Inflammation in injury-induced vascular remodelling; Functional Involvement and Therapeutical Options
Inhibition of Complement Component C3 Reduces Vein Graft Atherosclerosis in ApoE3Leiden Transgenic Mice.

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ABSTRACT

Background: Venous bypass grafts may fail due to development of intimal hyperplasia and accelerated atherosclerosis. Inflammation plays a major role in these processes. Complement is an important part of the immune system and participates in the regulation of inflammation. However, the exact role of complement in the process of accelerated atherosclerosis of vein grafts has not yet been explored.

Methods and Results: To assess the role of complement on the development of vein graft atherosclerosis, a mouse model, in which a venous interposition is placed in the common carotid artery, was used. In this model vein graft thickening appears within four weeks. The expression of complement components was studied using immunohistochemistry on sections of the thickened vein graft. C1q, C3, C9 and the regulatory proteins CD59 and Crry could be detected in the lesions four weeks after surgery.

Quantitative mRNA analysis for C1q, C3, CD59 and Crry revealed expression for these molecules in the thickened vein graft, whereas C9 did not show local mRNA expression.

Furthermore, interference with C3 activation using Crry-Ig was associated with reduced vein graft thickening, reduced C3 and C9 deposition and reduced inflammation as assessed by analysis of influx of inflammatory cells, such as leucocytes, T-cells and monocytes. Also changes in apoptosis and proliferation were observed.

When C3 was inhibited by Cobra Venom Factor, a similar reduction in vein graft thickening was observed.

Conclusion: The complement cascade is involved in vein graft thickening and may be a target for therapy in vein graft failure disease.
INTRODUCTION

Bypass graft surgery with venous grafts is one of the most frequently used therapies, both in cardiac as in peripheral vascular surgery, to treat atherosclerotic occlusive disease. However, graft patency is often compromised by formation of intimal hyperplasia (IH) and accelerated atherosclerosis, resulting in vein graft thickening. Failure rates as high as 50% after 10 years have been reported, and re-interventions are often required.

The process of vein graft thickening is characterized by adhesion and influx of inflammatory cells and migration of vascular smooth muscle cells to the intima of the vein graft. Moreover, macrophages in the intima take up oxidized lipoproteins and become foam cells contributing to the development of accelerated atherosclerosis. There is little information available about the mechanisms underlying these processes, but it is universally assumed that inflammation and, consequently the immune system plays a pivotal role.

A major component of the immune system is the complement cascade. Complement consists of a group of proteins, membrane-bound receptors and regulatory enzymes. Centrally in the complement cascade is complement component C3. Cleavage of C3 can be induced via three separate pathways; the classical pathway, alternative pathway and lectin pathway, all activated by specific substrates. Activation via one of the three pathways results in formation of C3 convertases. The convertases are capable of cleaving C3 into C3a and C3b, starting a cascade that ultimately results in activation of C5 and subsequently in formation of terminal complement component C5b-9 (also called Membrane-Attacking-Complex (MAC). Furthermore, cleavage of C3 eventually leads to formation of potent chemotactic factors, such as C5a.

The role of complement in several inflammatory conditions is well recognized and described. It plays a major role in host defense to micro-organisms, hyperacute rejection after organ transplantation, in ischemia-reperfusion injury and in several auto-immune diseases. Atherosclerosis, like vein graft disease, is a form of vascular inflammation accompanied by intimal thickening, and several studies have been published pointing at a role for the complement system in the process. This hypothesis is supported by the detection of complement components in human atheroma while expression of several of these complement components is upregulated in atherosclerotic tissue. Furthermore, animal studies using knock-out mice and rabbits deficient in complement have been performed; however these show conflicting results. Data concerning the role of complement activation in vein graft thickening are lacking.

As mentioned above, C3 is the central component in complement activation. Inhibition of C3 activation provides good insight in the role of complement as a whole, since formation of biologically active end-products (e.g. C5b-9, C5a and C3a) is blocked. Several substances can be used to modify activation of C3. One of
the most widely used compounds is Cobra Venom Factor, derived of venom of Naja species. It functions as a C3b-like molecule and leads to unregulated C3 activation resulting in depletion of the complement cascade, however it is associated with generation of phlogistic component fragments from C3 and C5 indirectly leading to tissue activation. A more elegant approach to block C3 activation is using Crry-Ig, a recombinant protein of the mouse membrane complement inhibitor Crry (complement receptor-related gene y) fused to IgG1-hinge. Crry-Ig demonstrates decay-accelerating activity for both the classical and alternative pathways of complement as well as cofactor activity for factor I-mediated cleavage of C3b and C4b and thus prevents increased activation of C3.

To study the role of complement activation in vein graft disease, we used a mouse model for vein graft disease in hypercholesterolemic ApoE3Leiden mice. This mouse model highly resembles graft morphology in patients regarding leukocyte adhesion and influx, foam cell accumulation, calcification in the vessel wall and development of a thin fibrous cap. Not only expression of several complement factors, on both RNA and protein level, in the thickened vein graft was shown, but it was also demonstrated that treatment with Crry-Ig, which inhibits C3 activation, resulted in a significant decrease of intimal hyperplasia and accelerated atherosclerosis in murine vein grafts. With this study we show that activation of complement cascade plays a pivotal role in the development of vein graft thickening.

**METHODS**

**Mouse model.**

All experiments were approved by the institutes’ Animal Welfare Committee. For all experiments male C57bl6 ApoE3Leiden mice, age between 15 and 20 weeks, were used. During the experiment, animals were fed a high-fat high-cholesterol diet, starting 3 weeks prior to surgery to induce hypercholesterolemia. All mice received water and food ad libitum. Cholesterol levels in serum were determined 1 day before surgery and at sacrifice. Mice were anesthetized by an intra-peritoneal (i.p.) injection with a combination of Midazolam (5mg/kg, Roche, Woerden, The Netherlands), Medetomidine (0.5mg/kg, Orion, Espoo, Finland) and Fentanyl (0.05mg/kg, Janssen, Berchem, Belgium).

Vein graft surgery was performed as described previously. In summary, caval veins were harvested from genetically identical donor mice to serve as grafts and were preserved in 0.9% NaCl containing 100U/ml of heparin at 4 degrees Celcius. The right carotid artery was cut in the middle and 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. Then the graft was sleeved over the two cuffs and ligated. Pulsations of the vein graft confirmed successful engraftment. Generally, this procedure is performed in 30min.
Crry-Ig.
Crry-Ig was produced as described\textsuperscript{12}. Crry-Ig treatment started one day prior to surgery and animals received 3mg of Crry-Ig i.p. every other day during the complete study period. The control group received a monoclonal murine IgG antibody (reactive with human CRP and no cross-reactivity in mice) of the same subtype in the same concentration every other day. This approach was previously described to be the proper control for Crry-Ig treatment\textsuperscript{17}.

Cobra Venom Factor (CVF).
CVF (Quidel Corporation, San Diego, USA) was dissolved in sterile 0.9% NaCl. Animals received daily i.p. injections with 20IU/kg/day of CVF to deplete C3 throughout the whole study period starting one day prior to surgery. Animals in the control group received daily injections with sterile 0.9% NaCl.

Morphometric assessment of vein grafts.
Mice were sacrificed after either 7 or 28 days after surgery. Vein grafts were in vivo perfused with 4% formaldehyde, harvested and embedded in paraffin. Serial perpendicular cross sections were made of the specimen. All samples 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 no morphological border exists 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.

Immunohistochemistry.
To detect expression of complement factors, immunohistochemistry was performed on paraffin embedded sections of vein grafts harvested after 28d. The presence of C1q was assessed using rabbit-anti-mouse primary antibodies (Roche Applied Science, Basel, Switzerland) and C3 was detected with a rabbit-anti-mouse antibody (developed in our laboratory\textsuperscript{18}). Anti-C9 (rabbit-anti-rat, cross reactive with mouse C9\textsuperscript{19}), was a kind gift of Prof. B.P. Morgan (Cardiff University, Cardiff, UK). The antibody used to detect the complement regulatory enzyme CD59a (monoclonal rat-anti-mouse) was a kind gift of Dr Harris, (Cardiff University) and Crry was detected with a rat-anti-mouse anti-Crry antibody (BD Biosciences, Alphen a/d Rijn, the Netherlands).

Complement components were quantified using computer assisted morphometric analysis (Qwin), and expressed as total immuno-positive area as percentage of total vein graft area in cross-sections.

In grafts, smooth muscle cells (SMC) were stained using mouse-anti-rat anti-SM \(\alpha\)-actin antibodies (cross reacts with mouse; Roche Applied Biosciences). Collagen was histochemically stained by Sirius Red. Leukocytes were detected using anti-
CD45 antibodies (Pharmingen, San Diego, USA). 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).

T-cells were detected using anti-CD3 antibodies (Serotec, Raleigh, USA).

Cellular proliferation was quantified using PCNA staining (Calbiochem, San Diego, USA), whereas apoptotic cells were identified by TUNEL (Roche Applied Biosciences).

Quantification of all stainings, except those for smooth muscle cells and foam cells, occurred by counting the positive number of cells/slide and expressed as percentage of total number of nuclei (determined by Nucleus Red staining). Smooth muscle cell, collagen and foam cell quantification, due to their high occurrence in the vein grafts, was performed similar as the quantification of complement components.

**RNA isolation, cDNA synthesis and RT-PCR.**

RNA was isolated from caval veins and vein grafts harvested on several time points (t=6 and 24 hours after surgery and 3; 7; and 28d after surgery, n=4 per time point), using RNA Isolation Mini Kits for Fibrous Tissue (Qiagen, Venlo, the Netherlands; using the manufacturers protocol). 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, Uppsala, Sweden).

Reverse-transcribed products were studied using semi-quantitative RT-PCR (Robocycler Gradient96, Stratagene, Cedar Creek, Texas, USA), with primers for C1q, C3, C9, CD59 and Crry (for sequences see Table 7.1). Samples were amplified for 35 cycles (30sec. at 94°C, 30sec at 56°C and 90sec. at 65°C) following an initial denaturation cycle for 2 min. at 94°C. The last cycle was followed by extension of 4 min. at 74°C. PCR products were visualized on 1.2% Agarose gel containing EthidiumBromide.

**Table 7.1:** Primer sequences and length of PCR product of genes analyzed in RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
<th>PCR-product</th>
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<tbody>
<tr>
<td>C1q</td>
<td>cagttgccgagaagatgtcgc</td>
<td>ccgrtgegtcgttgatgga</td>
<td>386</td>
</tr>
<tr>
<td>C3</td>
<td>gtagtgccgacaacaggagtrg</td>
<td>gtagtgccgcaaatgactg</td>
<td>566</td>
</tr>
<tr>
<td>C9</td>
<td>cgtatccctctacaagacgc</td>
<td>ctcatttagacatggctagc</td>
<td>402</td>
</tr>
<tr>
<td>Crry</td>
<td>cagctcagctctctctgcc</td>
<td>atcgtgctgctacgtagata</td>
<td>500</td>
</tr>
<tr>
<td>CD59</td>
<td>ttagctgaggagtgagac</td>
<td>ctcctgagactgagatac</td>
<td>405</td>
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</tbody>
</table>
Statistical Analysis.
All data were presented as mean±SEM. Statistical analysis was performed using SPSS 11.5 for Windows. Differences between groups were analyzed with a Student T-test. P-values<0.05 were regarded statistically significant.

RESULTS

Presence of complement components in intimal hyperplasia of murine vein grafts.
Twenty-eight days after surgery, mice (n=6) were sacrificed and vein grafts were harvested in order to detect complement factors and regulatory enzymes by immunohistochemistry (Figure 7.1). These factors were elicited on the basis that each represents a specific part of the complement cascade (e.g. C1q; early classical pathway, C3; central component, C9 part of the membrane attack complex, CD59 and Crry; regulatory molecules).

Regulatory enzymes CD59 and Crry in vein grafts demonstrate a different expression pattern. CD59 shows a diffuse expression throughout the thickened vein graft, whereas expression of the membrane-bound Crry shows a patchy, cell bound distribution, and is mainly localized in the media and adventitia of the murine vein graft. Magnification of all pictures 150x.

Massive presence of C1q was seen in thickened vein grafts, mainly deep in the intimal hyperplasia co-localizing with foam cells. Furthermore, inflammatory cells attached to the endothelium and in the adventitia of the veins showed positive staining. C3 was predominantly expressed in the same regions as C1q, being in macrophage derived foam cells, adhering and adventitial inflammatory cells and also in the endothelium. In addition, C9 co-localized highly with macrophages in the thickened intima but was not expressed by endothelial cells.

Complement inhibitor CD59 was detected diffusely distributed in all layers of the vessel wall. The membrane-bound Crry showed a patchy distribution in cells of the media and adventitia. Endothelial cells showed positive staining in about one-third of cells.

Presence of RNA coding for complement components in murine vein grafts.
Local production of complement component was examined by RNA analysis of vein grafts. Sixteen mice underwent vein graft surgery and were sacrificed on several time points (t= 6h, 24h, 3d, 7d and 28d). Also normal, not yet interposed, caval veins of donor mice were included. RT-PCR was performed for C1q, C3, C9, CD59 and Crry (Figure 7.2).
**Figure 7.1:** Expression of complement factors in thickened murine vein graft of ApoE3Leiden mice, 28 days after surgery. Massive intima hyperplasia formation vein graft thickening is observed, as indicated with arrows (HPS). The cellular composition (consisting of smooth muscle cells and macrophage-derived foam cells) of the thickened vein graft is shown. Immunohistochemical detection of complement factors C1q, C3 and C9. Both C1q and C3 are abundantly present in the deeper parts of the intimal hyperplasia co-localizing with foam cells. Furthermore, C1q and C3 are expressed in the endothelial layer and in inflammatory cells (mainly macrophages) adhering to the vessel wall and present in the adventitia. C9 is not detectable in the endothelium and inflammatory cells. However, like C1q and C3, it is present in the deeper parts of the intimal hyperplasia.
Minimal expression of C1q mRNA was seen in caval veins. In vein grafts, C1q mRNA was not detectable in the first 24 hours after surgery. However, 3 days after surgery, C1q mRNA was clearly present and expression remained constant for the rest of the study period.

C3 showed an explicit expression in vein grafts in all stages of remodeling. Interestingly, an equal expression was seen in caval veins, showing that also without a remodeling process going on in the vessel, local production of C3 occurs. mRNA expression of complement component C9 could not be detected in vein grafts, whereas expression in liver tissue (positive control) appeared clearly positive, indicating that C9-protein is not produced locally in vein grafts.

Crry mRNA was clearly detectable in normal caval veins. Down-regulation of Crry mRNA expression was seen in the first days after surgery, whereas after 7d an increased expression was seen to the level of normal caval veins.

Complement inhibitor CD59 showed robust expression in normal caval veins, whereas expression in the remodeling vein graft was present but appeared lower.

Effect of Crry-Ig treatment on vein graft thickening and complement expression. The studies above suggest involvement of the classical pathway on vein graft thickening. Earlier studies demonstrated efficient blockage of complement activation
by Crry-Ig. Twelve ApoE3Leiden mice were randomly divided into two treatment groups. One group received 3mg (i.p.) Crry-Ig every other day; controls received an injection with 3mg of non-relevant monoclonal antibody of the same isotype every other day. No significant differences were observed between groups in preoperative bodyweight, bodyweight at sacrifice and cholesterol levels before surgery and at sacrifice.

Vein grafts were harvested after 28d and vein graft thickening was quantified. Crry-Ig treated mice exhibited approximately 50% less vein graft thickening, when compared to controls (control 0.36±0.07mm², Crry-Ig 0.18±0.01mm², p=0.028). Luminal area was equal in both groups (control 0.53±0.04mm², Crry-Ig 0.40±0.06mm², p=0.28). Consequently, total vessel wall area was significantly larger in the control group (0.56±0.05mm²) when compared to Crry-Ig treated vein grafts (0.89±0.07mm², p=0.016), indicating reduced outward remodeling in the Crry-Ig treated group.

C1q quantity in vein grafts was not affected by Crry-Ig and demonstrated no differences with control IgG treated mice (control: 25±4%, Crry-Ig: 25±2%, p=0.48).

A significant decrease of C3 deposition was observed in Crry-Ig treated mice, compared to controls (control: 37±4%, Crry-Ig: 21±3%, p=0.007). Moreover, presence of C9 protein, was reduced in Crry-Ig treated vessels (control 19±3%, Crry-Ig 9±2%, p=0.007). Data are summarized in Figure 7.3.

**Figure 7.3:** Effect of Crry-Ig treatment on development of intimal hyperplasia and accelerated atherosclerosis in murine vein grafts. Quantitative analysis of the effect of Crry-Ig treatment on development of intimal hyperplasia and accelerated atherosclerosis in murine vein grafts and C1q, C3 and C9 deposition in the vein grafts.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Crry-Ig</th>
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<tbody>
<tr>
<td>Vein graft thickening (in mm²)</td>
<td>0.36±0.07 mm²</td>
<td>0.18±0.01 mm²*</td>
</tr>
<tr>
<td>C1q deposition (% of total IH)</td>
<td>25±4%</td>
<td>25±2%</td>
</tr>
<tr>
<td>C3 deposition (% of total IH)</td>
<td>37±4%</td>
<td>21±3%*</td>
</tr>
<tr>
<td>C9 deposition (% of total IH)</td>
<td>19±3%</td>
<td>9 ±2%*</td>
</tr>
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* p<0.05
Representative cross section demonstrating reduced vein graft thickening, similar amounts of C1q deposition, and reduced C3 and C9 deposition (magnification 150x).

Effect of Crry-Ig treatment on cellular composition of remodeling vein grafts.

To study the effect of blocking complement activation on cellular composition, 24 mice underwent surgery, were treated with either Crry-Ig or a non-relevant monoclonal antibody (as described above) and were sacrificed after 7 or 28d (n=6 in each group).

Overall, as expected, vein grafts harvested after 28d contained more cells than the ones harvested after 7d, and Crry-Ig treated grafts contained less cells as their untreated controls after 28d (control 7d: 228±59, Crry-Ig 7d: 227±32, n.s and control 28d: 2296±565, Crry-Ig 28d: 1143±69, p=0.044).

SMC content was analyzed in vein grafts harvested after 28d, since in the first week after engraftment all SMC disappear in this model. In the Crry-Ig treated group, thickened vein grafts had a significant higher relative SMC content than the control.
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The amount of collagen deposition, however, did not significantly differ between both groups (control 33±4%, Crry-Ig 29±2%, p=0.5).

Influx of CD45 positive leukocytes was especially seen after 7d and strongly reduced by Crry-Ig treatment (control: 31.3±10.2%, Crry-Ig: 9.6±1.5%, p=0.034). At 28d the relative amount of CD45-positive leukocytes was much lower and not significantly different between groups (control: 1.8±0.4%, Crry-Ig: 1.9±0.9%, p=0.44). In the seven-day vein grafts monocytes in both groups were predominantly adhering to the vessel wall and invading in the vein graft. Crry-Ig treatment resulted in a 42%, yet not significant reduction, in adhering monocytes (control: 7.0±1.8%, 4.1±0.9%, p=0.08). After 28d, AIA31240-immunostaining revealed mainly macrophage-derived foam cells in the plaque area. A significantly lowered foam cell content was seen in vein grafts of Crry-Ig treated animals (Crry-Ig: 16±1%, control: 25±5%, p=0.043) after 28d. Regarding CD3-positive T-cells, a significant difference could be seen after 7d (control: 1.9±0.6%, Crry-Ig: 0.7±0.1%, p=0.05), however after 28d, this difference could not be observed (control: 1.8±0.6, Crry-Ig: 1.2±0.3%, p=0.21). Results are summarized in Table 7.2.

Table 7.2: Effect of Crry-Ig treatment on cellular composition and cytokinetics of remodeling vein grafts, both 7 and 28 days after surgery (determined by immunohistochemistry, n=6 for each value). Positively stained cells are expressed as a percentage of total number of cells. np: analysis not performed; * represents p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>Control 7d</th>
<th>Crry-Ig 7d</th>
<th>Control 28d</th>
<th>Crry-Ig 28d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # cells</td>
<td>228±59</td>
<td>227±32</td>
<td>2296±565</td>
<td>1143±69*</td>
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<tr>
<td>Smooth muscle cells</td>
<td>n.p.</td>
<td>n.p.</td>
<td>33±5%</td>
<td>47±3%*</td>
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<tr>
<td>Collagen</td>
<td>n.p.</td>
<td>n.p.</td>
<td>33±4%</td>
<td>29±2%</td>
</tr>
<tr>
<td>CD45</td>
<td>31±9%</td>
<td>10±2%*</td>
<td>1.8±0.4%</td>
<td>1.9±0.9%</td>
</tr>
<tr>
<td>Monocytes/ foam cells</td>
<td>7.0±1.9%</td>
<td>4.1±0.9%</td>
<td>25±5.0%</td>
<td>16±1.0%*</td>
</tr>
<tr>
<td>CD3</td>
<td>1.9±0.6%</td>
<td>0.7±0.1%*</td>
<td>1.8±0.6%</td>
<td>1.2±0.3%</td>
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<tr>
<td>TUNEL</td>
<td>0.7±0.4%</td>
<td>3.0±0.6%*</td>
<td>0.2±0.01%</td>
<td>0.2±0.03%</td>
</tr>
<tr>
<td>PCNA</td>
<td>11±1.3%</td>
<td>6.7±1.2%*</td>
<td>2.3±0.7%</td>
<td>1.8±0.6%</td>
</tr>
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</table>

Effect of Crry-Ig treatment on cytokinetics of remodeling vein grafts.

To get insight on the possible mechanisms of the effect of complement inhibition on vein graft remodeling, we analyzed the effects of Crry-Ig treatment on cell
proliferation and apoptosis. Regarding proliferation, Crry-Ig treatment resulted in reduced cellular proliferation after 7d as assessed by PCNA immunohistochemistry (control: 10.6±1.3%, Crry-Ig: 6.7±1.2%, p=0.036) After 28d, cellular proliferation declined and no differences were seen between both groups (control: 2.3±0.7%, Crry-Ig: 1.8±0.6%, p=0.3).

Surprisingly, Crry-Ig treatment led to increased numbers of TUNEL-positive apoptotic cells in vein graft 7d after surgery (control: 0.7±0.4%, Crry-Ig: 3.0±0.6%, p=0.005). However, after 28d, the number of apoptotic cells was decreased in both groups to such a level that no conclusion was justified. All data are summarized in Table 7.2.

**Effect of CVF treatment on vein graft thickening.**

To demonstrate that the observed effect is not Crry-Ig-specific, an alternative treatment affecting C3 activation was tested. Therefore, Cobra Venom Factor (CVF) was administered. Vein grafting was performed in 12 mice and 6 were treated with CVF (20IU/kg/day). Mice were sacrificed after 28d. Quantification of vein graft thickening revealed a 63% reduction of intimal hyperplasia in CVF-treated animals (0.15±0.01mm²), when compared to controls (0.41±0.10mm², p=0.016). Luminal surface did not differ significantly between groups (CVF: 0.49±0.06mm², control 0.59±0.07mm², p=0.47).

**DISCUSSION**

This study argues for a causal role for complement activation in general, and C3 activation in particular, in the process of venous bypass graft thickening. To our knowledge, this is the first study that not only demonstrates presence of complement components in vein grafts but also provides evidence that inhibition of C3 activation results in a marked decrease of vein graft thickening in vivo.

Intimal hyperplasia formation and accelerated atherosclerosis in vein grafts is considered to be the result of an inflammatory process. This process is initiated by mechanical vessel damage and hypoxia during surgery and by altered shear stress that the vein is being subjected to after surgery. Several animal models are developed to mimic this process. In this study a mouse model is used, in which caval veins of donor mice are placed as interposition in the common carotid artery. When performed in hypercholesterolemic mice, vein graft thickening occurs with signs of accelerated atherosclerosis. The morphology of the observed lesions highly resembles what is seen in human vein grafts, underlining the relevance of this model.

Since the complement cascade is an important part of the innate immune system and is also involved in initiation of adaptive immune responses, it might be one of the mediators of inflammatory processes in remodeling vein grafts.
The presence of complement components (C1q, C3 and C9) and regulatory molecules (CD59a and Crry) in murine vein grafts was shown by immunohistochemistry. Furthermore, we analyzed local synthesis of complement components, defined as mRNA expression in vein grafts. Although synthesis of complement components is believed to be localized in the liver, recently evidence of extra-hepatic synthesis of complement components has been published. RT-PCR analysis of vein grafts at various time points after surgery revealed local expression mRNA coding for C1q, C3, CD59 and Crry. No local production of C9 could be detected, indicating that C9 protein deposited in vein grafts, as seen by immunohistochemistry, is produced elsewhere.

Although C3 mRNA expression was detectable in the vessel wall of caval veins, no up- or downregulation of C3 on mRNA level could be detected in vein grafts. Several possible explanations for this finding could be given. One might be that, although mRNA C3 is produced locally in vein grafts, this may not tell us much about C3 activity and protein presence. Furthermore, C3 is mainly produced in the liver, as most of the complement components, and is abundantly present in plasma. This liver derived C3 might be crucial for mediating the effects on vein graft thickening, whereas the role of locally produced C3 is unclear. In addition, factors involved in regulating local levels of (active) C3 and the effects of C3 down stream molecules on C3 levels in vein graft remodeling are not completely understood.

We were unable to detect activated complement factors or MAC, because of the lack of specific antibodies against murine activated complement components e.g. murine iC3b, C5b-9 or C5a. Since C3 is the central protein in the complement cascade, it was used as a specific target for our interventional experiments. C3 activation was blocked using Crry-Ig, an inhibitor for all activation pathways by the inhibition of C3 convertases. Crry-Ig treatment resulted in a significantly lower C3 and C9 deposition in vein grafts, accompanied by an inhibition of vein graft thickening of approximately 50%. Analysis of cytokinetics and cellular composition of vein grafts at 7 and 28 days, provided insight in the mechanism by which complement inhibition may affect vein graft remodeling. When C3 was inhibited by Crry-Ig, one of the first phenomena seen is reduced adherence and influx of leucocytes, possibly as a result of absence of (complement activation-derived) chemotactic stimuli. This points at tempered inflammatory activity in remodeling vein grafts. Reduction in inflammatory activation of the vein graft wall is thought to be the main cause of reduced vein graft thickening. Reduction of leucocyte adhesion and influx is accompanied by reduced numbers of proliferating cells in the remodeling vein graft and induction of apoptosis. This induction of apoptosis is unexpected, since reduced inflammation is usually accompanied by reduced apoptosis. No specific cell type or location could be associated to the observed apoptosis.

After 28 days, plaque composition was significantly altered in the Crry-Ig treated group, favoring SMC and reduced numbers of foam cells. Increase numbers of SMC in thickened vein grafts reflect a desirably state, since this is believed to be one of the main contributions to plaque stability. The observation that vein graft thickening
can also be hampered by CVF (another potent C3 activation inhibitor) shows that the phenomena seen after Crry-Ig treatment, are not Crry-Ig specific, but are caused by C3 inhibition. Inhibition of vein graft thickening following treatment with Crry-Ig also suggests that circulating complement components play a role in the remodeling process.

The demonstrable C1q in vein grafts suggest involvement of the classical pathway. Interestingly, we found a reduced C1q mRNA expression in the early phases after surgery, followed by enhanced expression in later stages. Regrettably, very little is known about in vivo C1q mRNA regulation in various cell types. However, since C1q in this model is predominantly expressed by monocytes and macrophages, and macrophage C1q mRNA expression has been shown to alter during the various stages of macrophage maturation in vitro, this reduced C1q mRNA expression in the early stages might be due to a temporary lowered C1q mRNA expression as a consequence of ongoing maturation during the transition of adhering monocytes into tissue macrophages.

Furthermore, we found that in the early phases after surgery, Crry mRNA expression was decreased, and therefore diminishing complement inhibition potential of the vein graft. This might be one of the causes responsible for complement activation in this model. However, other questions need to be considered. For instance, what triggers complement activation in venous bypass grafts, and which of the activation routes are accountable for activation? There are several possibilities.

A venous interposition can be seen as an autologous transplant; it undergoes ischemia-reperfusion injury during the procedure. In other models for ischemia-reperfusion the production of “natural antibodies” by CD5 positive B- cells is described, leading to deposition of IgM antibodies and thereby triggering the classical pathway. This is confirmed by the study of Fitzmaurice, he was able to detect immunoglobulins and C3 depositions in human saphenous vein grafts. We show the presence of C1q protein and mRNA in various phases of the remodeling process, suggesting that the classical pathway actually might play a role. Besides the classical pathway as the major activating pathway of the complement system in ischemia-reperfusion, recently Stahl et al described the role of the alternative pathway in (intestinal) ischemia-reperfusion injury and Thurman et al found that the alternative pathway alone was required for renal ischemia-reperfusion injury.

In addition, LDL depositions in the vessel wall might be responsible for complement activation in this model. Torzewski et al previously described co-localization of C5b-9 and enzymatically altered LDL in deeper parts of the intima of early atherosclerotic lesions. Furthermore, it was shown that this enzymatically modified LDL is atherogenic and can induce complement activation via the alternative route in vitro. However, since fat deposition and foam cell formation occurs relatively late in the model used in this study and the observed effects on cellular influx were present after 7d, it is not likely that this route of complement activation plays a role in this model.
Furthermore, the vein graft is liable to an arterial blood pressure and an increased shear stress, causing damage to the vein graft wall. Also the surgical procedure leads to damage of the vein graft. This results in a mechanical denudation of the endothelium and apoptosis of remaining endothelial cells. It has been described before, that apoptotic (endothelial) cells can induce complement activation both via the classical and alternative pathway. Which of the three activation pathways, alone or in combination, is responsible for the activation of complement and by what kind of injury this activation is triggered remains to be determined and further research is necessary to indicate the role of each of these modalities in vein graft disease.

When translating data from mouse studies to the human situation, several issues should be taken into account. Firstly, mice do not develop spontaneously atherosclerosis. Therefore usually genetically-altered hypercholesterolemic mice are used to induce atherosclerotic lesions, either spontaneously after prolonged exposure to a hypercholesterolemic environment or in an accelerated way after vascular intervention. Although the ApoE3Leiden mice used in this study suffered relatively mild hypercholesterolemia (serum cholesterol between 8-10 mmol/l), cholesterol levels still exceed serum levels seen in most of the patients. As previous reports have shown, severe hypercholesterolemia might induce increased vascular inflammatory reactions and consequently atherosclerotic lesion formation. Furthermore, due to anatomic variations between human and mice, it can be assumed that after engraftment, as a result to the 10-fold increase in blood pressure, more extensive graft distension and subsequent vascular damage and SMC apoptosis is occurring in (only a few cell-layers thick) murine vein grafts when compared to the human counterpart.

In conclusion, several complement factors (both on protein and mRNA level) are present in thickened vein grafts, and treatment with Crry-Ig (interfering in C3 activation) results in marked reduction of vein graft thickening. This reduction coincides with reduced numbers of leucocytes in early stages of vein graft remodeling, and increased numbers of smooth muscle cells in the later stage. Therefore, we have provided evidence that activation of C3, and thereby the complement cascade, is an important early trigger for vein graft thickening. Therapy that interferes in the function of C3 might be an interesting new target in order to overcome the clinical problem of vein graft disease.

REFERENCES

CHAPTER 7 - Inhibition of C3 Reduces Vein Graft Atherosclerosis in ApoE3Leiden Transgenic Mice.


