dimensions of organs and vessels, which enforce the use of other surgical techniques than those used on humans. Furthermore, the hemodynamic forces in mice are not fully comparable to humans. For example, the tensile stress of vessel walls is much lower, due to small vessel diameters. This is reflected in thinner vessel walls and it is especially evident in mouse veins.
The Animal Ethics Committee of Göteborg University approved all experiments.

Interposition graft technique (Paper I, II, III and IV)

The experimental set up was different in the studies but they were all based on a technique where grafts were interpositioned with cuff anastomoses to the common carotid artery (CCA).[94] Mice were anesthetized with inhalation of isoflurane during spontaneous breathing. The surgical procedure was performed under magnification using a dissecting microscope. A midline incision was made on the ventral side of the neck and the right CCA was exposed from its bifurcation to the thoracic aperture. The artery was cut at its mid portion and a cuff was externally placed around both ends. These ends were everted and anchored to the cuffs by ligation with 8-0 silk sutures. The vessel graft (inferior caval vein or the thoracic aorta) was harvested from a donor mouse and grafted

to the CCA in the recipient by sleeving its ends over the cuffs. The graft was held in place with additional 8-0 silk ligatures to the cuffs. (Figure 2)
This anastomose technique diverge from the procedure of bypass surgery in humans, in which veins are anastomosed with interrupted or continuous sutures. Consequently the vessel wall response in the anastomotic area in the mouse model may not be representative for the corresponding segment in human grafts. To avoid potential artefacts caused by the cuffs, mid-graft sections were consequently analyzed in the studies, except in study III in which a sewn end-to-end anastomose was assessed.

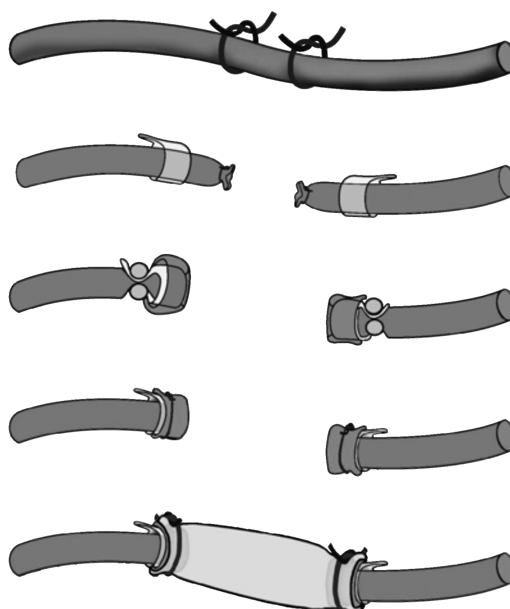

Figure 2.
Schematic representation of the surgical technique of interposition grafts to the common carotid artery.

Vein graft (Paper I, II and III)

Experiments on mouse vein grafts were obtained through a procedure where the inferior caval vein was harvested from a donor mouse, followed by implantation to a recipient animal. The donor mouse was anesthetized and the abdomen and thorax was opened. The inferior caval vein is easily accessible and it has no branches, which otherwise must be sealed before the grafting procedure. An alternative option is to use the ipsilateral jugular vein located next to the carotid artery.

Zou et al compared the ipsilateral jugular vein to the caval vein in the carotid position and could not find any difference in the morphology or in the inflammatory response.[94] The jugular vein is however more difficult to harvest.

As previously remarked, mouse vein walls are very thin and the vessel wall structure is limited to a few layers of cells and it has no distinct elastic laminae, which makes it difficult to define the media. These conditions imply that the morphological analyses of the subsequent vessel wall thickening must be interpreted with care. For example, the media layer is not distinguishable in histological sections of the arterialized mouse vein grafts, while it is present in human vein grafts. Thereby it is difficult to characterize the role of this layer in mice. However, towards the lumen a layer of α-actin positive cells establish when mouse veins are arterialized. The morphological features of this layer correspond well to the intimal thickening of vein graft stenoses in humans.

Composite graft anastomosed with interrupted sutures (Paper III)

An experimental setup with a composite graft was performed to investigate if SMCs migrated from the adjacent artery to the vein graft IH. Segments of SM22α-LacZ arteries and WT veins were anastomosed ex vivo. The vessel segments were anastomosed to each other with interrupted sutures forming the composite graft. This graft was then interpositioned to the carotid artery of WT animals (Figure 3). After six weeks the grafts were explanted and they underwent histological analyses including LacZ-staining of longitudinal sections, where SMCs with origin in the artery could be identified.

This experimental design had the ability to determine the significance of trans-anastomotic recruitment of neointimal SMCs in mouse vein grafts. The presence or absence of Lac-Z positive cells in the IH of the vein segment can clarify if there is a recruitment of arterial SMCs.

The weakness with this method is that the ex vivo procedure could have a negative impact on the arterial cells which could impair their migratory capacity. However, every anastomotic procedure demands separation of the artery from the perivascular tissue to some extent and this always results in discontinuity of vessel wall structures. These events are present in all vein graft models. Furthermore, we have morphologically analyzed multiple slides of the descending mouse aorta without finding any vasa vasorum (data not shown), which is supported by the literature.[95] Consequently the explantation should not have affected any nutrient supply of the vessel wall from the adventitia. Over all, we assume that the ex vivo procedure has no important impact on the final result.

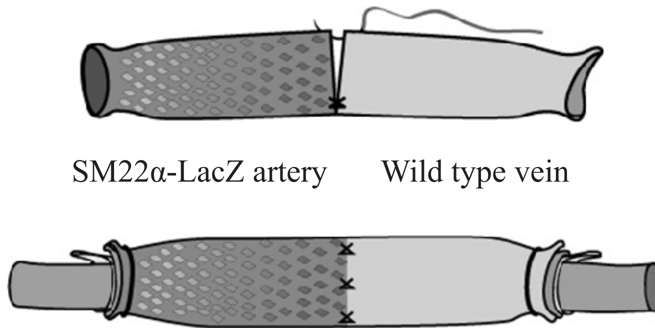

Figure 3. To investigate SMC migration across the anastomosis, an arterial segment from a SM22α-LacZ mouse was anastomosed to a vein from a wild type mouse ex vivo before grafting.

Decellularized grafts (paper II, IV)

Decellularized vessels were used in the mouse model to investigate the impact of different cellular sources on vessel wall remodeling. In paper II, we explored how the biology of the vessel wall thickening in vein grafts was affected, if all the vein-derived cells were excluded. In paper IV, decellularized arterial grafts were used, to allow exclusive recruitment of vessel wall extrinsic cells. This was performed to test the ability of these cells to restore morphology and function of the vascular wall.

Methods of removing the cellular population by decellularizing treatment and leaving the ECM unaffected have been utilized in research in connection to tissue engineering. Several studies report reestablishment of the cellular populations in acellular tissues from different organs.[96, 97] Mouse veins or arteries were first treated with 0.1 % sodium azide for 6 h to allow cell lysis followed by treatment with for 6 h to digest the DNA. Finally the vessels were treated with 4 % sodium desoxycholate for 6 h to extract the cellular membranes.

The vessels were then washed and kept in saline supplemented with heparine (250 U/mL), until surgery. For every set of decellularizing treatment, specimens were collected for histological analyses with Hematoxylin-Eosin (H&E) to confirm that all cells were successfully abolished. The treatment might have affected the structural integrity of the remaining ECM, which could impair the

possibility for cells to establish within the vessel wall. However, bladders in a canine model regain normal histological patterns after the same decellularizing treatment.[98] Further, microscopic analyses of the elastic fibers after van Giesson staining were performed and we could not detect any impact on the elastin framework. However, minor molecular changes of the matrix cannot be excluded.

Blockage of trans-adventitial migration (paper II)

To exclude external recruitment of cells through the vessel wall, vein grafts were shielded from their surroundings by a very thin tube shaped of polyethene film, which was impermeable for cells and fluids. The plastic tube was placed outside the vein during the grafting procedure and it was anchored to the anastomotic cuffs with additional silk ligatures.

There is a possibility that the polyethene film per se had an impact on the neointimal formation. The synthetic material might interfere with the inflammatory system and induce a reaction against the foreign material. Furthermore we cannot exclude that the external shielding interfered with the hemodynamic forces acting on the vessel wall. External support, surrounding vein grafts, has previously been shown to inhibit IH. [99, 100] However, if any impact due to the foreign material was present it did not influence the degree of IH in our study since there was no difference between vein grafts which were shielded or not.

Vein graft flow modulation (paper I)

The impact of blood flow on the development of IH in mouse vein grafts was explored with a new experimental set up. This was accomplished by adapting techniques from animal models in which the blood flow through the carotid artery can be regulated and then combine these methods with the mouse vein graft model.[101, 102] The animals were randomized either to a high flow-group (HF) or to a low-flow group (LF). In the HF-group the contralateral left CCA was ligated which resulted in an increased blood flow through the graft interpositioned to the right CCA. In the LF-group the flow was diminished by ligation of the ipsilateral right external carotid artery (Figure 4).

Only the volume flow was monitored in this model, the actual vessel wall shear stress was unknown. However, as volume flow is one of the determining variables for shear stress one can assume that different levels of shear stress was present in the two groups.

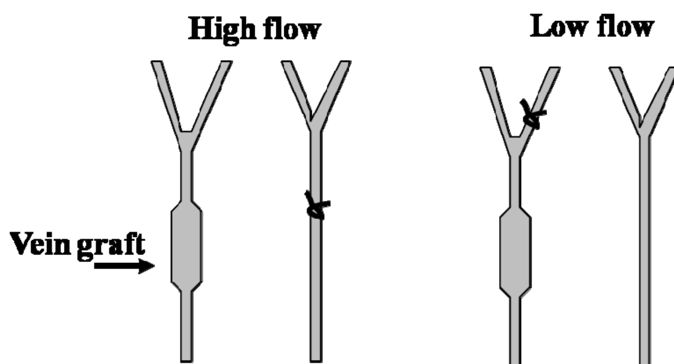

Figure 4. Schematic picture of the blood flow modulation.

Blood flow measurement (paper I)

Transit time ultrasound was used for measurements of vein graft blood flow. Mean blood flow was monitored before and after flow modulation and once again at the time of euthanization. Reliable flow registration with this technique is dependent on correct size and positioning of the probe. Our impression was that the diameter of the different grafts did not differ, although we did not measure the diameter of the implanted vein grafts. The probe was always positioned with great care and with the same angle to the graft in all animals to achieve adequate registrations.

Myograph analyses (paper IV)

Vasomotor function of arterial grafts and control vessels was investigated in myographs customized for micro vessels (Danish Myotechnology, Aarhus, Denmark). Segments of 2 mm length were positioned onto two stainless steel hooks, suspended in microvascular myograph baths for measurement of isometric tension as previously described.[103] The myographs were temperature controlled (37°C) and contained physiological salt solution (PSS) of the following composition (mM): NaCl 119, NaHCO3 25, glucose 5.5, KCl 4.7, CaCl2 2.5, KH2PO4 1.18, MgSO4 1.17, and ethylenediaminetetraacetic acid (EDTA) 0.026. KPSS was similar to PSS except that NaCl was exchanged with KCl on an equimolar basis. Solutions were equilibrated with 5% CO2 in O2 to maintain a pH of 7.4. The vessels were allowed to stabilize for 60 min. The isometric wall tension was recorded at well-defined internal circumferences. The contractile ability of the vessels was tested by stimulating with 124 mM KPSS until reproducible responses were obtained, i.e. when the active developed contraction was within 10% of the previous contraction to KPSS. In almost all

experiments this was reached with a third stimulation to KPSS. Contractile capacity was further tested with dose-response curves using noradrenaline (NA) After the NA-induced contraction had reached a plateau level, the potential of the vessels to dilate was tested. Acetylcholine (ACH) was used and added in a cumulative manner for this latter observation.

Histological techniques

Tissue preparation

At euthanization the mice were anesthetized and the grafts were explored through a neck incision in the mid line. The animals were exsanguinated, which was initiated through a cut in the abdominal caval vein. Simultaneously a perfusion with saline solution through cannulation of the heart was started and continued until the effluent was clear. For paraffin sections the mice were further perfused with 4% formalin and post-fixed in the same solution for 4-6 h. This was followed by dehydration and embedding in paraffin. For frozen sections, specimens were harvested after saline perfusion and mounted in O.C.T compound (Sakura Finetek, Netherlands), snap frozen in liquid nitrogen and stored in -70°C.

Transverse midgraft sections of paraffin embedded specimens were cut onto slides (5 µm) and frozen specimens were either transversely or longitudinally cut onto slides (8 µm) for further histological analyses.
Sections were routinely stained with (H&E) and when analyses of elastic fibers were needed sections underwent Van Giesson staining.

Immunohistochemistry (paper I, II, III, IV)

The morphological analyses were enhanced by identification of different cell types with immunohistochemistry. Briefly, sections were pretreated to uncover hidden antigens. Endogenous peroxidase activity was eliminated by pretreatment of the tissue sections with hydrogen peroxide prior to incubation with the primary antibody. To prevent non-specific background staining due to hydrophobic and electrostatic forces a blockage with serum or milk powder solution was applied.

Antibody labeling was performed with the indirect method, in which unlabeled primary antibodies react with the tissue antigen, followed by labeled secondary antibodies which attach to the primary antibodies. For detection of α-actin, the

sections were labeled with a mouse anti-human actin monoclonal antibody. A rat anti-mouse CD18 polyclonal antibody was used to detect leukocytes.
The secondary antibodies were either biotinylated or labeled with horseradish peroxidase (HRP). A third incubation with streptavidin-HRP or streptavidin-alkali phosphatase was performed on biotinylated antibodies. Sections were developed with diaminobenzidine or fast red to visualize the secondary antibodies.

To assure that the immunohistochemical protocol could detect the target antigen positive controls were included in the tests. As positive controls, mouse tissue with known cellular composition was used. Negative controls were performed to test the specificity of the antibody involved, intending to exclude unspecific binding. This was accomplished through omission of the primary antibody or replacement by a non-immune antibody.

LacZ staining (paper II, III, IV)

The bacterial lacZ gene, encoding the enzyme ß-galactosidase (ß-gal), is incorporated as a reporter gene in the genetically modified ROSA26 and SM22α-LacZ mouse strains. Tissues from these mice and wild type mice were assembled in special combinations in the different experimental setups. Identification of lacZ containing cells enabled us to determine cellular migrating pathways.

The enzyme was identified in cryosections by the substrate X-gal which resulted in a visible blue precipitate. First the tissue sections were fixed in formaldehyde supplemented with glutaraldehyd. The slides were rinsed in phosphate-buffered saline (PBS) and stained at 37°C overnight in X-gal solution. The specimens were thereafter rinsed and washed in PBS supplemented with Tween.

ß-galactosidase can endogenously be expressed in tissues which could result in non-specific background LacZ positivity.[104] Background staining was reduced by optimizing the pH of the phosphate buffers to assure that false identification of cells did not occur.

Blood vessel from ROSA26 and SM22α-LacZ mice were used as positive controls and vessels from WT animals were negative controls.
For double staining against actin filaments the X-gal treatment was followed by the immunohistochemical protocol for α-actin staining.

Morphometric analyses and cell counting (paper I, II, IV)

In paper I the morphometric analyses included measurement of lumen area, total vessel wall area, and neointimal area. Sections stained with H&E and with antibodies against α-actin were photographed in a microscope, followed by calculations of the areas in Kontron Electronic image analyzing system (KS 400version 2.0; Carl Zeiss). The areas in different groups were compared.

In paper II the extension of the IH was measured by cell counting. α-actin positive cells with positive staining of nuclei were counted in the different groups. In paper III the α-actin positive cells and the total number of cells within the arterial media were counted to assess the cellular repopulation.

Proliferation assay with BrdU (paper I, II)

Cellular proliferation was assessed with the method of bromdeoxyuridine (BrdU) labeling of replicating cells. The thymidine analog BrdU will after injection incorporate into newly synthesized DNA strands of actively proliferating cells which allow these cells to be identified in later histological analyses.

The mice were injected with BrdU up to 24 h prior to euthanazation. BrdU-incorporated cells were detected with immunohistochemistry by using a cell proliferation kit (Amersham Biosciences). The sections were nuclear stained with hematoxylin and BrdU positive and negative cells were counted. An index of proliferating cells was accomplished as a percentage of BrdU-positive cells in relation to all cells.

Statistics

The main aim of the statistical analyses in this thesis was to compare differences between different groups. As in many experimental studies the sample sizes were small and hence the observed variable values cannot be assumed to be normally distributed. Therefore non-parametric tests were used throughout the thesis. The Mann-Whittney-U test was used for comparisons of independent groups and the Wilcoxon signed rank test was used for paired observations. Kruskal-Wallis test was used for comparison of more than two independent groups.

SUMMARY OF RESULTS

Paper I

In this study the morphology in mouse vein grafts was analyzed and the impact of blood flow on the development of IH was investigated. Vein grafts interpositioned to the right CCA, exposed to either HF or LF, were harvested after 3, 14 and 42 days. Flow modulation resulted in a sustained change of blood flow in the two groups. The flow in the HF group was 2.7 times higher than in the LF group at euthanization.

A massive infiltration of leukocytes into the perivascular tissue was revealed three days after surgery with antibodies against CD18. Simultaneously there was an almost total loss of cells in the vessel wall including α-actin positive cells.

On day 14 the vessel wall had regained its cellular content and the thickness had increased without any significant difference between the groups. SMCs, demonstrated by positive α-actin staining, were found in the vessel wall towards the lumen.

On day 42 there was a further increase in the wall thickness. At this timepoint there was a defined vessel wall layer with α-actin positive cells towards the lumen, which was defined as IH. Morphometric analyses of the area revealed that there was a significant difference between the two groups. The neointimal area (α-actin positive) in LF animals was 74 % larger compared to HF animals, which demonstrates that IH in mouse vein grafts is regulated by the magnitude of flow.

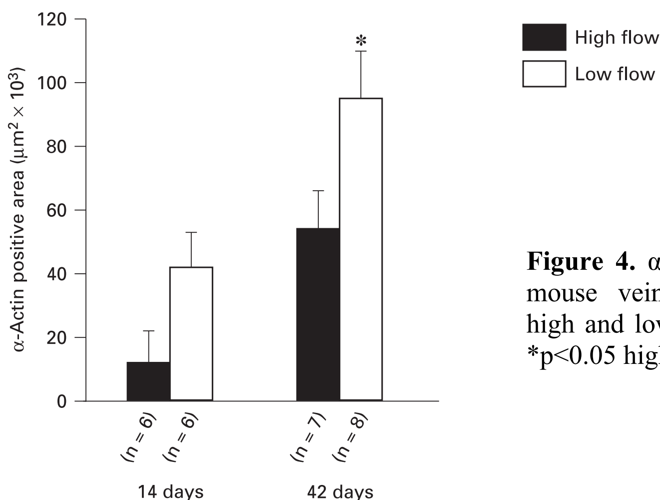

Figure 4. α-actin positive area in mouse vein grafts subjected to high and low flow. Mean ± SEM, *p<0.05 high flow vs low flow.

Paper II

The dynamics of SMC recruitment to IH in vein grafts was investigated in the mouse model. First, the contribution of SMC with origin outside the vessel wall was determined. Veins from WT animals were implanted into ROSA26 mice (n=6) for six weeks. Vein grafts were harvested and analyzed regarding the degree of IH and the origin of the neoitimal cells. The contribution from the recipient animals was at an average of 32 % but with a wide range (0.2% to 82%). The extent of IH, measured as total number of neointimal cells, was not influenced by the degree of recipient contribution. To examine if recipient derived cells was recruited through transadventitial migration, a group of WT grafts were implanted into ROSA26 mice (n=5) and shielded from the surrounding tissue by a polyethene film. With this setup the recipient contribution decreased to only 1.3% while the total number of intimal SMCs did not decrease.

To examine the role of the recipient derived (graft extrinsic) cells the cellular contribution from the vein itself was totally excluded through decellularizing treatment of the grafts before implantation. Control animals received ordinary cellular grafts and both groups were sacrificed after four weeks. Morphological analysis showed that the extent of neointimal formation was similar in the two groups. When acellular veins in addition received external shielding (n=4) and thereby excluding contribution from the vein wall and transadventitial migration, the numbers of intimal SMCs was reduced by almost 90%.

Together, this demonstrates that neontimal SMCs can be recruited from different sources with compensatory capacity. The extent of IH was not affected until the vein-derived cells were excluded together with simultaneous blockage of transadventitial recruitment.

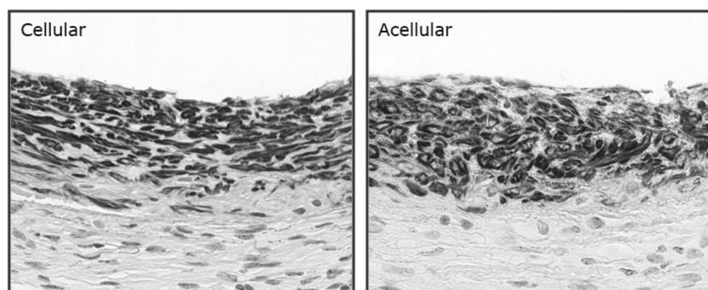

Figure 5. Representative sections from cellular and acellular vein grafts harvested after 28 days, demonstrating similar amount of intimal hyperplasia (α-actin positive area, dark cells).

Paper III

In this study the exclusive contribution of SMCs from the adjacent artery to the IH of vein grafts was investigated. Veins from WT mice were anastomosed to arteries from SM22α-LacZ mice ex vivo and then implanted as interposition grafts into WT animals. After six weeks the grafts were explanted (n=7) and were histologically analyzed.

In longitudinal sections, where the anastomose was identified, the morphology of the vein graft as well as the arterial wall was examined. Immunohistochemical staining with antibodies against α-actin demonstrated a several cell layers thick IH containing SMC in both the vein graft and the artery. Lac-Z staining revealed that the SMCs in the vein graft neointima did not have their origin in the arterial segment. However, when a composite (artery/vein) graft from WT animals was implanted into a SM22α-LacZ animal, the vein graft neointimal SMCs was positive for Lac-Z staining, which indicates that these cells were recruited from the recipient animal. In addition it proves that the neointimal cells have the ability to express SM22α.

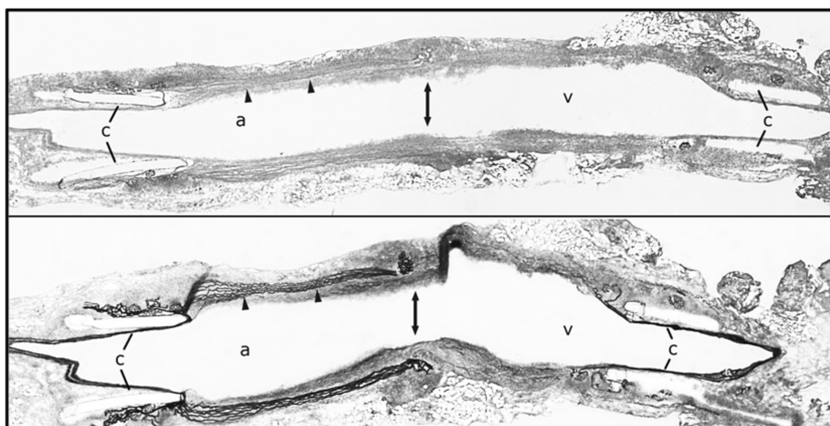

Figure 6. Composite graft morphology 42 days after surgery. a = arterial segment, v = vein segment, c = cuff anastomoses to the common carotid artery. Double arrows indicate the anastomotic lines between the arterial and vein segments. Arrow heads indicate elastic fibers in the arterial media, which are further enhanced by van Giesson staining (dark) in the lower picture.

Paper IV

In paper IV, the ability of externally recruited SMC progenitors to restore arterial morphology and function was explored in a mouse model. Acellular mouse aortas were interpositioned to the CCA in WT mice in order to exclusively analyze externally recruited SMCs. The grafts were harvested after 28 days (n=7) and 90 days (n=5). In one control group normal cellular aortas were grafted and harvested after 28 days (n=5). This control group was used to distinguish potential effects from the grafting procedure per se. In another control group non-implanted aortas were used (n=10). This group served as control for normal morphology and vasomotor function. The explanted grafts and non-implanted aortas were split; one segment underwent histological analyses, while the other segment was taken for examination of the vasomotor function.

At day 28 the morphology of cellular grafts was mainly unchanged compared to non-implanted aortas and they had almost normal vasomotor function, demonstrating both constrictive and dilatory capacity. Conversely, the acellular grafts had a different arterial wall structure in which the media was sparsely repopulated with α-actin negative cells. Towards the lumen a several cell-layers thick IH was formed. The majority of the cells were α-actin positive. The acellular grafts did not show any constrictive or dilatory function.

The observation time was extended to 90 days for acellular grafts, but the result was similar compared to 28 days, regarding both morphology and vasomotor response.

In summary we have shown that externally recruited progenitor cells have a limited ability to restore morphology and function in the decellularized arterial wall in mice.

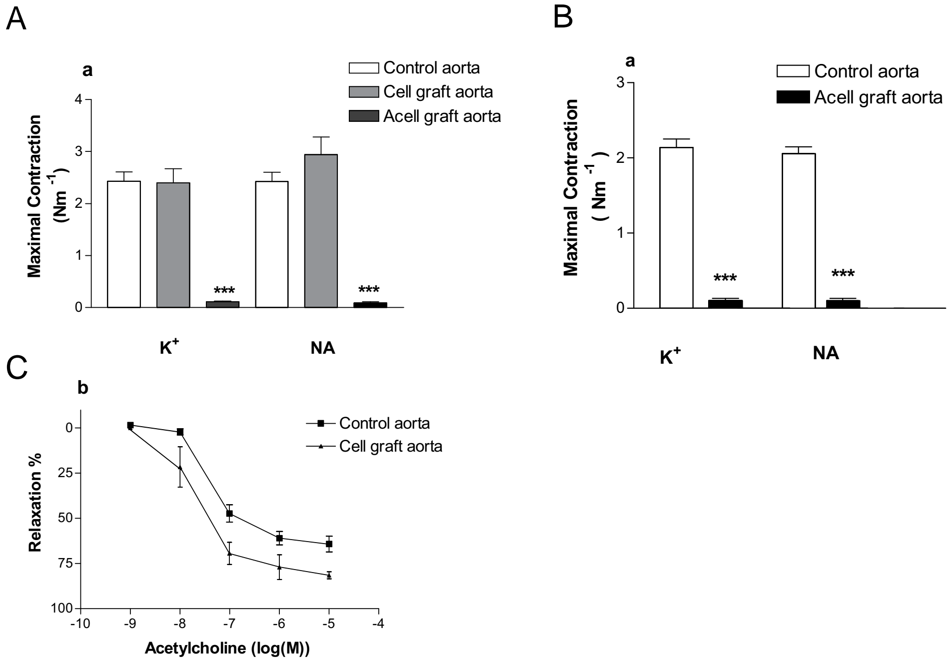

Figure 6. Result of the myograph analyses. A, Maximal contraction of control aortas, cellular grafts and acellular grafts at 28 days B, Maximal contraction of control aortas and acellular grafts at 90 days. C, Relaxation of control aortas and cellular grafts at 28 days. (***p <0.01 vs control aorta)

DISCUSSION

Vein grafts have been extensively used in bypass surgery for treatment of coronary artery disease and lower limb ischemia since the technique became available in the second half of the 20th century. The method has constantly been connected with a relative high frequency of graft failures. Great efforts have been done to improve the technique in order to reduce flow-restricting stenoses. Recent studies still demonstrate that the primary patency of vein grafts is not better than 60-80 % within the first year.[26, 28] The histological pattern of vein graft stenoses show similarities with restenoses and in-stent stenoses appearing after endovascular interventions. Morphologically the lesions are mainly composed of IH. SMC accumulation and matrix synthesis by these cells are key events in the formation of IH. Throughout the last decades numerous studies have tried to elucidate the pathogenesis of neointimal formation, but the molecular mechanism of IH is largely unknown. Consequently, there are no effective therapies to prevent vein graft failure.

Animal models have been widely used to study the biology behind IH and models of venous bypass grafts have been described in dogs, pigs, sheep, rabbits and rats.[52, 68, 105-107] Studies in these models have been used to describe the morphological changes appearing in vein grafts becoming arterialized and to investigate the associations between expressions of different molecular mediators and cellular events. In addition pharmacological interventions have been performed to study the role of different cellular mediators. Recent advances in gene-manipulating techniques have provided genetically modified mice models which have reached increased application in basic vascular research. The first vein graft model in mouse was presented by Zou et al and has been followed by an additional two.[94, 108, 109] Since the bypass models in mice became available the effect of different genes on vein graft neointimal formations can be studied. Another important advancement is that the genetically modified mice can harbor reporter genes which enable studies to identify the origin of cells that contribute to tissue regeneration.[93] Mice with identifiable cells can be used together with bone marrow transplantation or vascular grafting procedures. Results from such animals have been crucial for the extended and present view upon recruitment pathways of neointimal SMCs.

Flow rate and intimal hyperplasia

Vein grafts are subjected to changes by the hemodynamic factors after implantation to the arterial circulation. Progressive thickening of the vessel wall, including general IH and media thickening throughout the graft, is supposed to be an adaptive response to the forces exerted by the arterial circulation, mainly

due to increased wall tension. However, the cause of localized lesions of extensive neointimal formation leading to vein graft stenoses remains unexplained. Injuries to the vessel wall in connection to the dissection of the vein or the anastomotic procedure have been suggested. Another possibility is that the stenoses are caused by localized spots of disrupted flow patterns. It is well known that the vessel wall biology is influenced by the degree of shear stress; areas of low shear stress and turbulent flow are prone to develop IH. If a segment of the vein graft is influenced by low shear stress constantly it may lead to extensive neointimal formation in that segment. Models in dogs and rabbits have been used so far to study vein graft IH related to shear stress.[68, 110, 111] There are few models in mice reflecting the vessel wall biology related to different magnitude of blood flow. Kumar and Lindner presented a model where the CCA was ligated just proximal to the bifurcation resulting in a cessation of blood flow and extensive neointimal formation.[112] Even if they reported that pulsations were present at all times and the ligated vessels did not contain any major thrombus formation, this model is not optimal to investigate the influence of shear stress since it corresponds to a no-flow situation and not to a vessel with reduced flow. A mouse model of reduced flow in the CCA has been developed by Rudic et al.[101] They demonstrated a reduction in the vessel diameter, but without neointimal formation. No mouse model covers the biology of shear stress and vein graft stenosis.

In this thesis a new model that enables studies of the influence of blood flow on neointimal formation in mouse vein grafts is presented. The development of IH in mouse vein grafts seems to be regulated by the magnitude of blood flow as demonstrated in other species. This is in accordance with vein grafts in humans.[44] Recent studies, which performed high-resolution shear stress analysis of vein graft anastomoses, demonstrate low shear stress in areas prone to IH. [71, 113]The present vein graft model enables the study of shear stress and its effects on vascular biology in genetically modified animals.

Cellular renewal

Since the intima normally only contains single SMCs, recruitment of these cells to this location is a prerequisite for neointimal formation. Previous research has resulted in a strong opinion that intimal SMCs are derived from the underlying vessel wall media. If this were true it would be expected that the neointimal cells in the vein graft originate from the local vein wall. Our results challenge this conclusion in conformity with other studies in mice.[88, 89]

In this thesis early loss of cells in the vein grafts after 3 days was noted, which is in accordance with previous observations in the mouse and rat.[94, 114, 115] In

non-implanted veins α-actin positive cells positioned in the vessel wall was identified. When the same analysis was performed with grafts harvested after 3 days, no such cells could be detected within the graft vessel wall, which indicates a loss of these cells or a change of their phenotype. We assume that these cells disappeared, since hardly any cells at all could be found. This assumption is supported by a study by Mayer et al which demonstrated that an early apoptosis is induced in mouse vein grafts.[114] This phenomenon also seems to appear in humans. Loss of medial SMCs has been reported in a study of human vein grafts explanted within ten days after the bypass operation.[24] The question of the origin of the cells seen at later time points arises if the SMC in the graft vessel wall disappear.

Experiments with acellular grafts were performed in this thesis to determine if IH can be formed without contribution from the vessel wall. Four weeks after implantation acellular grafts contained a defined neointima with α-actin expressing cells. The IH of acellular vein grafts did not differ from cellular control grafts in regard to histological patterns and the extent of IH. The result from this experiment proves that cells, which are not derived from the local vessel wall, can contribute to neointimal formation in vein grafts.

Studies have been performed in genetically modified mice models harboring reporter genes to elucidate if there is a contribution of cells from a remote origin to the IH in normal veins. The first evidence of external contribution of neointimal SMCs of vein graft was provided by Hu et al who implanted veins from wild type donor mice to ROSA26 recipient mice which express LacZ in all tissue. The authors found that approximately 40 % of the neointimal SMCs had a recipient origin.[88] The contribution from the recipient animal to the vein graft IH has been investigated in two other mice studies. Zhang et al demonstrate that 60 % of the neointimal SMC population had a recipient origin.[89] The present thesis provides results which is in line with these studies. We implanted wild type vein grafts into ROSA26 mice and analyzed the origin. We found a great inter-individual variation in the amount of recipient derived SMCs, but with no effect on the extent of the neointimal formation. The mean recipient contribution was 32%.

In contrast to our and previous studies Coley report that vein graft neointimal cells arise predominantly from vein-derived cells.[108] The opposite results may be due to different experimental setups. Cooley grafted a smaller diameter vein to the femoral artery while the other studies are conducted with the caval vein inter-positioned to the carotid artery.

Our results show that cells both from the vein and recipient animal can contribute to the SMC population of IH without affecting the extent of it. This

indicates that different sources can compensate each other. It can be assumed that, regardless of the origin, the cells which accumulate into the intima respond to molecular factors and proliferate until the IH reaches a determined level.

Although we did not study renewal of the endothelium, this issue has been explored in the mouse vein graft model and should be mentioned in this context. Xu et al report that a large number of endothelial cells in mouse vein grafts was lost within the first few days and that the endothelium was regenerated by circulating progenitor cells. This result underlines the possibility that repair of the vessel wall can occur through recruitment of progenitor cells.[116]

We also demonstrate that medial SMCs in the arterial segments anastomosed to veins, did not contribute to the neointimal formation. This indicates that also the arterial IH has a contribution from other sources than the local vessel wall.

Recruitment pathways and cellular sources

There is evidence that other sources of SMCs than the medial cell population contribute to intimal thickening in vascular pathology. The origin and migrating pathways of these cells have not yet been established. Fundamentally these cells could either be recruited from the circulation, from locally derived cells in the surrounding tissue or from the adjacent artery.

This thesis shows that shielded vein grafts had significantly less SMCs of graft external origin compared to non-shielded grafts. This finding points towards transadventitial migration to be an important recruitment pathway in mice. The origin of these cells was not determined. During explantations procedures it was macroscopically evident that adherent healing tissue covered vein grafts and there is a possibility that cells from this tissue contributed to the formation of IH in non-shielded grafts. This hypothesis is supported by a study, conducted in a porcine vein graft model, which has demonstrated that perivascular myofibrobasts can contribute to the neointimal formation.[117] Another potential cellular source in the perivascular tissue are residing progenitor cells.

It cannot be excluded that the external shielding blocked migration of circulating progenitor cells since the experimental setup cannot differentiate recruitment from blood-borne cells that enter the graft via microvessels within the adherent healing tissue from cells with a true perivascular origin.

Circulating progenitor cells have been suggested to contribute to neointimal formation and cells with potential to differentiate to SMCs have been found in human blood, although their origin is unknown.[118]

Regarding the source of circulating progenitors, there has been a focus on hematopoietic derived cells. Intimal smooth muscle cells seem to be derived from donor bone marrow to a certain extent. This was demonstrated in a study of human atherosclerotic plaques in sex-mismatched bone marrow transplant subjects.[119] Mice models, including transplantation of bone marrow from sex-mismatched animals or animals with reporter genes, have been used to determine if progenitors of hematopoietic origin contribute to IH. Data obtained from arterial injury models indicate that a proportion of the SMC population of the neointimal formation have hematopoietic origin.[86, 87, 90] In mouse studies, which investigated the contribution of bone marrow derived cells to vein graft IH, this cellular source could not be confirmed.[88, 89]

Circulating progenitors may also originate from other tissues or remote vessel segments.[120, 121] Neointimal formation in mouse grafts can be caused by accumulation of such cells. Migration of cells from the adjacent artery is another potential recruitment pathway for neointimal SMCs in vascular grafts. These cells could originate from the SMCs in the media of the artery or from residing progenitors in the arterial wall. Hu et al demonstrated that progenitors are present in the adventitia of mouse artries.[121]

In the present thesis it was observed that vein graft intimal SMCs are not recruited from the artery. Our result contradict a previous study by Dilley et al, where they report, in a rat model, that SMCs in the arterial media proliferate and migrate across the anastomosis into the expanding vein graft intima.[105] Different recruitment pathways in mice and rats could explain the contradictory conclusions. We consider, however, that their experimental set up does not regard if SMCs are recruited from the adjacent artery or a pool of circulating progenitors.

The adjacent artery is discussed as a potential contributor of SMCs in nearly all publications using a grafting procedure in mice. Consequently we can assume that neointimal SMCs in vein grafts, which do not originate from the local vein wall, are recruited from circulating cells or through transadventitial migration.
The implication for human vein grafts is that the SMC population in stenoses located at the anastomotic region might have the same recruitment pathways as those located apart from the anastomoses.

Progenitor cells

Little is known about the significance of SMCs originating outside the vessel wall and their importance for future morphology and function. It is still unclear if these cells are SMC progenitors or if they are only reparative cells, which adopt SMC-like characteristics. In consequence of our results and others, one aim of this thesis was to explore the ability of these cells to regenerate vessel wall morphology and function. As mentioned above SMCs with external origin are interspersed with cells derived from the local vessel wall. This detail makes it difficult to specifically analyze externally recruited cells. By implantation of acellular arterial grafts, which assign progenitor cells to replace the cellular population of the graft, we could exclusively analyze these cells. Arterial grafts were chosen because arteries from mice are known to have a significant vasomotor function.[122]

The acellular grafts became repopulated by cells but without regaining their original anatomical features. Our results demonstrate that there is a contribution of SMCs (α-actin positive cells) to the vessel wall. These cells were mainly found in the neointimal formation. The media was sparsely repopulated with cells, the vast majority being α-actin negative.

The analyses of the vasomotor function corresponded well to the histology of the media. The repopulated grafts did not have any response to vasoactive agents compared to cellular controls. Our study demonstrates that progenitor cells in mice have the ability to contribute to the neointimal population of non-contractile SMCs but cannot restore the medial population of contractile SMCs. Even if we cannot exclude that the capacity to regenerate the medial SMCs in this study is affected by the artificial experimental set up, other studies support the finding. Similar morphological results are provided by studies of transplant arteriopathy in mouse, demonstrating a disappearance of medial SMCs in allografted aortas due to an immune reaction. In those studies recipient derived cells contributed to the IH but did not regenerate the medial SMC population.[83-85] In contrast to our study, Kaushal et al demonstrated in a sheep model that decellularized arteries seeded with extracted endothelial progenitor cells regain arterial characteristics including vasomotor responsiveness. Perhaps those progenitor cells are more prone to regenerate even the SMC populations in the vessel wall compared to spontaneously recruited progenitors.[123]

One explanation to our results is that SMC progenitors infiltrate the intima and media but the environment has been manipulated to such an extent that these cells are inhibited to differentiate into contractile SMCs. Another explanation is

that the repopulating cells are another kind of progenitors which only have the ability to express the α-actin protein without being true SMCs. We believe the latter explanation to be more probable, since the phenomenon is also present in mouse models of arterial injuries and transplantation vasculopathy where environmental alterations are assumed to be less. We suggest that there is a pool of mouse progenitor cells which are involved in vascular healing. These cells may contribute to the populations of lesser differentiated neointimal cells but cannot contribute to a population of differentiated contractile medial SMCs.

CONCLUSIONS

- The degree of intimal hyperplasia in mouse vein grafts is regulated by the magnitude of blood flow.

- Neointimal smooth muscle cells in mouse vein grafts can be recruited through transadventitial migration from external sources but not from the adjacent artery.

- Smooth muscle cells recruited exclusively from sources outside the vascular graft can form intimal hyperplasia. Externally recruited cells have a limited ability to regenerate normal morphology and function of the vessel wall.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Åderförkalkningssjukdomen är den enskilt vanligaste orsaken till hjärt-kärlsjukdomar. Sjukdomen kan orsaka förträngningar eller stopp i pulsådror (artärer). Detta kan leda till akut eller kronisk reduktion av blodflödet vilket ger upphov till syrebrist i målorganet. Blodkärl som ofta drabbas av åderförkalkning är hjärtats kranskärl och benens artärer. Kärlförträngningar i hjärtat kan leda till kärlkramp eller hjärtinfarkt medan förträngningar i benens kärl ger fönstertittarsjuka eller i värsta fall kallbrand. För patienter med uttalade kärlförändringar i hjärtat och benen är ofta bypass-operation nödvändig.

Operationen syftar till att leda blodet förbi det sjuka kärlavsnittet via det inopererade bypasskärlet. Vanligen används vener, tagna från patienten själv som bypass-kärl. Tyvärr är långtidsresultaten otillfredsställande då 20-40 % av bypasserna slutar att fungera inom något år. Den vanligaste orsaken till detta är att nya förträngningar bildas i själva bypass-kärlet till följd av överdriven bildning av läkningsvävnad på kärlets insida. Man vet idag relativt väl hur dessa förträngningar är uppbyggda, men de bakomliggande cellulära mekanismerna är till stora delar okända.

Avhandlingens mål var att kartlägga några av dess mekanismer, och därmed bidra till ökad kunskap, vilket i framtiden kan leda till att bypass-förträngningar kan förebyggas och behandlas.

Bypass-kärl implanterades i möss och blodflödet manipulerades och därefter mättes omfattningen på läkningsvävnaden. Försöket visade att blodflödets storlek reglerade läkningsvävnadens tjocklek, vilket stöder uppfattningen att förträngningar i bypass-kärl kan orsakas av lokala störningar i blodflödet, såsom turbulens.

Genom att implantera bypass-kärl, från normala möss, till mottagardjur av en genmodifierad musstam med identifierbara celler, har vi visat att ca 30 % av cellerna i bypass-kärlens läkningsvävnad har ett ursprung utanför kärlväggen. En ansenlig del av dessa celler tycks vandra in genom blodkärlets utsida. Vidare undersöktes specifikt om läkningsvävnadens celler härrör från artären som bypass-kärlet är kopplat till, vilket har diskuterats som en möjlighet i flera tidigare studier. Genom att foga samman en bit artär, från en mus vars celler kan identifieras, med en normal ven kunde vi visa att denna migrationsväg för celler i läkningsvävnaden inte är av betydelse.

Genom att implantera vener, som förbehandlats för att bli cellfria, har vi visat att utifrån kommande celler hade möjlighet att självständigt bygga upp den typiska läkningsvävnaden. I ytterligare ett försök testades om de utifrån kommande

cellerna kunde ersätta artärers muskelceller, vad gäller kärlväggens struktur och muskelfunktion. Cellfria artärer som implanterades som bypass-kärl fick ett tjockt lager av läkningsvävnad, medan det vägglager som normalt innehåller muskelceller förblev relativt cellfritt. Kärlen återfick inte sin normala muskelfunktion. Detta indikerar att, även om celler i läkningsvävnaden har likheter med kärlväggens muskelceller, så kan utifrån rekryterade celler vara av en annan celltyp.

Sammanfattningsvis har denna avhandling visat att bildningen av läkningsvävnaden i musvener regleras av blodflödets storlek. Cellerna i läningsvävnaden har flera källor och kan migrera in via bypass-kärlets utsida men verkar inte komma ifrån den sammankopplade artären. Celler med ursprung utanför bypass-kärlet har förmåga att bidra till läkningsvävnaden, men verkar ha begränsad förmåga att ersätta kärlväggens normala muskelceller vad gäller struktur och funktion.

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