Imaging of arterial wall inflammation

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SUPERIOR IN VIVO COMPATIBILITY OF HYDROPHILIC POLYMER COATED PROSTHETIC VASCULAR GRAFTS
ABSTRACT

Purpose: To compare protein adsorption, cell adhesion and graft patency of hydrophilic versus hydrophobic polymer coated prosthetic vascular grafts. We hypothesize that in vivo compatibility of hydrophilic polymer coated prosthetic vascular grafts is superior to in vivo compatibility of hydrophobic grafts.

Methods: Pairwise side-to-side common carotid artery interposition grafting was performed in 8 female landrace goats (mean weight 55kg). Protein adsorption was assessed using Western Blot in 2 hydrophilic and 2 hydrophobic grafts, harvested after 3 days. Graft patency was monitored during 28 days in 6 goats with continuous wave Doppler ultrasonography. Adherence of endothelial cells, leukocytes and platelets was determined with ELISA and compared between the two graft types after 28 days.

Results: After 3 days, more ApoA-I, albumin and VEGF and less fibrin adsorbed to hydrophilic grafts. After 28 days, compared to hydrophobic grafts, higher numbers of endothelial cells were present on hydrophilic grafts (p=0.016), and less thrombocytes and leukocytes (p=0.012 and 0.024, respectively). Two out of 8 hydrophobic grafts lost patency, while none of the hydrophilic grafts failed (p=0.157).

Conclusions: Hydrophilic polymer coated vascular grafts have superior in vivo compatibility when compared to hydrophobic grafts as characterized by reduced platelet and leukocyte adherence as well as higher endothelialisation.
INTRODUCTION

Over the past decades, the need for peripheral bypass grafts as well as arteriovenous fistulas (AVFs) for hemodialysis (HD) has increased dramatically. For all interventions in vascular surgery, the use of native blood vessels instead of synthetic grafts is preferred, given that both primary and secondary patency rates of the former are superior and native grafts have also been associated with a survival advantage [1–4]. However, there is a decreased availability of high-quality native blood vessels, due to the increasing age and vascular comorbidity in the general population affecting native donor vein quality [5,6]. Additionally, auto-transplantable vessel availability may be reduced due to use of arteries and veins in previous coronary or peripheral revascularization procedures. Consequently, the use of synthetic grafts increases steadily. Despite initial high blood flow rates following placement, synthetic grafts are frequently faced with the problem of continuous flow rate decline due to neo-intimal proliferation, pro-coagulant state and, eventually, occlusion [7]. Whereas the exact pathophysiology remains complex, parts of the adverse effects have been attributed to the absence of endothelial coverage leading to pro-thrombotic and pro-inflammatory effects [7]. In an effort to overcome lacking endothelial coverage, several strategies have been employed to improve endothelialisation. The ultimate surface adaption of synthetic materials seems pre-seeding of the graft with endothelial cells prior to implantation. However, such an approach is extremely expensive and without impressive patency improvements [8], partly due to poor retention of seeded cells.

Synthetic grafts are usually made from polyethylene terephthalate (PET) or polytetrafluorethylene (PTFE), which are both hydrophobic materials [9]. Recently, we challenged the dogma that hydrophilic surfaces are protein repellent by providing evidence that a hydrophilic coating attracts many proteins and protein particles that promote human microvascular endothelial cell (HMEC-1) proliferation on biomaterial surfaces in vitro [10–12]. Among these protein particles, high-density lipoprotein (HDL) appeared to be critical for dictating this HMEC-1 proliferation [13]. HDL and its primary protein constituent ApoA-I, exert vasculoprotective functions via different molecular pathways comprising anti-inflammatory, anti-thrombotic and anti-apoptotic effects [14–16]. In addition, both ApoA-I and HDL have been shown to enhance vascular function by promoting endothelial integrity [17]. Therefore, the use of a hydrophilic copolymer coating could be a promising method to improve patency rates of synthetic vascular grafts.

In the present study, we compared a hydrophobic copolymer-coated vascular graft with a hydrophilic copolymer-coated vascular graft in a goat model of arterial interposition grafting. The primary objective of this study was to compare protein adsorption and recruitment of leukocytes, platelets and endothelial cells to the surface of the prosthetic grafts. The secondary objective was to assess patency rates.
METHODS

Production of vascular grafts

The hydrophobic copolymer coating consists of 10% 1-Vinyl-2-pyrrolidinone (NVP) and 90% n-Butyl-methacrylate (BMA); the hydrophilic coating consists of 10% BMA with 90% NVP. Details about copolymer production and prosthetic graft coating have been reported previously [18] and are provided in supplemental material. In short, a stainless steel coil is coated with either hydrophilic copolymer coating or the hydrophobic equivalent, and is encapsulated in a Tygon sleeve to suture the common carotid artery on (see figure 1).

Goat model for arterial prosthetic graft implantation

The study protocol was reviewed and approved by the Institutional Animal Care and Research Committee at the Academic Medical Center, Amsterdam, The Netherlands. The goat carotid artery interposition graft model was chosen because of the adequate length and diameter of the artery matches with the graft’s dimensions and the model has been described before [19]. Details about the graft implantation are described in the supplemental material. In eight anesthetized female Dutch landrace goats, a hydrophilic and a hydrophobic coated graft are side-to-side implanted as interposition in the common carotid artery. Perioperative heparin was added to continuous administered acetylsalicylic acid to prevent thrombosis.

Prosthetic graft patency monitoring

On day 1, 2, 3, 7, 11, 14, 18, 21, 25, and 28 post-surgery, graft patency as reflected by pulsatile flow in the common carotid artery was assessed manually by use of continuous wave Doppler ultrasound at the proximal and distal site of the prosthetic graft.

Prosthetic graft harvesting

Two randomly chosen goats were sacrificed at three days after prosthetic graft implantation. The remaining 6 goats were sacrificed at 28 days after implantation. Before sacrifice, anaesthesia was initiated similarly to anaesthetic procedures at implantation. After Doppler ultrasound, patency monitoring of the common carotid artery, the 8 cm grafted section was prepared for prosthetic graft explantation. 200IU/kg heparin was injected intravenously to prevent acute thrombosis and both prosthetic grafts were explanted after marking proximal and distal in situ position.

The grafts were excised together with 1 cm of adjacent artery on both sides in order to include the graft’s anastomosis region in analyses. Immediately after explantation, the grafts were gently flushed and rinsed using 100 mL 0.9% NaCl with 200 IU heparin added to prevent thrombosis. Patency loss was confirmed by the inability to flush obliterated grafts. Grafts were incubated for preservation in Normal Saline for a maximum of 60 minutes before further processing.
Prosthetic graft sectioning and processing

Prosthetic grafts, harvested after three and 28 days, were sectioned into five pieces using a diamond belt saw (Figure 1). After dehydration in ethanol, the harvested pieces of the first, third, and fifth regions (corresponding with the proximal, middle, and distal section) of the prosthetic graft were fixated in neutral buffered formalin (NBF) for 24 hours. Fixated grafts were then embedded stepwise in methylmethacrylate and n-butylmethacrylate (MMA/BMA) mix in a 1:1 ratio and allowed to harden for 24 hours at a vacuum at 4°C. The intended diamond knifed microtome sectioning failed due to the large metal content. Diamond saw microtome sectioning failed due to the metallic coil breaking out of the sections, taking debris of coating and cells with it. Direct staining on the embedded tissue “en bloc” was also unsuccessful due to loss of contrast in imaging the tissue. The highly important three of five regions were lost.

Figure 1. A: Schematic overview of 5 graft sections, projected on a graft photo. The graft consists of a metal coil, coated with a hydrophobic- or hydrophilic polymer coating. The coil is encased in a silicon sleeve, which is end-to-end sutured to the common carotid artery (schematically visualized in the left). The coated coil thus protrudes into the carotid artery. The first, third, and fifth sections were used for histological and morphometric analysis, the second and fourth region was used for cytological and protein analysis. B: Photograph of a graft just after implantation in the carotid artery.
for histological and morphometric analysis, including the anastomosis regions where the artery is attached to the prosthetic graft.

**Protein elution from graft surface**

The second and fourth regions of the grafts, harvested after three days, were used for western blot analysis of adsorbed proteins. Adsorbed proteins after three days were eluded from the grafts surface using an elution buffer, consisting of 50 mM tris(hydroxymethyl)methylamine (Tris), 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% sodium dodecyl sulfate SDS in H₂O. Graft pieces were incubated in elution buffer for 45 minutes at 4ºC. Debris of cells was pelleted by centrifugation the eluate at 3000rpm at room temperature for 15 minutes, the supernatant was isolated and to purify, proteins were precipitated by adding 2:1 of ice-cold acetone for 10 minutes on ice. Precipitate was pelleted by centrifugation at 20000 g at 4ºC for 10 minutes. After decanting the supernatant, the pellet was allowed to dry and re-suspended in analysis buffer, consisting of 50 mM Tris, 2 mM EDTA and 50mM natriumchloride (NaCl) in H₂O.

**Western blot protein analysis**

To confirm the presence of specific pro-endothelial proteins on the second and fourth graft sections (Figure 1) of grafts harvested after three days, and to compare the amounts between eluates of the hydrophobic and hydrophilic coating, standard western blot analysis was performed. Details about the detection of albumin, fibrinogen, vascular endothelial growth factor and apo A-I are described in the supplemental material.

**Enzyme-linked immunosorbent assay**

Second and fourth graft sections (Figure 1) of the grafts harvested after 28 days were used to determine cell deposition on the grafts luminal surface using an enzyme-linked immunosorbent assay (ELISA) resembling analysis. Endothelial cells, thrombocytes and leucocytes were traced with antibodies against von Willebrand Factor (vWF), CD61 and CD11a respectively. Subsequently, the amount of bound antibodies was detected using a concentration dependent colour changing reagent. Details about this ELISA are described in the supplemental material.

**Scanning electron microscopy analysis of graft sections**

After performing the ELISA-like experiment, second and fourth graft sections (Figure 1) of the grafts harvested after 28 days were used to visualize cell adherence to the grafts and to compare the surface of implanted grafts with non-implanted grafts. Graft sections were washed extensively with PBS after the ELISA-like experiment. The pieces of coil were dehydrated in an ethanol and incubated with hexamethyldisilazane to allow for fast air-drying without loss of structure [20]. The samples were sputter-
coated with gold under constant rotation (108 auto/SE, Cressington Sci. Instruments, Watford, UK). Micrographs were made using a Philips XL30 SEM under an appropriate sample angle (20 to 45 degrees), looking into the coil at this angle.

**Statistical analysis and sample size calculation**

A non-parametric paired comparison between thrombocytes, leucocytes and endothelium adhered to the hydrophobic and hydrophilic coating was performed using a Wilcoxon signed-rank test. Patency comparisons between the hydrophobic and hydrophilic coated graft was performed using a log-rank test. Data will be presented as mean values with standard deviation added with error-bars.

**RESULTS**

**Graft patency results**

In eight goats, vascular prostheses were successfully implanted in both common carotid arteries (CCA). In one of the two goats that were sacrificed three days after implantation of the grafts, one hydrophobic graft had lost patency. In one of the six goats that were sacrificed after 28 days, another hydrophobic graft had lost patency. During weekly intermediate Doppler ultrasound monitoring of graft patency, no loss of patency was observed. Thus, two hydrophobic coated grafts lost patency compared to none of the hydrophilic coated grafts ($p=0.157$).

**Western blot analysis on eluate from grafts harvested after three days**

After three days, different proteins adsorbed to the grafts surface, depending on the hydrophilicity (Figure 2). As usual, albumin showed multi-band patterns on western blot, while fibrinogen, VEGF and ApoA-I gave single bands. Fibrinogen was more abundant on the surface of the hydrophobic coating, as were immunoglobulin fragments. Both proteins were hardly detectable on the hydrophilic surface. Together with ApoA-1, larger amounts of albumin and VEGF were detected on the surface of the hydrophilic coated grafts after three days.

**ELISA analysis of cells on grafts explanted after 28 days**

We observed large differences in cell adsorption after 28 days on the hydrophobic and hydrophilic surfaces of the grafts (Figure 3). Higher amounts of CD11a and CD61 were detected on the surface of the hydrophobic graft ($p=0.012$ and 0.024 respectively). Concomitantly, a lower number of thrombocytes and leukocytes adhered to the surface hydrophilic coated grafts after 28 days, compared to the hydrophobic coated grafts. In line, many more vWF positive cells were detected on the hydrophilic surfaces.
Figure 2. Western blot analysis of the eluate of the hydrophobic and hydrophilic graft, harvested 3 days after implantation. Standard molecular weights are indicated on the left in kilodalton (kD).

Figure 3. Thrombocyte, leukocyte and endothelial cell amounts on hydrophobic and hydrophilic coated grafts, harvested after 28 days. Data are presented as mean arbitrary values with standard deviation (SD).

(p= 0.016), indicating more adhering endothelial cells at the hydrophilic surface than at the hydrophobic coated graft.

SEM analysis of luminal surface of explanted after 28 days
The luminal surface of the grafts explanted after 28 days contained deposition of endothelial cells, thrombus and leucocytes (figure 4). Endothelial cells were visible especially on the hydrophilic graft surface, whereas thrombus formation and leukocyte
Figure 4. Scanning Electron Micrographs (SEM) of interior surface of hydrophobic graft section (left 2 columns) and surface of hydrophobic graft section (right 2 columns). Non-implanted graft sections are visualized in the upper row. Details of grafts explanted after 28 days are shown in the lower 3 rows. Endothelial cells are especially visible on the hydrophilic graft surface, whereas on the hydrophobic surface thrombus as well as leukocyte deposition and adherence is visible. Scale bars indicate size in micro meters (µm).

deposition and adherence were visible especially on the hydrophobic graft surface. The coating on surface of the non-implanted grafts was smooth.

DISCUSSION

In the present study, we demonstrate that in a goat model of arterial interposition grafting, vascular grafts with a hydrophobic coating result in indifferent patency rate when compared to grafts with a hydrophilic coating. Indeed, in vitro, cell- and blood-compatibility were superior on the hydrophilic coating, especially after pre-incubation with human plasma. The adsorbed plasma proteins on the hydrophilic coating, which turned out to be mainly HDL and some albumin, strongly increased endothelial cell growth and thrombo-resistance [13].
At three days after graft implantation in goats, hydrophilic coated grafts contained increased concentrations of ApoA-I and VEGF, whereas the hydrophobic coated grafts contained increased amounts of fibrinogen. After 28 days, the hydrophilic grafts were characterized by an increased number of endothelial cells, with reduced amounts of leukocytes and thrombocytes as compared to the hydrophobic coated grafts. These data imply that hydrophilic coated grafts may be less prone to patency loss than hydrophobic coated grafts.

**Protein adsorption after three days**

The presence of albumin, fibrinogen, VEGF and ApoA-I, is consistent with other findings describing protein adsorption on polymers facing whole blood. Albumin and fibrinogen are frequently described as proteins adsorbing on surfaces within seconds after contact with whole blood. Typically, the type and concentrations of proteins adsorbing to the surface of a graft after contact with whole blood depends on the polymer type and its physical properties [6,21].

In our analysis, albumin is the most prominent protein that differs in concentration between the eluates of the hydrophobic and hydrophilic coating. Albumin comprises up to 70% of human plasma proteins [22]. Albumin binding is generally considered to improve the functional profile of vascular grafts since it reduces the acute inflammatory response to the prosthetic material [22–25].

In contrast, fibrinogen was present in higher amounts in the hydrophobic eluate. Fibrinogen is the third most common plasma component and appears to play a major role in the coagulatory- and associated inflammatory response. Denaturation of fibrinogen after binding to a surface leads to induction of the coagulation cascade as well as adherence of white blood cells, which correlates strongly with the degree of acute inflammatory response [26–28].

The adsorption of VEGF on the hydrophilic coated grafts might contribute to a reduction of the inflammatory response since the role of VEGF stimulating graft endothelialisation has been suggested [29–31]. Whereas VEGF increase was relatively limited in hydrophilic grafts, ApoA-I was specifically adsorbed to the hydrophilic coated grafts. This is in line with our previous in vitro studies [13]. The retention of an increased level of albumin on the hydrophilic surface is somewhat different from the in vitro results. This may be the result of interaction with full blood, or the flow rate may have influenced adsorption and desorption rates of proteins to the coating. Overall, the hydrophilic coating adsorbed higher levels of vasculo-protective and endothelialisation-stimulating proteins.

**Analysis of cells on graft after 28 days**

The higher amount of endothelial cells on the hydrophilic coated graft after 28 days seems to be crucial for the grafts long-term performance. Indeed, the endothelium
acts as a first line of defence against vascular disturbances. The antithrombotic and anti-proliferative effects of the endothelial cells abidingly prevent thrombotic occlusions and occlusions due to intima hyperplasia of prosthetic grafts [32]. In line with these endothelial effects, the lower platelet and leukocyte count seems to be a logic counterpart of the increased presence of endothelial cells. Compared to the hydrophobic coated graft, the higher endothelial cell count and lower thrombocyte and leukocyte detection can be translated into better biocompatibility with lower inflammation and thrombogenicity of the hydrophilic graft.

ApoA-I links our in vitro findings [13] with the results of our present study. ApoA-I adsorbs specifically to the surface of the hydrophilic coated graft and exerts vasculoprotective functions. By inhibiting inflammation, locally increased ApoA-I can contribute to preventing intimal hyperplasia [33–35]. By promoting endothelial function and regenerative capacity, an intact endothelial monolayer may rapidly cover the luminal surface of the graft, thus preventing activation of the coagulation cascade and subsequent thrombus formation [36]. The presence of ApoA-I or the full lipoprotein HDL on the graft surface could also prevent thrombus formation via inhibition of platelet aggregation and activation of the protein C system [37,38].

The hypothesis that hydrophilic coatings specifically attract ApoA-I that mediates a pro-endothelial effect in vivo, has never been evaluated before. Locally increased ApoA-I, delivered intramurally to prevent in-stent thrombosis, significantly inhibited luminal narrowing in the porcine stent model [39]. In mice, carotid artery re-endothelialisation after endothelial denudation was promoted by HDL [40]. In humans, systemic elevation of ApoA-I and HDL-cholesterol is associated with anti-inflammatory and anti-thrombotic effects [41]. The in vitro analysis of our hydrophilic coating demonstrated that ApoA-I adsorbs to the surface, and that the presence of ApoA-I is associated with decreased thrombus formation and increased endothelial cell adherence [13]. The result of our current in vivo analysis confirms these observations.

**Study limitations**

Due to the coil’s high metal content, we were unable to perform thorough histological and morphometric analyses. The number of compared grafts was insufficient to confirm a statistical difference in patency rate between the two graft coatings. However, by western blotting and ELISA analysis, we provided support for the hypothesis that ApoA1 adsorption is potentially associated with increased endothelialisation and improved patency of the hydrophilic grafts. The polymer coated metallic wires were useful study objects to demonstrate the performance of hydrophilic coatings and the role of plasma protein adsorption. However the polymer coated metallic coil obviously does not fulfil clinical requirements. In the near future, we will investigate the performance of hydrophilic polymer coatings on different base materials that do possess the required flexibility.
CONCLUSIONS

Our hydrophilic polymer coated prosthetic vascular graft showed superior in vivo compatibility in goats when compared to hydrophobic polymer coated grafts. Most likely, the adsorption of endothelial-protective proteins such as ApoA-I and VEGF has facilitated the enhanced endothelialisation of the hydrophilic coating, which may promote improved patency rates in vivo. These data call for further research in humans to evaluate in vivo behaviour of hydrophilic graft coatings.

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