Post-interventional atherosclerotic vascular remodeling : preclinical investigation into immune-modulatory therapies

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Chapter 3

Small Animal Models to Study Restenosis and Effects of (Local) Drug Therapy

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Abstract

In-stent restenosis remains the major drawback of coronary interventions and is a highly complex process, initiated by the induction of vascular injury and stent deployment, ultimately leading to negative vascular remodeling and reoccurrence of symptoms. Development, testing and validation of new therapies strongly depends on the availability of animal models that closely mimic human restenotic pathophysiology. Moreover, for a better understanding of the pathophysiology of the restenosis process adequate animal models mimicking the human restenosis process are essential.

Here, we review various animal models, predominately humanized mouse models, currently used to study the pathophysiology of restenosis. Larger animal models such as pigs and rabbits that are used for testing new therapeutic strategies such as new drug-eluting stents, will not be discussed in this chapter.

We will discuss the involvement of inflammatory and immune modulatory factors in the development and progression of restenosis. Furthermore, we will discuss in detail several new therapeutic options based on modulation of their anti-proliferative, anti-inflammatory or proteinase-interference abilities.

We can conclude highly-reproducible animal models for post-interventional vascular remodeling remain essential for the development of future anti-restenotic therapies.

Introduction

Pathophysiology of Restenosis in Humans and Animals

Restenosis is defined angiographically in patients when neointimal tissue comprises over 50% of the luminal surface at the site of intervention of the affected artery. Restenosis is characterized by acute elastic recoil, negative remodeling and intimal hyperplasia due to inflammation, deposition of granulation tissue and extracellular matrix remodeling. Intracoronary stenting has virtually eliminated elastic recoil and negative remodeling. However, after balloon angioplasty and stent placement neointimal formation still occurs due to de-endothelization and injury of the vessel wall including the atherosclerotic plaque.

Together, these effects lead to activation of the remaining endothelium, platelet adhesion and subsequent activation, fibrin formation and the expression of adhesion molecules, leukocyte adherence and infiltration. Adhered leukocytes release an array of inflammatory cytokines, chemokines, proteases such as matrix metalloproteinases and growth factors which not only promote inflammation, but also cause medial smooth muscle cell migration and proliferation, matrix degradation, local proteoglycan deposition and subsequent extracellular matrix remodeling. Under hypercholesterolemic conditions this is accompanied by influx and accumulation of low-density lipoprotein (LDL) cholesterol in the vessel wall. This will be taken up by macrophages, that become foam cells and initiate a process of accelerated atherosclerosis in these vessel segments[1]. Patients receive platelet-inhibitors throughout interventional procedures to prevent arterial thrombosis and end-organ ischemia due to thrombotic occlusion of the affected vessel. Furthermore, this therapy partly prevents the initial pathophysiological events that eventually lead to restenosis development. Nonetheless, in patients limited local inflammatory processes are related to the healing of the vascular injury triggered by mechanical dilation, stent deployment and the continuous presence of stent struts against the arterial wall and their exposure to flowing blood[2, 3]. However, uncontrolled inflammatory processes may induce intimal hyperplasia.

Intravascular injury to diseased vessels with a hypercholesterolemic background is common in patients, but severely difficult to reproduce in healthy vessels of mainly young animals with a normocholesterolemic phenotype. Therefore, transgenic mouse models with a pro-atherosclerotic or hypercholesterolemic phenotype have attracted attention as humanized mouse models for mechanistic and pathophysiological restenosis research.

Animal Models for Restenosis and Vascular Remodeling

Various animal models, mainly in mice, are currently available to mimic human restenosis, extensively based on perivascular injury, aimed to induce local coagulation and subsequent inflammation, leading to accelerated atherosclerosis formation.

Balloon angioplasty of rat carotid arteries

A model of balloon-induced injury to the carotid artery in rats was described in 1983 by Clowes et al.[4] and used many studies there after, amongst others by Ohlstein et al.[5], in which an arterial embolectomy catheter is inserted into the common carotid artery and a balloon is inflated and drawn along the vessel wall to induce mechanical

injury. Afterwards, the external carotid artery is ligated. This leads to severe SMC migration and proliferation and intimal thickening.

Common carotid artery ligation in mice
Neointimal formation can be elicited by completely ligating the common carotid artery just proximal to the carotid bifurcation to disrupt blood flow as has been described by Kumar et al.[6] and since then has frequently been used by several groups. After 4 weeks, intimal thickening occurs and consists of both SMCs and leukocytes, indicating the pivotal role of inflammation in the formation of neointimal tissue. The model is hampered by its reproducibility, since morphometric analysis is very critical. The degree of the intimal hyperplasia depends on the position the analyzed section in regard to the point of ligation.

Mouse model of femoral artery denudation injury
Femoral artery transluminal injury can be induced by passage of a 0.25-mm diameter angioplasty guide wire in mice[7]. Four weeks after injury, neointima formation can be analyzed and consist predominately of migrated and proliferated SMCs, although inflammatory cells can already be observed early after injury.

Perivascular electrocoagulation injury
Electrocoagulation-induced injury to the femoral artery of mice was introduced in 1997 by Carmeliet et al.[8], leading to loss of all endothelial and medial smooth muscle cells (SMCs) and formation of a mural non-occlusive platelet-rich thrombus. This leads to inflammatory cell recruitment and SMC migration and proliferation, eventually leading to intimal thickening.

Photochemical intravascular injury
Photochemical endothelial injury to the femoral artery of mice injected with rose Bengal solution using transluminal green light has been used by Kikuchi et al.[9] in 1998 to induce a local thrombus formation, followed by endothelial denudation and medial SMC apoptosis and eventually intimal thickening. Unfortunately, the reproducibility of this model remains rather low.

Perivascular chemical injury
Chemical perivascular injury to the carotid artery of dyslipidemic mice using a filter paper saturated with a 10% ferric chloride solution was used by Zhu et al.[10] to induce formation of an occlusive platelet-rich thrombus due to endothelial cell loss and medial SMC necrosis, triggering inflammatory cell recruitment and SMC migration and proliferation. Eventually, this promotes intimal thickening.

Inter-arterial venous engraftment
Accelerated atherosclerosis and vascular remodeling also appear in engrafted vascular segments, which are performed frequently to bypass occluded arterial segments in patients. Although bypass models do not mimic the pathophysiology of restenosis completely, they can be used excellently to study various aspects of restenotic disease. Models have been developed for several animal species. Only relatively recently have mouse models for vein graft disease become available, de-

veloped by Xu et al.[7], most often based on the model described by Lardenoye et al.[11], in which a venous interposition is made within the carotid artery of a mouse. For this, an inferior caval vein is harvested from littermate donor mice and preserved in a 0.9% NaCl solution containing 100U of heparin at 4°C, to prevent thrombosis. Afterwards, the carotid artery is dissected free from its surroundings, ligated twice and cut between the two 8.0 silk ligatures. Clamps are placed proximally and distally from the ligatures to allow haemostatic control to the surgeon throughout the procedure, leaving free arterial ends over which a cuff can be placed. Next, the free arterial ends are everted over the cuffs and ligated with an 8.0 silk ligature, after which the harvested inferior caval vein is interpositioned between the ends of the artery. The connections are ligated together with an 8.0 silk suture and visible pulsations confirm successful engraftment.
When performed in hypercholesterolemic mice, concentric lesions are formed within 4 weeks and are friable, with extracellular lipid deposition and foam cell accumulation, underneath a poorly developed or absent fibrous cap. This morphology highly resembles the morphology observed in arterially-engrafted veins in patients. All aspects of rapid post-interventional vascular remodeling and accelerated atherosclerosis development are present in this very reproducible animal model.

Intravascular balloon dilatation injury
Intravascular injury models resembling invasive coronary procedures have been developed recently, but remain technically very demanding.
Kwak et al.[12] described a mouse model in which intravascular carotid balloon distension injury is performed, which a balloon catheter was introduced through an arteriotomy on the proximal external carotid artery and advanced into the common carotid artery and subsequently distended to induce controlled vessel wall distension. In this elegant model, distention could be matched to the animal's weight and the balloon was expanded using a water-filled inflation device. Vessel wall damage led to endothelial denudation, followed by leukocyte recruitment and medial SMC activation, leading to intimal thickening and luminal stenosis. Although this model is technically difficult to perform, vascular injury highly resembles the human situation and leads to a similar pathophysiological vessel wall response.

Inter-arterial stented-arterial engraftment
Ali et al.[13] have taken intravascular stenting in mouse models a step further, which they reported in 2007. Donor mice receiving aspirin underwent stenting using a stainless-steel stent crimped onto an angioplasty balloon catheter, which was guided into place retrograde through the thoracic aorta, leaving the balloon and stent in the descending thoracic aorta. The balloon was inflated for 30 seconds until 8 times atmospheric pressure to induce stent deployment. Next, the stented-aorta was removed and kept in heparinised PBS solution, before being engrafted within the ligated carotid artery of a hypercholesterolemic recipient mouse, similarly to the vein graft procedure. After 28 days, intimal thickening was significantly increased in the stented arterial graft, when compared to the aorta and balloon-inflated aortic grafts. Lesions consisted predominately of SMCs, macrophages and foam cells, similarly to human coronary lesions after interventions.

Perivascular femoral arterial cuff placement

Already in 1989, it was shown by Booth et al.[14] that placement of a perivascular non-constrictive plastic cuff around the common carotid artery of hypercholesterolemic rabbits results in intimal thickening based on SMC migration and proliferation, cholesterol deposition and foam cell formation as seen in human restenotic lesions. This is due to mechanical vascular damage and an inflammatory response evoked by the cuff. There was no need for endothelial cell damage and the formation of a transient occlusive thrombus in this model.

When this attractive model was downscaled to mice[15], this offered the unique opportunity to employ a mechanically-induced inflammatory-based restenosis model in an animal species of which an enormous range of strains with genetic variations existed, including atherosclerosis prone transgenic strains.

Since like the human genome, the murine genome has currently been completely mapped and multiple humanized-mouse models have been developed, this has allowed researchers to investigate the role of specific genotypic and general phenotypic traits in restenosis development. The aim was to identify pathophysiological changes leading to restenosis and accelerated atherosclerosis development, and subsequently identify key targets for prevention and treatment of restenosis in patients in a clinical setting.

The technical procedure of perivascular cuff placement[15] is described in detail in the next section. Before surgery, mice are anaesthetized with an intraperitoneal injection with a combination of 5 mg/kg Midazolam (Roche, Basel, Switzerland), 0.5 mg/kg Medetomidine (Orion, Helsinki, Finland) and 0.05 mg/kg Fentanyl (Janssen, Geel, Belgium). This combination of anesthetics gives complete narcosis, lasting minimally one hour and can be antagonized using Antisedan 2.5 mg/kg (Orion), Anexate 0.5 mg/kg (Roche) and Buprenorphine 0.08 mg/kg (Schering-Plough, Kenilworth, NJ, USA).

A microscope with 10-15x total magnification is used during the microsurgery procedures (Olympus SZX9 microscope). Basic instruments required are a blunt micro forceps (length: 10.5cm, height: 0.3mm, Medicon Instruments, Tuttlingen, Germany), a sharp micro forceps (length: 10.5cm, height: 0.3mm, Medicon Instruments), a micro scissor (length: 12.5cm, height: 10mm, Medicon Instruments) and a micro needle holder (length: 18.5cm, Medicon Instruments).

A longitudinal incision is made at the internal side of the thigh and the femoral artery is dissected from the femoral nerve and vein. The femoral artery is looped twice with a ligature (USP: 6/0, Metric: 0.7., Silkam natural silk, B. Braun, Melsungen, Germany) and a non-constrictive fine bore polyethylene tubing (0.40mm inner diameter, 0.80mm outer diameter, Portex, Kent, UK) is cut 2.0mm length and longitudinally opened and sleeved loosely around the femoral artery. The cuff is closed with two 6/0 ligature knots in the extremities of the cuff. Finally, the skin incision is closed with a running suture (USP: 6/0, Metric: 0.7., Silkam silk, B. Braun). After surgery, animals are placed in a clean cage on top of a heating pad for four hours. A schematic representation and photomicrograph of the femoral arterial cuff placement are shown in figure 1.

For histological analysis, animals are typically sacrificed 2-3 weeks after cuff placement. After anesthesia, the thorax is opened and a mild pressure-perfusion (100 mmHg) with 4% formaldehyde in 0.9% NaCl is performed for 5 minutes by cardiac

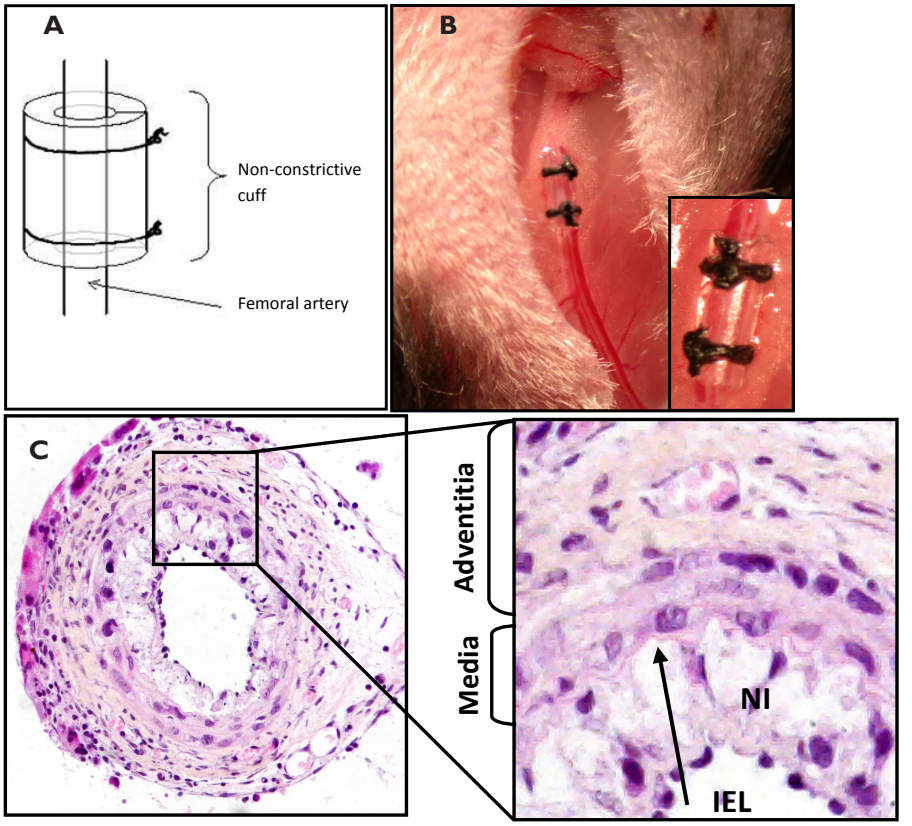

Figure 1. Schematic representation of non-constrictive cuff placed loosely around a murine femoral artery, held in place by two ligatures (A) and a photomicrograph (B) of a positioned cuff in vivo. (C) Photomicrograph of the restenosis lesion in the cuffed femoral artery in the mouse. Indicated are the internal elastic lamina (IEL) and the neointima (NI) formed within the vessel wall.

puncture. After perfusion, a longitudinal incision is made in the internal side of the thigh and the cuffed femoral artery is harvested as a whole and fixed overnight in 4% formaldehyde.

Preclinical application of drug-eluting stents and balloons

Intracoronary stenting decreased restenosis rates by preventing elastic recoil and negative remodeling, although in-stent restenosis due to neointimal proliferation remains the major limiting factor of the success rate for coronary interventions as treatment for coronary artery disease. Drug-eluting stents with a polymer coating have been developed which are loaded with various types of drugs designed to prevent in-stent restenosis. These drugs, some originally used as chemotherapeutic agents, against transplant rejection or as
immunosuppressive drugs, tend to prevent the local inflammatory reaction, SMC proliferation and migration or promote local healing due to a slow local release.
Drug-eluting balloons are coated with similar drugs, but are designed to deliver the drug only for a very short period of time whilst the balloon is left inflated, to prevent

solely the initial local responses to balloon inflation and vessel wall distention.
Limited (post-mortem) pathological data is available from stented human coronary arteries, since histology is usually not readily available. All in vitro and in vivo effects of new drug-eluting stents should be evaluated for safety and efficiency before being applied in human studies. Preclinical animal studies can also provide insight in the method-of-action, dose-response and side-effects of these new stents. Additionally, they can be used for investigation into specific genes involved in restenosis development. Genes of interest can be found in large prospective follow up studies, such as the GENDER study[16], in which the association between gene polymorphisms and clinical outcome can be studied. Additionally, these highly-reproducible models can be used to screen candidate compounds, without the need for expensive, large, long-lasting and time-consuming clinical trials.
Drug-eluting stents can be mimicked in mice by placement of a perivascular non-constrictive drug-eluting cuff[17], comprised of a poly(ε-caprolactone) (PCL) polymer and non-toxic polyethylene glycol, loaded with the candidate drug, shown in figure 2. This polymer cuff allows encapsulation and local sustained release of compounds over a longer period of time. Depending on the ratio between PCL and polyethylene glycol, the duration of drug-release can be extended up until 21 days after cuff placement, to allow local vessel wall drug-exposure throughout the entire study period, even in non-hypercholesterolemic mice which develop restenotic lesions relatively slowly.
An alternative for local delivery of active compounds to the mouse vessel wall is the use of local administration of the compound dissolved in pluronic gel or gelatin in and around the cuff. This allows short period of delivery of compounds locally in the murine cuff model as the gelatin or the pluronic gel will degrade in a short period of time. The set-up of this local application of pluronic gel in the cuff is shown in figure 3. In patients, drug-eluting stents release the drug intraluminally, whilst in this model drugs are released from the adventitial side of the vessel wall. However, the vessel wall in the mouse is smaller, therefore penetration of the compound will be efficient, although applied via the adventitia. Nonetheless, this drug-eluting PCL cuff is certainly an extremely useful and practical tool to evaluate the effects of new candidate anti-restenotic drugs on local vessel wall pathology and intimal thickening as part of post-interventional vascular remodeling.

Suitable mouse strains
Contrary to patients, wild-type mouse strains have low levels of pro-atherosclerotic low-density-lipoprotein and high plasma levels of anti-atherosclerotic high-density lipoprotein and therefore do not readily develop native atherosclerosis. Inbred mice used for studies into accelerated atherosclerosis and restenosis tend to respond to restenotic stimuli by displaying either a type 1 or type 2 helper T cell (Th1 or Th2) response. The Th1 response, typical of the C57BL/6 mouse strain, leads to macrophage differentiation into proatherogenic M1 macrophages and production of inflammatory cytokines and chemokines, which promote lesion formation[18]. The Th2 response, typical of the BALB/C mouse strain, promotes the differentiation of macrophages into anti-inflammatory M2 macrophages, which produce anti-inflammatory and anti-atherogenic cytokines, eventually leading to a healing response within the damaged vessel wall. For this reason, mouse strains with a C57BL/6 background

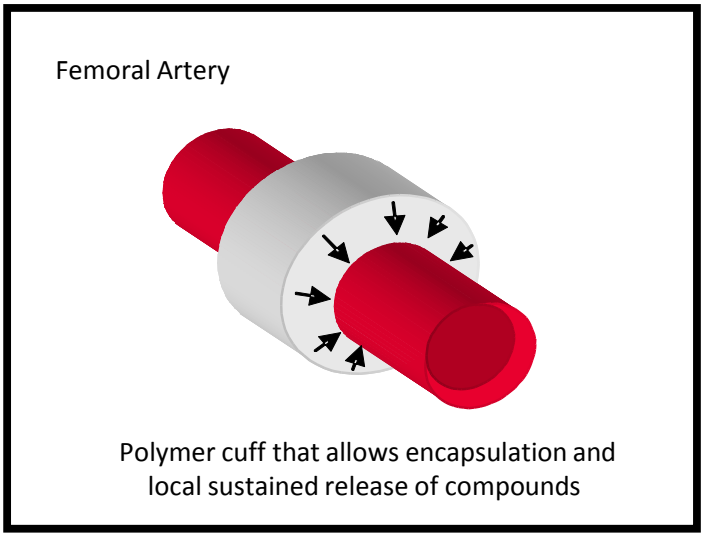

Figure 2. Schematic representation of a drug-eluting femoral arterial cuff and method-of-action.

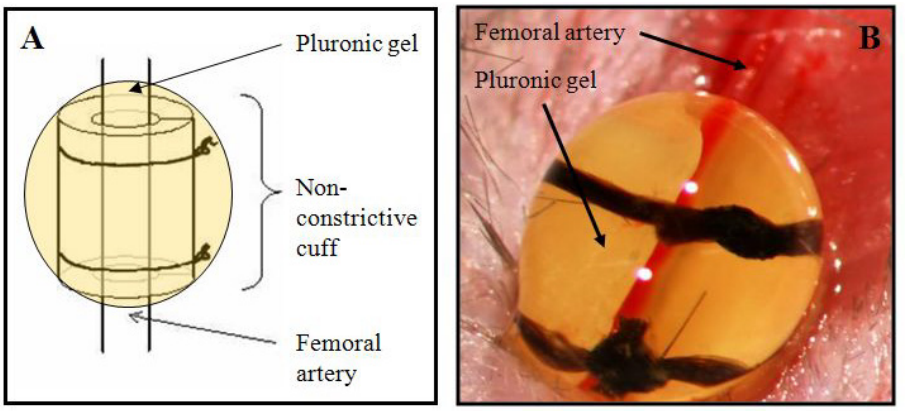

Figure 3. Schematic representation of non-constrictive cuff around a murine femoral artery, covered by hardened pluronic gel (A). Photomicrograph cuff in vivo, with hardened (40%) pluronic gel applied to the cuffed vessel segment, loaded with an anti-restenotic drug (yellow) (B).

a favored for studying pathophysiological changes leading to restenosis. When the perivascular non-constrictive cuff procedure is performed in this mouse strain[19], concentric lesions develop within 21 days after surgery and typically consist of collagen and α-actin positive SMCs.

Dyslipidemic mouse strains

Intravascular injury by balloon inflation and stent placement in diseased human vessels in hypercholesterolemic patients can be mimicked by cuff placement in hypercholesterolemic knock-out or transgenic mouse strains.
Apolipoprotein (Apo) E is an important part of circulating very (V) LDL and LDL cholesterol and acts as a ligand for the LRL-R, thus leading to uptake of proathero-

sclerotic cholesterol from the circulation. Genetic ApoE deficiency therefore leads to hypercholesterolemia due to insufficient LDL-receptor(R)-mediated VLDL and LDL clearance, especially when fed a high-cholesterol diet, and spontaneous atherosclerotic and interventional-induced lesion formation. Drawbacks of this mouse strain are plasma cholesterol levels that are mainly determined by the VLDL fraction, that exceed by far any physiological level and that all other atheroprotective (anti-inflammatory, anti-platelet and anti-proliferative) properties of ApoE are lost[20].
Patients with familial hypercholesterolemia display mutations in the LDL-R gene, leading to insufficient LDL clearance and dyslipidemia. Similarly, LDL-R knockout mice develop mild hypercholesterolemia, which increases strongly when fed a high-cholesterol diet. Since cholesterol elevation is mainly determined by the LDL fraction and spontaneous atherosclerotic lesions develop more slowly than in ApoE knockout mice, this is classified as a more moderate model. Nonetheless, both mouse strains can be used to study post-interventional restenosis development[20].
Mutations in the ApoE3 gene are associated with dysbetaproteinemia in patients. One of these mutations is the ApoE3Leiden mutation[21]. By introducing an ApoE3 and ApoC1 gene construct in the C57BL/6 mice, the transgenic ApoE3*Leiden mouse strain was generated[22]. Since the animals still express endogenous ApoE proteins, contrary to the ApoE knockout animals, the uptake of ApoE-containing lipoproteins is merely reduced and not completely inhibited. Animals have diet-induced increased plasma cholesterol concentrations, mainly in VLDL and LDL fractions. They are very responsive to high-cholesterol feeding. Desired plasma cholesterol concentrations can be obtained by varying dietary cholesterol content. Additionally, since both ApoE and LRL-R are still present, plasma cholesterol levels are subject to modulation by lipid-lowering drugs that influence endogenous chylomicron and VLDL production and indirectly affect LDL-R expression, such as statins. Restenotic lesions after cuff placement develop moderately and consist predominately of SMCs, foam cells and extracellular matrix formation, very similar to human lesions[15].

New mechanistic and therapeutic insights

Agents currently used or under investigation to prevent and treat restenosis can be divided between either drugs with cytotoxic/anti-proliferative and anti-inflammatory effects or drugs that target proteolytic systems. Here we give an overview how animal models may contribute to gain further insight into the mechanism of restenosis and to test new therapeutic strategies.

Proliferation

Rapamycin (sirolimus)

Immunosuppressive drugs like rapamycin are currently widely used in drug-eluting stents to prevent the development of in-stent restenosis. Rapamycin is a macrolide antibiotic drug with anti-proliferative and immunosuppressive effects that targets protein translation, resulting in a G1 arrest of the cell cycle which is known to inhibit vascular SMC proliferation and migration in vitro by inhibiting DNA synthesis and cell growth[23-25]. Rapamycin has been shown to effectively inhibit the arterial proliferative response after PCTA in a porcine restenosis model, without toxicity in low

doses. Local rapamycin application in the murine cuff model was performed using a rapamycin-eluting cuff and it was observed that locally released rapamycin led to an inhibition of neointima formation by 75±6% for all tested concentrations. Experiments demonstrated that perivascular sustained release was restricted to the cuffed vessel segment, with no systemic adverse effects. Moreover, when applying the rapamycin eluting cuff to a diseased atherosclerotic vessel segment in ApoE3Leiden mice, no progression of the atherosclerotic lesion development could be observed, nor any systemic side effects

Paclitaxel

Paclitaxel belongs to the taxanes, which are potent anti-proliferatives widely used to treat patients suffering from cancer. It leads to polymerization of the α- and β-units of tubulin and thus stabilizes microtubules, which are necessary for the G2 transition of a dividing cell into the M phase. Paclitaxel causes almost complete inhibition of cell growth and SMC proliferation and migration by targeting cytoskeleton structure. SMC proliferation and migration are both induced by balloon inflation and stent placement during interventional procedures. In a murine femoral arterial cuff model, drug-eluting cuffs containing high paclitaxel concentrations (1–5%) have been shown to reduce intimal thickening by 76±2%. When placing the paclitaxel eluting cuff in hypercholesterolemic mice or even over an existing atherosclerotic lesion in mice, dose dependent negative side effects were observed. High dosage of placlitaxel significantly increased apoptosis (also in the media), disruption of the elastic laminas and decrease medial and intimal smooth muscle cell as well as collagen content. These findings show elegantly the added value of testing drug eluting devices in atherosclerotic animal model. Many clinical trials[26-28] have investigated the effectiveness of (non) polymer-based paclitaxel-eluting stents and have shown to be very effective in the prevention of restenosis development, however, negative effects on the vascular pathology were not detected, emphasizing that the femoral arterial cuff model is of high predictive value in the screening for new candidate drugs for efficacy and local adverse side effects.

Inflammation

Although neointima formation is characterized by proliferation and migration of smooth muscle cells (SMC) and extracellular matrix turnover it is now broadly accepted that these processes are triggered by inflammatory activation of the vessel wall[16]. Evidence that inflammation is the initial trigger for vascular remodeling has accumulate over the past years and the role of various cytokines and chemokines as pro-inflammatory factors as well as immune modulation in general has been the focus of many studies on vascular remodeling and restenoses. In the next section we would like to illustrate this with a couple of representative examples. Starting with the description of the effects of the general anti-inflammatory factor dexamethason, we will further zoom in on the effects of specific cytokines (TNF α, IL10), chemokines (MCP-1) and the role of the innate immune system (complement, toll-like receptors) on restenosis in the mouse models.

Dexamethason

Dexamethason is a corticosteroid with strong glucocortocoid properties and is widely

used as a broad anti-inflammatory and immunosuppressant drug. Prolonged systemic delivery is associated with multiple side effects in humans and animals. These effect could be abolished by local delivery using a drug-eluting cuff (DEC), mimicking the potential effect of drug eluting stents. Local delivery of dexamethason via DEC delivery inhibited neointima formation drastically without systemic side effects, indicating a beneficial effect of local suppression of inflammation over systemic delivery as was tested by applying dexamethason via the drinking water in the same model. However pathobiological examination of the murine arteries revealed a dose-dependent medial atrophy, a reduction in vascular smooth muscle cells and collagen content, an increase in apoptotic cell count and disruption of the internal elastic lamina[29]. Short term systemic delivery in a model for vein graft remodeling showed a reduction in intimal hyperplasia formation without serious side effects probably due to the short period of delivery[30]. These results not only emphasize the importance of the immune system and the role of inflammation in restenosis more specifically and systematically, but also show that the use of small (hypercholesterolemic) animal models does have predictive value in regard to negative side effects on vascular pathology after local drug application.

Innate immunity

Innate immunity is very important in triggering inflammation and can be divided into a humoral and a cellular component. It comprises multiple cell types, receptors and mechanisms such as the Complement system and Toll like receptors (TLRs) that are very important in host defense. The innate immune system is considered to be highly involved in regulation of intimal hyperplasia and atherogenesis. This could very elegantly be demonstrated using specific mouse models in combination with cuff placement or vein grafting as described in the section below.

Complement

The complement system comprises the humoral mechanism of the innate immune system. C3 cleavage plays a central role in the complex regulation of complement activation and can be initiated via the classical, alternative or the lectin pathway. The role of complement was studied in the model of vein graft restenosis combined with accelerated atherosclerosis in the ApoE3*Leiden mouse. The expression of complement components C1q, C3, and the regulatory proteins CD59 and complement receptor-related gene y (Crry) could be detected on the protein and mRNA level in the grafts. A reduction in vein graft thickening and intimal hyperplasia was accomplished after interference with C3 activation by systemic administration of either Cobra Venom Factor or Crry-Ig protein. The latter was associated with a reduced number of inflammatory cells in the vessel wall[31], indicating that blocking the central factor in the complement activation cascade, C3, results in a profound reduction of vascular remodeling. This underscores a role of the innate immune system in restenosis related vascular remoding.

Toll-like Receptors

TLRs are membrane bound receptors located on a variety of immune and non-immune cells including macrophages, endothelium and SMCs. Cell stress and tissue damage may cause a release of Damage Associated Molecular Patterns (DAMPs)

that function as endogenous TLR ligands. Balloon inflation and stent placement or bypass grafting lead to injury to the vessel wall and may cause up regulation of DAMPs such as Heat Shock Protein 60 (HSP60), Fibronectin-EDA, Tenascin C and Biglycan. HSP60 binds directly to TLR2 or TLR4 thereby initiating proliferation of VSMC[32]. In response to peri-adventitial cuff induced injury TLR4 expression is up regulated in the vessel wall during at least 7 days (unpublished data). A causal role for TLR4 in restenosis was demonstrated by a reduced cuff induced neointima formation in TLR4 deficient mice[33]. Moreover, local adventitial TLR4 activation by LPS application strongly augmented neointima formation in both mouse models, so with and without involvement of accelerated atherosclerosis[34]. The same method was used to stimulate TLR2 with Pam3Cys and resulted in an increase in neointima formation in C57/B6 mice and in APOE$^{-/-}$ mice that develop atherosclerotic lesions[35]. We believe that the release upon vascular injury of specific DAMPs as endogenous TLR ligands is one of the earliest triggers in vascular remodeling in restenosis and there are convinced that the TLR signaling pathway has a crucial function in the restenosis process. This is supported by the SNPs found in the TLR4 gene that correlate with in increased risk for cardiovascular event and/or restenosis after an initial PCI[36-38].

Cytokines

Activation of the immune system results in secretion of multiple pro and anti-inflammatory cytokines. These cytokines can be seen as hormones of the immune system that communicate, can attract and activate different cell types importantly involved in restenosis and atherosclerosis like SMC and macrophages. A disturbance in the cytokine balance, locally or systemically, alters the inflammatory status thereby mediating inflammatory processes. Since many cytokines have their role in multiple inflammatory reactions it is important to know whether they are involved, can be a therapeutic target or may function as biomarker for the process of restenosis.

TNFα

Tumor Necrosis Factor alpha (TNFα) is a cytokine that regulates immune cells and promotes the inflammatory response. It is produced by many cells including endothelial cells, VSMCs and macrophages. The GENDER project systematically genotyped for six polymorphisms in the TNFα gene and found associations with an increased clinical and angiographic risk for restenosis in humans[39]. In a rat balloon-injury model blockade of the TNFα caused a reduction in neointima formation via acceleration of endothelium repair[40]. In mice, after common carotid artery (CCA) ligation, TNFα mRNA expression was found in intimal lesions itself. Application of the same model in knockout mice showed a decrease in lesion size[41]. After peri-adventitial cuff placement TNFα mRNA is rapidly up regulated to levels 4000 times to original mRNA levels and deficiency of TNFα in a murine study of restenosis with accelerated atherosclerosis, performed in ApoE*3-Leiden-TNFalpha knockout mice, caused a marked reduction in neointima formation. Interestingly, the use of a DEC for local delivery of thalidomide as a potent TNFalpha biosynthesis inhibitor demonstrated a powerful reduction of the neointima formation after cuff placement to levels similar to those observed in the ApoE*3-Leiden-TNFalpha knockout model[39]. TNFalpha is clearly an important cytokine in the inflammatory process that take place in the

vessel wall during restenosis and its role can be studied in detail using the specific mouse models for restenosis, cuff and drug eluting cuff placement.

CCR2/MCP1

The chemokine Monocyte Chemoattractant Protein1 (MCP1) or CCL2 is a cytokine that is capable of attracting immune cell types like monocytes that are known to infiltrate the vessel wall as a one of the first in neointima formation. Furthermore MCP1 influences SMC proliferation and is expressed in various stages of vascular remodeling. MCP1 binds to its receptor CC chemokine receptor 2 (CCR2) that belongs to the family of G-coupled receptors. Femoral artery transluminal injury by passage of a 0.25-mm diameter angioplasty guide wire was done in a CCR2$^{-/-}$ mice[7].Four weeks after injury, CCR2$^{-/-}$ mice showed a 61.4% reduction in neointima formation and a 62% reduction in intima/media ratio. The effects of MCP1 in vivo were studied in the murine vein graft model as well as in the femoral cuff model. Systemic overexpression of a dominant negative form of MCP-1, 7ND-MCP, by electroporation of a plasmid into the calf muscle resulted in circulating levels of this MCP-1 inhibitor that were sufficient to decrease intimal hyperplasia significantly, both in the cuff model[42] as well as in the vein graft model[43].

In addition, in the mouse vein graft restenosis model perivascular local vector application for lentiviral shRNA targeting CCR2 reduced intimal hyperplasia in the vein grafts by approximately 50%[44]. These studies demonstrate the role of these models in evaluation of experimental therapeutic strategies.

Interleukin-10

Interleukin-10 (IL-10) is one of the most prominent anti-inflammatory cytokines and functions pleiotropic. It may suppress antigen presentation and is capable of inhibiting pro-inflammatory cytokine production. These capacities make IL-10 a very attractive candidate for anti restenotic and anti atherosclerotic therapy. Three polymorphisms significantly increased the risk of restenosis in patients and demonstrate that IL-10 is associated with restenosis. This set interest for anti-inflammatory genes to be involved in the development of restenosis[45]. The functional role of IL-10 in restenosis was assessed by Feldman et al.[46] and showed beneficial effects of recombinant human IL-10 after balloon angioplasty or stenting in hypercholesterolemic rabbits. Per-adventitial cuff placement in hypercholesterolemic APOE*3-Leiden-IL-10$^{-/-}$ mice (hypercholesterolemic mice deficient for IL-10) resulted in an increased neointima formation indicating a protective role for IL-10 in a murine model for restenosis and accelerated atherosclerosis[47]. Electroporation of an IL-10 plasmid into the calf muscle resulted in IL-10 overexpression in ApoE3*Leiden mice and caused a reduction in cholesterol and neointima formation two weeks after cuff placement, underscoring the therapeutic potential of IL-10 in restenosis.

Protease Inhibition in Restenosis

Proteases of the Matrix metalloproteinase (MMP) system and of the plasminogen activator system are thought to play an important role in the matrix degradation and smooth muscle cell migration during vascular remodeling and are upregulated after coronary angioplasty[48]. Studies in mice showed a decreased neointima formation in MMP2$^{-/-}$, MMP9$^{-/-}$ mice and augmented neointima formation in TIMP1$^{-/-}$ mice[49-51]. By

use of the electrocoagulation vascular injury model Lijnen et al.[52] studied whether the plasminogen activation system, a system that also may activate the MMP system, and the MMP system itself play a role in neointima formation using knockout mice. Neointima formation was reduced in urokinase-type plasminogen activator knockout ($uPA^{-/-}$) and Plasminogen (Plg) knockouts ($Plg^{-/-}$) but no effect was seen in the tissue-type Plg activator knockout ($tPA^{-/-}$) mice.
To study the therapeutic potential of these findings a hybrid protein consisting of the receptor-binding amino-terminal fragment of uPA (ATF), linked to the potent protease inhibitor bovine pancreas trypsin inhibitor (BPTI) was constructed and cloned into an adenoviral vector[53] Mice were infected with the combined ATF.BPTI vector or single vectors for ATF or BPTI and cuffs were placed around the femoral arteries to induce neointima formation. Only the ATF.BPT showed a strong inhibition of neointima formation by selective binding to the uPA receptor and inhibiting plasmin activity[54]. Infection with the same vector in a balloon injury model also showed significant inhibition of neointima formation in rats[55] as well as in mice after cuff placement[53]. In 2002 a novel hybrid protein consisting of the tissue inhibitor of metalloproteinase-1 (TIMP-1) domain, as MMP inhibitor, linked to ATF (TIMP-1.ATF) was constructed. By binding to the u-PA receptor this protein blocks binding of u-PA and attaches TIMP-1 directly to the cell surface. This construct was able to inhibit SMC migration and neointima formation in vitro[56]. In vivo intimal hyperplasia combined with accelerated atherosclerosis was studied in murine vein grafts. Plasmids encoding ATF, TIMP-1, TIMP-1.ATF, were injected and electroporated (non-viral gene transfer) in both calf muscles of hypercholesterolemic ApoE*3Leiden mice. Although all constructs reduced vein graft thickening compared with the controls, the luminal area was best preserved in the TIMP-1.ATF-treated mice[57].
Finally a non-viral expression vector encoding the hybrid protein TIMP-1.ATF.BPTI (TAB) was constructed and validated. After four weeks, vein graft thickening was significantly inhibited in mice treated with the single domains TIMP-1, ATF or BPTI. In the TAB treated mice vein graft thickening was reduced and was also significantly stronger as compared to the individual domains[58].

Conclusions

For restenosis research animal models are definitely essential for testing new anti-restenosis devices, such as new drug eluting stent, as well as for unraveling the underlying pathophysiological mechanism and identifying new therapeutic targets.
It is important to work with models that mimic the human situation as good as possible, either in vascular anatomical aspects (size, diameter, wall thickness) or disease stage related aspects (hypercholesterolemia, vessel with atherosclerotic lesions).
In the current chapter we have focused on the later group of animal models, those humanized models that have the best predictive value for the pathophysiological process in the development of restenosis, intimal hyperplasia and accelerated atherosclerosis in the lesions. Various vascular interventions in transgenic mouse models have been described, with a strong focus on the mouse femoral artery cuff model and these mouse models have proven to be technically suitable for the study into restenosis development.
Next, to study effects of (local) drug therapy, animals should be susceptible to the

treatment of interest, have similar metabolic levels, coagulatory phenotype and react in a human-like fashion. The use of humanized (transgenic) animal models has extensively increased the similarity between human and animal lesions and the translation of new therapies into in the clinical setting.

Mechanistic and pathophysiologal studies have shown that local vessel wall inflammation, proliferation and proteolysis play important roles in the post-interventional vascular remodeling, both in humans and in the animal models used.

In addition, these animal models are extremely suitable to identify new potential therapeutic targets to prevent restenosis and test new experimental strategies for therapy, e.g. based on systemic or local gene delivery of inhibitory factors (anti-proliferative, anti-inflammatory or anti-proteolytic). These studies clearly demonstrate the importance and value of animal models for clinical medicine.

We can conclude that highly-reproducible animal models for post-interventional vascular remodeling remain essential for studying the process of restenosis and the development of future anti-restenotic therapies.

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