Inflammation in injury-induced vascular remodelling : functional involvement and therapeutical options

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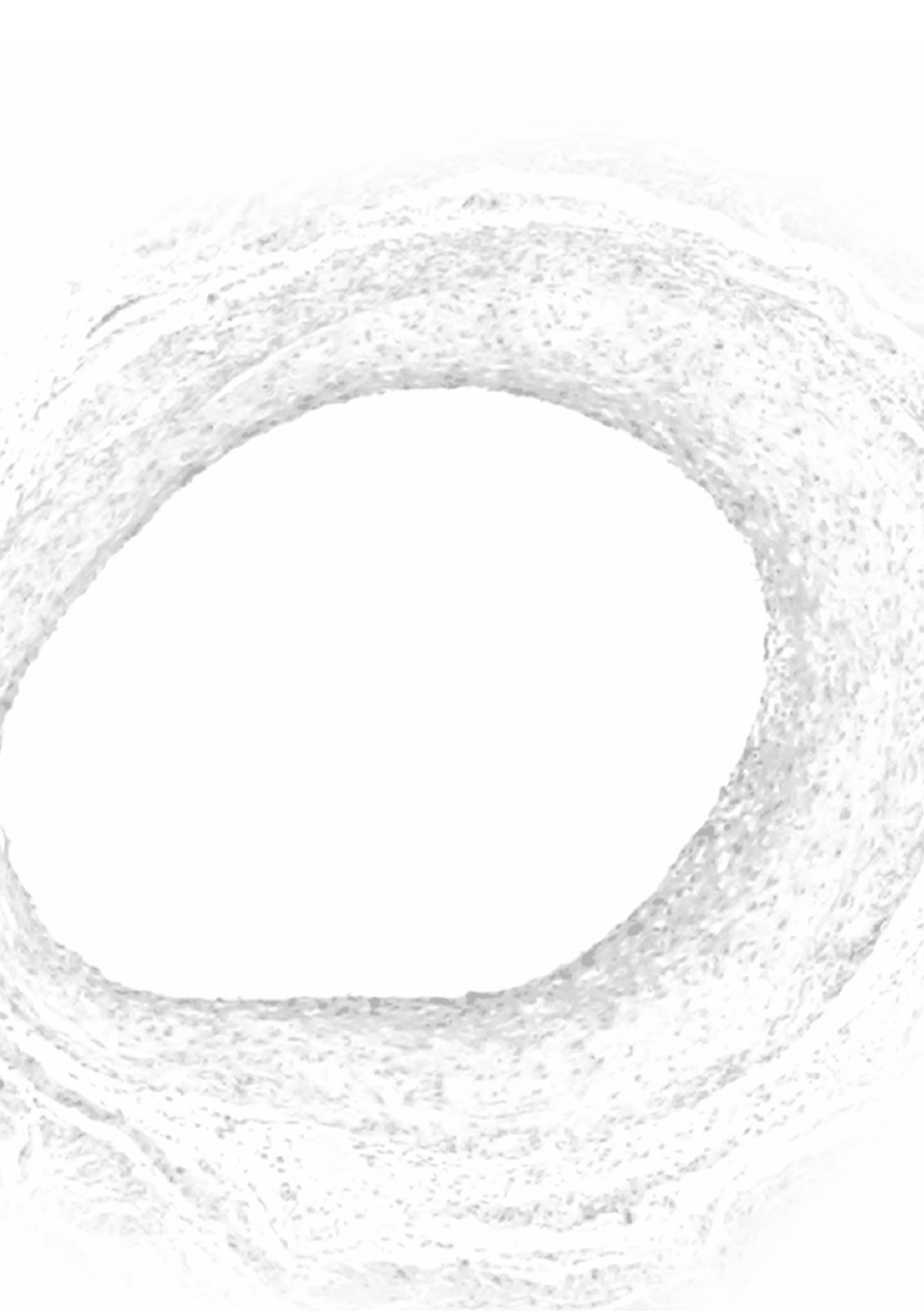

CHAPTER 1

General Introduction

1.1 ATHEROSCLEROSIS; DEMOGRAPHICS AND CLINICAL PROBLEM

Atherosclerosis is one of the major causes of morbidity and death in the Western World. The disease is characterized by narrowing of arteries due to development of so-called plaques, consisting of accumulations of lipids and fibrous elements in the vessel wall. Plaques are typically located in median and large arteries. Symptoms are usually related to type of the vessel involved and the resulting end-organ ischemia (varying from stroke, myocardial infarction, to intermittent claudicating and lower limb gangrene).

Complications and symptoms of atherosclerosis usually occur in the elderly, but early atheorsclerotic lesions, (fatty-streaks) can be detected already in the second and third decade and even at an earlier age. Fatty streaks consist largely of macrophages and T-lymphocytes and can develop to more advanced lesions in which lipid depositions and smooth muscle cell proliferation become more evident. An advanced atherosclerotic plaque is typically build up from a lipid core which often incorporates necrotic debris from apoptotic foam cells, a fibrous cap consisting of three fibroblasts and smooth muscle cells and "shoulders" containing various inflammatory cells[1].

It has been recognized that the origin of this process is inflammation and the role of the inflammatory processes in the vessel wall became more evident. The publication of the article of R. Ross definitively highlighted the role of inflammation and the contribution of the immune system as one of the main players in atherosclerosis[2].

Today's therapies, in order to reduce atherosclerosis-related morbidity by relieving end-organ ischemia, can be divided in preventive measures, such as lipid lowering, blood pressure control and anti-platelet therapy, and treatment of symptoms by revascularization of the end-organ by percutaneous interventions or reconstructive surgery.

Percutaneous interventions consist of percutaneous transluminal angioplasty (PTA) of stenotic vessel segments with or without the placement of a stent, whereas reconstructive surgery consist of bypassing the occluded vessel segment by a conduit. Conduits may be arterial, venous and prosthetic. Since this thesis describes processes after PTA and venous bypass surgery, arterial and prosthetic conduits are not further discussed in this work.

1.2 PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY

Cardiac catheterization in humans is a technique developed by Forssmann (1929, Dresden, Germany). Clinical applications of cardiac catheterization were introduced in 1941 by Cournand and Richards, utilizing catheter techniques to measure cardiac output in injured soldiers in World War II. In 1964, Dotter invented the transluminal angioplasty for occluded arteries in the lower extremities, using multiple catheters

of increasing diameter to open blocked arteries and improve blood flow. Gruenztig modified the Dotter catheters and added a balloon to dilate occluded vessel segments. The first balloon angioplasty in peripheral arteries was performed in 1974, whereas human coronary balloon angioplasty was performed in 1977[3].

Major improvements of the technique of PTA are the development of the Palmaz-Schatz stent in 1994, reducing elastic recoil of dilated arteries and the clinical introduction introduction of the first "drug-eluting"stent manufactured by Johnson & Johnson/Cordis in 2002[4], preventing in-stent restenosis. In time, PTA has become one of the most frequently used procedures in obstructive vascular disease.

1.2.1 RESTENOSIS

Long term success of PTA is limited by a phenomenon called restenosis. Restenosis is defined as closing or narrowing of an artery that was previously opened by a procedure such as angioplasty and occurs due to a combination of remodeling and intimal hyperplasia development. Physiologically, vascular remodeling might be either outward or inward directed (expansive versus constrictive remodeling), however in case of restenosis only constrictive remodeling appears to be a major determinant of luminal renarrowing[5-7]. Intimal hyperplasia describes the changes seen in the tunica intima of a vessel segment that underwent balloon angioplasty, endarteriectomy or surgical reconstruction. It consists of accumulation of smooth muscle cells and fibroblasts from different origins that migrate into the intima, and displays a pathological proliferation rate at that location, resulting in restenosis.

1.2.2 PATHOPHYSIOLOGY RESTENOSIS

The exact pathophysiology of development of restenosis is unknown; however several processes have been shown to be involved. It is has been established that restenosis occurs as "response to injury" to mechanical damage to the vessel wall after balloon angioplasty consisting of denudation of the endothelial layer and disruption of cell-architecture and extra-cellular matrix.

Directly after balloon dilatation, the stretched vessel segment remains its increased diameter for several days. A virtually complete denudation of the endothelial layer is seen and due to loss of endothelial integrity massive thrombi develop. In the tunica media damage due to stretching is characterized by disruption of the inner en outer elastic lamina (also called deep medial tearing) and increased levels of apoptotic smooth muscle cells (SMC) are demonstrable as early as 30 minutes after balloon dilatation[8, 9].

After this initial phase regeneration occurs, consisting of proliferation of the remaining endothelium and medial smooth muscle cells. Furthermore, cell migration results in the presence of smooth muscle cells and myofibroblasts in the intima, now

called “neointima”. Recent studies provided evidence for heterogeneous origin of the smooth muscle cells in the neointima. Some studies show that smooth muscle cells migrate from the media of the damaged vessel segment to the neointima[10], and lots of previous studies focused on inhibition of migration of smooth muscle cells[11-14]. Other studies demonstrate the influx of bone marrow derived progenitor cells as being the source of neointimal smooth muscle cells[15]. Once these cells have entered the neointima, proliferation indexes in the neointima are high, resulting in increase of neointima size (now called intimal hyperplasia) and in a gradually decreasing luminal size[16, 17].

Eventually, remodeling appears to be limited. Hypothetically, one can assume that restoration of the endothelial layer diminishes a great part of the drive for the inflammatory reaction. Furthermore, since smooth muscle cells proliferation results in a phenomenon called arterialisation, thereby adepting to the new situation and reducing the damage done by pulsatile stretching, the inflammatory reponse to that kind of damage is limited after surgery. However, clear data that can prove this hypothesis are lacking.

1.3 VEIN GRAFTING

Venous bypass grafting refers to the procedure where an occluded or injured arterial vessel segment is bridged by an autologous venous vessel segment. Historically, it was performed on war casualties suffering traumatic vascular injury of the extremities; a technique firstly described in the beginning of the 20th century. Venous bypass grafting, as known today, has its roots in 1950 as Holden publishes a reports in which he described to have used a saphenous vein graft to bypass an angiographically demonstrated atherosclerotic lesion in the lower limb[18], closely followed by a publication of Kunlin[19] who described approximately the same procedure from the Clinque of Leriche in Strassbourg. Coronary bypass grafting was firstly described in 1968 by Favaloro[20], when surgical procedures on the heart became technically possible.

Nowadays, coronary bypass surgery is more and more performed using arterial conduits, such as the left and right mammary artery and the gastroepiploic artery, since this technique displays less graft failure. Nonetheless, venous grafts remain frequently used for both coronary bypass surgery, if arterial conduits are not sufficient or available and in particular in grafting of peripheral arteries of the extremities.

1.3.1 VEIN GRAFT DISEASE

Failing of vein grafts can be divided in 3 phases. In the early phases after engraftment (days-weeks after surgery), 3-12% of the vein grafts fail due to acute thrombosis of the graft[21]. Usually technical problems, such as kinking of the graft, persistent valves, poor run-off or a technically insufficient anastomosis, are the cause of this acute bypass failure. Intermediate (30 days-2 years) and late graft failure (> 2 years) are caused by complete different pathophysological mechanisms, namely vein graft remodeling consisting of intimal hyperplasia formation and accelerated atherosclerosis. After 10 years up to 60% of the grafts has failed, depending on anatomical localization of the bypass[22-24.]

Intimal hyperplasia is believed to be responsible for intermediate graft failure (failure between 1 month and 1 year after surgery[21]) It refers to hyperplasia of the intima (and to a lesser extent the media), predominantly consisting of smooth muscle cells and increased amounts of extra-cellular matrix deposition. Typically intimal hyperplasia develops at the proximal and distal anastomosis of the grafts and and probably turbulent flow and accompanying altered shear forces contribute to intimal hyperplasia.

On the long term (beyond 1 year after surgery[21]), vein grafts are highly susceptible for development of a distinct, rapidly progressive form of atherosclerosis, generally known as accelerated atherosclerosis or vein graft disease, being the major cause of late vein graft failure. Vein graft atherosclerotic lesions are more diffuse, concentric and friable as compared with conventional atherosclerotic plaques; they are build up containing more foam cells and lipid depositions and have poorly developed fibrous caps.

It is generally believed that promoting factors for the development of accelerated atherosclerosis are mechanical injury at time of surgery, altered shear- and circumferential wall stress, and the occurrence of pulsatile flow in a vessel previously exposed to venous flow profiles. Furthermore, risk factors playing a role in spontaneous atherosclerosis, such as hyperlipidemia and smoking also play an important role in development of vein graft disease[25-28].

1.3.2 PATHOPHYSIOLOGY VEIN GRAFT DISEASE

The consecutive stages of vein graft disease development can be divided in 3 parts. First, there is the direct peri- and post-operative phase. Surgical trauma, particularly pressure distension[29] and preservation of the vein[30-32] results in damage of the endothelium and affects tissue connections. Furthermore, ischemia occurs once the vein graft is harvested from the body. The period after engraftment is characterized by ischemia-reperfusion injury at the luminal side of the vein. However, after engraftment ischemic damage might persist in the more distant layers of the vein due to removal from vasa vasorum[33]. Like in post-PTA restenosis, due to the non-intact endothelial layer and the overt collagen exposed to the blood stream, thrombi

form on the vein graft wall. These thrombi contain activated platelets, being an early source of various cytokines and growth factors that stimulate endothelial cells to express adhesion molecules but will also promote smooth muscle cell recruitment[34].

In days to weeks after surgery, the endothelial layer is restored. The source of endothelial cells is still a matter of discussion. Reports suggest that endothelial cells of vein grafts are derived from circulating progenitor cells[35], are build up of the remaining islands of endothelial cells after surgery[36] or migrated into the vein graft from the adjacent artery[37]. Although restoration of the endothelial layer is completed soon after engraftment, signs of endothelial activation can be recognized throughout the later stages of remodeling[38, 39].

As an early event, smooth muscle cell apoptosis occurs in all layers of the vein graft, but most extensively in the media where no viable cell can be detected some days after engraftment[40, 41]. Not only the surgical procedure is a cause for endothelial damage and smooth muscle cell apoptosis of vein grafts, it is also hypothesized that increased biomechanical forces after engraftment in the form of stretch stress plays a major role[42].

Inflammatory cells (predominantly polymorphonuclear and mononuclear cells) invade the vein graft wall as early as 24 hours after engraftment[38]. They localize throughout the whole vessel wall including the adventitia, but are mostly present in the direct subendothelial space.

In the later stages of vein graft remodeling "restoration" of the vein graft wall is accomplished by migration and proliferation of smooth muscle cells into the neointima and media, thereby contributing to vein graft thickening and arterialization. The origin of these cells remains topic of debate. Other than in spontaneous atherosclerosis and post-PTA restenosis, several studies in mice show that the majority of these cells are graft-extrinsic[43, 44]. Whether these data can be extrapolated to the human situation remains a matter of debate.

Later on, the graft may acquire an atherosclerosis-like morphology as the tissue-macrophages take up lipid and become foam cells[38, 45, 46]. Cellular analysis reveals more macrophages and other inflammatory cell infiltration in vein graft lesions than can be seen in regular atherosclerotic lesions[47-49].

1.4 MOUSE MODELS

Mouse models for studying restenosis or vein graft disease are of interest taking into account the availability of many transgenic mice, including those with an atherosclerotic phenotype, such as the apolipoprotein (APO) E knockout mouse ($APOE^{-/-}$), the low-density lipoprotein (LDL) receptor knockout mouse ($LDLR^{-/-}$) and the diet-dependent hyperlipidemic ApoE3Leiden transgenic mouse.

1.4.1 MOUSE MODEL FOR RESTENOSIS

(Partially adapted from "A Handbook of Mouse Models for Cardiovascular Diseases; Chapter 8: Perivascular cuff-, electronic and chemical injury-induced stenosis"

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Intravascular injury models to mimic the injury that is inflicted to the vessel wall during PTA are available, but are technically complicated and the reproducibility is not that good, e.g. due to the lack of balloon catheters of appropriately small sizes. As an alternative, models based on perivascular injury are developed to study neointima formation in mouse models.

In 1997, Carmeliet and colleagues described a model in which femoral arteries in mice were injured perivascularly via a single delivery of an electric current. After surgical exposure of the femoral artery, a single current pulse of two seconds (160μA) causes a complete loss of all medial smooth muscle cells (SMC) in the affected vessel over a length of 2-3mm. In addition, by this treatment the arterial segment is denuded of intact endothelium and mural (non-occlusive) platelet-rich thrombosis is present within two hours after injury. Via a vascular wound-healing response the mural thrombus degrades, transient infiltration of the vessel wall by inflammatory cells appears, and the necrotic debris diminishes progressively several days after the intervention. Simultaneously, SMC originating from the borders of the injured segment migrate towards the necrotic centre, ultimately leading to SMC accumulation.

Two mouse models of chemical injury-induced neointima formation, both adapted from thrombosis models, have been described in the past years. Kikuchi et al. adapted a (photo) chemical model of thrombosis to a model of neointima formation in the mouse femoral artery. In this model, endothelial injury is inflicted by photochemical reaction by a transluminal green light and intravenous administration of rose Bengal solution. An approximately 2mm long segment of the intact femoral artery is irradiated until blood flow completely stops due to a platelet- and fibrin-rich thrombus. Twenty-four hours later, spontaneous reflow is seen with denudation of the endothelium and medial SMC loss. Within seven days neointima formation starts and reaches a maximum after 21 days, mainly consisting of SMC. Zhu et al. adapted a thrombosis model developed by Farrehi and colleagues to a vascular injury model. In this model, the carotid artery is carefully exposed and a filter paper saturated with a 10% ferric chloride solution is placed on the adventitia for three minutes. This oxidative vascular injury leads to the formation of a transiently occlusive platelet-rich

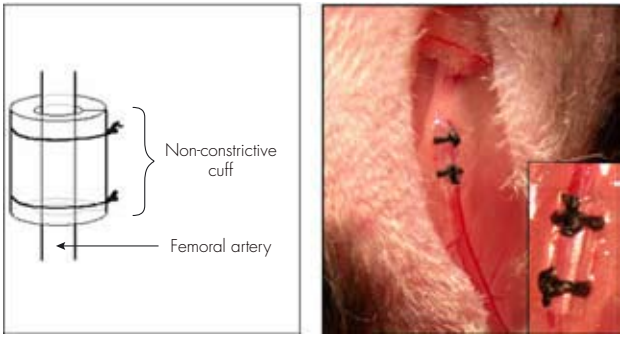
thrombus with endothelial cell loss and medial cell necrosis. After four weeks, intimal and medial hyperplasia is present in hyperlipidemic mice mainly consisting of SMC and foam cells.

Perivascular cuff injury-induced stenosis mouse model
In 1989, Booth and colleagues established a model for accelerated formation of atherosclerotic-like lesions in the carotid arteries of rabbits. This model is based on placement of a plastic perivascular collar in the rabbit common carotid artery which results in the development of an intimal lesion containing foam cells and SMC in the cuffed segment. Based on the success of this model, in the mid 90s, this model was downscaled to mice and modified using both non-constrictive and constricting tubes. These murine models are widely used to study both accelerated atherosclerosis phenomena and the process of restenosis.

Von der Thüsen et al. defined a mouse model in which a constricting silastic collar is placed around the common carotid artery of hypercholesterolemic mice. The development of collar-induced lesions is found to occur predominantly in the area proximal to the collar and to be dependent on a high-cholesterol diet. Lesions initially consist of monocyte-derived foam cells and as maturation progresses plaques become increasingly heterogeneous with the development of a necrotic core and a fibrous caps with typical shoulder regions.

Moroi et al. were the first to describe a model for inducing neointima formation in the femoral artery of mice. The murine femoral artery is isolated and loosely sheathed with a non-occlusive polyethylene cuff (Figure 1.1). In this model, the endothelial cells are not directly manipulated or removed, oppositely to what occurs in other intravascular injury models for induction of neointima formation. Placement of the cuff results in highly reproducible neointima formation within the cuffed vessel segment in a 2 to 3 week period and mainly consists of SMC on top of the internal elastic lamina underneath an endothelial monolayer. Remarkably, arteries dissected from surrounding tissues (sham-operated) but where a cuff is not placed, do not develop a neointima. The presence of the cuff seems to be essential for inducing the neointima formation after the initial perivascular injury inflicted during surgery. Moreover, if in a similar way a cuff is placed around the carotid artery in the mouse, no neointima formation is observed in the cuffed vessel segment. The reason for this most likely is the anatomic difference between the femoral artery and the carotid artery in the mouse.

Figure 1.1: *Schematic representation (A) and microphotograph (B) of femoral artery cuff positioning.*



For all experiments studying post-angioplasty restenosis in this thesis the perivascular cuff inury-induced stenosis mouse model was used.

(End citation "A Handbook of Mouse Models for Cardiovascular Diseases; Chapter 8: Perivascular cuff-, electronic and chemical injury-induced stenosis")

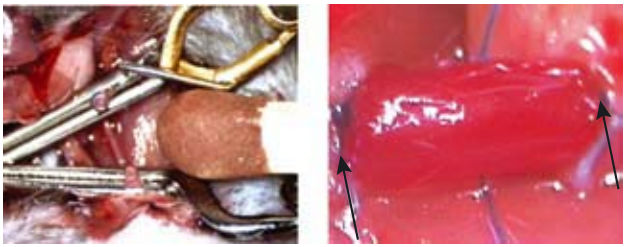
1.4.2 MOUSE MODEL FOR VEIN GRAFT DISEASE

Models to study vein graft disease have been developed for several species varying from rodents to quadrupeds to primates, each with their own pro's and con's. Initially, larger animals such as dogs, sheep, pig and rabbit were favored, facilitating the technical aspects of the anastomosis. For the same reason, venous interponates in peripheral arteries, such as carotids and femoral arteries, were used. In the last decade, mouse models for vein grafting have become of interest because of the availability of inbred, transgenic and knock-out strains. Using mice provides major advantages in studying vein graft disease, since they allow studying the effect of a single gene or protein.

However, the size of the animal requires an alternative approach to anastomose the vessels. The most frequently used murine model to study vein graft disease is the model of Xu[50]. In this model a venous interponate is anastomosed in the murine carotid artery. Therefore the caval vein of a donor mouse is harvested and preserved in heparin containing NaCl 0.9%. In a recipient mouse the right carotid artery is dissected free from its surroundings, ligated at two sides with a silk ligature and cut in the middle. Two clamps are placed distally and proximally from the ligatures, to maintain haemostatic control throughout the procedure, leaving a free end of artery at both sides (Figure 1.2A). Then two plastic cuffs are created and the vessel ends are sleeved through the cuffs. After releasing the ligatures the vessel is folded inside-out around the cuffs and fixed with a silk ligature. The donor caval vein is sleeved

over both cuffs and fixed with a second silk ligature. After removal of the clamps, the caval vein functions as a venous interponate in the carotid artery and pulsatile flow in the graft confirms a successful procedure (Figure 1.2B). When performed in hypercholesterolemic mice, vein grafts in this model undergo a striking remodeling with IH formation and atherosclerotic changes[45], resulting in formation of lesions that are concentric and friable, with lipid deposition and foam cell accumulation in the intima and media, and have a poorly developed or absent fibrous caps. This morphology is highly similar to the changes seen in human vein grafts.

Figure 1.2: *Bypass model in a mouse. Panel A: The common carotid artery is divided and occluded with 2 clamps. The inferior caval vein of a donor mouse will be implanted as interposition. Panel B: Venous interponate in situ, arrows indicate anastomotic side.*



A B

In this thesis this mouse model for vein graft disease is used. The cuff-assisted anastomosis, making the model feasible, is at the same time the main drawback of the model, being in fact a non-physiological anastomosis. Recently, in an attempt to make the model more human-like, mouse models are designed using either a side-to-side or an end-to-end anastomosis with interrupted sutures[51-53]. These models appear to have certain advantages over the "cuff-assisted" model. Since poly-ethylene cuffs are absent, less immunogenic, non-self material is present around the venous interponate and thereby the risk of inflammatory reactions other than those seen in daily practice (form surgical dissection and sutures), minimizes. Furthermore, in these models jugular veins are used to graft in the aorta, thereby overcoming the need for heterogenous grafts, harvested form donor mice. Heterologous grafts, although taken from inbred littermates carry the risk of immunological reactions similar to rejection. Finally, a more physiologically flow profile is claimed, when there is direct contact between the artery and vein at the place of anastomosis. However, this assumption can be doubted. Due to caliber difference between arteries and veins, turbulent flow is present at the side of anastomosis, just as it is near the cuff-assisted anastomosis.

Disadvantages of these models include the difficulty of surgery, the duration of the surgery (over 60 minutes instead of 30 minutes in the cuff-assisted model) and the fact that these models have not yet been assessed in hypercholesterolemic mice.

As mentioned above, the studies presented in this thesis were performed in the cuff-assisted model, mainly because of the fact that these new models, as described above,

for vein grafting have been described only recently and the cuff-assisted model is very well suited to study this type of pathology because sophisticated interventions and manipulations are relatively easy.

1.5 INFLAMMATION; INTRODUCTION

(For a general review see[54, 55])

Inflammation is defined as the response of an organism to tissue damage, either applied exogenously (e.g. chemical, thermal or immunological) or endogenously. Although stimuli inducing an inflammatory reaction may vary widely, the reaction of the host (in this case the mammalian body) tend to be similar. In general, inflammation consists of leukocyte exudation at the place of injury. Therefore, leukocytes need to pass the consecutive processes of margination, rolling along the endothelium, adhesion, transmigration thought the endothelial layer and chemotaxis to the location of the tissue damage. There, the inflammatory reaction is focused on trying to achieve elimination of the stimulus and repair of the defect by regeneration of parenchyma or replacement by fibro-elastic scar tissue.

For most part, the inflammatory reaction is anchored by the immune system. Grossly, it can be divided in the innate and adaptive immune response.

1.5.1 INNATE IMMUNITY

Immunity is complex. First, all animals possess a primitive system of defense against the pathogens to which they are susceptible, the so-called innate immunity. Innate immunity is nonspecific, being not directed against specific invaders but against any pathogens entering the body. It makes the difference between self and non-self, and includes two parts.

One part, called *humoral* innate immunity, involves a variety of substances found in body fluids and released by damaged cells. Inflammation is regulated by these chemical factors, including specialized chemical mediators, called cytokines. Cytokines can be either promote or diminish inflammatory reactions and are released by injured cells and leukocytes. The group of cytokines include interleukins (responsible for communication between leukocytes); interferons (anti-viral effects); chemokines (which promote chemotaxis). These cytokines and other chemicals serve to establish a barrier against the spread of infection, and to promote healing of any damaged tissue following the removal of pathogens.

The other part is called *cellular* innate immunity. The innate leukocytes include mast cells, eosinophils, basophils, natural killer cells, and the phagocytes (macrophages, neutrophils and dendritic cells) and function by identifying pathogens that might cause infection ultimately resulting in elimination of the pathogen by processes as phagocytosis, toxin release and induction of phagocytosis.

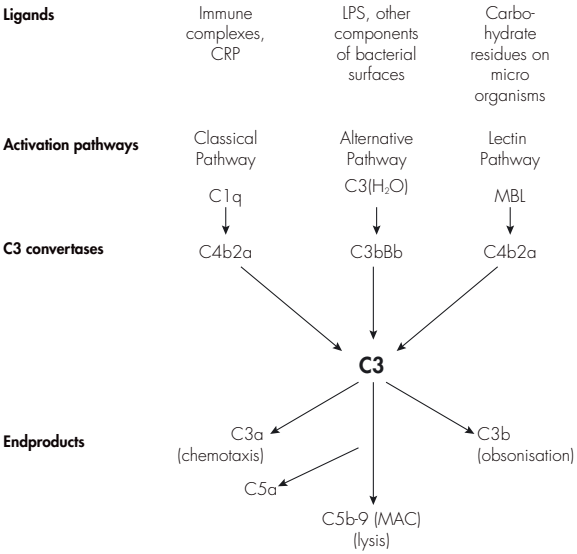
1.5.2 ADAPTIVE IMMUNITY

Only vertebrates have an additional and more sophisticated system of defense mechanisms, called adaptive immunity, that can recognize and destroy foreign invaders. The defensive reaction of the adaptive immune system is called the immune response. Antigens are not foreign microorganisms and tissues themselves, but substances, such as toxins or enzymes, in the microorganisms or tissues that the immune system considers foreign. Immune responses are normally directed against the antigen that provoked them and are said to be antigen-specific. Specificity is one of the two properties that distinguish adaptive immunity from innate immunity. The other is called immunologic memory. Immunologic memory is the ability of the adaptive immune system to mount a stronger and more effective immune response against an antigen after its first encounter with that antigen, leaving the organism better able to resist it in the future. In concrete, the adaptive immune response consists of antibodies (or immunoglobulins) produced by B-cells directed to a specific antigen, B-cells and the different subsets of T-cells.

1.5.3 COMPLEMENT

The complement system is a biochemical cascade of the immune system that helps clear pathogens from an organism. It is a major component of the innate immune system, but also contributes in adaptive immunity. The complement system consists of more than 35 soluble and cell-bound proteins, 12 of which are directly involved in the complement pathways. These plasma proteins can be enzymatically activated via a cascade reaction resulting in the generation of biologically active fragments. These end products have functions as cytolysis, chemotaxis, opsonization, as well as the marking of pathogens for phagocytosis. The complement system can be activated via 3 separate pathways, the classical pathway, the lectin pathway and the alternative pathway, each with its own activation mechanisms. Functioning of the complement system is depicted in Figure 1.3 and explicitly reviewed in[56-58].

Figure 1.3: Schematic overview of the complement cascade. *Depicted are the three pathways by which the cascade can be activated and their specific ligands. After activation of C3 convertases, the key molecule C3 is cleaved into active components. These active components (C3a and C3b) have inflammatory properties: chemotaxis of inflammatory cells and obsonisation of foreign invaders. Furthermore, cleavage of C3 results in the formation of various active endproducts more downstream in the cascade, such as C5a, being the most potent chemotactic agent of the cascade, and the Membrane Attack Complex (MAC, C5b-9) which is a composed molecule capable of cell lysis.*



Adapted from thesis L.A. Trouwe; used with permission of the author.

1.6 EVIDENCE FOR INFLAMMATION DRIVEN VASCULAR REMODELING

For a long time the problem of restenosis after percutaneous interventions and vein graft disease focused on the problem of smooth muscle cell migration and proliferation, based on the morphological findings of (re)stenotic lesions characterized by smooth muscle cells in the neointima.

However, as a result of the discussion regarding the role of inflammation in spontaneous atherosclerosis, the link to related processes such as restenosis and vein graft disease was quickly drawn, and many indications for involvement of inflammatory processes in restenosis could easily be found. As described above, leukocyte adhesion is seen in the early phases both in arteries after PTA as in vein grafts after engraftment. Furthermore, macrophages-derived foam cell accumulation is demonstrated in vein grafts. All these findings led to the nowadays general assuption that inflammation plays an important role in post-interventional vascular remodeling. In that line of thought, SMC migration and proliferation is seen as a result of the inflammatory reaction present in the vessel wall after vascular interventions.

A typical inflammatory reaction can be seen as a "response to injury" and usually follows a more or less common course. Initially cells of the vasculature of damaged tissue expresses chemokines and adhesion molecules to enable recruitment of leucocytes and subsequently rolling of the leucocytes on the endothelial layer, creating the conditions for diapedesis en invasion of the damaged tissue. Different subsets of leucocytes can contribute to various forms of reaction and "communicate" by the production of cytokines. Generally, macrorphages play an important role, since they are assumed to be the most potent phagocytes, responsible for forein or damaged tissue resorption. Production of growth factors and cytokines by the invaded cells ultimately results in stimuli to regenerate the damaged tissue.

Theoretically, the inflammatory reaction present after vascular intervention should follow such a path. Nevertheless, when putting together the scientific evidence for this hypothesis, one should notice that this is mainly circumstantial evidence. This is a logical consequence of the fact that the inflammatory reaction is to complex to study successive events as a whole in one experiment. Therefore, most studies focus on a specific part of the inflammatory reaction. In the following chapter, a recapitulation is given of the available evidence for the hypothesis that various components of the inflammatory reaction are involved in post-interventional vascular remodeling.

1.6.1 ADHESION MOLECULES

As described before, vascular interventions such as balloon dilatation, stent placement or handling during harvesting of a saphenous vein before grafting cause damage to the endothelium, thereby altering normal homeostatic properties of an intact

endothelial layer. Studies in animal models show that the remaining endothelial cells become activated and produce a variety of cytokines and upregulate adhesion molecules (P-Selectin, ICAM, VCAM) allowing leukocytes to migrate to the injured vessel wall and adhere to the endothelium[59, 59-61]. The same phenomenon can be seen in greater saphenous veins that were exposed to pressure distension during surgery[62].

In addition of endothelial cell activation, platelets massively adhere to the uncovered or injured areas of the intima, producing various pro-inflammatory cytokines, such as CD40L[63], RANTES[64] and P-selectin65, inducing production of growth hormones and cytokines and altering adhesive and chemotactic properties of vascular cells[66, 67]. Upregulation of soluble adhesion molecules can also be detected in the serum early after PTA, indicating that this reaction is not solely detectable in the treated vessel segment[68] and these levels appear to correspond with the extent of restenosis that can be observed in time[69-71].

1.6.2 CHEMOKINES

Inflammatory cells migrate to afffected vessel segments by a process called chemotaxis. Many chemotactic agents have been shown to be involved in chemotaxis of monocytes and neutrophils to the site of vascular intervention. Monocyte Chemoattactant Protein-1 (MCP-1) is one of the most studied chemokines in vascular remodeling. It expresses strong chemoatractant properties for monocytes, and to a lesser extent T-cells[72], and induces on monocytes the expression of integrins required for migration thought the vessel wall. Furthermore, it has been shown that is has pro-mitogenic properties for SMC[73, 74]. Its role in spontaneous atherosclerosis is well established, and MCP-1 is emerged as the possible molecular link between ox-LDL and foam cellsrecruitment. In the later stages, MCP-1 might also contribute to the pro-thrombotic aspects of advanced atherosclerotic lesion (all reviewed in[75]). Regarding post-PTA restenosis, human studies proved a correlation between serum MCP-1 levels after PTA and an increased risk of restenosis[76]. Animal studies underscore the pro-restenotic role of MCP-1 and show that lowering of MCP-1 expression results in decreased neointima formation and also reduces monocyte content in restenotic lesions[77-79].

Besides MCP-1, many other chemokines are involved post-angioplasty restenosis, although their role is less extensively studied. For instance Il-8, a potent neutrophil-specific chemokine, has been shown to be upregulated in the perivascular tissue after balloon dilatation of porcine coronary arteries[80]. RANTES, produced by accumulating activated platelets, has been shown to attenuate monocyte recruitment[64] and levels are correlated with restenosis[81] whereas MCP-3 mRNA is upregulated upon vascular injury, suggesting a role in the remodeling process[82].

1.6.3 CYTOKINES

Various cytokines has been shown to be involved in restenosis. After vascular injury in general endothelial cell and vascular smooth muscle cells are able to produce cytokines including interleukins Il-1β, Il-6, Il-8[83], TGF-β and TNF-α[84]. Cytokines are considered to be involved in regulation of the inflammatory response, eg chemotaxis, activation of leukocytes, inducing apoptosis and stimulation of smooth muscle cell proliferation. Since the enormous number of different kinds of cytokines, only some will be discussed in more detail.

TNF-α is a key pro-inflammatory cytokine produced by a number of cells, including macrophages, neutrophils, endothelial cells, and SMCs in response to a variety of stimuli such as LPS release or endothelial damage. TNF-α protein is present in atherectomy specimen of restenotic lesions[85] and in diseased vein grafts[86]. Furthermore, it was a independent predictor for restenosis after PTA[87]. Blockade of TNF-α has been proven useful in order to reduces restenosis in both a murine and rabbit model for restenosis[87, 88].

Il-1β and Il-6, like TNF-α, are also pro-inflammatory cytokines and known to be synthesized in injured vessel segments after balloon dilatation[89]. Il-1 receptor polymorphisms have been shown to be protective for development of restenosis[90], whereas serum Il-6 levels after PTA are predictive for the development of restenosis[91].

An exemple of an anti-inflammatory cytokine that is involved in vascular remodeling is Il-10. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th-1 cytokines, enhances B cell survival, proliferation, and antibody production, deactivates monocytes and can block NF-kappa B activity (a nuclear transcription factor involved in various inflammatory processes). Il-10 has been reported to inhibit post-injury restenosis in hypercholesterolemic rabbits and it reduced intimal hyperplasia after wire denudation in normocholesterolemic mice and rats[92-94].

1.6.4 INFLAMMATORY CELLS

As described above, various cell-types are capable of infiltrading inflamed tissue, and cell-types may differ in each type of inflammatory reaction (depending on the site of inflammation and the provocative factor). In the following alineas evidence for involvement of some inflammatory cells in vascular remodeling is summarized.

6.3.a Monocytes
Monocytes, alike their contribution in spontaneous atherosclerosis, appear to be crucial in the various stages of restenosis and vein graft disease. After angioplasty, monocytes are present in the vessel wall within 20 days after the procedure, as

shown in human autopsy specimen[95]. These results were confirmed by animal studies, in which adhering monocytes were detected in the first days after balloon angioplasty[96, 97] and vein grafting[38, 98]. Also in the later stages of arterial remodeling after PTA, monocytes and macrophages can be detected in the different layers of the vessel wall[80, 80, 99]. Functional involvement of monocyte recruitment is suggested by studies indicating that after PTA, circulating monocytes increase in blood in a time-dependent manner and the peak monocytes count relates to neointimal volume[100]. Furthermore, monocyte depletion after implantation of the vein graft results in reduced vein graft thickening in rat vein grafts[101].

6.3.b T-lymphocytes

Various subsets of T-lymphocytes are involved in atherogenesis, as reviewed in[102] and one might extrapolate this to restenosis development. Although not present in the normal tunica intima, T-cells have been shown to adhere and invade damaged vessel segments and remain present up to 10 days after injury in the developing neointima[60, 103]. However, studies providing direct evidecence for their involvement are lacking. Moreover, cyclosporine treatment, blocking activation and proliferation of T-lymphocytes did not have any effect on restenosis development in rabbits, thereby suggesting that T lymphocyte—mediated immune responses are not involved in neointima proliferation after balloon dilatation[103]. Clear evidence for a role for T-cell involvement or the lack thereof in post-interventional vascular remodeling remains to be provided.

6.3.c Neutrophils

In the first hours after angioplasty neutrophils adhere and invade the damaged vessel segment104, 105. Also neutrophil accumulation can be seen in the adventitia[80]. The same adherence and invasion can be detected in pressure-distended vein grafts[106]. Functional involvement of neutrophils is suggested since, upregulation of neutrophil adhesion molecules after PTA is associated with restenosis[107], combined with the findings that after stent placement activated neutrophils contribute to the oxidative burst, that was associated with occurrence of restenosis in the future[108].

1.7 AIM OF THE THESIS

The aim of this thesis was to study the functional involvement of various selected inflammatory processes in the development of post-PTA restenosis and vein graft disease. Therefore we evaluated the effect of specified anti-inflammatory interventions in various murine models of post-interventional vascular remodeling (perivascular cuff-induced femoral artery stenosis and vein graft accelerated atherosclerosis).

Stated that the influx of leucocytes is the main event in the development of both forms of remodeling, we furthermore focused on the effects of these specified interventions on inflammatory cell adhesion to the vein graft wall.

We aim to postulate a clear involvement of inflammatory processes in post-interventional vascular remodeling, and discover new opportunities for treatment in order to diminish (re)stenosis.

1.8 OUTLINE OF THE THESIS

Firstly, we established the role of inflammatory processes in cuff-induced vascular remodeling by studying the effect of both local and systemic administration of the corticosteroid dexamethasone in this model. Local dexamethasone delivery was achieved by placement of dexamethasone-eluting cuffs. This approach was chosen, since Dexamethasone is a very potent anti-inflammatory agent, possibly altering the inflammatory reaction in the vessel wall, and recently dexamethasone-eluting stents were introduced for clinical use. Taking these recent advances into account, not only the effect on intimal hyperplasia formation was studied, also histopathological alterations in the vessel wall after placement of dexamethasone-eluting cuffs were evaluated and results are described in **Chapter 2**.

In **Chapter 3**, the modulation of the restenotic response following perivascular cuff placement was assessed using both over-expression and inhibition of Il-10, a well known and potential anti-inflammatory cytokine (as described above). Since in human studies an inflammatory reaction can be detected in serum days after PTA, special attention was given to the systemic effects of Il-10 over-expression with regards to cytokine production.

Since inflammation is hardly studied in vein graft disease, a "proof-of-principle" study was performed, studying the hypothesis that if inflammatory processes are involved in vein graft disease, it should be inhibited by Dexamethasone treatment. Furthermore, since prolonged treatment with Dexamethasone is associated with unfavorable side-effects, it was decided to evaluate whether it is possible to temporarily block the inflammatory reaction and still inhibit vein graft disease in the long run, two treatments regimes of Dexamethasone were tested and results are presented in **Chapter 4.**

As discussed, one of the most important chemokines in vascular inflammation is MCP-1, a potent chemoattractant for monocytes. However, its role in vein graft disease was never studied. Therefore, in **Chapter 5**, a dominant negative receptor antagonist of MCP-1 (7ND-MCP-1) and a gene-therapeutic approach was used to block MCP-1 activity in remodeling vein grafts *in vivo*. In this particular study, the focus was not solely on the effects on vein graft thickening and monocyte invasion, but MCP-1s' direct effects on smooth muscle cell proliferation was also assessed.

Besides MCP-1, other CC-chemokines might be involved in vein graft disease. Out of the large group of CC-chemokines MIP-1α and RANTES were selected for their known chemotactic properties to monocytes. Their presence and functional involvement was studied and described in **Chapter 6**, using MetRANTES as a potent inhibitor of CCR1 and CCR5 downstream signaling.

Complement is a major contributor in many inflammatory processes, although its role in vascular inflammation, such as atherosclerosis and post-interventional remodeling, is still under debate. The goal of **Chapter 7** was to provide evidence for involvement of the complement system as a whole in the pathophysiology of vein graft disease. Several approaches were used to demonstrate the presence of complement components and to block complement activation in the *in vivo* mouse model of vein graft disease.

To further specify the involvement of the complement cascade in vein graft disease, component of the complement system, C5a, was selected for its potent chemotactic properties. The role of C5a in development of vein graft disease, particularly vein graft thickening and foamcell content, is described in **Chapter 8**, using a pharmacological approach to hamper C5a function.

All results are summarized and discussed in **Chapter 9**, Summery and General Discussion. Furthermore, future perspectives and some recommendations for further research are given for this interesting and clinically relevant field of science.

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