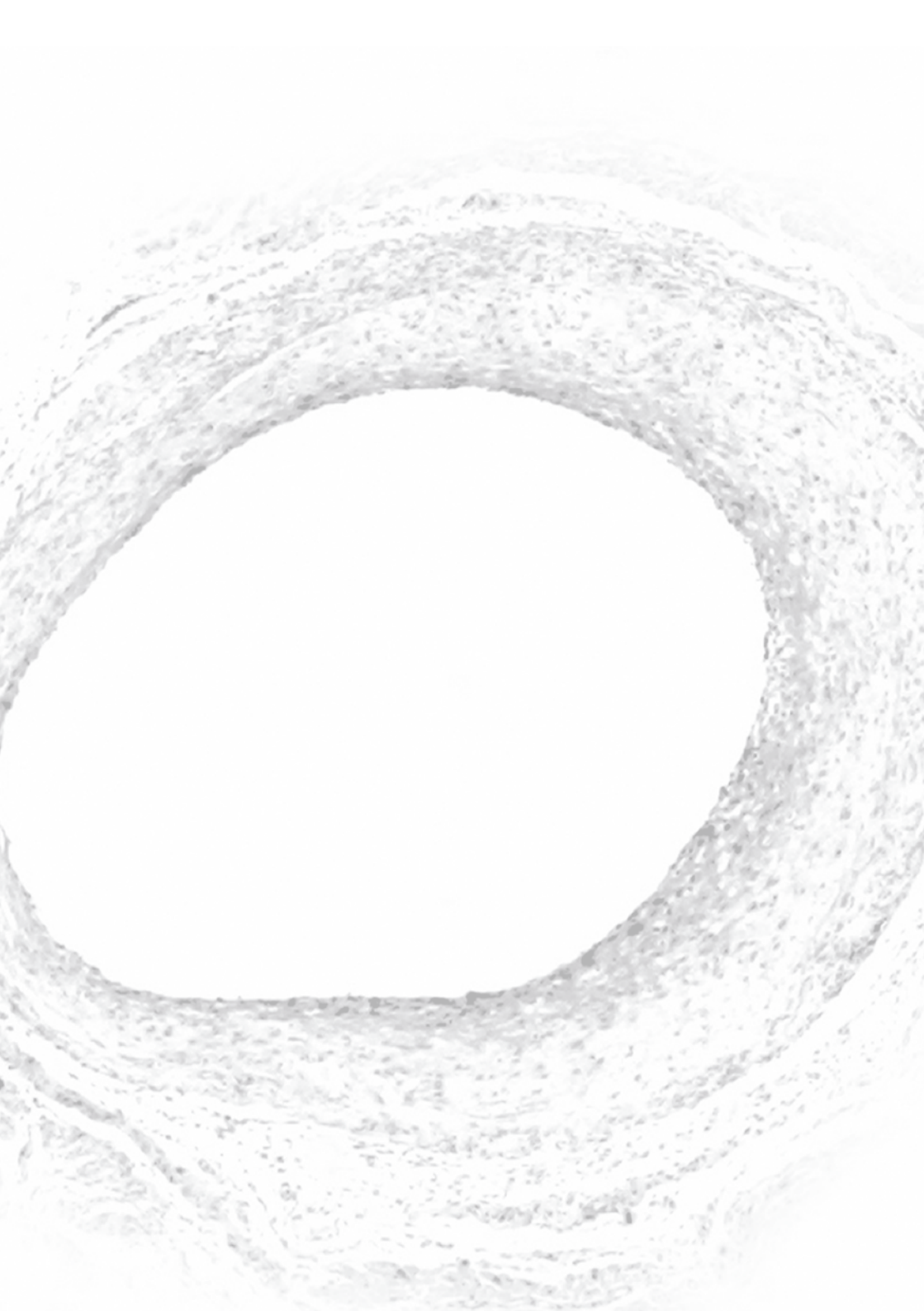
Inflammation in injury-induced vascular remodelling : functional involvement and therapeutical options

Schepers, A.



CHAPTER 8

Blocking of the Chemotactic Complement Endproduct C5a Inhibits Accelerated Atherosclerosis in Vein Grafts.

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Submitted

ABSTRACT

Objective: Vein graft thickening based on intimal hyperplasia formation and accelerated atherosclerosis is the main cause of vein graft failure. Influx of monocytes is one of the first phenomena seen in this process. C5a is a bioactive end-product of the complement cascade and a chemotactic factor for monocytes. Since evidence to support a definite role of C5a in vein graft remodeling is lacking, we sought to establish the functional involvement of C5a is involved in this process.

Methods and Results: Time-dependent C5 expression was demonstrated by immunohistochemistry in murine vein grafts (consisting of venous interpositions placed in carotid arteries) of hypercholesterolemic ApoE3Leiden mice, whereas mRNA-analysis revealed a up-regulation of C5a receptor (C5aR) mRNA post-surgery.

Functional involvement of C5a in vein graft remodeling was demonstrated by a dose-dependent increase of vein graft thickening upon C5a application. Conversely, blocking the C5aR with C5aR-antagonists inhibited vein graft thickening for 50%, accompanied by decreased numbers of adhering monocytes and reduced foam cell accumulation in the lesions.

Conclusions: These data establishes the important role of the complement cascade in general, and C5a in particular in vein graft thickening. This indicates that C5a-antagonists may be potential therapies in the prevention of vein graft failure in patients.

INTRODUCTION

Saphenous vein grafting remains the treatment of choice to overcome the symptoms of coronary and peripheral atherosclerosis. However, vein graft failure is a frequent outcome resulting in a relapse of symptoms. Late vein graft failure is predominantly caused by the development of intimal hyperplasia (IH) and accelerated atherosclerosis[1]. It is believed to be the result of an inflammatory reaction in the vein graft wall, initiated by mechanical damage during surgery, cyclic stretching and increased shear stress in the arterial circulation[2,3]. One of the first occurring phenomena in this remodeling process is chemotaxis followed by adhesion and migration of inflammatory cells, predominantly monocytes, into the intima of the vein graft[4]. Monocytes produce pro-inflammatory cytokines and growth factors, being potent stimuli for smooth muscle cell migration and foam cell accumulation[5], eventually resulting in a thickened vessel wall and a reduced luminal diameter. Interfering in chemotactic processes could potentially results in reduced monocyte influx and thereby ultimately in less vein graft thickening.

Recently, our group provided evidence for pivotal involvement of the complement cascade in the abovementioned processes[6]. Activation of the complement cascade leads to cleavage of complement component C3, which upon further activation results in formation of biological active end-products. Inhibition of C3 inhibited vein graft thickening and reduced inflammatory cell content in the thickened vein graft wall. To further clarify the mechanism and to identify the crucial proteins that led to this effect, complement component C5a was further studied in this setting. C5a is one of the biologically active components of the complement cascade and a potent chemotactic protein. C5a exerts its function via the C5a receptors (C5aR) and induces chemotaxis of numerous cell types including monocytes, T- lymphocytes, and granulocytes[7]. It has been shown to be of importance in several inflammatory mediated processes, such as SIRS response after extracorporal circulation[8,9], sepsis[10], bronchial asthma[11] and ischemia/reperfusion injury[12,13]. However, involvement of C5a in vascular inflammatory processes such as atherosclerosis and post-interventional vessel remodeling (e.g. post-angioplasty restenosis and vein graft thickening) is less clearly assessed.

In this study, we hypothesized that C5a plays a pro-inflammatory, pro-stenotic role in vein graft disease. In line with this hypothesis, interference of C5a function should lead to reduced vein graft thickening and decreased numbers of macrophage-derived foam cells in the lesion. This hypothesis was studied in a murine model for vein graft disease. When performed in hypercholesterolemic mice, within 28 days vein graft thickening occurs due to IH formation and accelerated atherosclerosis, thereby highly resembling the changes seen in human diseased vein grafts.

In this mouse model, the presence and expression of C5 and its receptor (C5aR) was assessed. To study the effect of C5a exposure on vein graft thickening, recombinant C5a was applied to the vein graft. Furthermore, C5a function was abrogated by treating mice that underwent vein graft surgery with two potent C5a receptor

antagonists (C5aRA), AcF-[OP-(D-Cha)WR] and HC-[OP-(D-Cha)WR])[14]. Previous reports demonstrate that these C5aRA are suitable to inhibit C5a function *in vivo*[15-17]. Furthermore, these compounds are highly stable and easy to work with, in contrast to C5a receptor blockage by e.g. monoclonal antibodies.

The results of this study provide clear evidence for the functional role of C5a in the development of vein graft thickening, and point to blockade of C5a as a potential target for therapy, in order to overcome vein graft failure in patients.

METHODS

Mice.
Experiments were approved by the Animal Welfare Committee of the institution. For all experiments 12 week old male C57Bl6-ApoE$^{-/-}$ mice, bred at the TNO laboratory, were used. Mice were fed a standard chow diet and received water and food *ad libitum*. Serum cholesterol levels were determined using a cholesterol-esterase, cholesterol-oxidase reaction (CholR1, Roche Diagnostics, Almere, The Netherlands) at sacrifice. Before surgery, mice were anesthetized by an intra-peritoneal injection with Midazolam (5 mg/kg, Roche, Woerden, The Netherlands), Medetomidine (0.5 mg/kg, Orion, Espoo, Finland) and Fentanyl (0.05 mg/kg, Janssen, Berchem, Belgium).

Vein graft model.
Vein graft surgery was performed as described previously[18]. Briefly, caval veins were harvested from (genetically identical) donor mice and before implantation preserved (at 4 degrees Celsius) in 0.9% NaCl containing 100U/ml of heparin. In recipients, the right carotid artery was dissected and cut in the middle. A polyethylene cuff was placed at both ends of the artery. The artery was everted around the cuff and ligated with a silk 8.0 suture. The caval vein was sleeved over the two cuffs, and ligated. Pulsations and turbulent blood flow within the graft confirmed successful engraftment.

At sacrifice, after 5 minutes of *in vivo* perfusion-fixation with 4% Formaldehyde, vein grafts were harvested and fixed overnight in 4% Formaldehyde, dehydrated and paraffin-embedded. Serial perpendicular cross sections (5µm) of the vessel were made through the entire specimen.

Detection of C5 in vein grafts by immunohistochemistry.
Twenty-four mice were sacrificed at several time points after surgery (6h, 24h, 3d, 7d, 14d and 28d after surgery). Vein grafts were harvested and processed as described above. Rat monoclonal antibodies against murine C5 were used to study presence of C5 in vein grafts by immunohistochemistry (1:25 dilution, HyCult Biotechnology, Uden, The Netherlands). Rabbit-anti-rat polyclonal antibodies were used as secondary antibody. Normal caval veins were included to assess baseline C5 expression.

RNA isolation, cDNA synthesis and RT-PCR.
Total RNA was isolated from 16 vein grafts harvested on several time points (24h, 3d; 7d; and 28d after surgery). Also, caval veins of donor mice were included.

RNA was isolated, including DNase treatment, using a RNeasy Fibrous Tissue Mini Kit and RNase Free DNase set according to the manufacturer's protocols (Qiagen, Venlo, the Netherlands). RNA (250 ηg) was reverse-transcribed using Ready-To-Go You-Prime First-Strand Beats (Amersham Biosciences, Roosendaal, The Netherlands).

Semi-quantitative RT-PCR (Robocycler Gradient 96, Stratagene, Cedar Creek, Texas, USA) was performed with primers for C5a receptor (Fw: gaccccatagataacagca Rev: cagaggcaacacaaaaccca[19]) and β-Actin (Perker-Elmar, Überlingen, Germany). Samples were amplified for 35 cycles following an initial cycle for 2 min. at 94°C (each cycle consisted of 30sec. at 94°C, 30sec at 56°C and 90sec. at 65°C), followed by an extension-cycle of 4 min. at 74°C. PCR products were visualized on a 1.2% Agarose gel containing EthidiumBromide.

Applying recombinant C5a in pluronic gel to vein grafts.
Twenty-one mice were randomly divided in three groups. In the treatment groups, either 0.5μg or 5μg of recombinant mouse C5a (E. Coli derived, HyCult Biotechnology) was dissolved in 0.1 ml of 20% Pluronic Gel (PG) and applied around the vein grafts at time of surgery. In the control group 0.1ml of 20% PG was applied without protein. Mice were sacrificed after 28 days.

Blocking C5aR by ACF-[OP-(D-CHA)WR] and HC-[OP-(D-CHA)WR] treatment.
In order to inhibit C5a function, 21 mice were randomly divided in three groups and were either treated with AcF-[OP-(D-Cha)WR] (AcF) and hydrocinnamate-[OP-(D-Cha)WR] (HC). Both compounds display potent antagonizing activity for the C5a receptor and were synthesized as described previously[20]. AcF was administered subcutaneously in a daily dose of 3mg/kg in 0.1ml 30% propylene glycol and 70% sterile water, starting one day prior to surgery. Since HC displays an increased *in vivo* potency over AcF, in a dose-dependent function, as recently described by Woodruff et al[21], it was administered in 2 dosages, 3 mg/kg/day and 0.3mg/kg/day, both in 0.1ml 30% propylene glycol and 70% sterile water. A control group (n=7) received daily injections of 0.1ml 30% propylene glycol and 70% sterile water.

All mice were sacrificed 28 days after surgery.

Quantification and histological assessment of intimal hyperplasia.
Cross sections of vein grafts were routinely stained with hematoxylin-phloxine-saffron (HPS). Morphometric analysis of vein grafts harvested after 28d was performed using image analysis software (Qwin, Leica, Wetzlar, Germany). Since only few layers of cells are in the media of murine veins and there is no morphological border between neointima and media, vein graft thickening, i.e. the region between lumen and

adventitia, was used to define lesion area. For each mouse five equally spaced cross-sections were used to determine vessel wall thickening.

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, Westbury, NY, USA). Granulocytes were detected using anti-mouse neutrophils antibodies (MCA 771GA, Serotec, Raleigh, USA).

Quantification of granulocytes occurred by counting the positive number of cells/slide and expressed as a percentage of total number of nuclei (determined by Nucleus Red staining). Foam cell quantification was performed by computer assisted analysis (Qwin, Leica) as AIA31240-positive area in the graft and expressed as a percentage of total vein graft surface.

Statistical analysis.
All data are presented as mean ± SEM. Statistical analysis was performed using SPSS 11.5 for Windows. To determine statistical significance overall comparisons were made using the one-way ANOVA. In case of significance, each group was separately compared to the control group using the student T test. Probability-values< 0.05 were regarded significant.

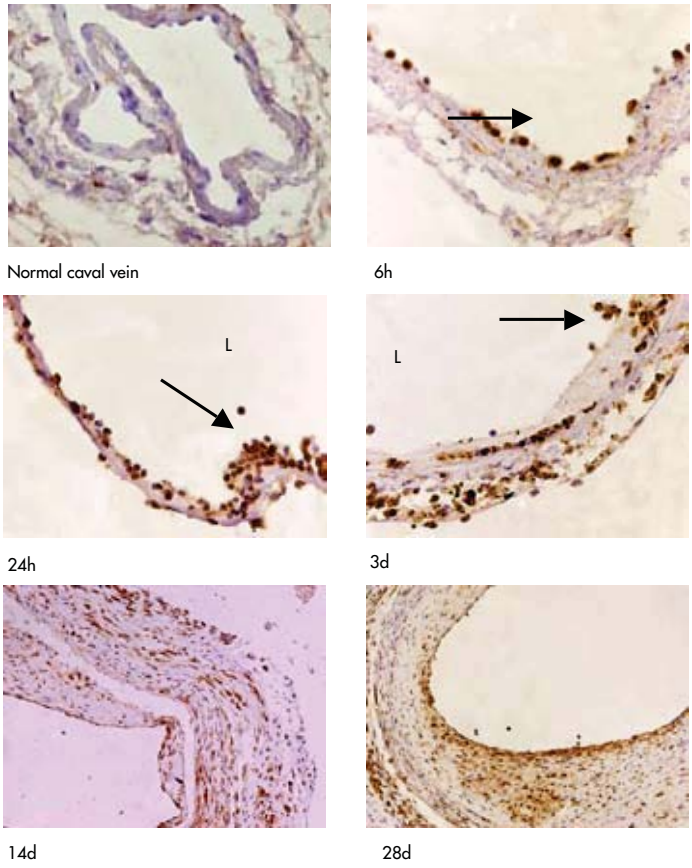
RESULTS

Presence of C5 protein in remodeling vein grafts.
Presence of C5 in remodeling vein graft was assessed in vein grafts (n=4 per time point), harvested at several time points after surgery (6h, 24h, 3d, 7d, 14d and 28d) by immunohistochemistry.

In normal caval veins no baseline expression of C5 could be detected. Interestingly, in vein grafts harvested 6 hours and one day after surgery, strong staining of C5 was detected in adhering monocytes and in adventitial fibroblasts. C5 was also detected in the regenerating endothelium as from 7 days after surgery. At this stage, staining appeared most pronounced and diffusely present, indicating high amounts of C5 in the vessel wall. At the later time points (14 and 28 days post-operatively), along with thickening of the graft, expression of C5 was seen in endothelial cells, adhering monocytes, adventitial fibroblasts and foam cells (Figure 8.1).

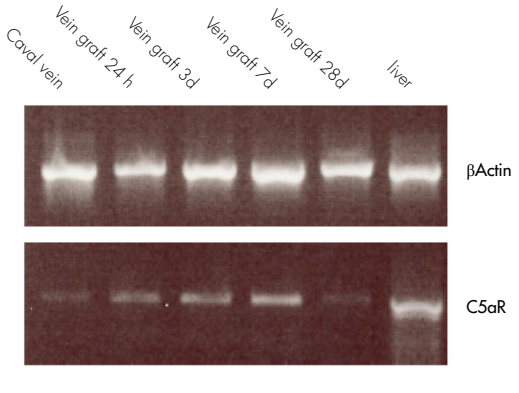
Expression of C5a receptor mRNA in remodeling vein grafts.
To assess whether mRNA of the C5a-receptor was present in vein grafts, total RNA was isolated and studied for presence of C5aR mRNA by RT-PCR. Vein grafts were harvested on several time points (24h, 3d, 7d and 28d, n=4 per time point) and normal caval veins served as controls. Amount of total cDNA in all specimens was studied by the housekeeping gene β-Actin.

Figure 8.1: *Detection of C5 in time in venous bypass grafts by immunohistochemistry. As soon as 6 hours after surgery, presence of C5 is seen in adhering leucocytes (appointed by arrows). Highest amounts of C5 are seen 3 to 7 days after surgery, and C5 remains present in later time-points predominantly in endothelial cells, foam cells and adventitial fibroblasts (magnification 150x (28d)-400x (6h)).*



Caval veins revealed minimal expression of C5aR. In vein grafts, C5aR mRNA expression gradually increased in a time-dependent fashion. Peak expression of C5aR was seen at 7 days after surgery, after which expression declined to levels seen in normal caval veins (Figure 8.2). These data confirm that the C5aR is present in vein grafts and up-regulated in the early stages vein graft remodeling.

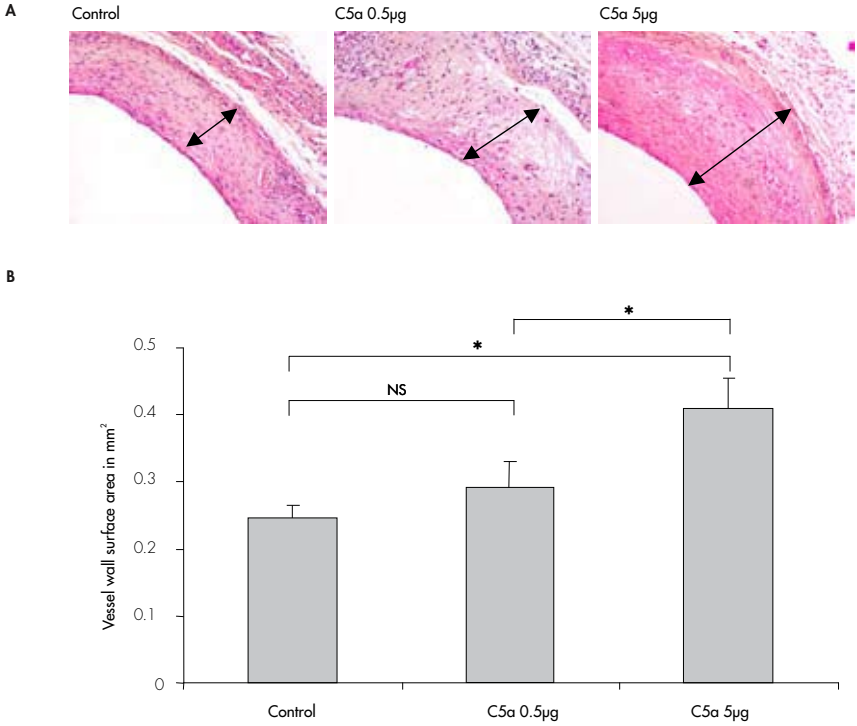
Figure 8.2: *Expression of C5a receptor mRNA by semi-quantitative RT-PCR, compared with basic expression in caval veins. Very small amounts of C5aR mRNA are present in caval veins. After surgery, an up-regulation of C5aR mRNA is seen with peak expression 7 days after surgery, after which the expression normalizes again.*



Effect of application of recombinant C5a on vein graft thickening.
To study involvement of C5a in development of vein graft thickening, we applied recombinant C5a in two concentrations (0.5µg and 5µg, dissolved in 100 µl 20% PG, n=7 per group, dose based on previous reports in literature[22]) directly to the vein graft at time of surgery. Pluronic gel was used as a vehicle to achieve a local, sustained delivery of C5a. In controls 100µl of 20% PG without C5a was applied. Serum cholesterol did not differ between groups and did not change during the experiment (data not shown).

Vein graft thickening, by measuring vessel wall surface, was quantified 28 days after surgery. Topical application of C5a resulted in a dose dependent increase of vessel wall surface, when compared to control vein grafts (control 0.24±0.02 mm^2, 0.5µg C5a: 0.29±0.03 mm^2, p=0.14, 5µg C5a: 0.41±0.04 mm^2, p=0.002 when compared to controls, p=0.037 when compared to 0.5µg C5a-treated group) (Figure 8.3A+B). No significant differences in luminal area were seen between the three different groups (control 0.64±0.06 mm^2, 0.5µg C5a: 0.60±0.05 mm^2, p=0.3, 5µg C5a: 0.58±0.06 mm^2, p=0.25 when compared to controls, p=0.39 when compared to 0.5µg C5a-treated group).

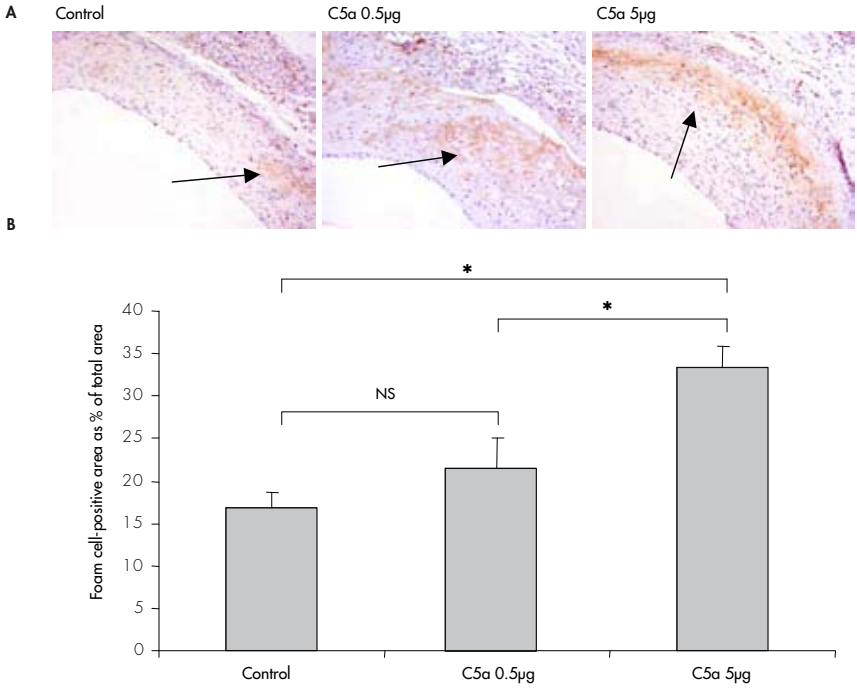
Figure 8.3: *Effect of C5a recombinant protein application to vein grafts of hypercholesterolemic mice. Representative cross-sections of vein grafts exposed to either 20% PG and 20% PG containing 0.5µ and 5µg C5a recombinant protein (panel A, HPS staining). A dose-dependent increase in vein graft thickening was seen in the C5a-treated mice (panel B) (0.5µg: p=0.1, 5µg: p=0.002; arrows indicate vessel wall surface, magnification 200x).*



Effect of application of recombinant C5a on cellular composition of vein grafts.

Since C5a is a potent chemotactic factor for monocytes/macrophages, numbers of macrophages and macrophage-derived foam cells in thickened vein grafts, harvested 28 days after surgery, were studied. A dose-dependent increase in macrophages contribution in thickened vein grafts was seen in C5a treated vein grafts (Figure 8.4A+B). In controls, approximately 17% (±2%) of the thickened vessel wall surface consisted of macrophages and macrophage-derived foam cells. When 0.5µg C5a was applied, this percentage increased to 22%±3% (p=0.11). Application of 5µg C5a to the grafts significantly increased macrophage/foam cell content of 33±2% (p<0.001 when compared to control grafts; p=0.008 when compared to grafts treated with 0.5µg C5a).

Figure 8.4: *Effect of C5a application to vein grafts on foam cell contribution in lesion. Increased exposure to C5a results in a significant increase in foam cell contribution in the lesions (panel B). Panel A displays representative cross sections of immunohistochemistry using antibodies against macrophage derived foam cells,, whereas arrows indicate positive staining against monocytes/ foam cell (0.5µg: p=0.1, 5µg: p<0.001; magnification 200x).*



Granulocytes contributed to a much lesser extent to the vessel wall surface of thickened vein grafts (quantified 28 days after surgery) and application of C5a did not appear to have a significant effect on presence of granulocytes (control 1.3±0.35%, 0.5µg C5a: 1.54±0.43%, p=0.31, 5µg C5a: 1.14±0.48%, p=0.29 when compared to controls, p=0.16 when compared to 0.5µg C5a-treated group).

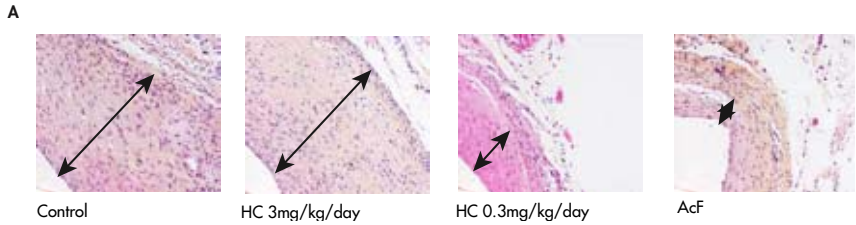
Effect of C5a receptor antagonists (C5aRA) treatment on vein graft thickening and foam cell contribution.

Twenty-eight mice received either AcF in 30% propylene glycol (3mg/kg/day subcutaneously (s.c.), n=7) or HC in 30% propylene glycol (3 and 0.3 mg/kg/day/s.c. n=7 each), or daily injections of 30% propylene glycol s.c. (n=7). No differences were observed in bodyweight or serum cholesterol between the different treatment groups (data not shown).

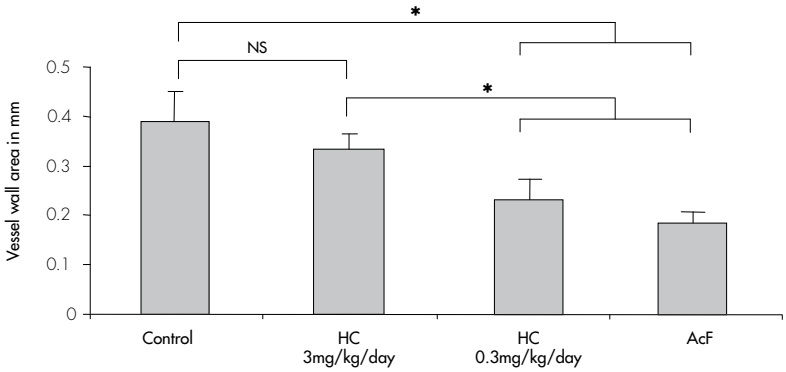
When mice were sacrificed after 28 days, analysis of the thickened vein graft revealed that treatment with AcF resulted in a 53% decrease in vessel wall surface, when

compared to control mice (AcF: 0.19±0.03mm², control: 0.39± 0.06mm², p=0.046), whereas treatment with similar doses HC did not result in decreased vessel wall surface (0.33±0.03mm², p=0.23). However, treatment with a 10-fold lower dose of 0.3 mg/kg HC did lead to significantly reduced vessel wall surface when compared to control animals (0.23±0.03 mm², p=0.035) (Figure 8.5A+B).

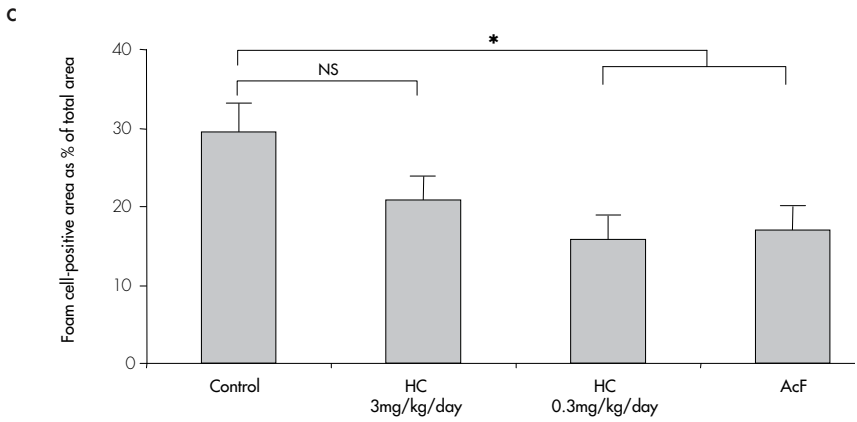
Figure 8.5: *Effect of treatment with C5a receptor antagonists, HC and AcF.*
Panel A: Representative cross sections of control and treated vein grafts 28 days after surgery; a decrease in vein graft thickening is seen in the AcF and HC 0.3mg/kg/day treated groups (HPS staining, magnification 200x).



*Panel B: Quantification of vessel wall surface in mm2 control and treated vein grafts 28 days after surgery (n=7 per group, *represents p<0.05); C5aRA treatment results in decrease in vein graft thickening 28 days after surgery.*



*Panel C: Quantification of macrophages-derived foam cells contribution in the vessel wall by immunohistochemistry, described as percentage of total vessel wall surface; treatment with C5aRA results in a decrease in foam cell content in the vessel wall (n=7 per group, *represents p<0.05).*



Regarding luminal size, no differences were seen between the control group, AcF and HC 3mg/kg, whereas treatment with HC 0.3mg/kg resulted in a significantly increased luminal area (control: 0.42±0.04mm², AcF: 0.41±0.08mm², p=0.48; HC 3mg/kg: 0.48±0.03mm², p=0.15; HC 0.3mg/kg: 0.61±0.04mm², p=0.005).

The thickened vessel wall of control, untreated vein grafts, analyzed 28 days after surgery, consisted approximately 30% (±4%) of foam cells. In AcF-treated vein grafts, a significant reduction of this foam cell contribution was seen (17±3%, p=0.01 when compared to controls). No significant reduction was seen in the HC 3mg/kg/day treated group (22±3% of foam cells in lesion, p=0.07 when compared to controls), whereas administration of HC 0.3 mg/kg/day, resulted in a significantly lowered contribution of foam cells (16±3, p=0.01) (Figure 8.5C).

DISCUSSION

The role of the complement system in vascular remodeling has not been studied in detail yet , regardless of its crucial role in the immune system and inflammatory processes. Recently, we demonstrated that complement activation is one of the crucial phenomena in vein graft disease[6]. Of all end-products of the complement cascade, C5a is one of the most important downstream proteins, exerting chemotactic properties for various inflammatory cells including monocytes, granulocytes and T-cells. It has been shown to modulate pro-inflammatory effects in several diseases (reviewed in[7]), and since chemotaxis of inflammatory cells is one of the first phenomena seen in bypass graft remodeling, it is highly interesting to study the role

of C5a in the context of vein graft remodeling. This report further specifies this role and demonstrates the involvement of C5a in the process of vein graft thickening.

The presence of C5 protein in vein grafts was shown by immunohistochemistry at several time points after surgery. In normal caval veins no C5 could be detected but a strong induction of expression occurred directly after surgery. C5 was predominantly expressed in adhering monocytes, adventitial fibroblasts, endothelial cells and foam cells and staining appeared to be most intense 7 days after surgery. We detected all forms of C5, including C5 and C5a, since the antibody used can not discriminate between these forms. In addition, expression of mRNA encoding for the C5a receptor was followed in time by RT-PCR. At baseline, in normal caval veins, very low levels of C5aR mRNA were detected. A fast up-regulation was seen in the first days after engraftment. Peak-expression occurred 7 days after surgery, coinciding with the highest levels of C5 staining seen with immunohistochemistry, after which expression declined to baseline levels.

In order to study functional involvement of C5a in vein graft thickening, the effect of increased exposure to C5a was studied by applying murine recombinant C5a protein to vein grafts, hypothesizing that increased exposure to C5a would result in increased vein graft thickening. Indeed, increased exposure to C5a dose-dependently aggravated vein graft thickening, and additionally also resulted in dose-dependently increased macrophage-derived foam cell content in the thickened vessel wall. Surprisingly, no significant effect on granulocyte content in the lesion could be observed after increased topical exposure to recombinant C5a. This might be explained by the relatively limited contribution of granulocytes in the thickened vessel wall 28 days after surgery, even in the control group.

When C5a function was blocked, using two potent C5aR antagonists (C5aRA), AcF and HC, the opposite effect was seen. Hampering C5aR function inhibited vein graft thickening and reduced contribution of foam cells in the lesion.

In this study we found a dose-dependent effect of HC. When administered in the highest dose of 3 mg/kg/day, treatment did not inhibit vein graft thickening in our model. This is in line with a recently published article of Woodruff et all[21]. Authors hypothesized that a lack of therapeutic effect, when HC was administered in high dosages, might be due to deleterious effects at unidentified receptors, to local toxicity or other factors not yet recognized. Similar results were found in the present study. The results of the present study, using both C5a overexpression as well as two separate C5a receptor antagonists, clearly indicate a role for the C5a in the pathogenesis of vein graft remodeling in the mouse. Regarding the role of C5a in other forms of inflammation related vascular remodeling, little is known.

Interestingly, because of the well known complement activation in extracorporal circulation[9,23], several reports discuss the role of C5a in patients undergoing cardiac surgery, especially coronary bypass grafts (CABG)[8,24]. However, all clinical studies focus on early mortality and adverse cardiovascular outcomes, instead of intimal

hyperplasia formation and accelerated atherosclerosis of the bypass. It might be of interest to follow these study populations with special regards to graft failure.

Several studies that argue for a role of C5a in spontaneous atherosclerosis have been published. However, these studies report inconclusive results. Yasojima and colleagues showed for the first time both C5 mRNA and protein present in normal arteries[25]. Moreover, an impressive increase of mRNA and protein was shown in atherosclerotic arteries. In addition, Patel and colleagues reported that ApoE/C5 double knock–out mice develop spontaneous atherosclerosis to a similar extend as their ApoE-/- littermates[26]. However, C5 knock-out mice are not suitable to solely study the role of C5a, since with C5 depletion, the formation of C5b-9 is also hampered. Speidl and colleagues reported that increased serum levels of C5a in patients with advanced atherosclerosis was associated with increased cardiovascular risk (as determined by the occurrence of major adverse cardiovascular events)[27], indicating that C5a might be a valuable marker for risk-assessment in patients.

Reports that describe the role of C5a in another form of vascular remodeling, namely post-angioplasty restenosis, are scarce. Recently, in line with their data on spontaneous atherosclerosis, Speidl and colleagues described the positive correlation between increasing C5a levels after PTA of the femoral artery with the development of restenosis during follow up. However, further research remains necessary to define the exact role of C5a in the broad perspective of vascular remodeling.

In conclusion, this report provides compelling evidence for involvement of the complement fragment C5a in vein graft thickening. Inhibition of complement activation in general, and specifically C5a, might be a valuable new approach in order to overcome the problems of graft failure in patients. In this point of view, the potent anti-restenotic effects of C5aR antagonists, such as HC-[OP-(D-Cha)WR] and AcF-[OP-(D-Cha)WR], make them attractive as potential candidates for human therapy.

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