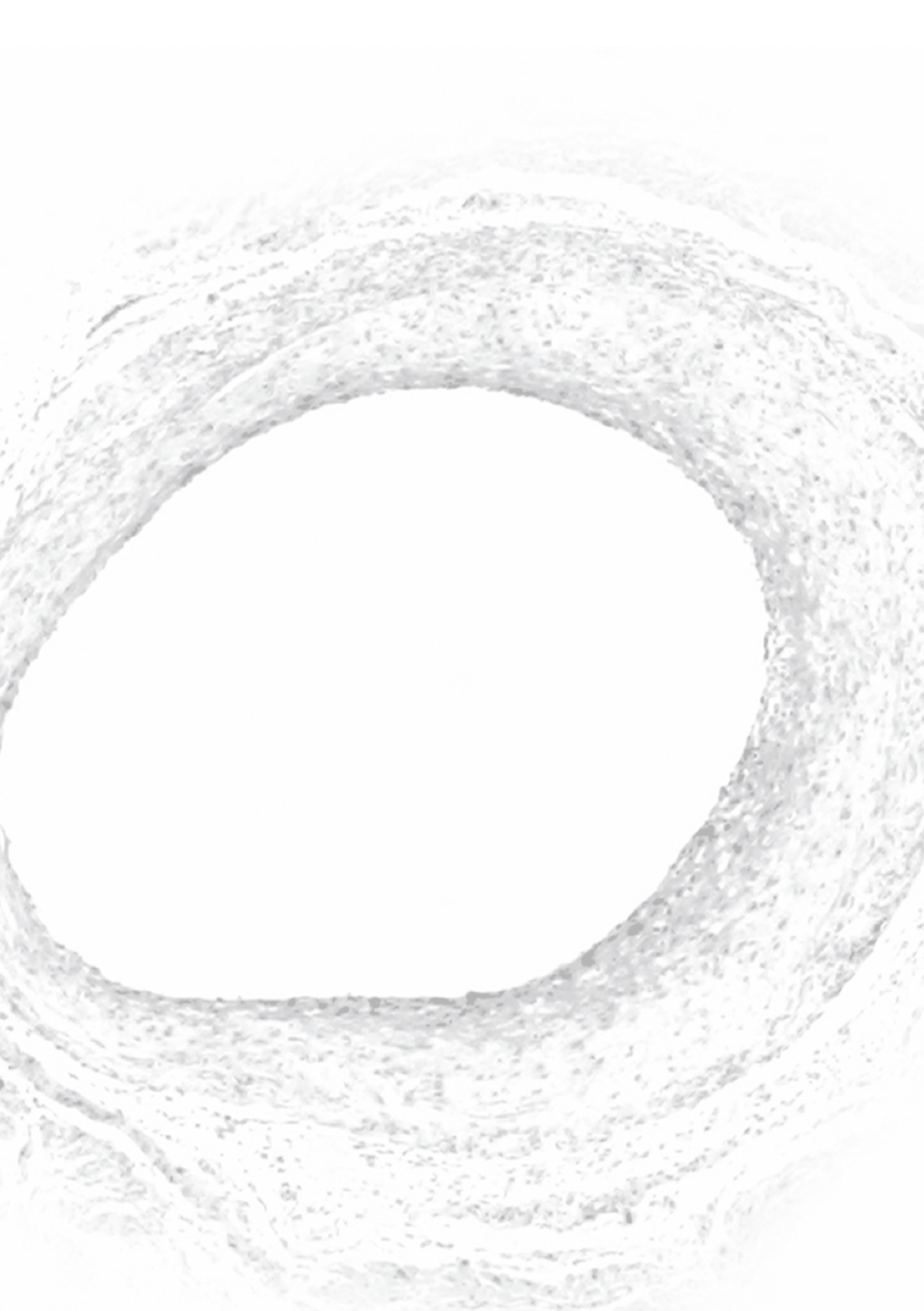
Inflammation in injury-induced vascular remodelling : functional involvement and therapeutical options

Schepers, A.



CHAPTER 6

Both MIP-1α and RANTES and their receptors are expressed in murine vein grafts and aggravate vein graft remodeling.

A. Schepers[1,2], M.R. de Vries[1], I. Bot[3], H.J. Anders[4], A.E.I. Proudfoot[5], J.H. van Bockel[2], P.H.A. Quax[1,2]

[1]Gaubius Laboratory, TNO, Quality of Life, Leiden, The Netherlands, [2]Department of Surgery, Leiden University Medical Centre, Leiden, The Netherlands, [3]Division of Biopharmaceutics, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands, [4]Nephrological Center, Medical Policlinic, University of Munich, Munich, Germany, [5]Serono Pharmaceutical Research Institute, Geneva, Switzerland

Submitted

ABSTRACT

Objective: Venous bypass graft patency is compromised by development of intimal hyperplasia and accelerated atherosclerosis. Both are thought to be inflammatory driven and are characterized by early influx of inflammatory cells. Here, the involvement of CC-chemoattractants RANTES and MIP-1α in vein graft thickening was studied.

Methods: Venous interpositions were placed in carotid arteries of hypercholesterolemic ApoE3Leiden mice. In this model massive influx of inflammatory cells is seen and vein graft thickening occurs within 28 days. Expression of MIP-1α and RANTES and their receptors was studied by immunohistochemistry and RT-PCR at various time points after surgery. Receptor-antagonist Met-RANTES was administered to study the effect of blocking RANTES and MIP-1α on monocyte adhesion and vein graft thickening.

Results: RANTES and MIP-1α protein was detectable in the various stages of vein graft remodeling and mRNA expression appeared to be regulated after engraftment.

Met-RANTES-treatment resulted in significantly reduced adherence of monocytes 3 days after engraftment. Twenty-eight days after engraftment vein graft thickening was significantly reduced in Met-RANTES-treated mice and their vein grafts consisted of less foam cells.

Conclusion: This study shows that RANTES/MIP-1α - receptor interactions are involved in monocyte-chemotaxis in the early phases of vein graft remodeling and blocking this axis reduces vein graft thickening. This might be an interesting new target in order to overcome the clinical problem of vein graft disease.

INTRODUCTION

Despite all recent advances in treatment of arterial atherosclerotic occlusions by percutaneus procedures like PT(C)A with of without (drug eluting) stent placement, bypass surgery is still considered the treatment of choice in occlusions in the distal vasculature or occlusions covering a long section of an artery. However, long term results of vein grafting are compromised by occlusion of venous bypass grafts. This often results in the necessity for renewed bypass surgery or amputation. Therefore bypass occlusion remains a major problem in clinical practice.

The main causes of occlusion of the vein graft, especially when occurring months to years after engraftment, are intimal hyperplasia (IH) formation and accelerated atherosclerosis. Both are believed to occur as a response to injury of the vein graft, e.g. the surgical procedure, altered shear stress after engraftment and other (pre-existing) causes of endothelial damage such as hypercholesterolemia, smoking or hyperglycemia. The response to injury can be characterized as an inflammation mediated process and consists of adhesion of various inflammatory cells and thrombocytes in the first days after grafting, followed by fibrin depositions and influx of inflammatory cells, predominantly monocytes. This finding points to a dominant role for the immune system at least in the early phases of vein graft remodeling.

Physiologically, monocytes and other leucocytes are attracted to the side of injury by the process of chemotaxis. Chemokines are a family of potent chemotactic cytokines that regulate the trafficking of leucocytes. One interesting group of chemotactic factors are CC-chemokines (also called β-chemokines), named after their capacity to attract leukocytes to the site of inflammation and the adjacency of the first two cysteines residues. CC-chemokines express their function via the CCR receptors, which are highly homologous, seven-transmenbrane –domain G-protein coupled receptors and most chemokines interact with more then one CCR receptor[1].

Monocyte chemoattactant protein 1 (MCP-1/CCL2) is the most studied CC-chemokine and its role in vascular inflammation is widely recognized[2-4]. Recently our group defined its role in vein graft thickening and demonstrated that MCP-1 has a pro-stenotic and pro-mitogenic effect in the process of vein graft thickening[5]. Other well known CC-chemokines are MIP-1α (CCL3) and RANTES (CCL5). Both have a potent chemoattractive effect on monocytes, but are also involved in the chemotaxis of activated T-cells, B-cells, dendritic cells and natural killer cells, of which monocytes and T-cells appear to be being the most important in vein graft thickening. Whilst the role of MCP-1 in vascular disease has been extensively studied, the role of other CC-chemokines is less clear. In this study we wanted to investigate the potential involvement of MIP-1α and RANTES and their receptors, CCR1, CCR3 and CCR5 in vein graft remodeling.

In this study, MIP-1α/RANTES pathways were blocked *in vivo* by the use of the CCR antagonist Met-RANTES. Met-RANTES is created by retention of the

initiating methionin in recombinant the RANTES protein[6] and has the capacity to block ligand-induced chemotaxis for human CCR1, CCR3 and CCR5[7]. It also displays antagonistic activity for murine CCR1 and CCR5, but not murine CCR3[8]. Furthermore, it has been shown to inhibit macrophage and T-cell accumulation in various (animal) models of disease[9-13]. In this study, Met-RANTES was used in a murine model for vein graft disease, in which a venous interposition is placed in the common carotid artery[14]. When performed in hypercholesterolemic mice, this vein graft undergoes a striking remodeling with IH formation and atherosclerotic changes[15], 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. More importantly, massive adhesion and influx of inflammatory cells is seen in the first days after surgery, making this model extremely suitable to study chemotactic factors, such as MIP-1α and RANTES.

METHODS

Mice.
All animal experiments were approved by the TNO Animal Welfare Committee and conform to the *Guide for the Care and Use of Laboratory Animals* (published by the US National Institute of Health, No 85-23, revised 1996). For all experiments male C57Bl6/ApoE3Leiden mice, age between 16 and 20 weeks, were used. Mice were fed a mild cholesterol-enriched diet (containing e.g. 0.5% cholesterol, 0.05%cholate[16]) *ad libitum*, aiming at plasma cholesterol levels of 10-15 mmol/l). Serum cholesterol levels were determined at time of surgery and sacrifice.

Vein graft surgery.
Vein graft surgery was performed as previously described[14]. In summary, caval veins were harvested from genetically identical donor mice and placed as an interposition in the common carotid artery of ApoE3Leiden recipients. Therefore, the artery was dissected free from its surroundings and ligated. After clamping the vessel, a plastic cuff was sleeved over both ends, the artery was everted over the cuff and ligated with a 8.0 Silk ligature. Subsequently, the caval veins were sleeved over the cuffs and ligated, thereby creating a venous interposition. After clamp removal, turbulent flow through the vein graft confirmed successful engraftment. At time of sacrifice, 5 minutes of in vivo perfusion-fixation at 100mmHg with 4% Formaldehyde was followed by harvesting of the vein graft.

Met-RANTES treatment.
Met-RANTES was produced as previously described[6]. Mice received daily intra-peritoneal injections of 30µg Met-RANTES dissolved in 0.1ml of sterile 0.9%NaCl (dosages based on protocols from literature[10]). Mice in the control group received daily injections of 0.1ml of sterile 0.9%NaCl.

Histological assessment of vein grafts.
Harvested vein grafts were fixed overnight in 4% Formaldehyde, dehydrated and embedded in paraffin. Serial perpendicular cross-sections (5μm) were made of the specimen and routinely stained with Hemotoxilin, Phloxin and Saffron (HPS).

Presence and distribution of MIP-1α and RANTES was studied by immunohistochemistry. MIP-1α was detected using antibodies against murine MIP-1α (Abcam) and for RANTES antibodies against murine RANTES (R&D Biosystems) were used.

To assess vein graft thickening, the vessel wall surface was measured in 6 cross-sections per specimen, using Computes Assisted Image Analysis (QWin, Leica) and averaged. Since the barrier between intima and media in murine veins is difficult to identify, vein graft thickening was defined as all tissue inside the external elastic lamina minus the luminal area.

Different subsets of leukocytes were specified. The amount of macrophage derived foam cells within the thickened vessel wall was visualized by AIA31240 antibody (Accurate Chemical) and T-cells were detected using anti-CD3 antibodies (Serotec).

Adherence of inflammatory cells was studied in vein grafts, harvested 3 days after surgery. T-cells and monocytes were stained by immunohistochemistry and all positive cells adhering to the endothelium were counted. Per specimen this procedure was performed in 6 cross-sections and scoring was performed by two blinded observers.

RNA isolation and PCR.
To study the expression of MIP-1α, RANTES and their CCR receptors in the remodeling vein graft, a time-course was made. Mice underwent vein graft surgery and were sacrificed at several time-points after surgery (6h, 24h, 3d, 7d, 14d and 28d, n=4 per time point). The vein grafts was harvested and snap-frozen. Also, caval veins of donor mice were included.

To isolate RNA, a RNA Isolation Mini Kit for Fibrous Tissue (Qiagen) was used, following the protocol provided by the manufacturer. To overcome the risk of DNA contamination, a DNase treatment was included (RNase Free DNase set, Qiagen). RNA (250 ηg) was reverse-transcribed using the Ready-To-Go You-Prime First-Strand Beats (Amersham Biosciences) according to the manufacturers protocol.

Gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems) using SYBR Green technology. PCR primers (Table 6.1) were designed using Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems; amplicon size: 68-150 base pairs). In a MicroAmp optical 96-well plates (Applied Biosystems), 19 μl SYBR Green mix (2.5 μl 10X reaction buffer, 1.75 μl 50 mM $MgCl_2$, 1 μl 5 mM dNTP's, 0.125 μl 5 U/μl Hot GoldStar enzyme, 0.75 μl SYBR Green 1/2000 dilution in DMSO and 12.875 μl sterile water; Eurogentec)

was added to 5 µl cDNA (25 ng) and 300 nM of forward and reverse primers. Plates were heated for 2 min at 50°C and 10 min at 95°C. Subsequently, 40 PCR cycles consisting of 15 at 95°C and 60 sec at 60°C were applied. Cyclophilin (CyP) and hypoxanthine guanine phosphoribosyl transferase (HPRT) were used as the standard housekeeping genes. Ratios of target gene and housekeeping gene expression levels (relative gene expression numbers) were calculated by subtracting the mean threshold cycle number (Ct) of the housekeeping gene Ct (mean of CpA and HPRT) from the target gene (=ΔCT) and raising 2 to the power of -ΔCT.

Table 6.1: *Primer sequences used for RT-PCR mRNA analysis.*

Gene	GenB Acces	Forward (5'-3')	Reverse (5'-3')
HPRT	J00423	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
CCR1	NM_009912	CAATCAGTGTGAGCAGAGTAAGCA	CACAACAGTGGGTGTAGGCAA
CCR3	NM_009914	TGCAGGTGACTGAGGTGATTG	CGGAACCTCTCACCAACAAAG
CCR5	NM_009917	GACTGTCAGCAGGAAGTGAGCAT	CTTGACGCCAGCTGAGCAA
MIP-1α	NM_011337	GCCACATCGAGGGACTCTTCA	GATGGGGGTTGAGGAACGTG
RANTES	NM_013653	CTTCTCTGGGTTGGCACACA	GCAAGTGCTCCAATCTTGCA

Statistical analysis.
Data are represented as mean ± SEM. For statistical analysis of morphometric data and data concerning adherence of cells, a non-parametric Mann-Whitney test was executed. The significance of differences in relative gene expression numbers measured by SYBR Green was calculated using a two-tailed T-test on the differences in Ct ($\Delta CT = Ct_{target\ gene} - Ct_{housekeeping}$). Probability values less than 0.05 were considered significant.

RESULTS

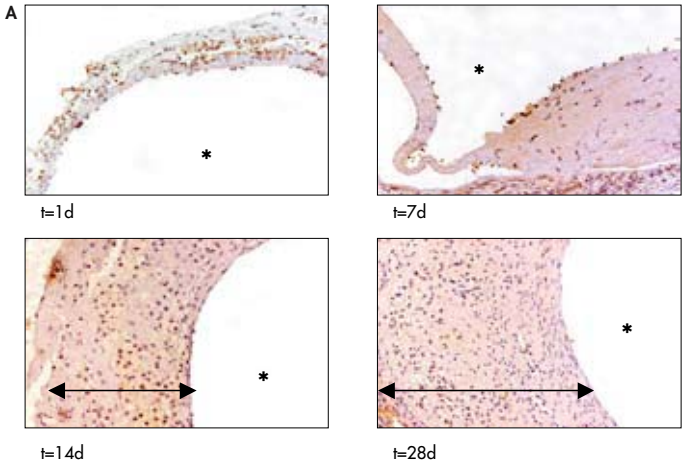
Presence of MIP-1α and RANTES in murine vein grafts.
The presence of MIP-1α and RANTES in remodeling vein grafts was studied by immunohistochemical staining. Therefore, vein grafts were harvested on several time-points (t= directly after surgery, t=3d, 7d, 14d and 28d, n=3 per time-point). In the early phases directly after surgery, massive adhesion of inflammatory cells was seen, existing pre-dominantly of macrophages as determined by immunohistochemistry. From day three on, the vessel wall appeared very thin, and little viable cells could be detected. After 7 days cellular accumulation could be distinguished, resulting in a markedly thickened vein graft wall, existing mainly of smooth muscle cells and macrophage-derived foam cells.

Directly after surgery MIP-1α could be detected in the adhering cells, whereas after a few days it was also expressed in cells of the native vein graft wall. Furthermore, MIP-1α was also detected in the regenerating endothelium. The presence of MIP-1α remained detectable until 14 days after surgery, and it subsequently declined and was not present in the 28 days vein grafts.

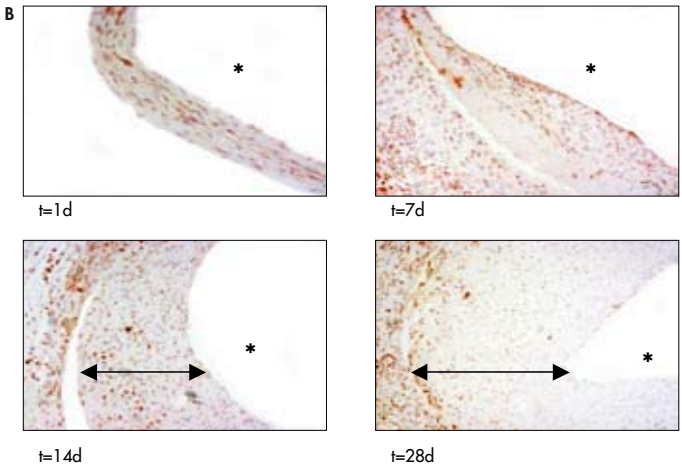
The distribution of RANTES was more concentrated in the adventitia of the remodeling grafts. A minority of the adhering cells in the specimen harvested directly after surgery were positive for RANTES protein just like approximately one third of the smooth muscle cells and fibroblasts of the vein graft vessel wall. In the later time points, RANTES could be detected diffusely in the thickened vein graft wall, but predominantly in the adventitia, up to 28 days after surgery.

Representative pictures are presented in Figure 6.1.

Figure 6.1: *Expression of MIP-1α and RANTES in remodeling vein grafts as shown by immunohistochemistry.*



Panel A *shows MIP-1α- expression in the adhering leucocytes 1 day after engraftment, and presence of MIP-1α in the thickened vessel wall at later time points. Panel B depicts RANTES presence in the vein graft wall at the early time-points. In the later stages of vein graft thickening diffuse distribution is seen in the intimal hyperplasia but predominantly in the adventitia of the vein grafts. (n=3 per time-point, magnification 125-300x, * represents lumen, arrow indicates vessel wall thickening).*



Expression of MIP-1α, RANTES and their receptors in murine vein grafts.

Since MIP-1α and RANTES protein appeared to be present in remodeling vein grafts, the relative mRNA expression of these factors and their receptors CCR1, CCR3 and CCR5 were studied, to evaluate the presence of a possible time-dependent up- or downregulation.

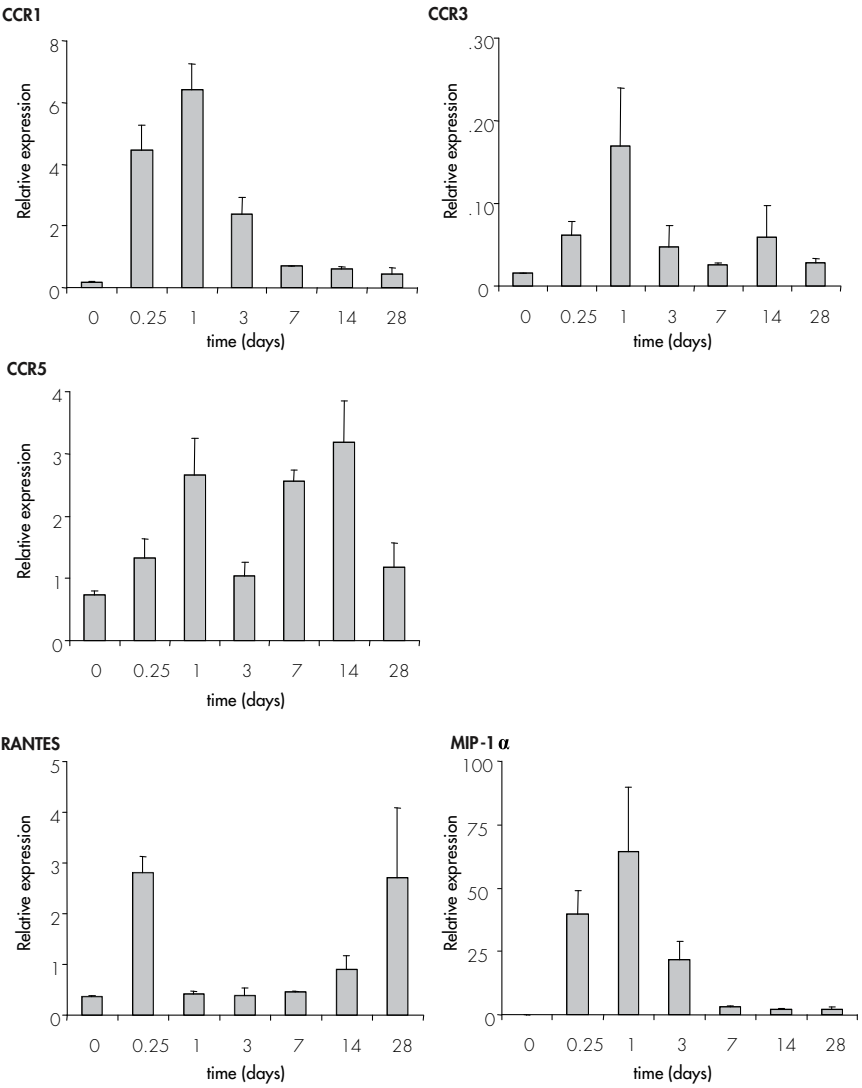
Therefore total mRNA of vein grafts, harvested at several time points, was analyzed. Real time RT-PCR was performed with specific primers for CCR1, CCR3, CCR5, MIP-1α and RANTES and expression was correlated to expression of the housekeeping gene. The following data are summarized in Figure 6.2.

All three CCR receptors tested had baseline mRNA expression in normal caval veins and all displayed induction of mRNA expression in grafted veins. CCR1 showed a rapid upregulation as early as 6h after engraftment, when compared with expression in normal caval veins. Peak expression was seen after 1d (relative expression 6.4±0.85, $p<0.0001$)) after which expression declined to baseline level after 28d.

CCR3 expression was much lower than CCR1 expression (relative expression between 0.015 and 0.16, as compared to the housekeeping gene) however mRNA induction upon engraftment followed a similar pattern with peak expression after 1d (relative expression 0.17±0.07, $p=0.03$).

CCR5 also demonstrated significant mRNA upregulation after engraftment (relative expression when compared with expression in normal caval veins after 1d 2.6±0.59, $p=0.01$), but expression remained very variable over time and no evident pattern could be detected.

Figure 6.2: *RNA synthesis of MIP-1α, RANTES and their receptors at various time-points of vein graft remodeling.*



MIP-1α followed an expression profile that confirmed the immunohistochemistry results and was not detectable in normal caval veins, but after surgery a swift and strong upregulation occurred, with peak expression at 1d after surgery (relative expression 64.1±24.7, p=0.0002). Thereafter expression was reduced to a stable level from 7d.

RANTES mRNA was upregulated to peak expression after 6h (relative expression 2.8±0.31, p=0.0001), after which the expression diminished and then surprisingly was upregulated again at 14d and 28d.

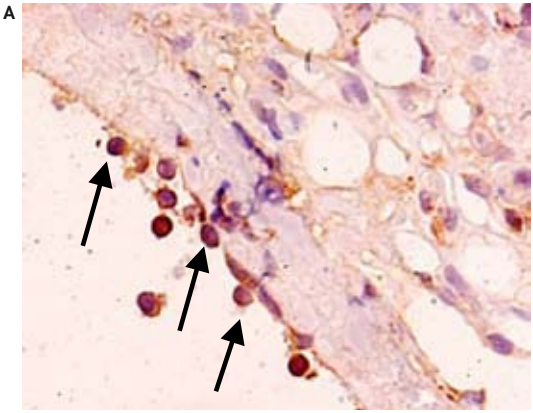
Effect of Met-RANTES on leukocyte adhesion.
Since leukocyte adhesion is believed to be one of the first phenomena occuring in remodeling vein grafts and chemotaxis plays a vital role in this process, the effect of blocking CCR1 and CCR5 signaling on the early adherence of leukocytes was studied. Therefore, 16 mice were randomly divided in 2 groups. One group was treated with Met-RANTES (30µg/day, starting one day prior to surgery), the other group served as a control group and received daily injections of sterile 0.9%NaCl. Mice underwent vein graft surgery and were sacrificed after 3 days. Monocytes were identified using immunohistochemistry and adhering monocytes were counted on 6 cross-sections per vein graft.

As shown in Figure 6.3, massive adhesion of monocytes could be detected in the control group (31.4±3.4 per cross section). In the Met-RANTES treated mice, adherence of monocytes was still detected, however a significant reduction in adhering monocytes was seen (16.7±3.8, p=0.007).

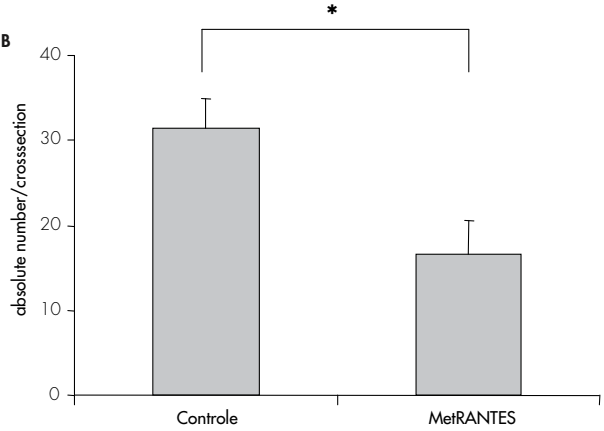
Interestingly, almost no adhering CD3-positive T-cells were present in the vein grafts harvested after 3 days (1-2 positive cells/ 6 cross-sections). This phenomenon was seen in both groups and therefore, no significant difference could be demonstrated between both treatment groups.

Figure 6.3:

Panel A: *Representative picture showing adhering AIA31240 positive cells (some are indicated by arrows; magnification 400x).*



Panel B: *Number of adhering AIA31240 positive cells (monocytes) 3 days after engraftment in Met-RANTES-treated and control vein grafts (analysis by immunohistochemistry, n=8 per group, at least 6 cross-sections per mice were analyzed). Data are expressed as mean±SEM.*



Effect of Met-RANTES on vein graft thickening.
To study the effects of inhibiting CCR1 and CCR5 on vein graft thickening, 8 mice were randomly divided into two groups; one group treated with Met-RANTES as described above, and one control group. Serum cholesterol (measured at sacrifice) did not differ between the two groups (control 14.6±1.4mmol/l, Met-RANTES 15.4±4.1mmol/l, p=0.82). Mice were sacrificed after 28 days and vein graft thickening was quantified.

In the control animals, vein graft thickening occurred (0.41±0.08mm^2). Met-RANTES treatment resulted in a significantly decreased vein graft thickening (0.16±0.01mm^2, p=0.042 when compared to controls; Figure 6.4). To see whether treatment with Met-RANTES changed the inflammatory status of the thickened vein graft, read out by the presence of macrophages and macrophage-derived foam cells in the vessel wall, the AIA31240-positive area, was determined. The thickened vein graft wall in the control group consisted for 21±2% of AIA31240-positive cells, compared to 11±5% in the Met-RANTES-treated group (p=0.032; Figure 6.4). This finding indicates that upon Met-RANTES treatment not only adherence of monocytes is decreased, it also results in reduced foam cell content in the vessel wall. Immunohistochemical staining for T-cells, in vein grafts harvested after 28 days, disclosed that T-cells appeared pre-dominantly in the adventitia, whereas staining in the thickened vessel wall was clearly present but less abundant (data not shown).

DISCUSSION

The process of vascular remodeling has been thoroughly explored over the past decade. Multiple studies show a major role for inflammatory processes in the pathophysiology of atherosclerosis and post-interventional restenosis. Although it is often assumed that vein graft thickening can be seen as a similar inflammatory process, the important differences in the structure of vein grafts compared to arteries, require a separate approach.

Leukocyte migration to the site of vascular injury involves a concerted interaction of adhesion molecules, chemokines and their receptors. Although the role of chemokines in general, and CC-chemokines specifically, has been elaborately studied in atherosclerosis (as reviewed in [17]), reports about their role in vein graft disease are scarce. Recently, our group demonstrated the important role of MCP-1 in the development of vein graft thickening and its effects on smooth muscle cell proliferation[18]. Furthermore Ali et al described the potent inhibitory effects of a single intravenous injection of the broad spectrum CC-chemokine inhibitor 35K on accelerated vein graft atherosclerosis[19]. Their study demonstrated very elegantly the potential therapeutic options of this approach, however the role of individual CC-chemokines was not further addressed. Therefore, the functional involvement of MIP-1α and RANTES and their receptors CCR1, CCR3 and CCR5 in the process of vein graft thickening was studied.

The presence and expression of CC-chemokines MIP-1α and RANTES has been demonstrated in various forms of vascular lesions. In advanced atherosclerotic plaques, for instance, RANTES is predominantly expressed in (approximately 5% of all) activated T-cells[20]. In contrast, within the plaque area of accelerated atherosclerosis in organ transplants, RANTES was highly expressed in various cell types (e.g. macrophages, endothelial cells, myofibroblasts)[21]. In this study we found expression predominantly in the later stages of vascular remodeling, in the adventitia of the grafts, co-localizing with T-cells.

Expression of MIP-1α is shown in the remodeling vein graft, predominantly by invading monocytes/macrophages and leukocytes in the adventitia. To our knowledge, there are no previous reports describing the presence of MIP-1α in atherosclerotic plaques or other vascular lesions, but its presence has been demonstrated *in vitro* in various processes related to atherogenesis and vascular inflammation[22, 23] Additionally, in this manuscript a time-dependent upregulation of RANTES and MIP-1α mRNA upon engraftment was demonstrated in the remodeling vein grafts, and a similar pattern was detected by immunohistochemistry.

Moreover, a time-dependent upregulation of CC-receptors mRNA was demonstrated in these grafts. These findings are in line with the report of Hayes and colleques[24] who showed upregulation of CCR mRNA in cultured smooth muscle cells originating from atherosclerotic tissue and with the report of Veillard and colleques[25], who showed expression of various CC-receptors in endothelial cells and monocytes.

These data show that besides MCP-1, other CC-chemokines might be involved in vascular remodeling in general and vein graft thickening in particular.

To obtain more insight in the functional involvement of these CC-chemokines and their receptors on chemotaxis and inflammatory cell adhesion to the vein graft wall in vivo, the receptor antagonist Met-RANTES was used to block signal transduction. Met-RANTES it is an established CCR1 and CCR5 receptor antagonist and has been used to study the involvement of RANTES in atherosclerosis and arterial post-interventional neointima formation[26, 27] in the past. Treatment with Met-RANTES resulted in significantly decreased numbers of adhering monocytes in vein graft harvested 3 days after surgery.

After 28 days, Met-RANTES treatment not only reduced vein graft thickening, but a reduction in foam cell content could also be detected. This morphology (with less foam cells in the plaque area) is assumed to be associated with plaque stability[28] and therefore a desirable situation.

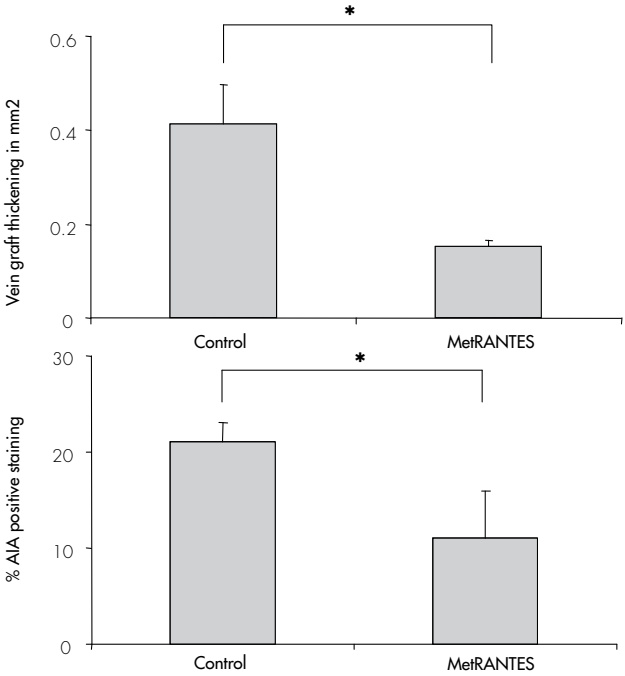
Interestingly, we found in vein grafts harvested after 3 days, hardly any T-cells present and this was not related to Met-RANTES-treatment. Although this study was not designed to study the involvement of T-cells in vein graft thickening, this observation might indicate that T-cells are less involved in the earlier stages of vascular remodeling in this model. Exploring the exact value of this finding, however, requires further studies.

Recently, Zernecke and colleagues demonstrated that CCR5 is more crucial than CCR1 for neointimal plaque formation. This might also be the case in our study. With the use of the CCR1/ CCR5 antagonist Met-RANTES it is not possible to discriminate the individual contribution of both. The same question can be posed for the individual contribution of RANTES, MIP-1α or other (unknown) CC-chemokines sharing the CCR1 and CCR5 receptor. Further studies in CC-chemokine knock-out mice with hypercholesterolemic features are needed to answer these questions.

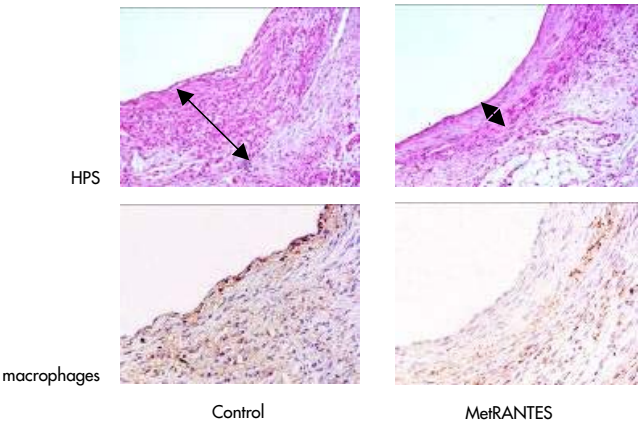
In conclusion, we have demonstrated a causal involvement of the CCR1/CCR5 pathway in vein graft thickening in a mouse model using hypercholesteremic ApoE3Leiden mice. This was demonstrated by the profound inhibition of vein graft thickening by Met-RANTES and was further underscored by the upregulation of both the CC-chemokines MIP-1α and RANTES and their receptors.

Therefore, we have provided evidence that RANTES/MIP-1α - CCR receptor interactions are an important early trigger for vein graft thickening. This establishes the hypothesis that inflammatory pathways are involved in the development of vein graft thickening. Therapy that interferes in the RANTES/MIP-1α - CCR axis might be an interesting new target in order to overcome the clinical problem of vein graft disease.

Figure 6.4: *Effect of Met-RANTES on vein graft thickening and plaque composition (n=8 per group). Panel A displays the quantified data of both vein graft thickening and macrophage content of the plaque as defined by percentage of staining positive for AIA31240.*



Panel B shows representative pictures of HPS and AIA 31240 staining (magnification 200x, arrows indicate thickened vein graft wall).



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