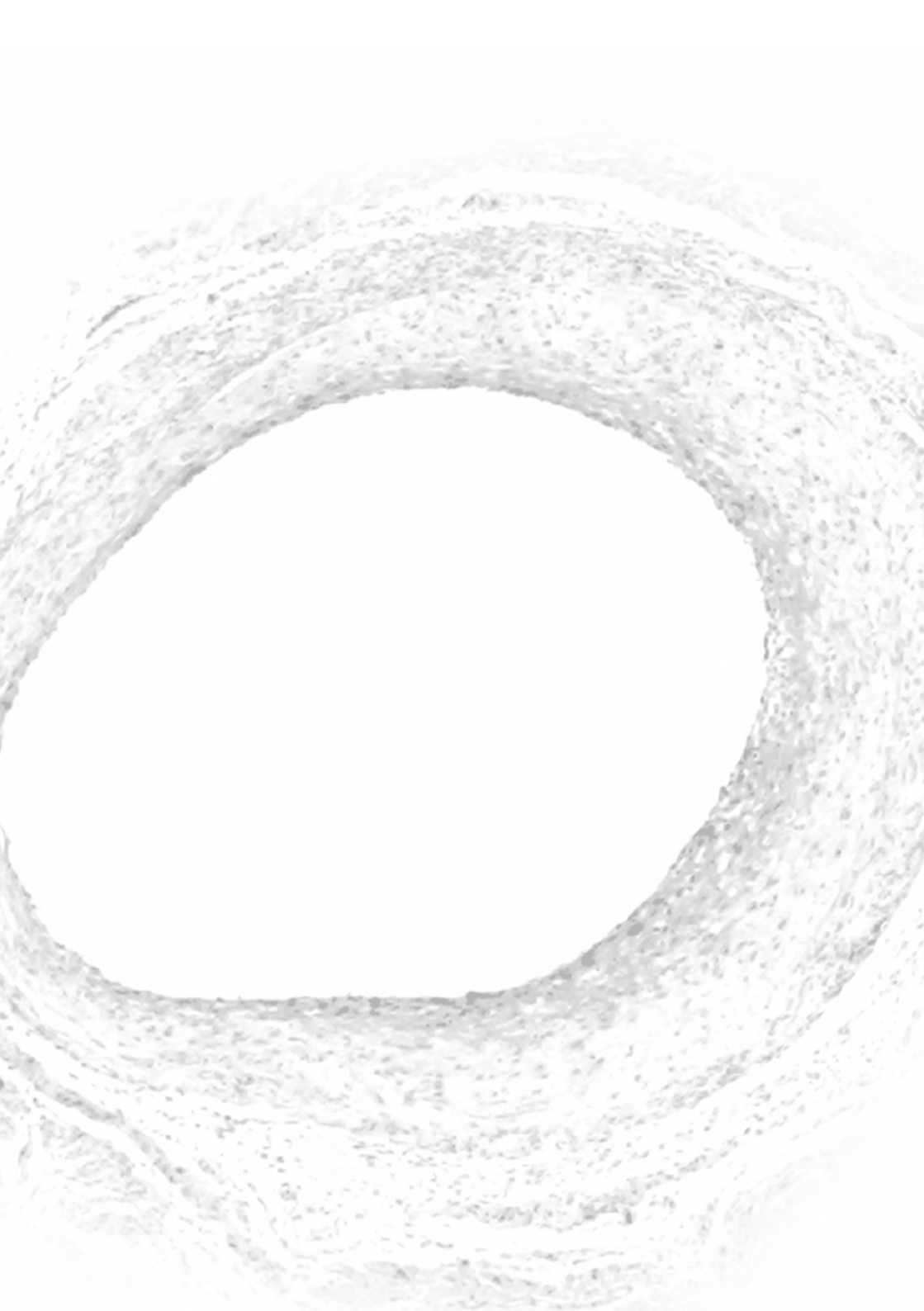
Inflammation in injury-induced vascular remodelling : functional involvement and therapeutical options

Schepers, A.



CHAPTER 9

Discussion

SUMMARY

The aim of this thesis was to gain more insight in the involvement of inflammatory processes in vessel wall remodeling seen after PTA or bypass surgery and put these processes in the perspective of restenosis, vein graft failure and potential therapeutic preventive strategies. Therefore, we firstly focused on inflammation in general, using the anti-inflammatory agent Dexamethasone, assessing the effects of such a broad approach on restenosis and vein graft remodeling. Then, we further focused on some specific parts of the immune system, namely Interleukin 10 (IL10), chemokines and the complement cascade. Il10 was chosen because it is one of the most studied anti-inflammatory cytokines and this property makes it a potential candidate for ant-restenosis therapy. Furthermore, it was hypothesized that chemokines are involved in vascular remodeling, since they are generally known for their regulatory properties regarding influx of inflammatory cells to tissues and this is one of the first phenomena seen in vascular remodeling.

The complement cascade was studied in this context since it contains pro-inflammatory activity and some end-products of the cascade, like chemokines, are potent chemotactic agents.

Several preclinical and clinical studies have assessed the anti-restenotic properties of the glucocorticoid Dexamethasone, showing inconsistent outcomes. Recently, several studies have been performed with Dexamethasone-eluting stents. However, none of them focused on the (possible adverse) pathophysiologic changes in the vessel wall. In **Chapter 2**, the anti-restenotic properties of Dexamethasone were assessed, and more importantly the pathophysiologic effects of local application of Dexamethasone to the vessel wall were studied in a mouse model of cuff-induced stenotic remodeling. Both systemic and local treatment with Dexamethasone resulted in reduction of intimal hyperplasia in the cuffed vessel segment, indicating an inflammatory component in vascular remodeling regulation. However, systemic treatment with Dexamethasone was accompanied by serious side-effects (mice showed reduced daily activity, gained less weight, reduced fur quality). Local delivery of Dexamethasone to the cuffed vessel segment in the mouse, prevented these side-effects, but was associated loss of vascular integrity, particularly at high concentrations of Dexamethasone, as was revealed by medial atrophy, reduced SMC and collagen content, increased apoptosis and internal elastic lamina fracture in the treated vessel segments. The results of this study indicate that although local Dexamethasone delivery, for instance via Dexamethasone-eluting stents, has the potential to inhibit restenosis, the toxic-therapeutic window is relatively narrow and loss of vascular integrity is already detected at relatively low doses of Dexamethasone. This loss of vascular integrity may reflect potential “weak spots” in the vessel wall, hypothetically vulnerable for thrombus- or aneurysm formation. For daily practice, caution should be taken when using overlapping stents. Studies that study this phenomenon of loss of vascular integrity after drug-eluting stent placement should be performed with other anti-restenotic drugs that might influence vessel wall composition.

The role of inflammation in regulation of vascular remodeling is clarified in more detail in **Chapter 3. Chapter 3** deals with the effects of the cytokine Il10 on post-angioplasty restenosis. Generally, Il10 displays anti-inflammatory activity and therefore might be an interesting target in the search towards anti-restenotic therapy, in which for example over-expression of the Il10 protein might reduce post-angioplasty restenosis. Effect of Il10 on cuff-induced vascular remodeling was studied in two models. In the first the effect of absence of Il10 on cuff-induced restenosis was studied in ApoE3Leiden-Il10 knock-out mice. Then, over-expression of Il10 was achieved using an electroporation-mediated gene transfer in the calf muscle of ApoE3Leiden mice and effects on restenosis were studied similar as in the knock-out experiment. These approaches both show that IL10 indeed is significantly involved in the regulation of neointima formation and accelerated atherosclerosis and has anti-restenotic potential: absence of Il10 aggravated intimal hyperplasia, whereas in contrast over-expression of Il10 inhibited intimal hyperplasia formation. The pathophysiologic mechanism of this effect is presumably due to induction of a more Th2-like response as was suggested by mRNA expression of various Th2-related cytokines in the spleen; several pro-inflammatory cytokines were down regulated in response to Il-10 over-expression. Furthermore, over-expression of Il10 resulted in reduced serum cholesterol levels in hypercholesterolemic ApoE3Leiden mice, a phenomenon that also might partially contribute to inhibition of intimal hyperplasia.

Both **Chapter 2** and **Chapter 3** further underscore the role of inflammation in post-angioplasty restenosis and discuss some potential therapeutic options and safety considerations, in order to prevent restenosis in patients that underwent PTA of a stenotic vessel. Using the glucocorticoid Dexamethasone, as a general inhibitor of inflammation to prevent vascular remodeling, at a first glance is a promising, but definitely has adverse effects, which indicates that refinement in Dexamethasone treatment is required. Il10-overexpression on the other hand seems to be a potentially suitable anti-restenotic strategy.

In the remaining part of the thesis, the role of inflammation in vein graft remodeling was studied. As in **Chapter 2**, we started with a general approach, a proof of principle of the hypothesis that inflammatory processes are involved in vein graft remodeling, using Dexamethasone as a potent and broadly active anti-inflammatory agent.

In **Chapter 4** ApoE3Leiden mice that underwent vein graft surgery were treated with 28 days of orally administered Dexamethasone treatment and the effect in vein graft thickening was assessed 28 days after surgery. Treatment resulted in reduced vein graft thickening, however the 28 days treatment was accompanied by severe side-effects related to prolonged use of corticosteroids. As an alternative, a short term treatment with Dexamethasone for 7 days was evaluated. Short term treatment did not lead to any side-effects, but led to a similar reduction of vein graft thickening as the 28 days treatment. mRNA analysis of vein grafts harvested on different time-points after engraftment revealed that treatment with Dexamethasone resulted in reduced expression of various pro-inflammatory cytokines in the vein graft wall,

already as fast as 24 hours after engraftment. For testing the applicability in human, we used human saphenous vein organ culture, in which neointima formation is mimicked. When Dexamethasone was applied to human saphenous veins in culture, either 7 or 28 days, the same results as in mice were observed, namely a similar reduction in neointima formation unrelated to the duration of exposure to Dexamethasone. This indicates that these effects of short term treatment are not mouse-specific. Extrapolating this to clinical practice, where long term treatment of patients with Dexamethasone is not acceptable, it seems that Dexamethasone can be used for a limited period of time in order to prevent vein graft thickening. In that way, serious side effects of prolonged Dexamethasone-treatment can be avoided whereas the anti-restenotic properties, most likely by reducing the inflammatory response directly after surgery, might be already present.

More generally, these data suggest that inhibiting inflammatory pathways in the very early phases of vein graft remodeling might result in reduced vein graft thickening on the long run.

Having established the hypothesis that indeed inflammatory processes play a role in vein graft remodeling, some specific parts of the inflammatory reaction were further studied, namely the role of chemokines (**Chapter 5 and 6**) and of the complement cascade (**Chapter 7 and 8**).

CC-chemokines are a group of pro-inflammatory cytokines, which have been shown to be involved in various inflammatory processes. They display chemo-attractant properties to a variety of (inflammatory) cell types and therefore it was hypothesized that they might well be involved in vein graft remodeling, as one of the first phenomena seen in remodeling grafts is influx of inflammatory cells.

MCP-1 is the most potent and most frequently studied chemokine in the CC-family. It has been shown to be a major participant in various remodeling processes such as spontaneous atherosclerosis, post-angioplasty restenosis and transplant accelerated atherosclerosis. In **Chapter 5** the role of MCP-1 in vein graft disease is studied. In this chapter its role in vein graft thickening was assessed in ApoE3Leiden mice, using both the murine vein graft model and in human saphenous veins organ cultures. MCP-1 was shown to be both present in the vein graft as well as in the human saphenous vein, in various stages of the remodeling process. A significant reduction of vein graft thickening was seen after inhibition of MCP-1 activity by electroporation mediated over-expression of a dominant receptor antagonist, 7ND-MCP-1. Similar findings were seen in human saphenous vein organ culture, wherein neointima formation was significantly reduced in the presence of 7ND-MCP-1 protein in the culture medium. Furthermore, in addition to its known effects on macrophage influx and subsequent macrophages presence in the vascular lesion, also a direct inhibitory effect of 7ND-MCP-1 on smooth muscle cell proliferation was demonstrated, as assessed by proliferation assays on cultured smooth muscle cells.

From these data we conclude that MCP-1 is an important mediator in vein graft remodeling and it might be a potential target for anti-restenosis therapy in vein grafts.

Other CC-chemokines are less extensively studied in the field of vascular remodeling as a whole. MIP-1α and RANTES also belong to the CC-chemokine family and have chemoattractant properties for macrophages, T-cells and (to a lesser extent) granulocytes, and share receptors (predominantly CCR1, CCR3 and CCR5, whereas MCP-1 binds to CCR2). Therefore they were studied together in **Chapter 6**.

A possible causal involvement of the MIP-1α/ RANTES-CCR1/CCR5 pathway in vein graft thickening was studied in the murine vein graft model using hypercholesteremic ApoE3Leiden mice. Alike MCP-1, MIP-1α and RANTES expression could be detected in the vein graft wall, shortly after engraftment, whereas they were absent in normal caval veins and time-dependent upregulation of MIP-1α and RANTES mRNA and their receptors was observed after surgery. When mice were treated with the CCR1/CCR5 antagonist Met-RANTES, inhibition of adherence of monocytes was seen three days after surgery. Furthermore, a profound inhibition of vein graft thickening occurred after treatment with Met-RANTES after 28 days, coinciding with reduced numbers of foam cells in the lesion. These data indicate that not only MCP-1, but also other CC-chemokines are involved in vein graft remodeling, and therefore blocking function of these chemokines by (receptor) antagonists in the direct post-operative phase might be a rational, and possibly additional, approach to prevent vein graft disease.

Another part of the innate immune system is the complement cascade. Complement, being a group of proteins, membrane-bound receptors and regulatory enzymes, regulates inflammation by several different biological functions (opsonisation, chemotaxis etc.) and is involved in many (patho-) physiological responses to stimuli. However, regarding its role in the regulation of vascular remodeling only limited information is available. In **Chapter 7**, we demonstrated that several complement factors (both on protein and mRNA level) are present in thickened vein grafts, including complement component C3, the key player in the complement cascade. Treatment of mice that underwent vein graft surgery with Crry-Ig (interfering in C3 activation) results in marked reduction of vein graft thickening. This reduction coincides with reduced numbers of (adhering) leucocytes and reduced proliferation indexes in early stages of vein graft remodeling. Furthermore, treatment with Crry-Ig resulted in a increased relative contribution of smooth muscle cells in the thickened vein graft in the later stage. A similar reduction in vein graft remodeling as after treatment with Crry-Ig, was seen after treatment with Cobra Venom Factor, another inhibitor of C3 activation, indicating that the observed effects are really due to inhibition of C3 activity and not Crry-Ig specific. These results suggest that activation of the complement cascade is one of the crucial events in initiation of vein graft remodeling.

In **Chapter 8**, the involvement of the most potent chemotactic factor of the complement cascade, C5a, was studied in our murine model of vein graft disease. Immunohistochemistry showed that C5 appears to be predominantly expressed in adhering monocytes, adventitial fibroblasts, endothelial cells and foam cells in the thickened vein graft, whereas it is not detectable in normal caval veins. Furthermore, increased exposure to C5a (during surgery by topical application of recombinant C5a dissolved in pluronic gel directly to the vein graft) dose-dependently aggravated vein graft thickening, and also dose-dependently increased macrophage-derived foam cell content in the lesion. Oppositely, hampering signal transduction of the C5a receptor (C5aR) using specific C5aR-antagonists resulted in a decreased vein graft thickening with reduced contribution of foam cells to lesion formation. These results demonstrate that besides CC-chemokines, also other chemotactic factors (in this case C5a) are involved in vein graft remodeling, and that these factors can be used to hamper vein graft remodeling.

The above-mentioned results in **Chapter 7 and 8** clearly specify at least one of the pathways by which complement is involved in vein graft remodeling, being induction of influx of various inflammatory cells to the vessel wall and cytokinetic processes such as proliferation. More specifically we show that C5a is an end-product of great importance in the process of vein graft remodeling and it might actually be the predominant end-product of the complement cascade involved in the influx of inflammatory cells.

CONCLUSIONS

Occurrence of restenosis after PTA or development of vein graft disease is a significant clinical problem, often requiring new interventions. Insight in the pathophysiology of vascular remodeling will provide possible targets for therapy. This thesis has focused on the role of inflammation in post-interventional vascular remodeling in general. However, since inflammation can be seen as an orchestra, with contributors acting at different time points and influencing each other, the several components were studied separately. Taking all the studies together, this thesis clearly shows that specific parts of the inflammatory reaction (chemokines, as well as the complement cascade) are involved in the early phases of post-interventional vascular remodeling and that inhibition of the inflammatory response, by counteracting separate components, results in reduced vascular remodeling.

Therefore, we believe that we did not only gained more insight in the mechanism of post-interventional vascular remodeling, but also indicated several potential targets for therapy to defeat the clinical problem of restenosis. Future research will reveal whether these targets are truly applicable in patients.

FUTURE PERSPECTIVES

In the last years, a great amount of work generated valuable data that provided insight in the pathophysiology of post-interventional remodeling. As endorsed by this thesis, inflammation is importantly involved in the early steps of the remodeling process and the regulation of influx of inflammatory cells is performed by several different factors. However, it remains difficult to put all events in place and to survey the complete process. We believe that future research should aim at a better understanding of the consecutive events in vascular remodeling and seek for hierarchical series herein. One of our major challenges is to appoint which of all inflammatory factors that have been shown (either described in this thesis or by other groups) to be involved in the process of vascular remodeling in murine or other animal models, the one to be chosen for further therapy development in patients. Given the complex and multi-factorial character of the problem, a close collaboration between clinicians and basic researchers appears to be of utmost importance. This represents the area of *translational research*. In this thesis, we pursued this by attempting to test data gained from murine experiments for applicability in human tissue **(Chapter 4 and 5)**. Another study (although not included in this thesis) that nicely illustrates a translational approach is the one wherein TNF-α-polymorphism is identified as a risk factor for coronary restenosis in patients that underwent PTA and this hypothesis is conformed in various murine experiments[1].

Taking all recent advances in the field of atherosclerosis and vascular remodeling research together, what will be the benefit for the patient? What changes in therapy can be expected for patients suffering from the clinical consequences from advanced atherosclerosis? We believe that therapies aiming at modulation of the immune response will enter the clinical field of vascular medicine.

One of the first questions that need to be asked is: which patients should be considered for therapy?

Since the firsts signs of atherosclerosis can already be detected in the second and third decade of life and these early stages of the disease are not accompanied by clinical symptoms, therapies aiming at *prevention of atherosclerosis* do not appear feasible as it implies that virtually the whole population should be treated protractedly, not knowing whether the specific individuals will ever develop clinically overt atherosclerosis. Clearly, this approach results in serious over-treatment of a lot of healthy adults. However, *secondary prevention*, defined as treatment to prevent recurrent cardiac and vascular morbidity and mortality and to improve quality of life in people who had a prior manifestation of complicated atherosclerosis, overcomes the problem of over-treatment and selects patients that might benefit from treatment. Therapies for secondary prevention will change in the upcoming years. It is this kind of therapies this thesis has focused on and since this thesis dealt solely with post-interventional remodeling, we will further discuss future therapeutic measures aimed at inhibiting post-PTA restenosis and vein graft disease.

The last years, prevention of restenosis after PTA has evolved using the drug-eluting stents, and with good results (although long-term studies show conflicting results). Until now, coatings has focused on inhibition of proliferation, using Paclitaxel and Sirolimus. In the upcoming years, anti-inflammatory agents might be introduced as a coating to stents, taking into account the growing amount of evidence pointing at an important role for inflammatory processes in the early stages of post-PTA restenosis. Furthermore, pre-intervention assessment by micro-array analysis of snips and other polymorphisms can indicate patients with high risk of restenosis and new complications of atherosclerosis, and identify individualized targets for therapy, an approach demonstrated in the GENDER study[2]. Ideally, multiple coated stents are available, each with a different coating (for example antagonists of MCP-1, TNF-α or C3, Il10 overexpression, anti-proliferative agents, lipid lowering compounts etc.) and pre-intervention analysis allocate the proper coated stent to a specific patient, thereby customizing treatment in the individual patient undergoing PTA.

In case of bypass surgery, a similar approach can be made. Specific groups of patients with high risk of bypass failure should be identified, either by experiences from previous bypasses, or based on micro-array data as described above. Once high-risk patients are indicated, one can hypothesize that these patients can be treated post-operatively with regular (corticosteroids) or newly developed anti-inflammatory agents (e.g. anti-MCP-1, anti-C5a).

One other approach would be to treat the bypass locally. Although the phenomenon of drug-eluting stents does not exists in the field of bypass surgery, theoretically vein grafts are highly suitable for ex-vivo manipulation. In this line of thinking, coatings containing therapeutic agents can be directly applied or gene transfer can be used to over-express or block expression of certain proteins involved in vein graft remodeling.

Finally, aside of the future perspectives, one remark regarding studying vein graft remodeling should be brought under attention. Insight in the pathophysiology of vein graft diseases is to a large extent gained by extrapolating observations in other forms of vascular remodeling (post-angioplasty restenosis, spontaneous atherosclerosis) to the failing bypass, thereby assuming that pathophysiologies in the different forms of vascular remodeling are alike. This assumption even led to publications of review papers dealing about vein graft disease, wherein the majority of the cited references concerns studies of atherosclerosis and post-interventional restenosis.

There are multiple arguments to oppose this assumption. Firstly, venous endothelium is distinct from arterial endothelium with regards to origin, response to hemodynamic changes and inflammatory stimuli. Secondly, the vessel wall of vein grafts exist of less smooth muscle cells in the media, making them more vulnerable to circumferential forces. Finally, restenosis occurring after PTA, is developing in diseased tissue (an underlying atherosclerotic plaque is present) and therefore response to mechanical injury may be different than that of a principally healthy greater saphenous vein that is used as a venous bypass graft.

To our opinion, although a lot of the processes and mediators in vein graft disease, post-PTA restenosis and spontaneous atherosclerosis might be alike, no remarks about pathophysiology of vein graft disease can be made, unless studied in a proper model. For the reasons mentioned above, good research aimed specifically on vein graft disease remains necessary in the future.

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