C1-esterase inhibitor protects against early vein graft remodeling under arterial blood pressure

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Objectives: Arterial pressure induced vein graft injury can result in endothelial loss, accelerated atherosclerosis and vein graft failure. Inflammation, including complement activation, is assumed to play a pivotal role herein. Here, we analyzed the effects of C1-esterase inhibitor (C1inh) on early vein graft remodeling.

Methods: Human saphenous vein graft segments (n=8) were perfused in vitro with autologous blood either supplemented or not with purified human C1inh at arterial pressure for 6h. The vein segments and perfusion blood were analyzed for cell damage and complement activation. In addition, the effect of purified C1inh on vein graft remodeling was analyzed in vivo in atherosclerotic C57Bl6/ApoE3 Leiden mice, wherein donor caval veins were interpositioned in the common carotid artery.

Results: Application of C1inh in the in vitro perfusion model resulted in significantly higher blood levels and significantly more depositions of C1inh in the vein wall. This coincided with a significant reduction in endothelial loss and deposition of C3d and C4d in the vein wall, especially in the circular layer, compared to vein segments perfused without supplemented C1inh. Administration of purified C1inh significantly inhibited vein graft intimal thickening in vivo in atherosclerotic C57Bl6/ApoE3 Leiden mice, wherein donor caval veins were interpositioned in the common carotid artery.

Conclusion: C1inh significantly protects against early vein graft remodeling, including loss of endothelium and intimal thickening. These data suggest that it may be worth considering its use in patients undergoing coronary artery bypass grafting.

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1. Introduction

Vein graft failure remains a major clinical problem. As many as 50% of coronary artery vein grafts fail within 10 years after coronary artery bypass grafting (CABG). The main inducer of vein graft damage is presumed to be the exposure of the vein grafts to arterial blood pressure, inducing endothelial injury. Vein graft failure then develops through accelerated atherosclerosis, characterized by foam cell formation, influx of inflammatory cells and migration of vascular smooth muscle cells to the intima of the vein graft. Inflammation is assumed to play a pivotal role in propagating vein graft failure [1].

The complement system is a large family of effector and regulatory proteins that forms a prominent component of the innate immune system. The involvement of complement in cardiovascular disease is well accepted, including atherosclerosis of arteries and aortic valves [2,3]. Furthermore, a role for complement in vein graft failure is acknowledged. In patients undergoing CABG the complement system is activated. This can occur as a result of, amongst others, the surgical trauma, contact of the blood with non-endothelial surfaces during extracorporeal circulation and...
ischemia–reperfusion injury [4–6]. This activation of the complement system in CABG patients is characterized by the plasmatic release of potent vasoactive anaphylatoxins C3a, C4a and C5a and the membrane attack complex C5b-9 [7]. On a tissue level, expression of complement factors C1q, C3, and C9 was found in failing venous grafts in mice [8], indicating direct involvement of complement activation in venous graft failure. Indeed, complement factor C3 inhibition via Ctry-lg-antibody treatment reduced vein graft atherosclerosis in mice [8] and antibody-based inhibition of C5a (pexixulimab) or the serine protease inhibitor C1-esterase inhibitor (C1inh) resulted in better short term clinical outcomes, such as decreased mortality and improved cardiac function, in patients undergoing CABG [9–12]. Although, of these latter two, their effect on the venous graft itself remains to be established.

C1inh is a natural occurring protease inhibitor of the serpin family [13]. C1inh inactivates a variety of proteases such as the complement proteases C1r and C1s, thereby inhibiting the alternative pathway of complement [14]. In addition, the contact system proteases factor XII and plasma kallikrein and the coagulation protease factor XI are inactivated by C1inh [13]. As such, C1inh treatment was shown to exert favorable, anti-inflammatory effects in sepsis patients [15] and animal sepsis models [16], to protect against LPS-mediated increased vascular permeability and endothelial cell injury in vitro [17] and to inhibit atherosclerotic lesion development after arterial injury in mice [18]. These data suggest that C1inh treatment may be beneficial in CABG and protect the venous graft against acute injury to the endothelium and against atherosclerotic lesion development. In this study therefore, the effects of C1inh on acute vein graft injury were analyzed in perfused human saphenous veins and on early vein graft atherosclerosis development in a venous bypass model in mice.

2. Materials and methods

2.1. Human vein graft tissue

Surplus segments of harvested saphenous veins of patients who underwent coronary artery bypass grafting (CABG) (n=8) were used with the patients consent. These vein segments were collected in the operating room under sterile conditions for histopathological examination. Part of these vein graft segments were fixed in 4% (m/v) buffered formaldehyde immediately after harvesting. The remaining part was cut in half. One half was perfused with autologous heparinised blood of the same patient and the other half was perfused with this blood supplemented with purified human C1inh (4U/mL). The veins were perfused in an experimental set-up at arterial pressure (60 mmHg) for 6 h [19] and fixed in 4% (m/v) buffered formaldehyde thereafter. The vein sections were then embedded in paraffin for immunohistochemical analysis. Samples (±0.5 mm) of the perfusion blood were taken every hour. The perfusion blood was centrifuged at 1300 × g for 10 min and the obtained plasma was stored at –80°C until analysis. Our study was approved by the ethics committee of the VU Medical Centre and the OLVG, Amsterdam.

2.2. Antibodies

The following antibodies were used: mAb mouse-anti-human C1inh (RIL; Sanquin Research at CLB, Amsterdam, The Netherlands; 10 µg/mL), mAb mouse-anti-human CD34 (Dako, Glostrup, Denmark; 1:25 dilution), mAb (mouse) against Bcl-2 (Dako; 1:150 dilution), pAb rabbit-anti-active caspase-3 (Promega, Madison, WI, USA; 1:250 dilution), pAb rabbit-anti-human C3d (Dako; 1:1000 dilution), mAb mouse-anti-human C4d (Serotec, Düsseldorf, Germany, 1:200 dilution), pAb rabbit-anti-human Myeloperoxidase (MPO) (Dako; 1:500 dilution), pAb rabbit-anti-mouse macrophage (Accurate Chemical, Westbury, NY, USA; 1:3000 dilution) for the detection of macrophage derived foam cells within the thickened vessel wall and mAb mouse-anti-rat smooth muscle α-actin (SMA) (cross-reacts with mouse; Roche, Almere, The Netherlands: 1:750 dilution).

2.3. (Immu)n histochemistry

Paraffin embedded human- or mouse vein graft cross sections (4 µm thick) were deparaffinised for 10 min in xylene and dehydrated with ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% (v/v) H2O2 in methanol for 30 min. The antigen retrieval step for CD34, C1inh, C3d, C4d, macrophages and MPO staining was boiling in 10 mmol/l sodium citrate buffer, pH 6, for 10 min. For caspase-3 and Bcl-2 staining the antigen retrieval step was boiling for 10 min in buffer prepared by dissolution of Tris and EDTA. For the SMA staining no antigen retrieval was used. All antibodies and normal sera were diluted in PBS containing 1% (w/v) bovine serum albumin (BSA). After pre-incubation with normal swine serum (for C3d and active caspase-3) (Dako; 1:10 dilution) or normal rabbit serum (CD34, C1inh and Bcl-2) (Dako; 1:50 dilution) for 10 min, slides were incubated with the primary antibodies for 1 h. After a wash in PBS the slides were incubated for 30 min with Envision (Dako) for C4d and MPO; with a biotin-conjugated swine-anti-rabbit antibody (Dako; 1:300 dilution) for C3d and active caspase-3; or with a biotin-conjugated rabbit-anti-mouse (Dako; 1:500 dilution) for CD34, C1inh and Bcl-2. For SMA, rabbit anti mouse HRP (Dako: 1:300 dilution), and for macrophages biotin-conjugated donkey-anti-rabbit (GE Healthcare 1:300 dilution) were used. After washing in PBS, the slides that were incubated with a biotin-conjugated secondary antibody, were incubated with streptavidin–biotin complex (Dako; sABC; 1:200 dilution) for 1 h. All slides were visualized with 3'–diaminobenzidine (DAB; 0.1 mg/mL, 0.02% H2O2). Slides were counterstained with hematoxylin, covered and scored.

2.4. Immunoscopying

In human vein grafts, the percentage of residual endothelium was determined by dividing the measured part of the lumens surface area that was still covered with CD34-positive endothelial cells by the total lumens surface area × 100%. The area positive for C1inh, C4d, C3d, Bcl-2, MPO and active caspase-3 staining in each vein cross section was measured by computer-assisted morphometry (Image-Pro Plus, version 4.5) and then expressed as the percentage of the total vein graft cross section surface area.

In mouse vein grafts, for morphometric analysis the cross sections were stained with hematoxylin–phloxine–saffron (HPS) and then analyzed using image analysis software (Qwin, Leica, Wetzlar, Germany). Since the media in murine veins consists only of a few layers of cells, there is no morphological border between neointima and media. Therefore, to define vein graft thickening, the region between lumen and adventitia was used as the lesion area. For each mouse six equally spaced cross-sections were used to determine vessel wall thickening. Quantification of the different subsets of cells was performed by computer assisted analysis (Qwin) as positive stained area in the graft and expressed as a percentage of total vein graft cross section surface area.

2.5. Measurement of functional C1inh in serum

Functional human C1inh in the blood was measured according to a previous study [20]. In short, microtiter plates, coated with the mAb against C1inh (2 µg/mL in PBS overnight at 4°C), were washed with PBS containing 0.02% (w/v) Tween20,
incubated with plasma samples diluted in PBS, 0.1% (w/v) Tween20, 0.2% (w/v) gelatine (PTG) for 90 min at 4 °C. The plates were washed and incubated with biotinylated C1s (1 μg/mL in PTG), final volume 100 μL, for 60 min. After a wash the plates were incubated with 0.001% (w/v) HRP-labeled streptavidin (Amersham International plc, Amersham, UK) for 30 min, washed and incubated with 0.1 mg/mL 3,5,3′,5′-tetramethylbenzidine (TMB) (Merck, Darmstadt, Germany) in 0.1 M sodium acetate containing 0.003% (v/v) H2O2, pH 5.5. Serial dilutions (in PTG) of pooled normal human plasma containing 230 μg/mL active C1inh were used as a standard.

2.6. Mouse experiments

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) [18] ad libitum, aiming at plasma cholesterol levels of 10–15 mmol/L. Serum cholesterol levels of C1inh in blood decreased significantly to 1273 ± 468 μg/mL (p < 0.001). Thereafter, the levels of C1inh in the supplemented blood did not change significantly anymore. In the un-supplemented blood the levels of C1inh did not change significantly between the different time points. At all time points the concentration of active C1inh was significantly higher in the supplemented blood versus the un-supplemented blood (p < 0.01).

3. Results

3.1. C1inh levels in the perfusion blood

The saphenous vein graft segments of patients were perfused with autologous blood either or not supplemented with purified human C1inh (4U/mL). The perfusion blood was analyzed for the concentration of active C1inh before perfusion and at 1 h intervals until 6 h after perfusion.

In the blood supplemented with C1inh, the concentration of active C1inh was 2854 ± 1248 μg/mL prior to perfusion, compared to 146 ± 56 μg/mL in the un-supplemented blood (p < 0.001; Fig. 1). After 1 h of perfusion, the C1inh concentration in the supplemented blood decreased significantly to 1273 ± 468 μg/mL (p < 0.001). Thereafter, the levels of C1inh in the supplemented blood did not change significantly anymore. In the un-supplemented blood the levels of C1inh did not change significantly between the different time points. At all time points the concentration of active C1inh was significantly higher in the supplemented blood versus the un-supplemented blood (p < 0.01).

3.2. C1inh protects against acute perfusion-induced endothelium loss

Saphenous vein graft segments of patients were analyzed before and after perfusion. Of each patient part of the vein was fixed before perfusion, part was perfused with autologous blood and part was perfused with autologous blood supplemented with C1inh. Early
changes in veins perfused at arterial pressure include substantial loss of endothelial cells and cell damage in the media [19]. Therefore, the putative protective effects of C1inh on endothelial loss, cellular apoptosis and infiltration of granulocytes were analyzed using immunohistochemistry.

In non-perfused veins, the percentage of residual endothelium was 83 ± 8% (Fig. 2). After 6 h of perfusion with un-supplemented blood the percentage of residual endothelium was 25 ± 14%, which was significantly lower than in non-perfused veins (p < 0.001). Supplementation of C1inh to the perfusion blood resulted in a significant increase in residual endothelium to 47 ± 17% (p < 0.001), indicating that C1inh protects against endothelial loss in vein grafts under arterial pressure.

3.3. C1inh in the vein graft wall

The amount deposited C1inh in the vein wall was subsequently analyzed, whereby we differentiated between the longitudinal and circular layers of the media. In non-perfused veins the percentages of C1inh-positive area were low with 1.7 ± 0.7% in the longitudinal layer and 2.5 ± 0.7% in the circular layer (Fig. 3). These percentages did not differ significantly. Perfusion with un-supplemented blood induced a significant increase in C1inh deposition to 6.8 ± 1.9% in the longitudinal layer (p < 0.001) and 16.5 ± 4.1% in the circular layer (p < 0.001). Notably, the C1inh-positive area in the circular layer was significantly larger than in the longitudinal layer (p < 0.001). In veins perfused with C1inh-supplemented blood, the C1inh-positive area was 14.3 ± 1.8% in the longitudinal layer and 20.4 ± 1.4% in the circular layer, which was significantly larger than in non-perfused veins (p < 0.001) and veins perfused with un-supplemented blood (p < 0.001). Furthermore, also here the C1inh-positive area was significantly larger in the circular layer than in the longitudinal layer (p < 0.001). Thus, C1inh supplementation resulted in an increase in C1inh deposition in the media of the vein wall.

3.4. C1inh reduced complement deposition in the vein wall

The effect of C1inh supplementation on complement deposition (factors C3d and C4d) in the vein wall was also analyzed as a marker for cell damage in the media. In non-perfused veins the C3d-positive area was 0.5 ± 0.2%, whereas no C4d was detected (Fig. 4). After 6 h of perfusion with un-supplemented blood the percentage C3d-positive area increased significantly to 5.5 ± 1.8% (p < 0.001) as did the percentage of C4d-positive area to 7.9 ± 0.9% (p < 0.001). These complement depositions were found not only in the media but also on residual endothelial cells. Supplementation of C1inh to the perfusion blood resulted in a significant decrease in the C3d-positive area 0.5 ± 0.2% (p < 0.001) and the C4d-positive area to 2.4 ± 0.3% (p < 0.001), which did not differ significantly compared to non-perfused veins. C1inh thus led to reduced complement depositions in the vein wall, indicating an inhibitory effect on complement.

3.5. Neutrophilic granulocytes in the vein graft wall

Complement activation can lead to chemo-attraction of neutrophilic granulocytes. Therefore, the infiltration of MPO-positive granulocytes was quantified. In non-perfused veins no neutrophilic granulocytes were found (not shown). Perfusion, both with or without supplemented C1inh, resulted in a minor, not significant increase in MPO-positive cells (1.3 ± 3.5% for un-supplemented; 1.4 ± 3.9% for C1inh-supplemented).
3.6. Apoptosis in the vein graft wall

It has been shown that C1inh can prevent cellular apoptosis directly via an effect on the Bcl-2/Bax ratio [22]. Therefore, the effect of C1inh on apoptosis in perfused veins was analyzed via immunohistochemical detection of activated caspase-3 and Bcl-2. However, neither in non-perfused veins, nor in veins perfused, with or without supplemented C1inh, staining of activated caspase-3 or the anti apoptotic Bcl-2 was found (not shown).

3.7. Mouse vein perfusion

We show here in perfused human saphenous veins that C1inh protects against acute vein graft injury. Subsequently, the effects of C1inh treatment on vein graft atherosclerosis development were studied in a venous bypass model in APOE<sup>−/−</sup>-Leiden mice. Intravenous administration of C1inh resulted in a significant decrease in vein graft thickening 28 days after engraftment, from 0.39 ± 0.05 mm<sup>2</sup> in the vehicle group to 0.27 ± 0.03 mm<sup>2</sup> in the C1inh group (Fig. 5A–C; p = 0.04). The vessel wall circumference, a measure for in- or outward remodeling, also decreased significantly upon C1inh treatment from 0.78 ± 0.04 mm<sup>2</sup> (vehicle group) to 0.64 ± 0.03 mm<sup>2</sup> (C1inh group) (Fig. 5D; p = 0.03), whereas the luminal surface was not different between the groups (0.38 ± 0.05 mm<sup>2</sup> in the vehicle group and 0.36 ± 0.03 mm<sup>2</sup> C1inh group (p = 0.4)).

In the C1inh treated group thickened vein grafts displayed equal amounts of smooth muscle cells when corrected for total vessel wall area (vehicle: 18.1 ± 3.5%, C1inh: 27.1 ± 2.6%, p = 0.120; example Fig. 5F–G). Also in difference in relative foam cell contribution was seen (control: 12.6 ± 4.14%, C1inh: 10.8 ± 1.8%, p = 1.000; example Fig. 5H–I). Serum cholesterol did not differ between the vehicle- and C1inh groups (not shown). Thus, also on the long term, administration of C1inh shows a protective effect on vein grafts.

4. Discussion

Bypass graft surgery using venous grafts is one of the most used therapies in cardiovascular surgery to treat atherosclerotic occlusive disease of coronary arteries [23]. However, vein grafts frequently fail because of acute damage or through accelerated development of atherosclerosis. Here we found that C1inh treatment protected vein grafts under arterial pressure against acute endothelial loss and against atherosclerosis development.

We have shown before that perfusing human saphenous veins at arterial pressure induced acute damage characterized by the loss of virtually all endothelium and considerable ultra-structural damage in smooth muscle cells of the media [19]. shear stress as a result of over-distension of the thin-walled vein grafts under arterial pressure appears to be the main inducer of early vein graft remodeling. Indeed, in animal vein graft models peri-venous support to counteract vein distension significantly reduced neointima formation and intimal hyperplasia [24,25]. And in the perfusion model used also in the present study, peri-venous support almost completely attenuated endothelial loss [19,26]. Here we show in this model that C1inh supplementation to the perfusion blood reduced endothelial loss by approximately 50%, indicating that the mechanisms underlying shear stress-induced acute endothelial loss include inflammatory mechanisms inhibited by C1inh. An important inflammatory system inhibited by C1inh is the complement system. It is possible that C1inh protects the endothelium via this inhibitory ability as complement activation products, including C5a, have been shown to induce apoptosis in different cells and tissues, including endothelial cells [27,28]. Indeed, in our study complement depictions were found on residual endothelial cells, indicating a possible involvement of complement in vein graft endothelial cell death. On the other hand C1inh has been shown to inhibit apoptosis independent of complement in cardiomyocytes [22] and we cannot exclude C1inh exerting this effect in our perfusion model. However, we found no staining of activated caspase-3 or the apoptotic regulator Bcl-2, indicating the absence of apoptosis in the perfused veins. It has to be noticed that the loss of endothelial cells in this perfusion model was seen earlier to occur mainly within the first hour of perfusion [19], which may explain the lack of apoptosis detection after 6h of perfusion.

In the media, earlier studies using this perfusion model showed ultra-structural changes, such as vacuolization of smooth muscle cells and collagen fragmentation. Interestingly, these ultra-structural changes predominantly occurred in the circular layer of the media, the layer experiencing the highest blood pressure [19,26]. These early changes apparently do not lead to apoptosis of smooth muscle cells within 6h of perfusion, as suggested by the absence of active caspase-3. Furthermore, only small depictions of complement were found in the media of perfused veins, coinciding with a very limited non-significant increase in neutrophil infiltration, indicating limited cell death of smooth muscle cells this early after perfusion. Remarkably though, we found considerably more extensive depictions of C1inh in the media, also in veins perfused without supplemented C1inh. Interestingly, most of the C1inh deposited in the circular layer. It is therefore plausible that endogenous mechanisms play an important role in the induction of local C1inh deposition in the vessel wall. So far we do not know whether it originates from the perfusion blood or is produced locally in the media. The fact that significantly more C1inh deposits there in veins perfused with blood supplemented with C1inh suggests that at least part of it originates from the perfusion blood. Therefore, most C1inh deposits in the circular layer coinciding with most ultra-structural cellular damage [19,26] in the perfused human vein, suggesting that C1inh may protect the media. Indeed, in the vein graft model in mice we found that infusion of C1inh in the circulation significantly protected the whole vein, both intima and media, against arterial pressure induced vein graft remodeling. It has been shown before that C1inh can protect against neointimal plaque formation after arterial injury in mice [18]. Now we show similar effects of C1inh on early atherosclerotic changes in vein grafts in mice.

In conclusion, it is known that loss of vein graft endothelium leads to a high risk of vein graft failure [29] and that strategies to accelerate re-endothelialization of the graft via infusion of endothelial cells reduces early vein graft remodeling [30,31]. In this study we show that C1inh significantly reduced endothelial loss in perfused human saphenous veins and protected vein grafts against early atherosclerotic changes in mice. It may therefore be worth considering its use in CABG patients, especially since C1inh has already been used in patients for the treatment of hereditary angioedema, sepsis and myocardial infarction [32]. Herein, a therapeutic strategy of C1inh administration during surgery and intermittent in the first 3 weeks after surgery would, according to our data, protect the graft and would be practicable for the patients.

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