Novel Strategies to Improve the Patency of Vascular Prostheses

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Nieuwe strategieën om de duurzaamheid van vaatprothesen te verbeteren
Met een samenvatting in het Nederlands

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht, op gezag van de rector magnificus, prof. dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 29 september 2006 des middags om 12.45 uur

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Aan mijn twee gezinnen.
Het ene waarin ik ben opgegroeid, het andere waarin ik verder groei.

Contents

Chapter 1 Introduction and Objectives

Almost a third of patients that need peripheral arterial reconstructive operations do not have suitable autologous veins available for grafting [1]. For that reason, prosthetic grafts, such as polytetrafluoroethylene (PTFE) grafts, are frequently used in arterial bypass procedures. The patency of autologous veins however, is better when compared to prosthetic grafts. In a recent review comparing venous and PTFE above-knee femoropopliteal bypasses, 5-year primary patency rates of 74% and 39% respectively, were reported [2].
The absence of a functional endothelial monolayer on the prosthetic grafts is an important stimulus for intimal hyperplasia (IH). To improve the outcome of synthetic vascular bypass surgery, cell seeding is a promising concept that has extensively been investigated and is still evolving [3-8]. Alternative techniques to reduce the thrombogenicity of prosthetic grafts might also improve the outcome of synthetic vascular bypass surgery.
Until now results of cell seeding in humans have been disappointing, either due to IH, thrombus formation and/or the laborious techniques necessary to realize endothelialization of synthetic grafts. In search for less laborious techniques to improve the patency of vascular prostheses, the studies described in this thesis are performed. The emphases of the studies are: 'auto-endothelialization' and 'thrombogenicity-reduction' of the PTFE graft.

The central questions of this thesis are:
1. Can 'auto-endothelialization' of grafts be realised by precoating of these grafts with anti-CD34 antibodies?
2. Does this coating reduce intimal hyperplasia?

And
3. Does heparin immobilization reduce thrombogenicity of vascular grafts?
4. Is there a systemic effect of the locally immobilized heparin on the vascular grafts?

To answer these questions *in vitro*, *ex vivo* and *in vivo* studies are performed, which are described in this thesis.
In **Chapter 2** a review describes the evaluation of the concept of cell seeding and other tissue engineering concepts to improve the outcome of non venous vascular bypass surgery. The hypothesis of cell seeding, where different types of cells are described, is explained in detail. A truthful description of cell seeding using different types and sources of cells is given. The most promising cell type for seeding turned out to be the endothelial cell (EC). EC must be harvested before it can be used to cover the prosthetic endoluminal surface. An endothelialized endoluminal surface is – in theory- comparable to veins, as native ECs cover the inner surface of veins.

The drawbacks of the different cell types and techniques used for endothelialization are also described in this chapter.
Because of the drawback of the different techniques described in chapter 2, a new concept of 'auto-endothelialization', is described in **chapter 3,** which circumvents the laborious harvesting techniques to endothelialize prosthetic grafts. Joris Rotmans et al. developed a porcine model for graft failure [9]. This setup allows evaluation of techniques –like auto-endothelialization- in a rapid *in vivo* model focussing on intimal hyperplasia (IH). IH is one of the reasons why vascular grafts fail, due to a progressive stenosis at the anastomosis [10].
Endothelial progenitor cells (EPCs) are a subset of CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells. In recent animal models, *in vitro* seeding of prosthetic vascular grafts using CD34+ progenitor cells increased graft endothelialization [11;12]. We hypothesized that *in vivo* auto-seeding using immobilized anti-CD34 antibodies establishes a confluent mature endothelial cell monolayer which may attenuate IH formation at the AV anastomosis.
Chapter 4 describes the concept of auto-endothelialization with EPCs using immobilized anti-CD34 antibodies in an *in vitro* setup using human blood. To study the adhered cells in detail, both glass coverslips and pieces of expanded PTFE (ePTFE) coated with anti CD34 antibody are used. This gives us the opportunity to study the adhered cells not only by scanning electronic microscopy on the ePTFE graft, but also by techniques like standard light microscopy and immunofluorescent staining on the glass coverslips. The study was also designed to find a mechanistic explanation for the adverse effect of the coating and/or the adhered cells on the induction of IH as was demonstrated in our *in vivo* study.

In **chapter 5**, we give an overview of the up to date techniques concerning graft cell seeding with emphasis on its newest era: seeding with endothelial progenitor cells.

Prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade. Heparin is a potent anticoagulant drug widely used to prevent and treat thrombosis. A new ePTFE graft with long-term covalent bonding of heparin is now commercially available in several European countries, but a basic analysis of its mechanism of action in humans has never been described. In **chapter 6** we describe a study which evaluates the thrombogenicity of heparin-bonded ePTFE grafts compared to standard ePTFE in a newly developed human *ex vivo* model.
Chapter 7 describes the results of a human *in vivo* study using heparinized ePTFE grafts in comparison with standard ePTFE grafts for patients undergoing femoro-popliteal bypass grafting. This human *in vivo* study

examines systemic effects of the endoluminal heparin, and addresses whether graft implantation results in:

1) A measurable reduction of systemic markers of haemostasis activation as compared to control grafts?
2) Antibody formation against heparin, potentially responsible for HIT (= heparin-induced thrombocytopenia)

A summary and general discussion is described in **chapter 8**.

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Chapter 2 Improving Small-Diameter Vascular Grafts: From the Application of an Endothelial Cell Lining to the Construction of a Tissue-Engineered Blood Vessel

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JMM Heyligers, CHP Arts, HJM Verhagen, PhG de Groot, FL Moll

One of the main reasons why vascular reconstruction with synthetic small diameter grafts has limited success is the absence of endothelial cells. To improve the outcome of non venous vascular bypass surgery, cell seeding of vascular grafts and other tissue engineering techniques were developed. In this article we give an overview of the artificial blood vessel as an alternative for venous vascular bypass surgery.

Introduction

Vascular surgery was practiced long before synthetic grafts were available, but only by a limited number of surgeons, and their clinical results was seldom evaluated. Nevertheless, the early vascular surgeries inspired the search for better techniques and materials. In 1912, Carrel was the first to describe the use of glass or metal tubes to bypass arterial defects in dogs [1]. In the early fifties Vinyon N was introduced as the first synthetic graft to replace arteries [2;3]. Different types of 'ethylene' were introduced as materials to replace human arterial vessels in the following years. Outstanding results were achieved in large vessel grafting. However, it became apparent that they were poorly suited for small-caliber vessel grafting. The large difference in patency between prosthetic and autologous vein grafts might partially be attributed to the presence of viable endothelial cells on the luminal surface of autologous veins [4]. In a recent review article the primary patency rates after two years of venous and PTFE above-knee femoropopliteal bypasses were 80 and 69%, respectively, and after 5 years they were 74 and 39% [5].

Endothelial cells were once thought to be a monolayer of passive cells lining the vasculature. We now know that endothelial cells inhibit thrombosis and intimal hyperplasia actively, and that they serve as an anticoagulant surface [6;7]. Endothelial cells are therefore interesting as coverage of prosthetic grafts. The original idea behind endothelial cell (EC) seeding was to improve the patency of small diameter vascular grafts by establishing a functional biological lining on the luminal surface.

In this article we describe an overview of the development of an artificial blood vessel, from the application of an (endothelial) cell lining, to the construction of a tissue engineered blood vessel. In the first part, the unique properties of EC are depicted. In the second part, the evolution of EC seeding is described. And in the third part up to date techniques to line blood vessels *in vivo* and the development of complete blood vessels by tissue engineering (TE) are given.

Endothelial cells: physiology in platelet aggregation, coagulation and their protective function in intimal hyperplasia

Platelet aggregation

When vessel wall damage occurs, proteins from the subendothelial matrix (von Willebrand Factor, collagen and fibronectin) are exposed [8;9] and proteins from the circulation (particularly fibrinogen) are absorbed to the subendothelial matrix. Platelets adhere to the exposed proteins present in this matrix and become activated [10].

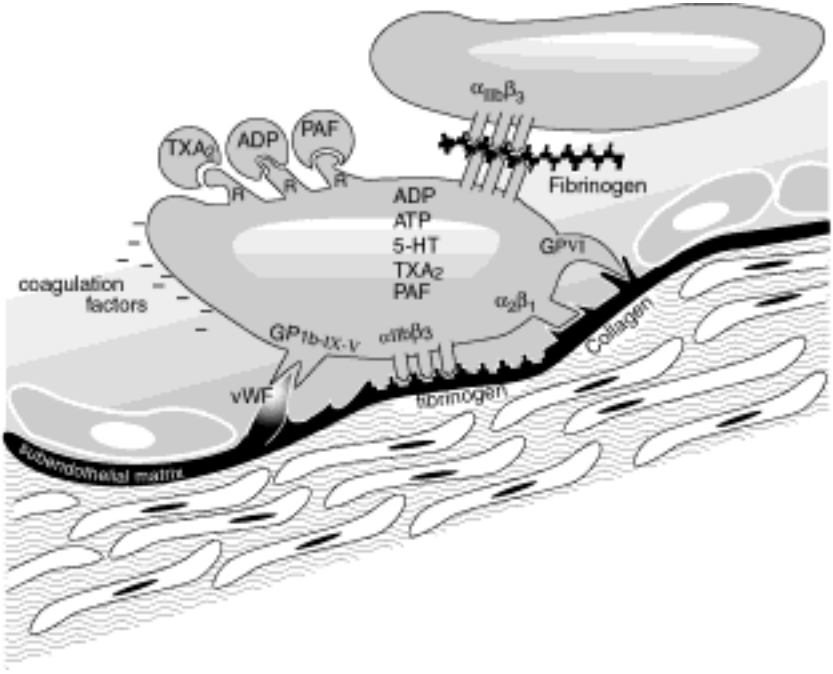

Figure 1: platelet adhesion, aggregation and activation
ADP, adenosine diphosphate; ATP, adenosine triphosphate; 5-HT,5-hydroxytryptamine; PAF, platelet activating factor; TXA2, thromboxane A2; vWF, van Willebrand factor; GP, glycoprotein.

Under normal circumstances EC have an antithrombogenic function. First, the surface characteristics of EC formed by glycocalyx prevent platelets to adhere [11]. Second, nitric oxide (through cyclic guanosine monophosphate) and prostacyclin (through cyclic adenosine monophosphate) production, inhibit platelet adhesion and aggregation and cause blood vessel dilatation. Third the EC surface ectonucleotidases metabolize ADP to prevent platelet recruitment [10;12;13].

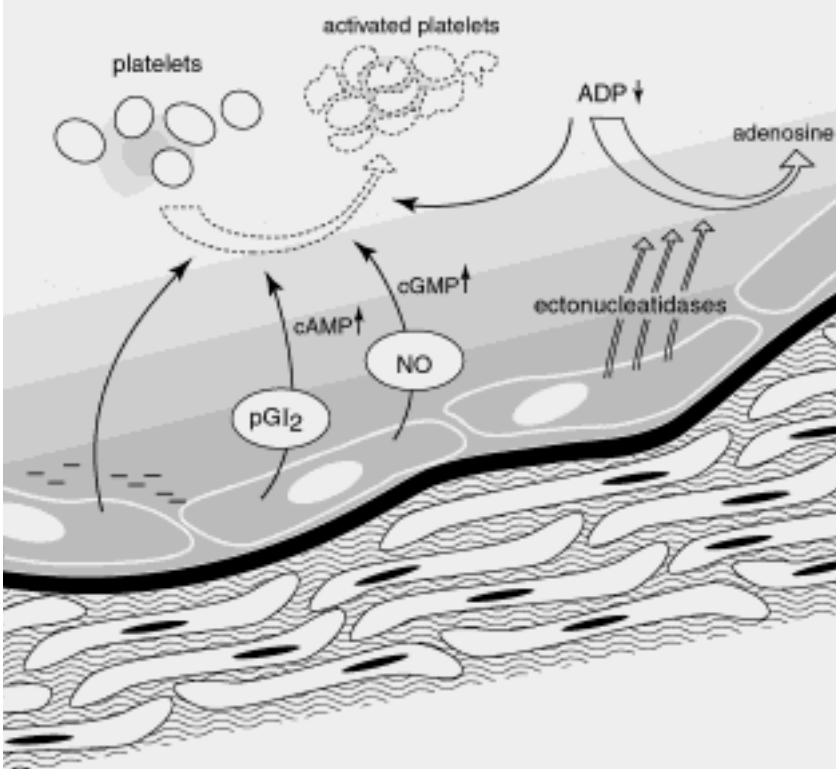

Figure 2: endothelial cell inhibition of platelet activation.
ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic 3',5'-guanosine-monophosphate; pGI, prostacyclin.

Coagulation
Situated between the blood and the vascular wall, endothelium serves as an anticoagulant surface [7]. *In vivo*, the initiation of coagulation in response to trauma occurs via the exposure of tissue factor TF (the extrinsic pathway) to blood [14;15]. Clot formation occurs when fibrinogen is converted into fibrin by thrombin. EC have binding sites for different coagulation factors [16]. An intact layer EC does not express TF [17]. Tissue factor pathway inhibitor is an important inhibitor of coagulation produced by EC. Moreover, EC expresses thrombomodulin, a protein that converts thrombin from a thrombotic into an anti-thrombotic protein.

Protective function in intimal hyperplasia
The initial events of intimal hyperplasia, smooth muscle cell proliferation in the adventiatia and media following migration into the intima, are triggered by two different growth factors, basic fibroblast growth factor

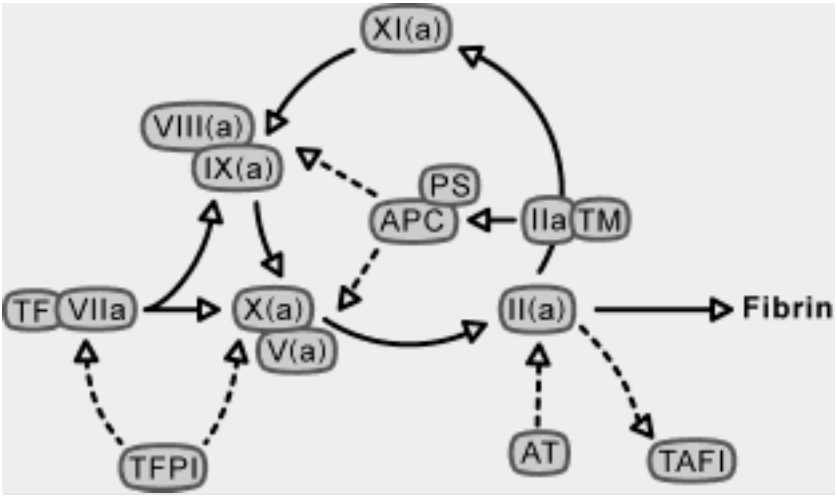

Figure 3: coagulation cascade.
XIa, factor XIa; IXa, factor IXa; VIIIa, factor VIIIa; TF, tissue factor; VIIa, factor VIIa; Xa, factor Xa; TFPI, tissue factor pathway inhibitor; AT, antithrombin; TAFI, thrombin activatable fibrinolysis inhibitor; APC, activated protein C; PS, protein S; TM, thrombomodulin.

(bFGF) and platelet derived growth factor (PDGF). Uninjured endothelium has a protective function with respect to intimal hyperplasia [18]. Cells form a barrier to the influx of growth factors and release inhibitors of smooth muscle cell proliferation [19].

Endothelial cell seeding

Mansfield and co-workers started EC seeding in 1975 [20]. They used granulation tissue from the bed of a pedicled skin flap as a cell source to seed a mixture of EC, fibroblasts and macrophages onto patches of polyester. In the early seventies, it was possible to isolate and culture EC and progress was made [21]. Since then, different sources and types of cells, isolation methods and graft materials have been developed and used.

Venous endothelial cells
In 1978, Herring at al. reported the first successful seeding of prosthetic grafts in dogs.[22] Mechanical isolated venous EC were mixed with blood. This mixture was used to preclot Polyester grafts, implanted as infrarenal abdominal aorta interposition grafts. After 2-8 weeks, the thrombus-free surface area was found to be significantly smaller in the seeded than in the nonseeded grafts.

Subsequently, many additional studies with dogs have been performed [23-32]. EC were no longer harvested mechanically but enzymatically with a solution of trypsin and collagenase, first from everted vessels and later from clamped cannulated vessels. It was soon shown that not only preclotting of polyester grafts with a mixture of EC and blood, but also seeding of EC onto the luminal surface of PTFE resulted in surface endothelialization in dogs. A condition for successful seeding was a precoating of the graft with a protein matrix [24;26;29].

A compilation of the results of EC seeding in animal trials using 4mm inner diameter polymer grafts showed that although not all animal experiments provided statistical significant results, cell seeding was able to accelerate the formation of an EC lining on the luminal surface of polymeric grafts and to complete luminal healing within 4-6 weeks [33].

The first human study was reported by Herring et al. in 1984 [34]. Isolated venous cells were seeded onto preclotted polyester prosthetic grafts. Only in non-smokers seeded femoropopliteal grafts performed better than nonseeded grafts. A randomized trial of EC seeding versus no seeding was initiated, using 6mm ePTFE grafts into the femoropopliteal position [35]. After 1 and 2 years, the patency rates of the seeded grafts were significantly better compared to the nonseeded grafts. In a multi-center study comparing autologous vein grafts and endothelial cell seeded PTFE grafts, the patency after 30 months was better for vein grafts. PTFE grafts failed due to anastomotic hyperplasia [36].

Orthenwall et al. seeded one limb of a preclotted polyester aortobifemoral graft as an intra-patient comparative study. Radiolabelled platelet uptake after 12 months was significantly lower in the seeded limb.[37] Similar experiments using PTFE femoropopliteal grafts showed a lower platelet deposition in the seeded grafts at 1 and 6 months after implantation [38]. Zilla et al. found no significant difference between EC-seeded and nonseeded ePTFE femoropopliteal grafts [39].

Reason for failure of most of the human studies with vein derived EC seeded grafts, was the amount of seeded cells that might have been too small since a high instantaneous cell loss of freshly seeded EC could have occurred [40]. Furthermore, the growth potential of human EC could be lower than that of canine EC [41].

Experiments with cultured adult EC began in the 80s after the discovery of two substances that stimulate EC growth: endothelial cell growth factor (ECGF) derived from the bovine pituary gland [42] and heparin as a necessary cofactor for heparin binding growth factor [43].

Leseche et al. implanted ePTFE grafts as femoropopliteal bypasses with cultured EC [44]. The two-stage technique proved to be feasible in 69% of the patients that did not require emergency reconstruction.

In addition to culturing EC before seeding, *in vitro* culturing of cells

already seeded on the prosthetic graft was introduced. In this way, the amount of cells was increased and, at the same time, maturation of the cytoskeleton, deposition of extracellular matrix [45] and resistance of seeded cells to shear stress was achieved [28]. Both a canine [46] and a primate model [47] showed that *in vitro* lined endothelium on fibrin glue coated ePTFE grafts was persistent and of low thrombogenicity.

Between 1989 and 1991 a randomized clinical trial was performed with seeded and nonseeded ePTFE grafts being used for femoropopliteal bypasses. Seeded grafts were cultured *in vitro* for at least 9 days. The total period between EC isolation and graft implantation was 37 days. After 32 months the patency was 85% for the seeded and 55% for the nonseeded graft [48]. The persistence of a confluent endothelium was proven from two specimens [49;50]. In 1999 the 9-year follow-up of the initial randomized study by Zilla et al. and the 5-year follow-up of the routine clinical endothelialization program were reported [51]. The patency rates were significantly higher for the seeded grafts (65%) than for the nonseeded control group (16%). The 5-year patency rate for all *in vitro* endothelialized infrainguinal reconstructions was 68% (66% for above the knee and 76% for below the knee grafts) and comparable to the rates of reversed saphenous vein grafts (68% after 5 years)[52].

This group continued their work and reported in 2001 that their data provided strong evidence that autologous endothelial cell lining distinctly improved the patency of small diameter vascular grafts. They based their conclusion on the implantation of 153 endothelialized ePTFE in the infrainguinal position in 136 patients [53].

Magnometschnigg et al. performed a non randomized study. *In vitro* seeded ePTFE grafts were used for crural repeat reconstructions. A 30 days patency of seeded grafts of 92% and of nonseeded grafts of 53% was found [54].

Swedenborg et al. evaluated in a pilot-study the feasibility of *in vitro* endothelialized PTFE grafts, used as interposition arteriovenous (AV) fistulas in uraemic patients. In this highly problematic patient group, results were promising with EC coverage after 5 weeks of implantation [55].

Despite strong evidence that *in vitro* EC lining improves the patency of small-diameter vascular prostheses, a major shortcoming of all studies is its labor intensity. It takes a 4- to 5-week delay between cell harvest and graft implantation, growth- and infection problems can occur and the costs of cell culture are substantial especially when performed under 'good manufacturing practice' (GMP) [48;56]. Furthermore, the seeded cells have been cultured under all sorts of growth factors, inducing the risk of unwanted growth after implantation. To avoid these problems, a search for other cell types and for other sources of EC has been started.

Mesothelial cells

In search for an alternative for endothelial cells, mesothelial cells were studied, since they are abundantly available in the omentum, they can be cultured in large amounts in only a few days, and they have many properties in common with endothelial cells. In 1984, Nicholson et al. were the first to discover that pieces of mesothelium exposed to *in vitro* blood flow, are non-thrombogenic.[57] They implanted polyester grafts seeded with mesothelial cells (MC) in dogs. One month later, a confluent layer of MC without platelets was found.[58] After the discovery that MC secreted high amounts of tPA,[59] additional dog experiments with MC-seeded polyester prostheses in the femoral position were performed [60]. No improvement in thrombus-free area nor luminal cell cover was found. An analysis of the results suggested the amount of seeded cells was too low and the time for the cells to attach to the graft too short.

Pronk et al. and Verhagen et al. focused on the use of MC as an alternative for seeding EC. A method was developed to isolate enough pure cells from omental fat [61]. The amount of cells which remained attached to the prosthesis was large enough and the cells appeared to have the same flow-resistant properties *in vitro* as EC [62]. Unfortunately, the short period of culturing, necessary to enlarge the yield, increased the expression of TF on the cells [63]. Changing the culture conditions reduced TF expression with 90%, to the same level as that of EC [64]. Furthermore, MC express a large amount of the antithrombogenic thrombomodulin on their surface, even more than EC [65]. However, *in vivo* experiments in dogs did not show any antithrombogenic or profibrinolytic properties of MC-coated grafts nor absence of intimal hyperplasia [66].

Microvascular endothelial cells

In 1986, Jarrell and Williams applied a modification of a technique to harvest MVEC from fat tissue previously developed by Wagner [67] to synthetic graft seeding [68]. The theoretical advantage of MVEC over EC is that high amounts of MVEC can be obtained easily. Also, MVEC can be used directly without a culture period.

Baitella et al. concluded that omental fat-derived MVEC seeded polyester prostheses were able to generate a vascular wall resembling the wall of a normal artery [69]. Pasic et al noticed an improvement of the patency of small diameter polyester grafts in 30 dogs after 26 weeks, with a one-stage EC-seeding procedure with omental fat [70]. A mechanical method for the isolation of EC provided comparable results to the manual method.

A major improvement in the isolation of MVEC was the use of liposuction-derived subcutaneous fat as a source of MVEC [71]. These fat-derived cells were characterized to be 85% endothelium prior to tissue digestion, and pure endothelium following digestion and separation of adipocytes [72].

MVEC have been used successfully to endothelialize prosthetic grafts in dogs. Since dogs do not have subcutaneous fat, falciform ligament fat was used. Four millimeter diameter ePTFE grafts were implanted as carotid interpositions. After 12 weeks of implantation all control grafts were occluded while 86% of the cell-sodded grafts remained patent. Statistical evaluation of the data revealed a significant improvement in patency of cell sodded grafts. A lining with characteristics of EC was present. But underneath this lining a distinct multilayer of non-EC was found [73]. Synthetic Arterial Venous (AV) shunts were also sodded with MVEC, and these also showed an intact EC lining, but also a non EC layer underneath it [74].

Hedeman Joosten et al. showed that MVEC keep their antithrombogenic properties *in vitro*, also after seeding onto prosthetic graft material [75].

Seeded MVEC's showed to be resistant against physiological shear forces and showed to express little prothrombogenic activity after perfusion with non-anticoagulated blood [76]. Seeded grafts, implanted as carotid interpositions in dogs, showed a confluent EC layer above a multilayer of myofibroblasts, without fibrin and platelet deposition. Nonseeded grafts, on the other hand, were covered with platelets and fibrin.

Despite successful patency rates obtained in animal experiments, human studies have been sparsely performed and the results were disappointing. In a case-report, a seeded mesoatrial polyester graft was implanted [77]. Histology of the midportion, obtained after 9 months because of a mechanical stricture, showed a substantial subendothelial layer. Grossly, the luminal surface of the resected graft was free of thrombus, with a smooth, glistening, white surface. Light microscopy demonstrated a surface layer of cells morphologically consistent with an endothelial cell monolayer, a subendothelial layer composed of extracellular matrix and spindle-shaped cells, and granulation tissue around the Dacron fabric. Immunohistochemistry and electron microscopy confirmed the presence of vascular endothelium on the luminal surface.

Another group used PTFE grafts seeded with subcutaneous fat derived MVEC for peripheral vascular reconstruction in 34 patients [78]. The cumulative patency at 36 months was 19%, similar to other studies using nonseeded grafts. Williams published the results of an initial safety study with 11 patients with significant vascular disease who received MVEC sodded ePTFE bypasses in the ilio- or femoro-peroneal or tibial region [79]. Of the original 11 patients, seven were available for follow up at four years, while four expired prior to this time due to nongraft related causes. The cumulative patency at 4 years was 55%.

The results of human studies with fat derived MVEC have been less convincing than those of animal studies with fat derived MVEC, and of human studies with cultured vein derived EC. Two causes have been suggested. First, the formation of a confluent endothelium might be delayed in a sin-

gle-stage procedure. Second, a high percentage of contaminating non-endothelial cells might be present [80;81]. Although a purity of 100% has been claimed for fat derived EC after isolation [71], this purity was not always obtained by other groups [82].
Arts et al. pointed out that contaminants from the transplant contribute to intimal hyperplasia [83]. They developed a novel method to isolate pure MVEC from subcutaneous fat tissue, which is ideal for direct cell seeding [84]. It was also concluded that the reduction of non-endothelial cell contamination of MVEC seeded grafts decreases thrombogenicity and might prevent intimal hyperplasia [85].

Endothelial Progenitor Cells as an alternative source of cells
In 1997 the isolation of putative progenitor endothelial cells for angiogenesis was described [86]. The authors were the first to isolate a subtype of circulating, bone-marrow-derived $CD34^+$ cells, now known as endothelial progenitor cells (EPCs). Peripheral blood of adults contains these cells. They have the potential to proliferate and differentiate into mature endothelial cells. As they circulate in peripheral blood, these cells can be isolated easily.
EPCs have been shown to participate in neovascularization of ischemic limbs [87]. EPCs also contribute to reendothelialization of prosthetic grafts in animals [88]. EPC can be mobilized from bone marrow using angiogenic cytokines (e.g. vascular endothelial growth factor (VEGF) [87]. In a recent publication Griese et al. describe the transplantation of EPCs to play a crucial role in re-establishing endothelial integrity in injured vessels, thereby inhibiting neointimal hyperplasia. They also show that EPCs have the ability to endothelialize vascular bioprosthesis. After sodding grafts with EPC, 40-60% of the graft was covered with EC after 4 weeks *in vivo* [89].
The advantage of using EPC is that they can be obtained by venopuncture rather than by an operation. A disadvantage is that *ex vivo* expansion of EPC takes on average two weeks. The drawbacks of *in vitro* culture are the same as mentioned before for mature EC. This includes the risk of infection, change of phenotype, the need for a GMP culture facility, and limitations for emergency situations [48;56;90].

Tissue engineering
Other advances have been made in the field of tissue engineering. In fact, EC seeding conceptually can be considered a form of tissue engineering, although it does not form a blood vessel with all its components. Recently, the first studies have been published about completely engineered blood vessels. Three models have been investigated [91].
The collagen based model consists of an adventitia-like layer of fibroblasts, a media-like layer of smooth muscle cells, and a monolayer of EC

[92]. However, Dacron sleeves are required to withstand physiological pressures. In the self-assembly model, cells are cultured to form a continuous sheet of cells and extracellular matrix and then rolled over a permeable solid bar. Later, the EC can be seeded [93]. These constructs exhibit adequate mechanical strength, but the long time to culture these cells is a considerable limitation. In the third model, a polymeric biodegradable scaffold is seeded with cells and matured using dynamic culture. This allows cells to proliferate and produce an organized extracellular matrix as the scaffold degrades [94]. After the maturing process, EC are seeded. This model remains patent up to 24 days in Yucatan miniature swine. Also, for this third type of construct several issues must still be addressed. Eight weeks of *in vitro* development are required to exhibit the desired characteristics. This caries all of the disadvantages previously mentioned. From current reports, it is not clear whether such a construct is able to support a stable EC layer under flow conditions. And the presence of residual polymer might cause an inflammatory response. So far only short term studies have been performed [95]. A human study has been done in a child [96].

The advantage of a tissue engineered vessel over a cell seeded prosthetic graft is its ability to dilate and constrict. In fact, the behavior of a normal vessel can also be expected from a seeded denuded vessel after PTA and endarterectomy. But, a disadvantage of seeding a denuded vessel is that the EC layer must be applied *in vivo*, so that temporary occlusion of the vessel is necessary.

At this moment, we do not know whether intimal hyperplasia develops in tissue engineered vessels. This might be expected since a combination of smooth muscle cells (SMC), (myo)fibroblasts, and EC are used. In fact this combination of cells is comparable to nonpurified fat derived MVEC, which have been shown to induce seeding related intimal hyperplasia. Maybe, intimal hyperplasia will not develop in tissue engineered vessels since cells are layered instead of mixed.

Conclusions

Endothelial cell seeding to achieve a natural layer inside a prosthetic graft to improve the outcome of small grafts has a greater than 25 year history. The techniques of stem cell seeding and tissue engineering have developed only recently.

EC seeding has proven its beneficial effects on the outcome of patency of vascular bypass surgery for small caliber grafts. The disadvantage is that the technique can not be used in the emergency setting, as EC need to be expanded *ex vivo*. Mesothelial cells as an alternative are not suitable as they did not show any antithrombogenic or profibrinolytic properties in *in vivo*

experiments. MVEC might be a suitable alternative as long as contaminating cells can be removed from the transplant.
Although the use of stem cells for tissue regeneration is promising, the use of stem cells as an alternative for seeding adult EC is not yet possible. Currently, the major drawback is the necessity of *ex vivo* expansion and differentiation of EPC into mature EC.
The first tissue engineered blood vessels have been constructed using EC, SMC and fibroblasts obtained from veins after expansion. In the future, it may be possible that all components of tissue engineered vessels can obtained from fat tissue instead of peripheral veins. This would make expansion of the individual cell types, harvested from peripheral veins, unnecessary. Still, maturation of the total vessel under dynamic circumstances will be needed to obtain the necessary burst strength.

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Chapter 3 In vivo Cell Seeding Using anti-CD34 Antibodies Successfully Accelerates Endothelialization but Stimulates Intimal Hyperplasia in Porcine Arteriovenous ePTFE-grafts

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Abstract

Background. The patency of arteriovenous expanded polytetrafluoroethylene (ePTFE) grafts for hemodialysis is impaired by intimal hyperplasia (IH) at the venous outflow tract. The absence of a functional endothelial monolayer on the prosthetic grafts is an important stimulus for IH. In the present study we evaluated the feasibility of capturing endothelial progenitor cells (EPCs) *in vivo* using anti-CD34 antibodies on ePTFE-grafts to inhibit IH in porcine arteriovenous ePTFE-grafts.
Methods and results. In 11 pigs, anti-CD34-coated ePTFE-grafts were implanted between the carotid artery and the internal jugular vein. Bare ePTFE-grafts were implanted at the contralateral side. After 3 days (n=2) or 28 days (n=9), pigs were sacrificed and AV-grafts were excised for histological analysis and scanning electron microscopy. At 3 and 28 days after implantation, 95% and 85% of the coated graft surface was covered by endothelial cells. In contrast, no cell coverage was observed in the bare graft at 3 days while at 28 days, bare grafts were partly covered with endothelial cells (32%, p= 0.04). Twenty-eight days after implantation, IH at the venous anastomosis was strongly increased in anti-CD34-coated grafts (5.96±1.9 mm^2) compared to bare grafts (1.70±0.4 mm^2, p = 0.03). This increase in IH coincided with enhanced cellular proliferation at the venous anastomosis.
Conclusions. Autoseeding using anti-CD34 antibodies results in rapid endothelialization within 72 hours. Despite persistent endothelial graft coverage, IH at the outflow tract is increased profoundly at 4 weeks after implantation. Further modifications are required to stimulate the protective effects of trapped endothelial cells.

Condensed abstract

The patency of AV-grafts for hemodialysis is impaired by intimal hyperplasia (IH) at the venous outflow tract. We studied if IH could be prevented by *in vivo* EPCs capturing using anti-CD34 antibodies on ePTFE grafts. Already at 3 days after AV-graft implantation, 95% of the coated-graft surface was covered by endothelial cells. At 28 days, IH at the venous anastomosis was 3-fold increased in the coated grafts. Autoseeding using anti-CD34 antibodies seems a highly effective strategy to enhance graft endothelialization in AV-grafts. Further modifications are required to stimulate the protective effects of trapped endothelial cells.

Introduction

The patency of arteriovenous (AV) expanded polytetrafluoroethylene (ePTFE) grafts for hemodialysis is severely compromised by intimal hyperplasia (IH) at the venous outflow tract, ultimately leading to graft thrombosis [1]. The clinical relevance of AV-graft failure is illustrated by the limited 1- and 2-year primary patency rates of 50% and 25%, respectively [1;2]. To date, there is no effective intervention available to improve graft patency. Successful development of new strategies requires closer insight into the pathogenesis of IH formation. The latter is thought to reflect a cumulation of several separate entities, comprising inflammatory, coagulatory and hemodynamic factors [3-5]. A crucial factor in both activation of coagulation and inflammation is the lack of a functional endothelial monolayer on the prosthetic graft, since the endothelium constitutes the first line homeostatic defense mechanism by exerting anti-coagulatory and anti-inflammatory effects [6-8]. Consequently, endothelial cell (EC) seeding at the luminal surface of prosthetic vascular grafts is a valuable strategy to improve graft patency [9]. Indeed, graft seeding with autologous ECs has been shown to increase patency rates of prosthetic bypass grafts in clinical trials [10]. However, implementation of *in vitro* EC seeding is hampered by the laborious procedures for harvesting, expansion and application of ECs obtained from autologous veins or adipose tissue.

Bone-marrow derived endothelial progenitor cells (EPCs) have emerged as a promising source of autologous ECs. EPCs are a subset of CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells [11]. Recent studies have emphasized that circulating EPCs have the capacity to home to sites of vascular injury, thus promoting the process of reendothelialization [12;13]. Moreover, *in vitro* seeding of prosthetic vascular grafts using CD34± cells markedly increased graft endothelialization in animal models [14;15].

In the present study we evaluated the feasibility of capturing EPCs *in vivo* using immobilized anti-CD34 antibodies on expanded polytetrafluoroethylene (ePTFE) grafts. If proven valid, this novel technique circumvents the laborious *in vitro* procedures mandatory for handling ECs. We hypothesized that *in vivo* 'autoseeding' using immobilized anti-CD34 antibodies establishes a confluent mature endothelial cell monolayer which may attenuate IH formation in the outflow tract of porcine arteriovenous ePTFE-grafts.

Materials and methods

Study design

Eleven female Landrace pigs weighing 50.8 kg ± 0.7 kg were used. In each pig, an anti-CD34 coated ePTFE graft was implanted between the carotid artery and the internal jugular vein at a randomly determined side. The same commercially available bare ePTFE-graft was implanted at the contralateral side, which served as an internal control. To study graft coverage, 2 pigs were terminated 72 hours after surgery. The remaining 9 pigs were sacrificed at 28 days of follow-up to evaluate cell retention on the graft and intimal hyperplasia in the venous outflow tract. The study protocol was approved by the Ethical Committee on Animal Experimentation of the University Medical Center Utrecht and animal care and conforms to established guidelines [16].

Graft coating

Anti-human CD34 monoclonal antibodies (IgG2a, epitope class III) were immobilized to the ePTFE graft material (Orbus Medical Technologies, Fort Launderdale, FL) using a proprietary, multi-step process (Ssens, Hengelo, the Netherlands). The first step involves functionalization of the surface. To this functionalized surface, a biopolymer is covalently coupled in a reaction that leaves immobilized polymer without adding any new species to the graft or the polymer. Next, the anti-human CD34 monoclonal antibody is covalently coupled to the polymer-coated graft, through stable peptide linkages. The anti-CD34 immobilized ePTFE grafts have been shown to exhibit cross-reactivity in porcine graft explants, which were observed to have a rich population of EC marker positive cells after only four hours [17].

Anesthesia

Before operation and termination, the animals were fasted overnight and pre-medicated with intramuscular ketamine hydrochloride 10 mg/kg, midazolam 0.4 mg/kg, atropine 0.5 mg and intravenous thiopental sodium 4 mg/kg. They were then intubated and ventilated with a mixture of O_2 and air (1:2). An ear vein was used for continuous administration of 0.3 mg/kg/h midazolam, 2.5 µg/kg/h sufentanil and 50 µg/kg/h pancuronium.

AV-graft implantation

Starting six days before the operation the pigs received acetylsalicylic acid 80 mg/dd. Clopidogrel 225 mg was given one day prior to operation and continued at a dose of 75 mg/day until termination. The ePTFE AV-grafts were created bilaterally between the carotid artery and the internal jugular

vein as described previously [18]. In short, heparin 5000 IU i.v. was administered prior to manipulation of the vessels. After dissection of the common carotid artery and the internal jugular vein, papaverin 5 mg/ml was applied locally to prevent vascular spasm. Next, the carotid artery was clamped and a standardized 8-mm arteriotomy was performed. An end-to-side anastomosis was created at an angle of 45 degrees by use of a continuous suture of 8-0 polypropylene (Ethicon, Somerville, NJ, US). All ringed ePTFE-grafts were 5 mm in diameter and 7 cm in length. The venous anastomosis was created in a similar fashion.

Tissue preparation and histological analysis

After 72 hours (2 pigs) or 4 weeks (9 pigs) of follow-up, pigs were anesthetized as described previously. Heparin 10000 IU was administered prior to manipulation of the vessels. Next, the carotid artery was cannulated whereupon the grafts and adjacent vessels were perfused with saline for 3 minutes. Subsequently, the pigs were sacrificed and the grafts and adjacent vessels were excised. The ePTFE grafts were then cut in 2 pieces: (1) the first 2 cm from the arterial anastomosis was fixated in 2% glutaraldehyde for scanning electron microscopy (SEM); (2) the remainder of the ePTFE graft and the adjacent jugular vein were immersed in formalin for histological analysis. After 24 hours of formalin fixation, the graft and jugular vein were cut in 5 mm blocks and embedded in paraffin. Histological analysis was performed on five-mm-thick sections obtained from 4 different locations: (1+2) at the center of the ePTFE-graft with an in-between distance of 5 mm to determine graft coverage; (3) at the center of venous anastomosis and (4) 5 mm proximal (i.e. caudal) to the venous anastomosis, to determine intimal and medial areas. For morphometric analysis, sections were stained with Elastin van Gieson (EvG). With the highest magnification that allowed visualization of the entire vein section in one field, the intimal and medial areas were manually traced at the venous anastomosis and proximal of the anastomosis. The intima was defined as the tissue area encompassed by the internal elastic lamina. Thrombus formation was discriminated from intimal hyperplasia using Hematoxylin-Eosin and EvG-stained sections. In the sections of the venous anastomosis, two parts of the intima area were distinguished: (1) the intima covering the graft (shoulder region) and (2) the intima located at the venous part of the anastomosis (cushioning region). For immunohistochemical analysis, sections were incubated in 1.5% hydrogen peroxide in methanol to block endogenous peroxidases. Next, sections were incubated in boiling 10 mM citrate acid for 15 minutes and subsequently pre-incubated with 10% horse serum (Vector laboratories, Burlingame, CA). Serial sections were stained with murine antibodies against α-smooth muscle actin (Sigma, St. Louis, Missouri, US), for vascular smooth muscle cells (VSMCs), at 1:1500 dilution; Ki-67 (Immunotech,

Marseille, France) for cellular proliferation, at 1:100 dilution and MAC387 (Serotec, Raleigh, NC) for macrophages, at 1:10 dilution. Subsequently, sections were incubated with a biotinylated anti-mouse IgG antibody (Vector laboratories, Burlingame, CA) for 1 hour. To visualize endothelial cells, sections were incubated with lectin from bandeiraea simplicifolia BS-1, at 1:100 dilution (Sigma) for 1 hour [19]. Immunoreactive materials were visualized by the use of streptavidin labeled HRPO, diaminobenzidine in 0.05 M Tris-Cl mixed with 0.01 M imidazole and 0.1% hydrogen peroxide. Sections were counter-stained with hematoxyline. Ki-67-stained sections from each venous anastomosis were used to measure the amount of proliferating cells within the intima. Therefore, 100 cells were counted per high power field (2 at the shoulder region and 2 at the cushioning region). A proliferation index was defined as the number of positive cells divided by the sum of Ki67-negative and positive cells, and expressed as a percentage.

Scanning electron microscopy
The integrity of the cellular coverage of the ePTFE-grafts was also visualized by SEM. Therefore, the ePTFE grafts were fixed in 2% glutaraldehyde and then dehydrated through increasing concentrations of ethanol (80% to 100%). The samples were dried with the use of Hexamethyldisilazane. Next, the ePTFE discs were sputter-coated with a thin layer of Platinum/Palladium and analyzed with a scanning electron microscope (Philips XL30, Eindhoven, the Netherlands).

Statistical evaluation
Data are presented as mean ± standard error of the mean (SEM). SPSS 11.0 was used for all statistical calculations. To ascertain the significance of differences we performed the Wilcoxon test. A probability value of <0.05 was considered significant.

Results

In total, 22 grafts were successfully implanted in 11 Landrace pigs. In the 2 pigs which were sacrificed after three days, all grafts (n=4) were patent at time of harvest. In the 9 pigs which were terminated at 4-weeks grafts were patent in 6 pigs, whereas bilateral occlusion was observed in 3 pigs. Histological analysis of the occluded grafts showed recent thrombotic occlusion on top of extensive intimal hyperplasia in the venous outflow tract. These occluded grafts were excluded for further analysis in view of the impossibility to reliably perform morphometric analysis of intimal hyperplasia in occluded grafts. The remaining patent grafts (n=16) were included for final analysis.

Scanning Electron Microscopy

Three days after graft implantation, the anti-CD34 coated grafts showed a confluent monolayer of cells. These attached cells had an inhomogeneous phenotype, ranging from round to spreading and flattened cells. In contrast, the bare grafts showed only little cellular adherence. Twenty-eight days after graft implantation, the majority of the attached cells on the coated graft were flattened in shape. In some areas, cells with a platelet-like phenotype were observed on top of the cellular layer on the coated graft. At this time point, partial coverage of cells was observed in the bare grafts as well (Fig 1).

Histological analysis

Graft coverage

Histological analysis of the grafts confirmed complete cellular coverage of the anti-CD34 coated grafts, 3 days after graft implantation. The adhered cells were identified as endothelial cells by lectin-staining (Fig. 2). Endothelial cell coverage at 3 days after implantation was ≈95% in the coated graft and <5% in the bare grafts. Four weeks after implantation, 88% ± 5% of the surface in the coated grafts was covered by lectin-positive cells which was significantly higher than in the bare grafts (32 ± 8%, p= 0.04). (Fig. 3ab).

Intima and media areas

EvG-stained sections from the venous anastomosis revealed prominent intimal thickening at 4 weeks after graft implantation (Fig. 3cd). At this location, a strong increase in intimal area was observed in coated grafts compared to bare grafts (Table 1). Overall, the amount of microvessels in the intima is relatively low in both groups. The vast majority of microvessels are located in the adventitia of the recipient vein. Also in this vessel layer, microvessel formation was comparable between groups. Approximately 90% of the intimal cells express the VSMC-marker α-actin (Fig. 3e), while at shoulder region of the venous anastomosis, a small amount of macrophages was observed in both groups. Thus, except for the size of the intimal area, no differences in composition of the intimal hyperplastic lesions at the venous anastomosis were observed between groups. At the proximal vein (5 mm proximal to the toe of the venous anastomosis), the intimal and medial areas did not differ significantly between the 2 groups (Table 1).

Cellular proliferation

Ki-67 stained sections obtained from the venous anastomosis showed prominent cellular proliferation in the intima (Fig. 3f). At the venous anastomosis the proliferation index was significantly higher in the coated grafts (20.9 ± 2%) when compared to the bare grafts (13.3 ± 2%, p = 0.027). The higher proliferation rate in the coated-grafts was mainly due to increased proliferation at the shoulder region (Fig. 4).

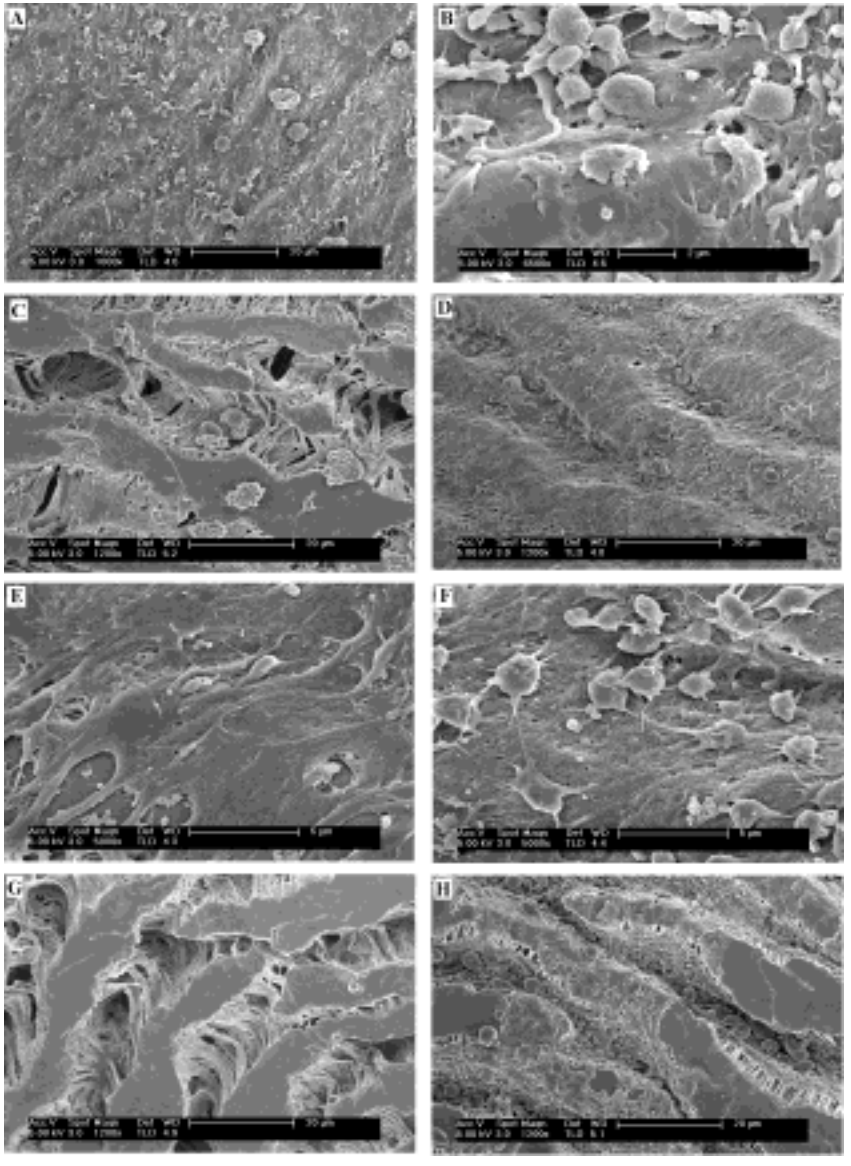

Figure 1. Scanning electron micrographs of ePTFE-grafts, 3 days (A-C) and 28 days (D-H) after graft implantation. Already at 3 days after implantation, the anti-CD34 coated grafts were completely covered with cells (A). The attached cells showed various appearances ranging from round to spreading and flattened cells (B). The bare graft shows only sporadic cellular attachement (C). At 28 days after implantation, almost complete cell retention was observed in CD34-coated grafts (D). At this time point, the majority of the attached cells on the CD34-coated grafts were flattened in shape (E). In some areas, cells with a platelet-like phenotype were observed on top of the cellular layer on the coated graft (F). In the bare-grafts, areas without (G) and with partial (H) cellular coverage were observed.

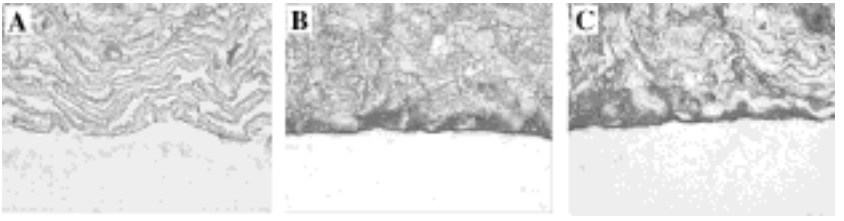

Figure 2. Representative Hematoxylin-eosin-stained sections of bare (A) and CD34-coated grafts (B) obtained from the center of the graft at 72 hours after implantation. The adhered cells on the CD34-coated grafts were identified as endothelial cells by lectin-staining (C).*

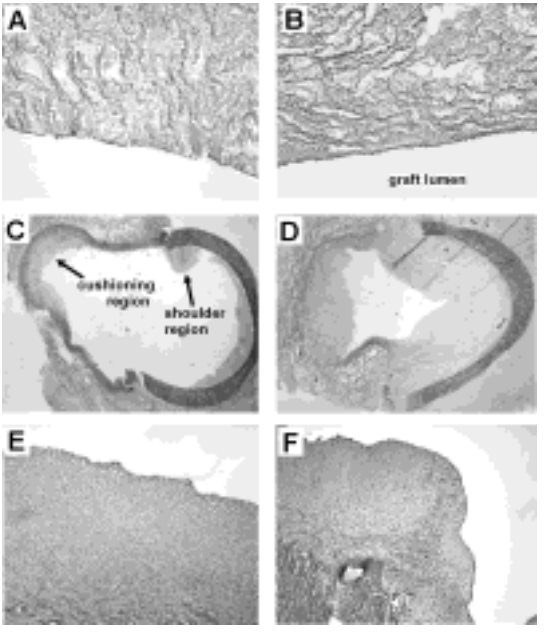

Figure 3. Representative sections obtained at 4 weeks after graft implantation. Lectin-stained sections of bare (A) and CD34-coated grafts (B) obtained from the center of the graft. EvG-stained sections of the venous anastomosis of bare-graft (C) and CD34-coated grafts (D). Detail of α-actin smooth muscle cell-stained section of the venous anastomosis (E). Detail of Ki67-stained section of the venous anastomosis. Extensive proliferation is observed at the shoulder region (F).*

* See color supplement, starts at page 141.

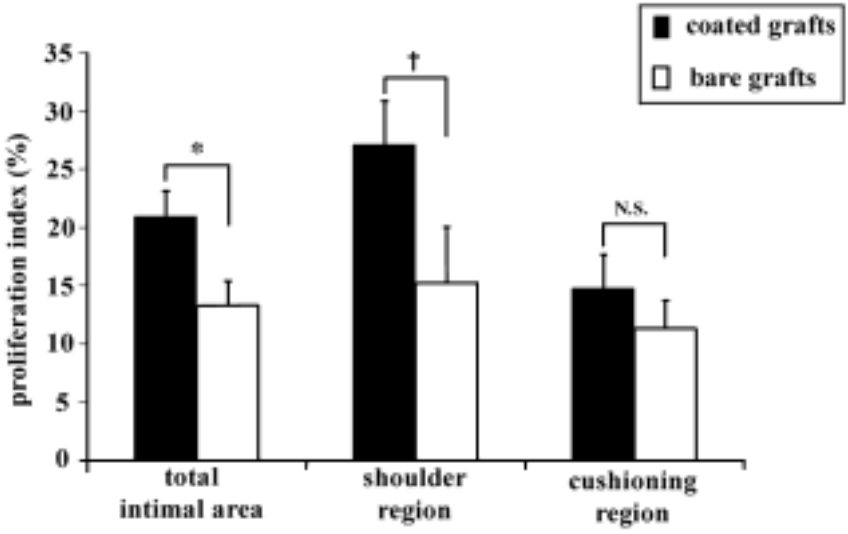

Figure 4. Cellular proliferation at the venous anastomosis obtained by Ki67-stained sections. Proliferation index was defined as the number of positive cells divided by the total amount of cells in 4 high power-fields, and expressed as a percentage. Two parts of the intima area were distinguished: (1) the intima covering the graft (shoulder region) and (2) the intima located at the venous part of the anastomosis. The proliferation index was significantly higher in the coated-grafts, mainly due to increased proliferation at the shoulder region. * p = 0.027, † p = 0.028.

	anti-CD34 coated grafts (n=6)	bare-grafts (n=6)	p-value
center of venous anastomosis			
total intimal area (mm²)	5.96 ± 1.9	1.70 ± 0.4	0.03
shoulder region (mm²)	2.47 ± 0.8	0.84 ± 0.4	0.03
cushioning region (mm²)	3.48 ± 1.6	0.86 ± 0.3	0.08
medial area (mm²)	2.95 ± 0.8	1.49 ± 0.2	0.05
intima/media ratio	1.95 ± 0.4	1.23 ± 0.4	0.03
jugular vein (5 mm proximal to venous anastomosis)			
intimal area (mm²)	2.49 ± 0.6	2.39 ± 1.0	0.75
medial area (mm²)	2.75 ± 0.3	2.29 ± 0.3	0.25
intima/media ratio	0.85 ± 0.2	0.88 ± 0.3	0.75

All values are expressed as the mean value ± the standard error of the mean (SEM).

Table 1. Summary of morphometric analysis of EvG-stained sections obtained from the center of the venous anastomosis and from the proximal jugular vein.

Discussion

In the present study, we show that anti-CD34 coating of arteriovenous ePTFE-grafts results in a rapid and almost complete coverage of the graft with lectin-positive cells within 72 hours after implantation in pigs. This cellular coverage of coated grafts persists for at least 4 weeks. In spite of cellular coverage of the luminal surface, IH is augmented at the venous anastomosis of anti-CD34 coated grafts at 28 days of follow-up, when compared to uncoated grafts. The present findings show that anti-CD34 coating is successful in promoting cellular coverage of grafts with lectin-positive cells, whereas these trapped cells are unable to attenuate IH formation at the venous outflow tract.

Graft coverage with endothelial (progenitor) cells

The concept of endothelial cell seeding of grafts is based on the assumption that these cells will constitute a biologically active lining that will attenuate activation of blood passing through the graft. Unfortunately, the complicated procedures for harvesting, expansion and seeding of ECs *in vitro* have prevented this method from finding broader application in humans. EPCs are bone-marrow derived CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells [11;20]. Circulating EPCs have recently been shown to actively provide new endothelial cells from the circulating blood to sites of endothelial denudation and/or injury [12;13]. By promoting adherence of these 'endogenous' circulating endothelial progenitor cells, the need for *in vitro* seeding procedures could theoretically be avoided. In the present study, we observed rapid coverage with lectin+ cells from 3 days onwards. Four weeks after implantation, the cellular coverage of anti-CD34 coated grafts remained almost confluent, whereas the histological appearance of the cells gradually changed into flattened cells. The degree of cellular coverage compares favorably to previous studies on *in vitro* graft seeding techniques with CD34+ cells, in which graft coverage did not exceed 65% [15]. The efficiency of *in vitro* seeding techniques using mature ECs has also been limited by low levels of endothelial cell retention [21;22] Combined, these data illustrate that antibody-assisted autoseeding of CD34+ cells is associated with more confluent coverage of grafts, whereas it simultaneously offers a safer and easier technique for clinical use.

Graft coverage and intimal hyperplasia

Increased graft coverage should result in less activation of circulating blood cells and potentially contribute to paracrine mediators conveying anti-inflammatory and anti-proliferative effects downstream at the venous outflow tract. This line of events assumes that the attached cells are able to

exert effects similar to the physiological actions of a normal endothelial lining. In contrast, despite complete graft endothelialization in the anti-CD34 coated grafts, we observe a 3-fold increase in IH at the venous anastomosis. Concomitantly, a strong increase in cellular proliferation was present, predominantly at the shoulder region of the venous outflow tract. These data imply a proliferative, rather than anti-proliferative effect of the cells covering the graft. Our results are consistent with those of Kang et al., who showed aggravated restenosis after intracoronary infusion of peripheral blood stem-cells in patients who underwent coronary stenting [23]. Several factors may have contributed to the adverse effects in the anti-CD34 coated grafts. First, it has been previously reported that CD34+ cells are able to differentiate into various cell types, amongst which endothelial cells [24]. Thus, VSMCs can originate from bone marrow–derived CD34+ progenitor cells [25;26], implying that differentiation of CD34+ captured cells into VSMCs can contribute to the increased proliferation index in CD34-coated grafts [27]. Whereas differentiation of CD34+ progenitor cells into macrophages [28] has been reported, we observed no significant difference in macrophages numbers between coated- versus bare-grafts. Second, EPCs have the capacity to release potent proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [29]. The proliferative and migratory effects of these cytokines are not restricted to endothelial cells, but include VSMCs as well [30-32]. Enhanced secretion of proliferation factors from cells adhered to anti-CD34 coated grafts may have contributed to increased intimal hyperplasia at the venous anastomosis. Third, interaction of ECs with subendothelial matrix components has been shown to be of pivotal importance for the protective function of ECs [6]. Therefore, the absence of a physiological micro-environment may also contribute to the apparent lack of protective effects. Fourth, laminar shear stress has been shown to upregulate EC-specific markers such as VE-cadherin in EPCs [33]. Therefore, the turbulent flow pattern in AV-grafts may have deteriorated differentiation into ECs, especially in the anastomotic region. Finally, adequate maturation of captured CD34+ cells may have been hampered by the binding of the immobilized antibody to the CD34-epitope.

Study limitations

The adhered cells were characterized as endothelial-like cells by their morphological appearance on SEM and by immunohistochemistry using lectin from bandeiraea simplicifolia, a marker for porcine endothelial cells [19]. However, extensive characterization of the adhered cells was not feasible due to the unavailability of monoclonal antibodies for porcine endothelial (progenitor) cells markers such as CD133, VEGFR-2, CD31 and VE-cadherin. As a consequence, we cannot exclude that the adhered

cells contain a heterogeneous population including CD34- cells. Still, the uniform lectin-positive staining of the graft-covering cells and their morphological appearance on SEM suggest efficient and specific binding of cells with an endothelial phenotype.
We used an AV-graft model because of the poor patency rate of hemodialysis access grafts. The process of neo-intimal formation in AV-grafts is characterized by specific pathophysiological stimuli such as high turbulent flow and graft-vein compliance mismatch, which coincide with the arterialization process of the vein. Therefore, extrapolation of these data to arterial bypass grafts may not be valid.
The uremic milieu has a clear impact on the progression of vascular diseases in ESRD patients [34;35]. In this respect, the decreased number and impaired function of EPCs in patients with chronic renal failure, has recently been suggested to contribute to the accelerated progression of cardiovascular diseases in these patients [36;37]. Since this study was performed using non-uremic pigs, one should be cautious in extrapolating these results to the human situation.

Clinical implications and future directions
The present findings indicate that autoseeding with EPCs offers a potential strategy to establish graft endothelialization in AV-grafts. It remains to be established whether and to what extent these captured cells can be stimulated to regain their functional capabilities. In this respect, it is interesting to note recent developments demonstrating the regulatory role of vascular endothelial growth factor[38], erythropoietin[39] and angiopoietins[40] on maturation of endothelial progenitor cells. In addition, capturing a mixture of both CD34+ and CD14+CD34- cells also holds a promise for optimal endothelial cell differentiation and functionality of EPCs[41]. These options need to be addressed in future studies.

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Chapter 4

Immobilization of an Antibody against Human CD34 Results in Recruitment of Cells with Endothelial Properties from Blood under Flow Conditions

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Background. The absence of a functional endothelial layer on prosthetic grafts is thought to be an important pathogenic stimulus for small diameter graft failure. Graft endothelialization might therefore improve graft survival. Endothelial progenitor cells (EPCs) are a subset of CD34+ cells with the potential to differentiate into mature endothelial cells. The present study was performed to evaluate the concept of auto-endothelialization with EPCs using immobilized antibodies against human CD34.
Methods. Glass coverslips were coated with a monoclonal antibody directed against human CD34. As controls, coverslips were coated with the isotype of the CD34 antibody and with polymer base-coating only. Coverslips were perfused with anticoagulated human whole blood in a validated *in vitro* flow model at a shear rate of 25/sec and a perfusion time of 90 minutes. Adhered cells on the coverslips were counted (May Grunwald Giemsa staining) and characterized using immunofluorescent stainings. Anti-CD34-coated and uncoated ePTFE discs were also perfused using a similar procedure. Cells adhered to these ePTFE discs were studied by scanning electron microscopy.
Results. The amount of cells adhered to the anti-CD34 antibody coated coverslips was significantly increased compared to basecoated and isotype-coated coverslips (330 cells/mm^2 vs 141 cells/mm^2 and 189 cells/mm^2, respectively $p<0.001$ and $p<0.01$). A subset of adhered cells on the CD34 antibody coated coverslips stained positive for CD34, CD31, KDR and/or VWF, indicating cells with an endothelial phenotype. These cells did not stain positive for CD133, suggesting that mature EPCs are attracted. Other cells stained positive for CD14 and CD66b, suggesting adherence of mononuclear and granulocytic features. Adhered cells to the CD34 coated ePTFE discs had a variety of characteristics. A subset of these cells can be classified as endothelial-like.
Conclusions. Cells with endothelial characteristics from peripheral human blood adhere to surfaces coated with an antibody against human CD34 under flow conditions. This coating technique may be a potential tool to improve graft survival since it offers the possibility to use prosthetic grafts off the shelf that auto-endothelialize after implantation. However, simultaneous adherence of granulocytes might hamper application.

Introduction

The patency of small diameter prosthetic vascular grafts is limited when compared to bypass grafting using venous conduits [1]. This is probably due to the absence of endothelial cells at the inner surface of the vascular grafts, ultimately leading to intimal hyperplasia in the anastomotic region and to acute thrombosis [2]. Indeed, the vascular endothelium plays an

important role in regulating coagulation, inflammation and the response to vascular injury [2-4]. Graft seeding with autologous endothelial cells (EC) has been shown to increase patency rates of prosthetic bypass-grafts in clinical trials [5]. However, *in vitro* EC seeding is time consuming and therefore not suitable for (semi)acute vascular bypass grafting.

Bone-marrow derived endothelial progenitor cells (EPCs) might serve as an alternative source for ECs used in graft endothelialization. EPCs are a subset of CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells [6]. Circulating EPCs have been shown to home to sites of vascular injury, where they contribute to reendothelialization [7;8]. *In vitro* coated grafts, using CD34+ cells isolated from bone-marrow [9] or peripheral blood [10], markedly increased graft endothelialization in animal models. Our recently published data also show an increased and accelerated endothelialization, using immobilized anti-CD34 antibodies to capture EPCs on porcine vascular grafts *in vivo*. Unfortunately, this process of autoseeding also resulted in enhanced intimal hyperplasia (IH), but the mechanisms by which anti-CD34 antibody coating enhanced IH are unclear.

In the present *in vitro* study, cells from human blood that adhered under flow conditions to the immobilized anti-CD34 antibodies immobilized on glass coverslips or on ePTFE discs were studied. We hypothesized that the cells adhered in the early phase are responsible for the development of an endothelial coverage, as a start of the process of 'auto-endothelialization'. The present study was also designed to delineate the adverse effect of the coating and/or the adhered cells on the induction of IH as was demonstrated in our *in vivo* study [11].

Materials and methods

Square glass coverslips (surface area, 3.2 cm^2) and circular expanded polytetrafluoroethylene (ePTFE) discs (surface area 2.5 cm^2, Bard, Tempe, AZ) were coated with antibodies against human CD34 (IgG2a, epitope class III). Uncoated, IgG2a-isotype coated and base-matrix coated glasses and discs were used as controls. Base-matrix is the coating attached to the surface which is required for optimal attachment of antibodies. Preparation of coverslips and discs was performed by OrbusNeich Medical, Inc. (Fort Launderdale, FL) using a proprietary, multi-step process (Ssens, Hengelo, the Netherlands).

In vitro perfusion model

Human whole blood anticoagulated with low molecular weight heparin

(LMWH 20U/ml) was perfused over the coverslips and ePTFE discs at a shear rate of 25/sec, using a single-pass perfusion chamber as described previously [12]. The blood was aspirated over the glass coverslips and ePTFE pieces at a constant flow rate with a syringe pump (Harvard Apparatus, South Natick, MA). After 90 minutes of perfusion, the ePTFE discs were removed from the perfusion chamber processed for scanning electron microscopy (SEM) as described below while the glass coverslips were processed for May–Grünwald Giemsa staining and immunocytochemical analysis as described below.

Cell count and characterization on glass coverslips

To count adhered cells and study their morphology, the coverslips were rinsed with 10mM HEPES buffered saline and fixed with 2% glutaraldehyde in PBS for 30 minutes and postfixed with methanol for 5 minutes. Next, the cells were stained with May–Grünwald and Giemsa and analyzed using light microscopy.

For immunofluorescent analysis, glass coverslips were rinsed with 10mM HEPES and fixed in 4% paraformaldehyde for 30 minutes. The cells were then permeabilized in 0,1% Triton-X100 in PBS and rinsed in PBS. Next, FITC or TRITC labeled antibodies were incubated for 1 hour at room temperature in the dark. Anti-CD34 FITC labeled (BD Pharmingen, San Diego, California), anti-CD31 FITC labeled (BD Pharmingen, San Diego, California), anti-CD133 PE labeled (Miltenyi Biotec, Amsterdam, The Netherlands), anti-CD14PE labeled (BD Pharmingen, San Diego, California) anti-CD66b FITC labeled (Sanquin, Utrecht, The Netherlands), anti-VWF FITC labeled (US Biological, Swampscott, Massachusetts) and anti-VEGF-R2 (Santa Cruz Biotechnology, Santa Cruz, California) using GAM FITC (BD Pharmingen, San Diego, California) or TRITC labeling kit (Molecular Probes, Inc, Eugene, OR) were used to characterize the adhered cells.

As stated above, the adhered cells on the coated glasses were stained with antibodies against markers for EPCs (CD34, CD133), mature ECs (CD31, KDR and VWF), monocytes (CD14) and granulocytes (CD66b). These markers were chosen because of the morphology of some of the adhered cells as observed with the May–Grünwald Giemsa staining (see results). Cells were double-stained using the following combinations of antibodies: CD34 and KDR; CD31 and KDR; VWF and KDR; CD133 and KDR; CD14 and CD66b.

Scanning Electron Microscopy (SEM)

For Scanning Electron Microscopy (SEM), discs were washed with HEPES buffer and fixed in 2% glutaraldehyde in PBS. After washing with distilled water, discs were dehydrated with increasing concentrations of ethanol (80% – 100%) and subsequently treated with hexomethyldisylazane (Fluka

Chemie, Buchs, Switzerland). Discs were embedded on a stub in carbon glue. Samples were coated with a thin layer of Platinum/Palladium using an Emitech K-575X Sputter Coater and were examined by SEM (XL30 SFEG, Philips, Eindhoven, The Netherlands).

Statistical evaluation
Statistical analysis was performed using the GraphPad InStat (San Diego, CA) software package. Statistical differences in surface coverage were analysed by standard one-way analysis of variance (ANOVA) using Tukey's multiple comparison test. *P* values <.05 were considered statistically significant.

Results

Cell count and morphology
The amount of cells on the anti-CD34 coated surface was significantly higher when compared to both basecoat (330 cells/mm² vs 141 cells/mm²) and isotype (330 cells/mm² vs 189 cells/mm²) respectively $p<0.001$ and $p<0.01$ as is shown in figure 1. On all types of coated glasses (basecoat, isotype and anti-CD34), adhesion of cells with mononuclear and granulocytic features were observed (figure 2).

Cell characterization
The adhered cells on the anti-CD34 coated glasses stained positive for CD34, CD31, KDR and VWF (figure 3). Seventeen percent stained positive for CD34, 29 % stained positive for CD31, 20% stained positive for KDR and 83% of cells expressed VWF. Cells did not stain positive for CD133 (data not shown). In concordance with the observation done in the May–Grünwald Giemsa staining, cells showed mononuclear (CD14) and granulocytic (CD66b) features as well (figure 4). The cells on the basecoat and isotype coated glasses had –in part- the same characteristics as the cells adhered on the anti-CD34 coated glasses, but fewer cells adhered and hardly any cells stained positive for the endothelial markers (figure 5).

SEM scanning
Adhered cells on the ePTFE discs were visualized using SEM. Here, uncoated, basecoat and anti-CD34 coated ePTFE discs were used to study cell adherence.
The bare discs show extensive platelet adherence and fibrin strands. The basecoat discs show some adhered platelets as well. The rounded cells that adhere to the anti-CD34 coated discs resemble EPCs as published in an ultrastructural study on progenitor cells [13] (figure 6).

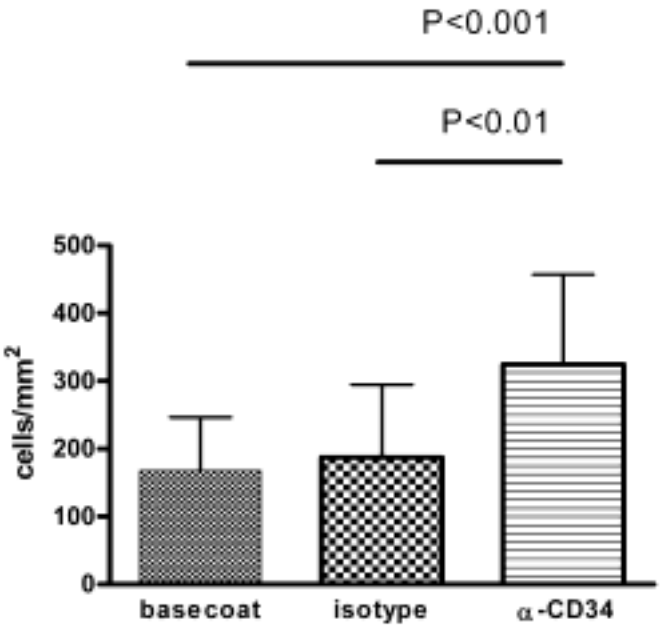
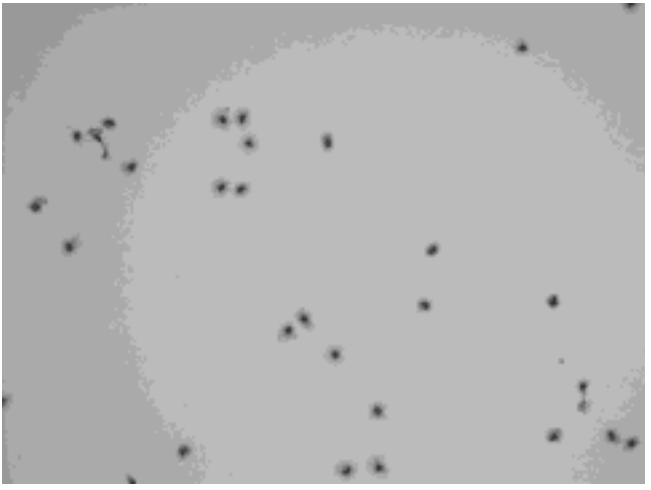
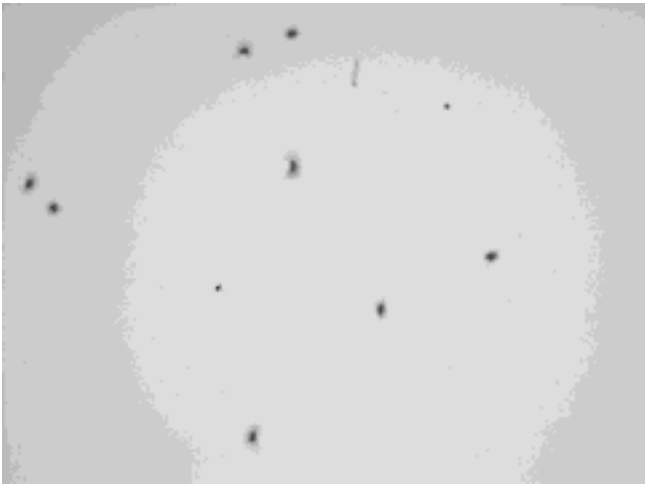


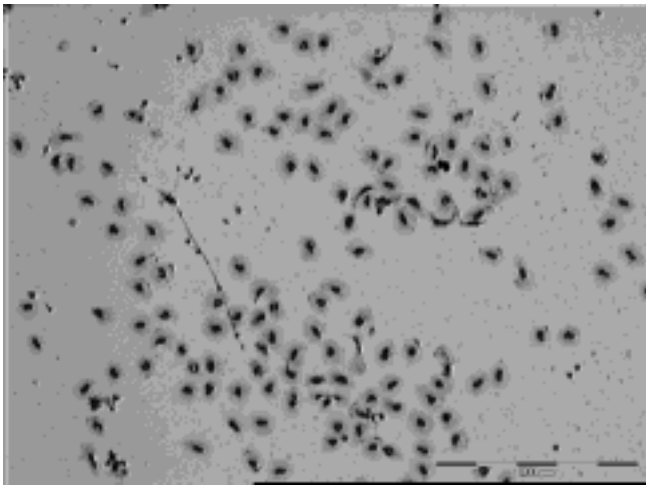
Figure 1: The amount of cells/mm² attached on the different types of coated glass coverslips (n=4 perfusions per type). LMWH-anticoagulated human blood was perfused for 90 minutes at a shear rate of 25/sec, after which the glass coverslips were prepared for May–Grünwald Giemsa staining. Anti-CD34 coated, basecoat and isotype glass coverslips were studied. There is a significant increase of attached cells on the anti-CD34 coated glasses compared to both basecoat ($p<0.001$) and isotype ($p<0.01$).



A Isotype coated coverslip



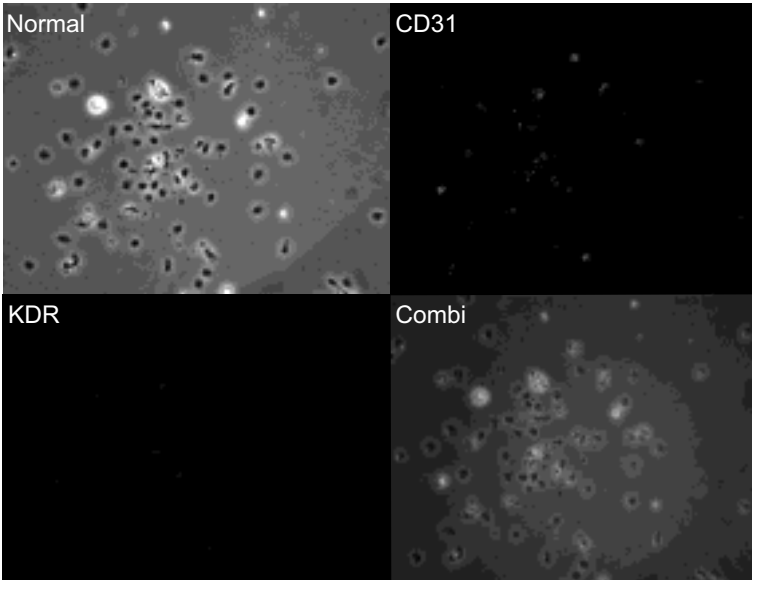
B Basecoat coverslip



C anti-CD34 coated coverslip

Figure 2: Representative pictures of Light Microscopy after May–Grünwald Giemsa staining. LMWH-anticoagulated human blood was perfused for 90 minutes at a shear rate of 25/sec, after which the glass coverslips were prepared for May–Grünwald Giemsa staining. A is an example of isotype coated coverslip, B of basecoat coverslip and C of anti-CD34 coated coverslip. The cells show morphologic mononuclear and granulocytic features.*

* See color supplement, starts at page 141.


Normal
CD31
KDR
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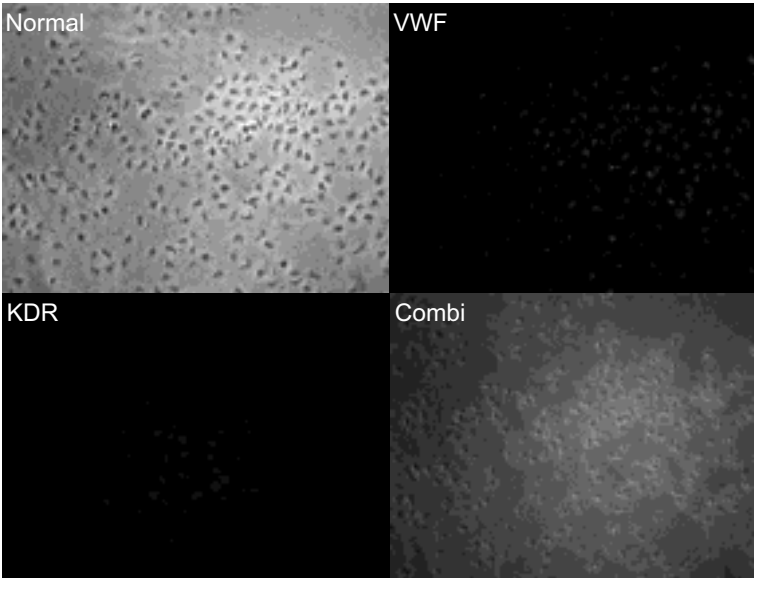
Normal
VWF
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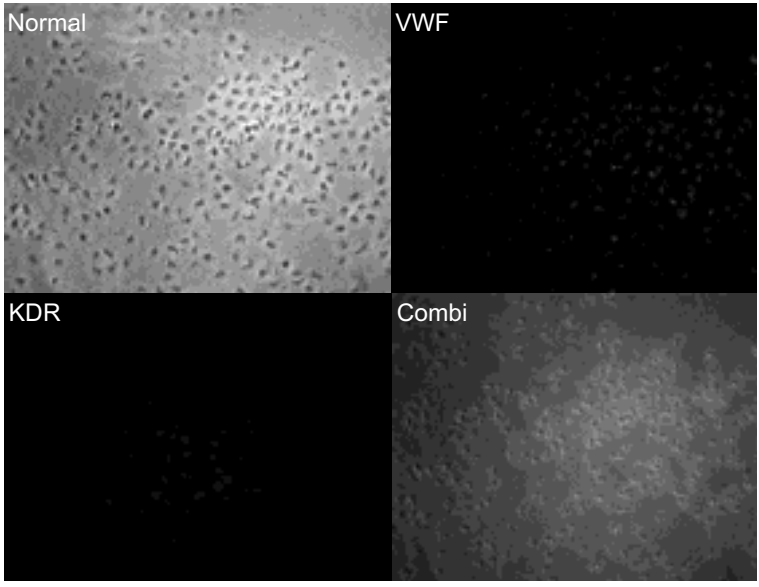


Figure 3: Characterisation of cells adhered to coverslips coated with an antibody against CD34. LMWH-anticoagulated human blood was perfused for 90 minutes at a shear rate of 25/sec, after which the coverslips were stained with antibodies against CD31, KDR, vWF and CD34. The blue colour indicates the nucleus (DAPI staining). Note that the cells stain positive for CD31, KDR, vWf and CD34. These are all endothelial characteristics.*

* See color supplement, starts at page 141.

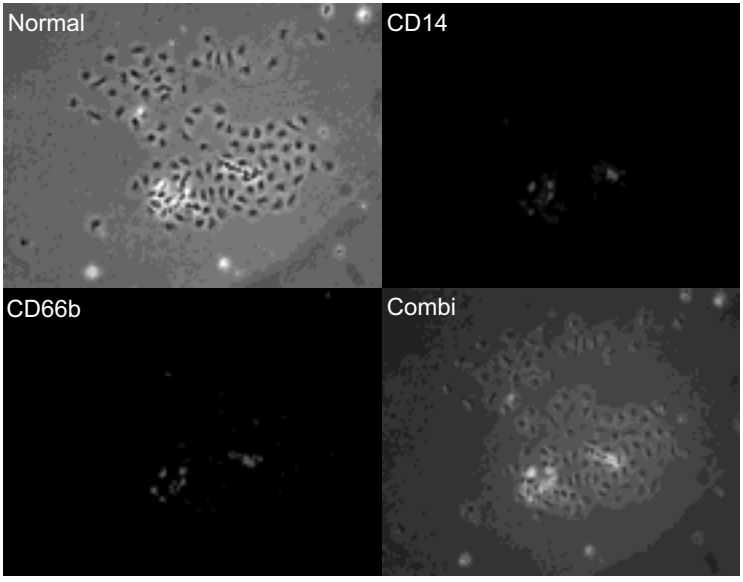


Figure 4: In concordance with the observation done in the May Grunwald Giemsa staining, the cells also show characteristics of mononuclear and granulocytic features, as they stained positive for CD14 and CD66b.*

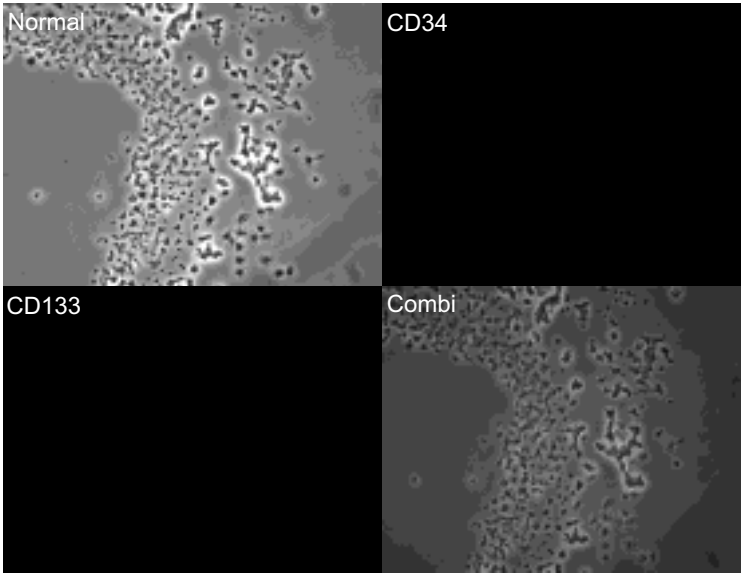
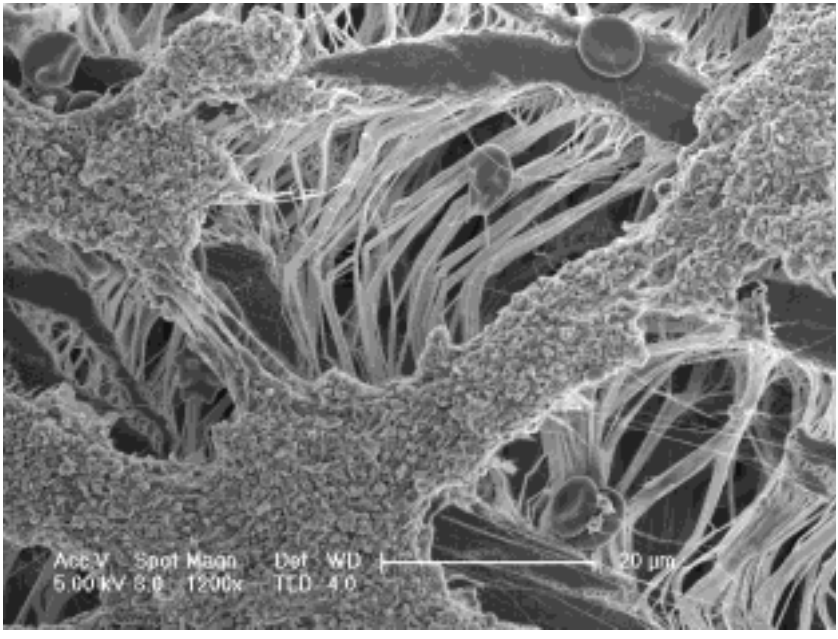
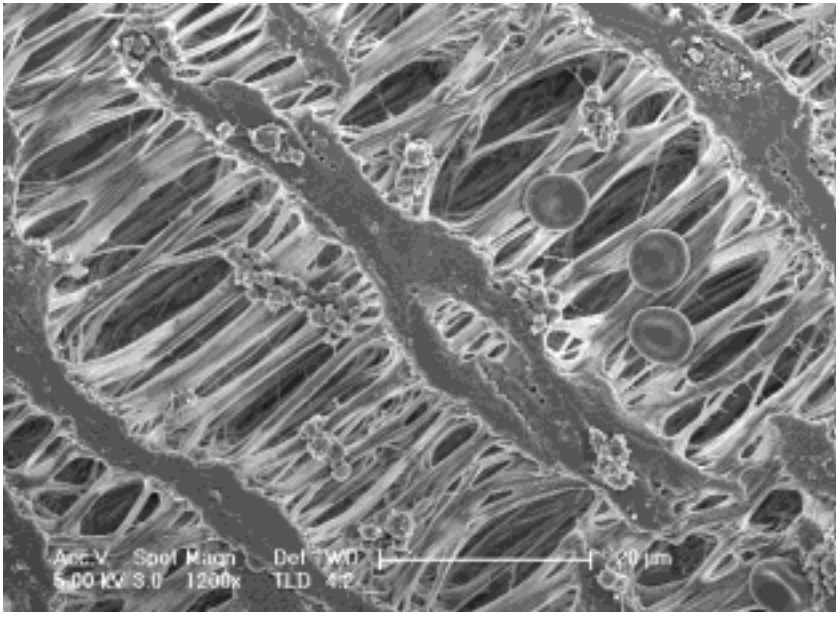


Figure 5: Example of adhered cells to the isotype of CD34 coated glass coverslip. Note that none of the adhered cells stain positive for either CD34 or CD133. Also note that only the blue DAPI stained dots, are cells. The structures around are probably lysed cells or debris.*

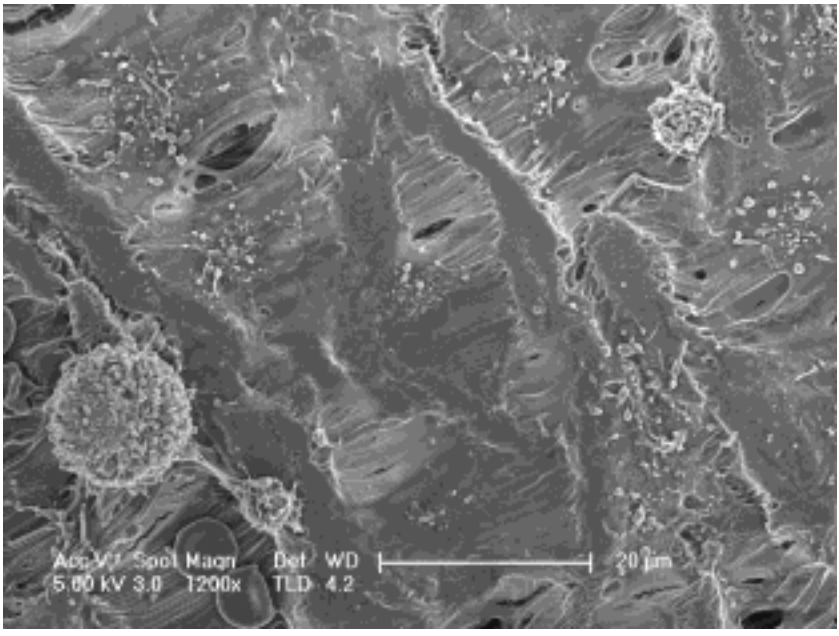
* See color supplement, starts at page 141.



A: non-coated ePTFE showing many platelets and fibrin strands adhered.



B: basecoat ePTFE showing less platelet adhesion.



C: anti-CD34 coated ePTFE showing surface coverage. Note that the surface is not 'visually permeable' as is seen at the uncoated and base-coated ePTFE. This 'permeability' is the normal architecture of the ePTFE. Hardly any platelets are seen. The rounded cells resemble EPCs as published in an ultrastructural study on progenitor cells [13].

Figure 6: SEM scanning after 90 minutes of perfusion with LMWH-anticoagulated human blood at a SR of 25/sec, showing three different types of coated or non-coated ePTFE discs (A, B and C).

Discussion

In the present study we show that immobilisation of an antibody against CD34 on glass or ePTFE enhances attraction of cells with endothelial characteristics. This was demonstrated by May–Grünwald Giemsa staining, immunofluorescence and scanning electron microscopy. However, the adhered cells also showed granulocytic features.

Endothelial cell seeding

The concept of endothelial cell seeding of grafts is based on the assumption that ECs will form an active cell-lining that will soothe activation of blood passing through the graft. Clinical *in vitro* endothelialization provided strong evidence that autologous endothelial cell lining improves the patency of small-diameter vascular grafts [14]. The complicated procedures for harvesting, growth and seeding of ECs *in vitro* have prevented broad clinical application in humans.

EPCs are bone-marrow derived CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells [6]. Circulating EPCs are suggested to have beneficial effects to sites of vascular injury and to lead to enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34+ bone marrow cells[8-10]. The disadvantage of the laborious *in vitro* procedures required to endothelialize vascular grafts, can be avoided when circulating EPCs adhere to surfaces coated with an antibody against CD34 and the process of 'auto-endothelialization' is realised. This endothelialization technique might therefore imply a broader application in humans. Pre-coated grafts can simply be picked of the shelf by the surgeon at the very moment a vascular bypass is needed.

In a recent study, Rotmans et al. demonstrated accelerated endothelialization but enhanced intimal hyperplasia (IH) in anti-CD34 coated ePTFE grafts in a porcine model of AV-graft failure [11]. The present study was also designed to elaborate this adverse effect of the coating and/or the adhered cells on the induction of IH.

Cell adherence to the anti-CD34 coated surface

In the present study we investigated the adherence of cells to the anti-CD34 coated surface. A significant increase in cell adherence was shown in benefit of the anti-CD34 coating when compared to the isotype of the CD34 antibody and the polymer base-coating only. This is in concordance with our recently published *in vivo* porcine study. There, we have demonstrated that anti-CD34 coating of ePTFE results in an accelerated endothelialization, already at 72 hours after implantation, when compared to uncoated ePTFE grafts. Although graft endothelialization should theoretically inhibit neo-intima formation as a result of the anti-proliferative and

anti-inflammatory effects of ECs, enhanced IH was observed in the anti-CD34 coated grafts which were completely endothelialized in the *in vivo* porcine model.

In the SEM studies we show a noticeable difference in surface coverage comparing the different coated or uncoated ePTFE discs after flow (see figure 6). The anti-CD34 coated discs show a complete surface coverage with a number of cells that resemble EPCs as published in an ultrastructural study on progenitor cells [13]. This suggests a specific binding of cells to the anti-CD34 antibody.
A subset of cells that was captured in this study on the anti-CD34 coated surfaces, expressed established markers of endothelial cells (CD34, CD31, KDR, and VWF).
Surprisingly, a substantial amount of CD34- cells adhered to the CD34-coated surface as well. These cells stained positive for monocytic (CD14) and granulocytic (CD66b) markers.
Perhaps, the adherence of granulocytes might be explained by an immunoreaction against the mouse anti-human CD34 antibody. Granulocytes can produce cytokines which directly function as a chemo-attractant for cells like smooth muscle cells that lead to the development of IH [15;16]. This mechanism may explain the observed increase in IH in the anti-CD34 coated ePTFE grafts in our porcine model.

Study limitations
The *in vitro* set-up mimics the clinical situation only partially. The fact that the blood was used in a flow model mimics the normal situation where blood flows through a vascular graft. The advantage of using both glass coverslips and pieces of ePTFE gave the opportunity to investigate the adhered cells in three different ways. Characterising adhered cells in such a basic setup creates the opportunity to study different types of antibody coating as we have proven that the concept is promising, although the 'perfect antibody coating' isn't found yet.

Clinical implications and future directions
The present findings imply that autoseeding with EPCs offers a valuable strategy to enhance graft endothelialization. With this study we might have found an explanation for the increased IH that was found in the *in vivo* study, as granulocytes adhered to the coated surface as well.

Future studies should reveal if further optimization of the coating-technique is able to improve the functional profile of captured cells. Recent studies suggest that an adequate balance between CD34+ and CD14+CD34- cells is required for optimal endothelial cell differentiation

and function of EPCs [17-19]. Also, other data indicate the potential beneficial effects of treatment with CD14+ cells that improve healing and vascular growth [20]. Therefore, a carefully-designed mixture of anti-CD34 and anti-CD14 antibodies might result in a combination of trapped cells with a higher potential to improve vascular homeostasis. This might imply a more specific binding of EPCs resulting in a smaller amount of granulocytes being attracted. Maybe an alternative for the basecoating, and replacement of mouse antibodies by humanised counterparts, might also result in a reduction of adhered granulocytes.

Further studies are needed to delineate whether and to what extent autoseeding of a combination of cell types is able to result in a functional graft-coverage layer, which exerts beneficial effects on the use of small diameter vascular grafts. This *in vitro* model offers a valuable set-up to investigate the (dis)advantages of antibody coating to favour 'auto-endothelialization'.

Conclusions

This *in vitro* study demonstrates the feasibility to attract cells with endothelial characteristics from human blood under flow conditions, on glass coverslips and ePTFE, coated with a monoclonal antibody against human CD34. However, concurrent adherence of granulocytes may deteriorate functional capacity of the adhered EPCs and its clinical applicability.

Acknowledgements

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Chapter 5 Endothelial Progenitor Cell Coated Grafts: Rash and Risky

Accepted for publication. Can J Cardiol
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ESG Stroes, G Pasterkamp

Clinical context

The patency of prosthetic vascular grafts is impaired by intimal hyperplasia (IH) near the anastomotic regions. The absence of a functional endothelial monolayer on the prosthetic grafts is an important stimulus for IH. To improve the outcome of synthetic vascular bypass surgery, cell seeding is a promising concept that has extensively been investigated and is still evolving. In this manuscript cell seeding is discussed with emphasis on its newest era: seeding with endothelial progenitor cells (EPCs). Although experimental studies on prosthetic graft seeding using EPCs showed excellent result on graft endothelialization, none of these studies reported favourable effects on the more important endpoints such as intimal hyperplasia or graft patency.

Introduction

The patency of prosthetic vascular grafts is impaired by intimal hyperplasia (IH) near the anastomotic regions. The absence of a functional endothelial monolayer on the luminal side of prosthetic grafts is an important stimulus for IH. Indeed, the endothelium acts as a first line defense against vascular disturbances. In order to fulfill this role successfully, endothelial cells have been shown to produce a wide array of auto-, para- and endocrine substances with vasodilatory, anti-thrombotic and anti-proliferative effects [1]. Consequently, endothelial cell (EC) seeding at the luminal surface of prosthetic vascular grafts is a valuable strategy to improve graft patency. Recent progress in the research on the potency of bone-marrow derived endothelial progenitor cells (EPCs) have emerged these cells as a promising source for graft seeding. In the present review we will address the clinical problem of prosthetic graft failure and the rationale for graft seeding. Subsequently, we provide an overview of previous experimental studies on graft seeding with ECs and new insights in the applicability and the risks of EPCs for seeding of prosthetic vascular grafts.

Prosthetic vascular graft failure

In patients requiring peripheral arterial bypass grafting or vascular access for chronic hemodialysis, autologous veins are currently the conduit of choice in view of their superior patency rates. For peripheral arterial bypass grafting, the 5-year primary patency rate of venous bypasses is 74% compared to 39% for prosthetic bypass grafts [2]. In addition, the 1-year primary patency rates of native arteriovenous (AV)-fistulas utilized as vascular access for hemodialysis is approximately 75%, which is superior to the

50% 1-year primary patency rate of prosthetic AV-grafts [3]. However, many patients utilize prosthetic grafts when a suitable vein is unavailable, which is more often the case in the aging and diabetic population. Failure of prosthetic hemodialysis access grafts is predominantly due to a progressive intimal hyperplastic response near the venous anastomosis which ultimately leads to graft thrombosis. Currently, AV-shunt-related morbidity accounts for 20% of all hospitalizations in end stage renal disease (ESRD) patients, surmounting to more than 1 billion dollars per year being spent on AV-shunt-related care in the US alone.

Rationale for graft seeding

To date, there is no effective intervention available to improve graft patency. Successful development of new strategies requires closer insight into the pathogenesis of IH. The latter is thought to reflect a cumulation of several separate pathogenic entities, comprising inflammatory, coagulatory and hemodynamic factors [4].

A crucial factor in both activation of coagulation and inflammation is the lack of a functional endothelial monolayer on the prosthetic graft, because the endothelium constitutes the first-line homeostatic defence mechanism by exerting anticoagulatory and anti-inflammatory effects (figure 1). With regard to the latter, healthy ECs produces nitric oxide (NO), heparan sulphate, and c-type natriuretic peptide, which exert potent anti-proliferative as well as anti-migratory effects on vascular smooth muscle cells (VSMCs). In addition, implantation of ePTFE material is associated with a 'foreign body' response. This inflammatory response includes infiltration of leucocytes into the graft. These inflammatory cells secrete cytokines such as tumor necrosis factor-alpha (TNF-α) with clear proliferative effects. Therefore, graft seeding might suppress this inflammatory response.

In humans, complete prosthetic graft endothelialization does occur rarely and usually does not extend beyond 1-2 cm of the graft edges. Subsequently, the bare prosthetic graft provides a continuous adhesive surface for activated platelets that release thromboxane A2, serotonin and platelet-derived growth factor (PDGF), all known promoters of VSMC proliferation. The concept of endothelial cell seeding of grafts is based on the assumption that a functional endothelial layer attenuates activation of leucocytes and platelets passing through the graft and potentially contributes to paracrine mediators conveying anti-inflammatory and anti-proliferative effects down-stream at the outflow tract.

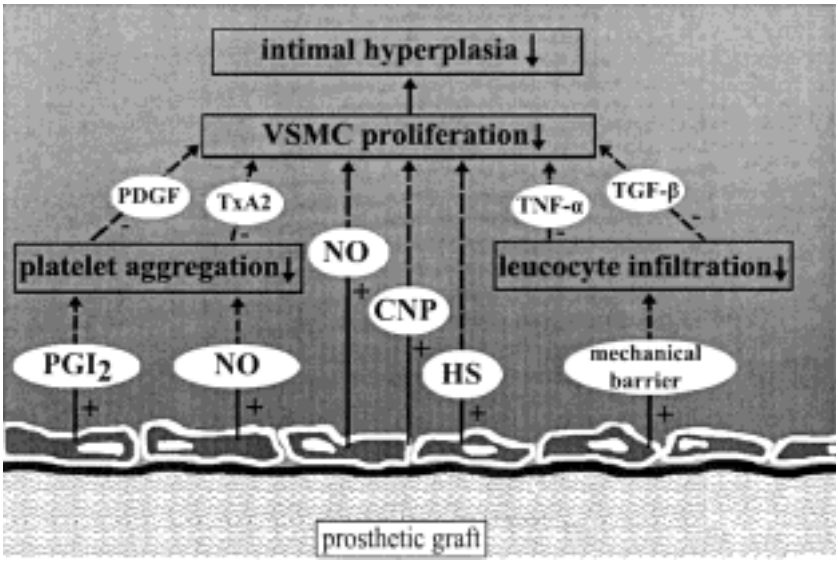

Figure 1: Potential mechanisms of protective effects of seeded endothelial cells on intimal hyperplasia in vascular prosthesis.1. Production of NO, CNP and HS by ECs leads to inhibiton of profileration and migration of VSMCs. 2. Production of NO and pGI_2 by ECs inhibits platelet aggregation, with subsequent reduction in release of PDGF and TxA2 bij platelets. 3. The mechanical barrier formed by ECs prevents infiltration of leucocytes, thereby reducing the production of growth-stimulation cytokines including TNF-α and TGF-β. NO: nitric oxide; CNP: c-type natriuretic peptide; HS: heparan sulphate; pGI_2: prostacyclin; TNF-α: tumor necrosis factor alpha; TGF-β: transforming growth factor beta; PDGF: platelet-derived growth factor; TxA2: thromboxane A2; VSMC: vascular smooth muscle cell.

Previous studies on in vitro graft seeding with mature ECs

Various studies have been performed to evaluate the efficacy of *in vitro* graft seeding using mature ECs. Herring et al were the first to show the benefits of seeding ECs on polyethylene prosthesis in a canine *in vivo* model [5]. Since then, different sources and types of cells, isolation methods, graft materials, graft sizes, coatings of grafts, animal models, antithrombotics and methods of follow up have been used. In subsequent randomized clinical trials, endothelial cell seeding of PTFE-grafts resulted in increased patency rates of arterial bypass-grafts after 3 and 9-year follow up, respectively [6]. However, broader clinical implementation of *in vitro* EC seeding techniques is hampered by the laborious procedures for harvesting, expansion and application of ECs obtained from autologous veins or adipose tissue. These procedures preclude application of *in vitro* graft seeding techniques for acute interventions. In addition, ECs that are seeded onto the luminal surface of prosthetic grafts prior to surgery do not retain com-

pletely after implantation *in vivo* due to a lack of adhesive strength. Moreover, seeded microvascular ECs on macrovascular prosthetic grafts may require phenotypic modulation for optimal functioning, since ECs from diverse tissues and vascular beds are heterogeneous with respect to their surface phenotype and protein expression [1].

Endothelial progenitor cells: a new source of ECs for graft seeding

Bone-marrow derived endothelial progenitor cells (EPCs) have emerged as a promising alternative source of autologous ECs. EPCs are a subset of CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells [7]. Previous studies on *in vitro* seeding of prosthetic vascular grafts using CD34+ progenitor cells revealed marked enhancement of graft endothelialization in animal models. Bhattacharya and coworkers [8] were the first to demonstrate that CD34+ cells, isolated from bone marrow led to increased endothelialization of vascular grafts in dogs. The procedure however was still laborious as 120 mL bone marrow was aspirated, and CD34+ cells needed to be enriched using an immunomagnetic bead technique.

Griese and cowokers [9] described a method for the isolation and expansion of circulating EPCs from peripheral blood and evaluate their therapeutic potential for autologous cell-based therapy of injured blood vessels and prosthetic grafts. The cells needed to be expanded *in vitro* to yield sufficient numbers for therapeutic applications. These cells were successfully transplanted into balloon-injured carotid arteries and into bioprosthetic grafts in rabbits. This technique also led to rapid endothelialization of denuded vessels and graft segments.

Both studies describe a promising concept, but laborious procedures are required to harvest and expand ECs prior to graft seeding. Moreover, both these studies did not report on beneficial effects on IH in the anastomotic region of prosthetic grafts.

In an effort to circumvent the limitation of cell culture prior to graft seeding, we evaluated the efficacy of anti-CD34 antibody coated prosthetic grafts which are thought to bind bone-marrow derived CD34(+) EPCs *in vivo* [10]. By promoting adherence of these endogenous circulating EPCs to the prosthetic graft, the need for *in vitro* seeding procedures can be avoided. This study was conducted in a validated model of prosthetic AV-graft failure in pigs [11]. In this model, AV-grafts are created bilaterally between the carotid artery and the jugular vein. This approach provides a potent model for investigating therapeutic approaches, since one graft can be experimentally manipulated while the other graft is used as a control. Histological and electron-microscopical analysis of anti-CD34 coated prosthetic-grafts showed almost complete coverage of the graft within 3 days after implantation. However, in spite of cellular coverage of the lumi-

nal surface, the intimal hyperplastic response at the venous anastomosis of anti-CD34 coated grafts dramatically increased at 28 days of follow-up (figure 2). These findings show that anti-CD34 coating is successful in promoting cellular coverage of grafts with endothelial-like cells, whereas these trapped cells failed to exert protective effects for IH formation at the venous outflow tract. Although, the exact mechanism of this adverse effect is still unclear, release of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) by adhered ECs may have contributed to the observed increase in VSMC proliferation. Alternative explanations for this proliferative response relate to the capacity of CD34+ pluripotent progenitor cells to differentiate into various cell types. The CD34-epitope is not a specific marker for lineage-committed EPCs. Besides ECs, these cells include platelets, macrophages, granulocytes and VSMCs [12]. The latter cell types have been identified as active participants in the process in neointima formation. Furthermore, adequate maturation of captured CD34+ cells may be hampered by the binding of the immobilized antibody to the CD34-epitope, the turbulent flow pattern at the anastomotic region or the lack of a required micro-environment for optimal function.

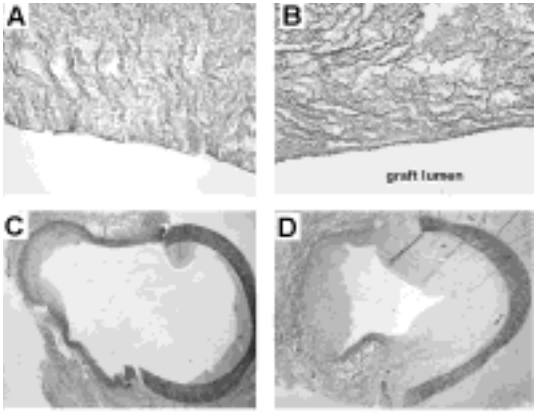

Figure 2: Representative sections obtained at 4 weeks after AV-graft implantation in pigs. Enhanced endothelialization in the anti-CD34 coated grafts coincided with profound increase in IH at the venous anastomosis. Lectin-stained sections of bare (A) and CD34-coated grafts (B) obtained from the center of the graft. Lectin is a marker for ECs. EvG-stained sections of the venous anastomosis of bare-graft (C) and CD34-coated grafts (D). Reproduced with permission. JI Rotmans et al. *Circulation*. 2005;112(1). Fig. 3, p15 (10).*

* See color supplement, starts at page 141.

Future directions for EPC seeding of prosthetic grafts

As described in the previous paragraph, various animal studies on graft seeding using EPCs revealed accelerated graft endothelialisation after *in vitro* or *in vitro* EPC seeding [8-10]. However, in none of these studies, a beneficial effect on intimal hyperplasia or graft patency was observed. Thus far, the exact mechanism of this lack of effect is unclear. The process of progenitor cell differentiation is poorly understood. Various studies revealed that function and amount of EPCs depend on several internal and external entities. Risk factors for coronary artery disease, such as diabetes, smoking and chronic renal failure but also hypercholesterolemia and hypertension are associated with impaired number and function of EPCs [13]. On the other hand, several therapeutic modalities have been developed to counteract the reduction of EPC number and the decreased functional activity in patients with coronary artery disease. Recent studies showed that vascular endothelial growth factor (VEGF), the PPAR-gamma agonist rosiglitazone and HMG-CoA reductase inhibitors (statins) promote differentiation of CD34+ cells into mature ECs [14]. Therefore, adaptation of the matrix composition of the prosthetic grafts using these molecules may ameliorate the vasculoprotective function of seeded EPCs. In addition, accumulating data suggest that an adequate balance between CD34+ and CD14+CD34- cells is required for optimal endothelial cell differentiation and function of EPCs [15]. Therefore, a mixture of CD14+ and CD34+ cells may result in EC coverage with a high potential to improve vascular homeostasis.

Finding the right balance in atheroprotective factors, thereby improving EPC number and function may theoretically ameliorate vascular graft patency when EPC-coated grafts are used. However, this hypothesis needs careful consideration since animal experiments are executed in an environment lacking risk factors that influence EPC number and function.

In conclusion, EPCs have emerged as a promising source for prosthetic graft seeding. The expanding knowledge of EPC function and differentiation should stimulate further research on the therapeutic application of EPCs to improve the poor patency rates of prostethic grafts.

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Chapter 6 Heparin Immobilization Reduces Thrombogenicity of Small-Caliber Expanded Polytetrafluoroethylene Grafts

J Vasc Surg. 2006 Mar;43(3):587-91
JMM Heyligers, HJM Verhagen, JI Rotmans, C Weeterings, PhG de Groot, FL Moll, T Lisman

Background. The patency of small diameter expanded polytetrafluoroethylene (ePTFE) grafts for vascular reconstruction is impaired by acute thrombotic occlusion. Prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade. Heparin is a potent anticoagulant drug widely used to prevent and treat thrombosis. A new ePTFE graft with long-term bonding of heparin is now commercially available in several European countries, but a basic analysis of its mechanism of action in humans has never been described. The present study was performed to evaluate the thrombogenicity of heparin-bonded ePTFE grafts compared to standard ePTFE in a newly developed human *ex vivo* model.

Methods. Non-anticoagulated blood was drawn from antecubital veins of 10 healthy donors with a 19-G needle. The proximal end of a 60 cm ePTFE vascular graft with a diameter of 3 mm was connected to the needle, while the distal end was connected to a syringe, which was placed in a syringe pump. Every volunteer served as its own control, by using a heparin-bonded ePTFE graft on one arm, and a standard ePTFE graft on the other arm. The perfusions were performed over 6 minutes with a flow rate of 20 ml/minute corresponding to a shear rate of 74/sec. Serial samples were taken at the distal end of the graft for determination of prothrombin fragment F_{1+2}, fibrinopeptide A (FPA), and P-selectin expression on perfused platelets. Fibrin deposition and platelet deposition were studied using Scanning Electronic Microscopy (SEM).

Results. FPA production over time was significantly reduced on the heparin-bonded ePTFE grafts compared to standard ePTFE grafts ($p<0.05$). There was no increase in the production of F_{1+2} or P-selectin over time on either type of graft. SEM scanning showed platelet deposition and fibrin formation on standard ePTFE grafts, while no platelets or fibrin were observed on heparin-bonded ePTFE grafts.

Conclusions. Heparin immobilization substantially reduces thrombogenicity of small diameter ePTFE in a newly developed human *ex vivo* model. Here, we provide evidence that the mechanism of action of the heparin bonding is not only due to anti-coagulant, but also to anti-platelet effects. Heparin bonding may be an important improvement of ePTFE, expectantly resulting in better patency rates for vascular reconstructions.

Introduction

It is well known that an autologous vein graft is the surgeons' first choice in peripheral arterial bypass procedures, because of superior patency rates when compared to prosthetic grafts. In a recent review on venous and polytetrafluoroethylene (PTFE) above-knee femoropopliteal bypasses,

5-year primary patency rates of 74% and 39% respectively, were reported[1]. However, almost a third of patients who undergo peripheral arterial reconstructive operations do not have suitable autologous veins available for grafting[2]. Therefore, prosthetic grafts, such as PTFE grafts, are frequently used in arterial bypass procedures.

Apart from this clinical evidence, laboratory models also have shown that PTFE grafts are substantially more thrombogenic compared to autologous veins. Prosthetic materials cause platelet adhesion and activation of the coagulation cascade on the graft[3]. One potential strategy for reducing thrombogenicity of prosthetic material is to bind heparin to the endoluminal surface. Heparin is a polysaccharide anticoagulant with potent inhibitory effects on coagulation and a long history of clinical use in prevention and treatment of thrombosis[4]. Heparin-bonded (or heparinized) grafts have shown favourable results in animal models and humans, compared to untreated vascular grafts [5-7]. A new expanded PTFE (ePTFE) graft (GORE-TEX Propaten® vascular graft, W.L. Gore and Associates, Flagstaff, AZ) with long-term bonding of heparin accomplished by covalent linkage of the anticoagulant is now commercially available in those European countries (except France) that accept the
CE certification.

An *in vivo* canine study showed measurable heparin activity on these heparinized ePTFE grafts throughout a 12-week observation period, with only a marginal drop in activity after 12 weeks[5]. These results indicate that this particular heparin immobilisation method yields grafts that express anticoagulant properties for prolonged periods after implantation. Although the anticoagulant mechanism of action of intravenous heparin is well established, little is known about the precise mechanisms by which immobilization of heparin on vascular grafts reduces the thrombogenicity of ePTFE in humans. In this study we evaluated the potential mechanism of action by analysis of the thrombogenicity of standard ePTFE and heparinized ePTFE grafts by a comprehensive investigation of clotting parameters and deposition of platelets and fibrin in a newly developed human *ex vivo* model.

Methods

Ex vivo model

In a newly developed *ex vivo* model, non-anticoagulated blood was drawn from the antecubital veins of 10 healthy donors with a 19-G needle. A 60 cm vascular graft with a diameter of 3 mm was directly connected to the needle (figure 1). During 6 minutes, the blood was aspirated with a constant flow rate of 20 ml/min using a syringe pump (Harvard Apparatus,

South Natick, Mass). This flow rate and graft diameter results in a shear rate of 74/sec, which reflects venous flow conditions and favors fibrin-rich clot formation. A cuff was wrapped around the upper arm to ensure a constant pressure of 45 mm Hg, resulting in a continuous bloodflow through the graft during the experiment. Volunteers denied taking any medication 2 weeks prior to the experiment, and gave informed consent.

Every volunteer served as its own control. In the first run, standard GORE-TEX® ePTFE graft was perfused using blood drawn from one arm. In the second run, which was performed within half an hour of the first, GORE-TEX® ePTFE graft treated with Carmeda® BioActive Surface (CBAS) technology was perfused with blood drawn from the other arm. CBAS® is a clinically used heparin binding technology. This technology is based upon covalent end-point attachment of heparin to a biomaterial surface, enabling maintenance of functional heparin bioactivity.

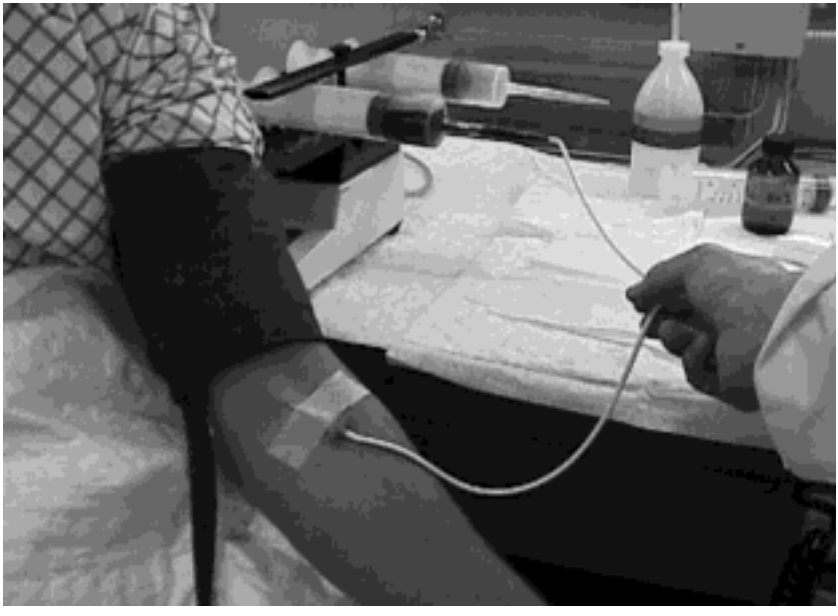

Figure 1: The newly developed *ex vivo* perfusion system using a cuff at a constant pressure of 45 mm Hg to ensure bloodflow. Blood is aspirated through the graft with a constant flow of 20ml/min, resulting in a shear rate of 74/sec.*

* See color supplement, starts at page 141.

Heparin-bonded ePTFE grafts

Standard ePTFE GORE-TEX® vascular grafts (internal diameter, 3 mm; length, 60 cm; W. L. Gore and Associates, Flagstaff, Arizona) were used in our study. The luminal microstructure of the heparinized grafts was treated with the Carmeda BioActive Surface® in which heparin molecules are covalently bound via endpoint aldehyde linkages to free amino groups in an underlying polyethyleneimine (PEI) sublayer. Endpoint attachment permits the pentasaccharide active sites on the heparin to remain available for binding anti-thrombin III (AT-III). The procedure to modify heparin with free aldehyde moieties has been described previously[5, 6]. Standard non-heparinized ePTFE grafts were steam sterilized; heparinized ePTFE grafts were sterilized with ethylene oxide.

Blood samples and assays

Blood samples (900 µL) were collected at the end of the graft, starting directly after connection to the vein and thereafter every minute until 4 minutes. After 4 minutes, samples were collected every 30 seconds until the end of the perfusion (total perfusion time = 6 minutes per arm). The samples were mixed immediately with 100-mL of ethylenediamine tetraacetic acid (500 mmol/L) and centrifuged at 3500 rpm for 5 minutes, and aliquots of plasma were stored at –20°C until assayed.

A commercially available enzyme-linked immunosorbent assay (ELISA) was used for the fibrinopeptide A (FPA) measurements as an indicator for fibrin formation (Zymutest FPA, Hyphen Biomed, Andresy, France). Prothrombin fragment 1+2 (F_{1+2}), an indicator for thrombin formation, was also measured using an ELISA (Enzygnost–F_{1+2}, Dade Behring, Marburg, Germany). P-selectin expression on perfused platelets, as an indicator for activation of platelets, was determined in whole blood (=non-anticoagulated blood). Blood was collected in paraformaldehyde (4%) by flow cytometry analysis using an R-phycoerythrin conjungated monoclonal antibody according to the instructions of the manufacturer (Dako, Glostrup, Denmark, Code No R7200).

Scanning Electron Microscopy

The deposition of platelets and fibrin onto the grafts was visualized by scanning electron microscopy (SEM). Therefore, the distal end of the graft was cut into small pieces (5x5 mm), fixed in 2% glutaraldehyde, and then dehydrated through increasing concentrations of ethanol (80% to 100%). The samples were dried with the use of Hexamethyldisilazane. Next, ePTFE pieces were sputter-coated with a thin layer of Platinum/Palladium and analyzed with SEM (Philips XL30, Eindhoven, The Netherlands).

Statistical analysis
Results are expressed as the mean ± the standard error of the mean. A paired t-test was used to determine the significance of differences between groups. A p-value of <.05 was considered significant.

Results

In our newly developed *ex vivo* model, non-anticoagulated blood was drawn directly from the antecubital veins over either standard GORE-TEX® ePTFE vascular grafts, or grafts to which heparin was covalently bonded by means of CBAS® technology. All 10 perfusions were performed without any complications, concerning the volunteers as well as the set-up itself.
On standard grafts, platelet deposition and fibrin formation was observed by SEM, whereas on heparinized grafts no platelets or fibrin deposits were observed (figure 2).
On standard grafts, a progressive increase in the production of FPA was observed in time. FPA production on the heparinized grafts was substantially depressed compared to the standard grafts. A significant difference in FPA values between standard and heparinized grafts was observed at timepoint 5 minutes and later (figure 3).
No increase in F_{1+2} levels in time was observed on either graft (figure 4), nor was an increase of P-selectin expression in time noted on platelets in the perfusate of either graft (figure 5).

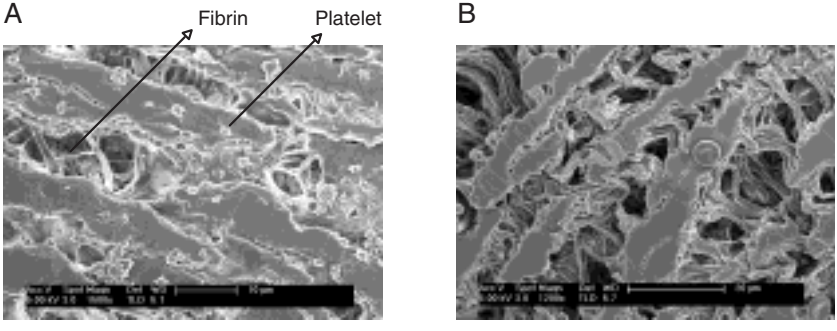

Figure 2: Scanning Electronic Microscopy showing platelet adhesion and fibrin deposition (arrows) on the untreated GORE-TEX® ePTFE vascular graft perfused for 6 minutes with non-anticoagulated whole blood (A) and no such adhesion or deposition on the GORE-TEX® graft treated with CBAS® technology (B). Representative pictures of a single volunteer are shown.

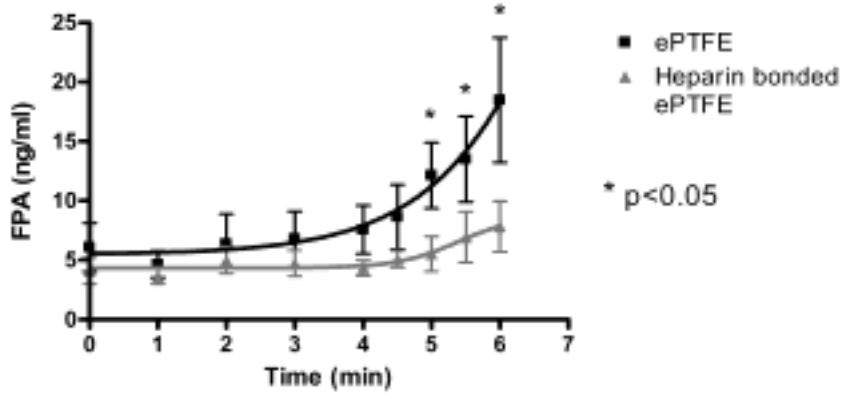

Figure 3: Heparin immobilization reduces fibrin formation during *ex vivo* perfusions. Perfusions were performed in 10 volunteers, using both a non-coated and heparin-bonded graft in each volunteer. Serial samples were taken during perfusion, in which levels of FPA were measured by ELISA. Error bars indicate standard error of mean.*

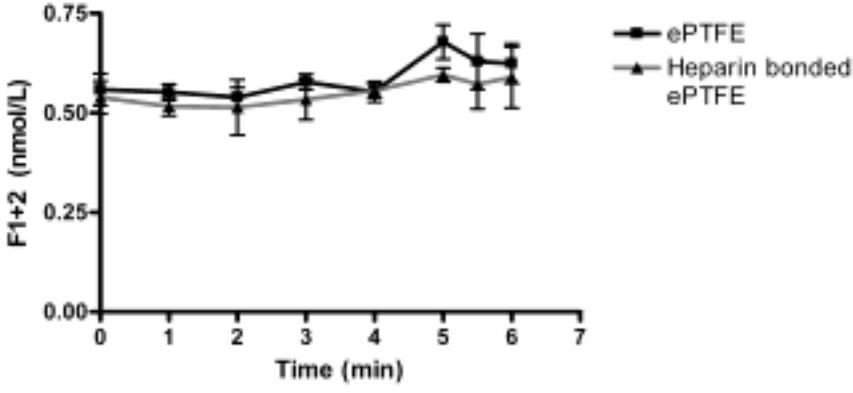

Figure 4: Progression of F1+2 levels in the perfusate over time. There was no increase in F_{1+2} in the perfusate over time on both types of vascular grafts, indicating that no measurable amount of thrombin was generated. Error bars indicate standard error of mean.*

* See color supplement, starts at page 141.

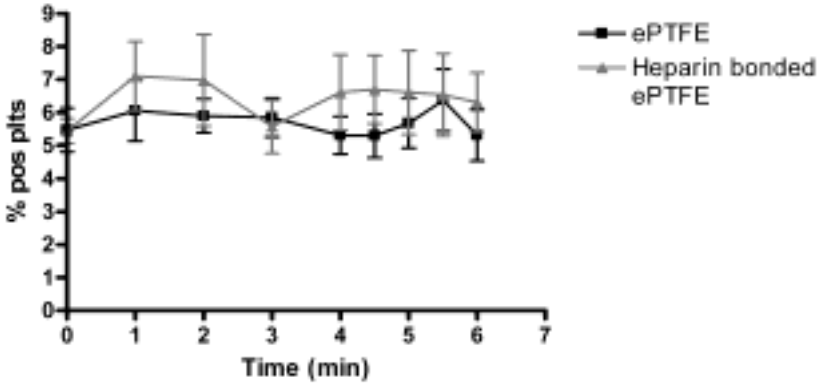

Figure 5: Progression of the percentage of P-selectin positive platelets in the perfusate over time. P-selectin expression on the perfused platelets did not increase over time on either graft surface, indicating that no measurable amount of platelets was activated. Error bars indicate standard error of mean.*

Discussion

In the present study, we evaluated in a novel *ex vivo* set-up the thrombogenicity of small diameter (3 mm) ePTFE grafts treated with CBAS® technology compared to standard GORE-TEX® ePTFE vascular grafts. The advantage of this model, in contrast with other *ex vivo* models, is that the blood of the volunteer does not come into contact with other surfaces than the materials investigated. In this set-up every volunteer was its own control.

We assessed the standard untreated ePTFE always in the first run. We hypothesized that if there was any *in vivo* activation of the coagulation cascade as a consequence of the procedure after the first run, the outcome of the second run might be influenced. Therefore the condition in which inhibition of thrombogenicity was anticipated was always performed last. However, we did not observe any differences in baseline F_{1+2} and FPA levels in the first and second run, indicating that systemic coagulation activation by the procedure is unlikely. Moreover, we did observe a substantially decreased production of FPA by heparinized grafts in the second run, indeed indicating that thrombogenicity of heparinized grafts is reduced. If an unmeasurable activation of hemostasis was present after the first run, the results of this study might even be underestimated.

* See color supplement, starts at page 141.

We found a substantial reduction in thrombogenicity of the ePTFE grafts as a result of the heparin bonding. Platelet adhesion and fibrin deposition were only observed on the surface of the standard grafts. Moreover, a significant reduction in FPA production was observed on heparinized grafts when compared to standard grafts. There was no increase in F_{1+2} in the perfusate over time on both types of vascular grafts, indicating that no measurable amount of thrombin was generated. P-selectin expression on the perfused platelets did not increase over time on either graft surface, indicating that no measurable amount of platelets was activated.

The discrepancy between the increase in FPA levels and the absence of significant F_{1+2} production can be explained by the fact that one single thrombin molecule cleaves many fibrinogen molecules into fibrin. Apparently, the amount of thrombin generated is too small to be detected by the F_{1+2} assay used.

In a recently published study by Keuren et al., comparing heparinized and non-heparinized collagen surfaces, it was noted that thrombin activity became detectable after 12 minutes, and peaked after 20 minutes[8]. In contrast with our study, these experiments were performed with recalcified anticoagulated blood. The time course of coagulation in this more or less artificial system will be different from the time course *in vivo* or in our *ex vivo* experiments. Although detectable thrombin generation might have occurred with longer perfusion times, this was not feasible due to the substantial amounts of blood that were used in this experiment (2 times 120 ml of blood was drawn). The clinical extrapolation of our results might therefore be limited to the mechanism of action by which the heparinized graft reduces acute graft thrombosis, although the mechanism by which delayed thrombosis is accomplished is presumably highly similar.

The fact that P-selectin expression did not increase over time on either graft surface may be explained by the limited perfusion time in this model. During the 6 minutes of perfusion, only a small percentage of platelets become activated as a result of the platelet activating properties of ePTFE. These activated platelets are able to adhere to the graft surface, as shown by SEM. It is conceivable that those platelets that are activated by ePTFE also adhere to the graft, explaining why no activated platelets are found in the blood collected at the distal end of the graft.

Two potential mechanisms may be responsible for the reduced thrombogenicity of the heparinized grafts. First, the bonded heparin reduces the formation of thrombin due to the inhibition of coagulation. As a result, both fibrin formation and thrombin-mediated platelet activation are diminished. Second, the negatively charged surface directly prevents platelet interaction with the graft due to electrostatic repulsion, as the net charge of the platelet is also negative[9]. The phenomenon of reduction of platelet adhesion using covalent heparin immobilization has not been shown in humans before.

As the patency of small diameter ePTFE grafts for vascular surgery is impaired by graft occlusion due to acute thrombosis, the application of the CBAS® technology to vascular grafts may reduce this acute problem. This study is the first to prove that thrombogenicity is reduced in a human *ex vivo* model using the CBAS® technology on GORE-TEX® ePTFE vascular grafts. In concordance with our results, a recent study on small-caliber CBAS® heparin-coated ePTFE grafts showed significantly reduced platelet deposition in arterial grafts in baboons[5]. In addition, reduction of anastomotic neointimal hyperplasia and cell proliferation, without measurable side effects was observed. A recently published prospective randomized trial describes patients with femoropopliteal bypass, using a different kind of heparin bonding technique on vascular grafts. The authors show significantly increased patency rates after 3 years of follow up and a reduced incidence of major limb amputation, compared with non heparin-bonded vascular grafts[7].

In conclusion, heparin bonding reduces the thrombogenicity of ePTFE grafts. Here we provide evidence that the mechanism of action of the heparin bonding is not only due to anti-coagulant, but also to anti-platelet effects. Heparin bonding may be an important improvement of ePTFE, expectantly resulting in better patency rates for vascular reconstructions.

Acknowledgements

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Chapter 7

A Heparin-Bonded Vascular Graft Generates no Systemic Effect on Markers of Haemostasis Activation or Detectable HIT-Inducing Antibodies in Humans

JMM Heyligers, T Lisman, HJM Verhagen,
C Weeterings, PhG de Groot, FL Moll

Background. In peripheral bypass procedures, almost a third of patients do not have suitable veins making the use of prosthetic materials necessary. Prosthetic materials can cause platelet adhesion and activation of the coagulation cascade on the graft. One potential strategy to reduce this thrombogenicity is to covalently bind heparin to the endoluminal surface of grafts.
This human *in vivo* study examines systemic effects of the endoluminal heparin, and addresses whether graft implantation results in:
1) A measurable reduction of systemic markers of haemostasis activation as compared to control grafts.
2) Antibody formation against heparin, potentially responsible for HIT (= heparin-induced thrombocytopenia).

Methods. Sixteen patients undergoing femoro-popliteal bypass grafting were included in the study. Eight patients received a standard GORE-TEX® Thin Walled Stretch Vascular Graft and 8 received a Heparin-bonded ePTFE Graft (GORE-TEX® Propaten Vascular Graft). Blood samples were drawn pre- and directly postoperatively and at days 1, 3, 5 and week 6 after surgery. Established markers of *in vivo* activation of platelets and blood coagulation (F_{1+2}, FPA, sGPV, TAT complexes, and D-Dimers) were measured using standard commercially available techniques. Furthermore, anti-PF4/heparin antibody titres were measured using a commercially available ELISA, and platelet counts were determined.

Results. No statistical differences were observed in any of the markers of *in vivo* activation of platelets and blood coagulation between patients receiving PROPATEN or control ePTFE. Moreover, no antibodies against heparin could be demonstrated up to six weeks after implantation.

Conclusions. No measurable effect of heparin immobilization on systemic markers of haemostasis was found using a heparin-bonded ePTFE graft *in vivo*. Also, no antibodies against heparin could be detected up to 6 weeks after implantation.

Introduction

Almost a third of patients that need peripheral arterial reconstructive operations do not have suitable autologous veins available for grafting [1]. For that reason, prosthetic grafts, such as polytetrafluoroethylene (PTFE) grafts, are frequently used in arterial bypass procedures. The patency of autologous veins, however, is better when compared to prosthetic grafts. In a recent review comparing venous and PTFE above-knee femoropopliteal bypasses, 5-year primary patency rates of 74% and 39% respectively, were reported [2].
Apart from this clinical evidence, laboratory models also have shown that

PTFE grafts are substantially more thrombogenic compared to autologous veins. Prosthetic materials can cause platelet adhesion and activation of the coagulation cascade on the graft [3]. One potential strategy to reduce this thrombogenicity is to covalently bind heparin to the endoluminal surface of grafts.

Heparin is a polysaccharide anticoagulant with potent inhibitory effects on coagulation and a long history of clinical use in prevention and treatment of thrombosis [4]. Long-term systemic use of heparin is hampered by the immunogenicity of the heparin-platelet factor 4 complex. Heparin-induced thrombocytopenia without (HIT) or with thrombosis (HITT) is a serious complication of heparin administration. Even short term administration of heparin may be associated with HIT or generation of antibodies against the heparin-platelet factor 4 complex without clinically overt symptoms. These antibodies lead to platelet activation resulting in thrombocytopenia as a consequence of immune-mediated clearance and consumption. Furthermore, these antibodies may lead to activation of the coagulation cascade resulting in thrombosis, which occurs in approximately one-third of patients with HIT [5]. These thromboses are clinically relevant in about 50% of the patients, and these patients have a poor prognosis; 20% will lose a limb, and 30% eventually dies [6].

Heparin-bonded (or heparinized) grafts have shown favourable results with respect to graft patency in animal models and humans, compared to untreated vascular grafts [7-9]. In fact, a recent analysis showed that the heparinized graft used in this study gives patency rates comparable to that of autologous veins [10]. The beneficial effects of heparinized grafts appear particularly caused by a substantial reduction in acute graft thrombosis, within weeks after implantation. Clinical evidence thus suggests heparin-bonded grafts to be superior over untreated grafts. Studies on the mechanism by which heparin immobilized on the grafts prevents graft thrombosis are scarce. A new expanded PTFE (ePTFE) graft with long-term bonding of heparin accomplished by covalent linkage of the anticoagulant is now commercially available in several European countries. We recently developed a human *ex vivo* model comparing this heparin-bonded with untreated ePTFE and concluded that heparin bonding substantially reduces the thrombogenicity of ePTFE grafts [11]. Whether this translates in a systemic anticoagulant effect is unknown, and this will be the focus of our study.

The fact that the heparin is covalently bonded to these grafts raises an important question with respect to antigenicity. It is unknown whether surface-bonded heparin, like heparin in solution, is able to elicit an immunogenic response.

Thus, this study will therefore examine systemic effects of the local presence of heparin, and specifically addresses whether graft implantation results in:

1) A measurable reduction of systemic markers of haemostasis activation as compared to control grafts.
2) Antibody formation against heparin, potentially responsible for HIT.

The thrombotic markers used in the proposed study are generally accepted as predictors of thrombotic complications *in vivo* [10].

Material and methods

Study population. The study population included sixteen human subjects all of whom have signed a patient informed consent form to participate in the study. Each study subject received an infrainguinal bypass with a GORE-TEX e-PTFE Vascular Graft. Eight subjects received a GORE-TEX® Propaten Vascular Graft and 8 subjects received a GORE-TEX® Thin Walled Stretch Vascular Graft. Standard intraoperative and postoperative medication regimens applied. Intraoperative heparin, if deemed necessary by the surgeon, was not reversed.

Vascular grafts. All vascular grafts used in the study were GORE-TEX e-PTFE Vascular Grafts from standard hospital inventory. Patient assignment to a specific graft-type treatment group, GORE-TEX Propaten Vascular Graft or GORE-TEX Thin Walled Stretch Vascular Graft, followed a systematic, alternating-type methodology initiated at the beginning of the study to establish the starting treatment group assignment and the order of subsequent treatment group assignments.

Blood sampling. Peripheral blood samples were collected preoperatively, directly postoperatively prior to administration of postoperative thrombophrophylactic low molecular weight heparin (LMWH, Fragmin 2500 or 5000 IU once daily), and at subsequent postoperative intervals of 1, 3, and 5 days and also at 6 weeks. All postoperative blood samples were collected immediately prior to administration of daily LMWH during the hospital stay. All blood samples were obtained from the antecubital vein according to standardized venapuncture procedures. Blood was collected in tubes containing sodium citrate. Although this study was not performed blinded for the surgical team, the samples were blinded prior to the execution of the laboratory analyses.

Systemic marker assays

- F1+2 in blood samples were measured with an F1+2-specific ELISA (Enzygnost F1+2 Micro, Dade Behring, Marburg, Germany).
- FPA was measured with an FPA ELISA assay (Zymutest FPA, Hyphen Biomed, Andresy, France).
- TAT was measured with a TAT-specific ELISA (Diagnostica Stago, Asnieres-Sur-Seine, France).

- Soluble GPV was measured with an sGPV ELISA Kit (Diagnostica Stago, Asnieres-Sur-Seine, France)
- Levels of D-dimer were measured with an Asserachrom D-DI ELISA kit (Diagnostica Stago, Asnieres-Sur-Seine, France)
- Antibodies against the heparin-platelet factor 4 complex were measured by a sensitive ELISA assay, which is able to detect low-titre antibodies. Anti heparin antibody assay (Diagnostica Stago, Asnieres-Sur-Seine, France)
- Platelet counts were determined according to standard hospital methods.

Statistical analysis

Data for systemic markers in the peripheral blood samples from patients with GORE-TEX Propaten and GORE-TEX Thin Walled Stretch Vascular Grafts were compared at each individual time-point with a standard t-test. Progression of platelet counts in time was assessed by repeated one-way analysis of variance (ANOVA) with Tukey's post-test. P values <.05 were considered statistically significant.

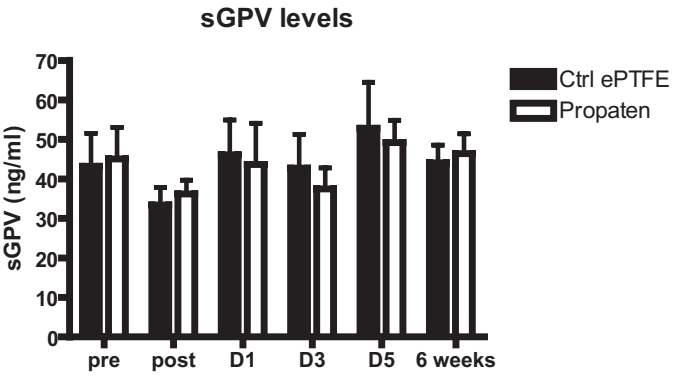
Recruitment of patients

- Patients underwent physical examination by a medical doctor directly after operation, before discharge and at the outpatient clinic after 6 weeks, checking the status of the graft implanted.
- Patients were 18 years or older and capable of making the decision whether to participate or not.
- Patients with a known allergy to heparin were excluded.

Results

Seventeen patients were initially included. Of them, 6 were women. The average age of this population was 72 (range 62-78). All patients had uncomplicated operation procedures and all bypasses were realised infrainguinal. All patients were discharged within one week after operation. One patient was excluded from the analysis, as systemic heparin infusion was needed during four days postoperatively, due to anticipated risk of progressive leg ischemia after the procedure. However, we did measure all markers in this patient as a decline in platelet count or an antibody against heparin might be detected. Therefore, 16 patients were analyzed as a group and the one patient was analyzed separately for the above mentioned reasons.

At the long term follow up (six weeks) all grafts were patent, as was concluded from the physical exam at the outpatient clinic.
No substantial or statistically significant differences were observed in any of the markers of *in vivo* activation of platelets and blood coagulation between patients receiving Propaten or control ePTFE grafts (figure 1). Platelet counts stayed relatively constant after surgery. In the Propaten group, a 20% decrease in platelet count was observed at day 1 after surgery as compared to baseline values, but this difference was not statistically significant, and platelet counts were already normalised again at day 3. At day 5, platelet counts were 23% higher as compared to baseline values in the Propaten group ($p<.05$, figure 2). In contrast, the one patient that was not included in the study population because of administration of i.v. heparin, yet analyzed, showed a progressive decrease in platelet count after surgery, with a percentual nadir value of 55% compared to the platelet count prior to surgery (figure 3). Moreover, antibodies against heparin were detected at day 3 and 5, without clinically overt manifestations of HIT. At week 6, the amount of platelets almost returned to the preoperative count. At that time point antibodies against heparin could still be detected. On the other hand, no antibodies against heparin could be demonstrated up to six weeks after implantation in the primary study population.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sGPV (ng/ml)
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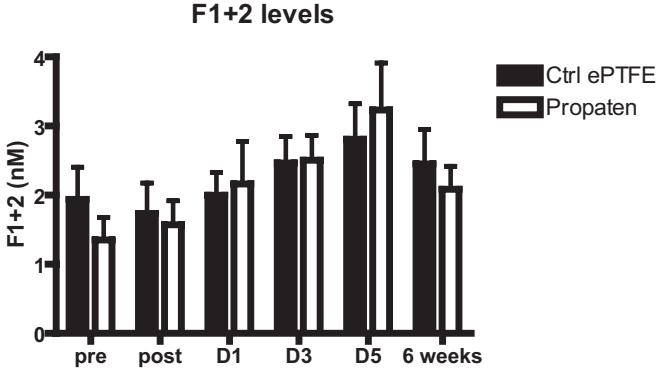
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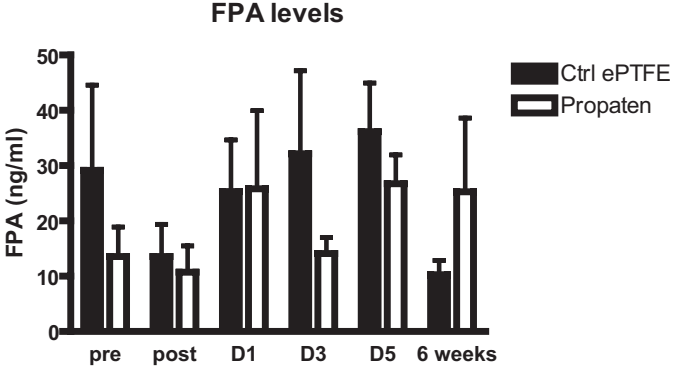
FPA levels
FPA (ng/ml)
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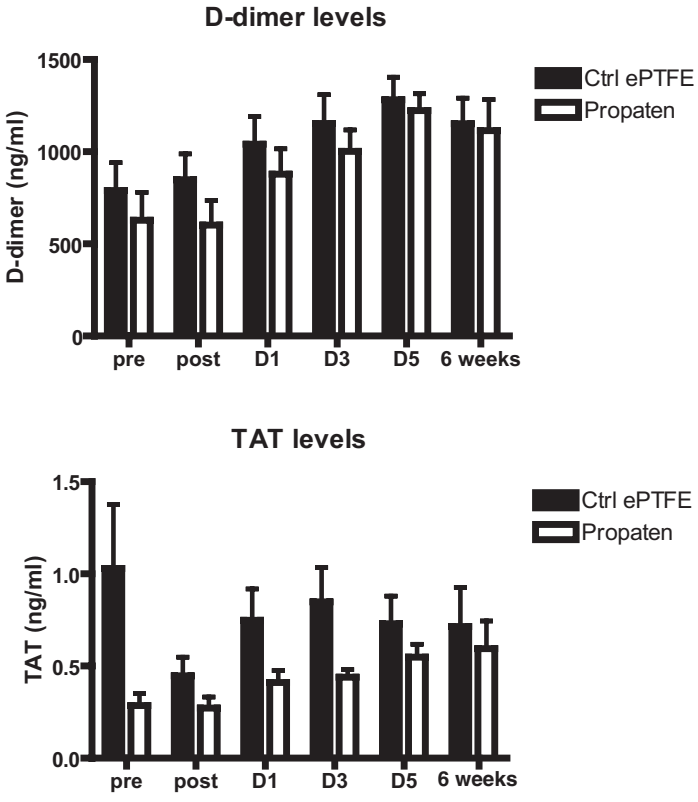

Figure 1: Mean levels of markers of coagulation or platelet activation in patients receiving plain ePTFE or Propaten grafts at various timepoints after surgery as compared with preoperative levels. Error bars indicate standard error of mean (SEM).

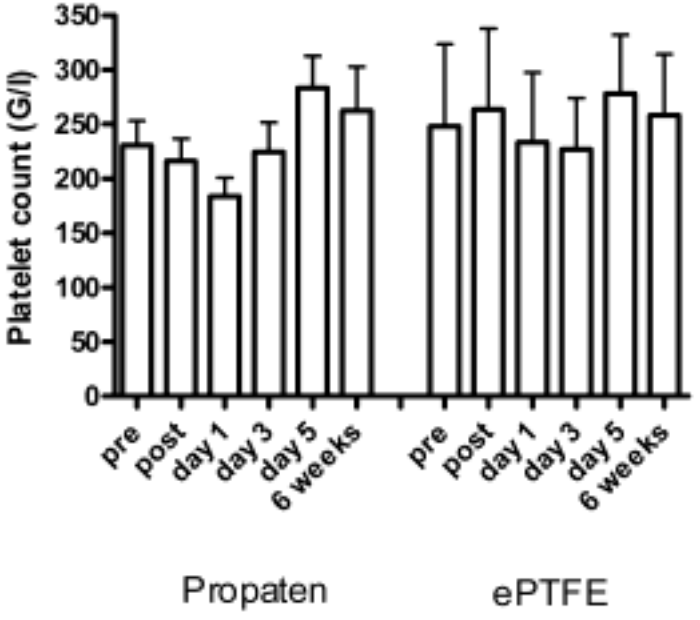

Figure 2: Mean platelet count of patients receiving either a Propaten or ePTFE graft at various time-points before and after surgery. Error bars indicate standard error of mean (SEM).

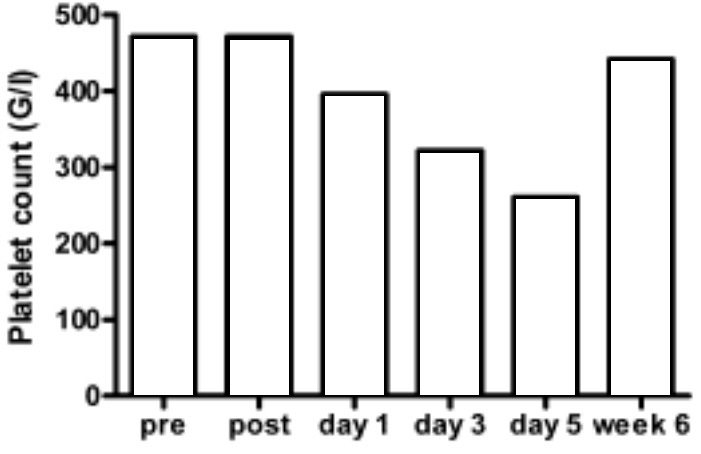

Figure 3: Absolute platelet count of one patient who received an ePTFE graft that was excluded from the analysis as systemically heparin was needed during four days postoperatively. A 45% decline in platelet count was observed at day 5, which recovered up to normal at week 6. Moreover, she did develop antibodies against the Heparin/PF4 complex.

Discussion

In the present study, we evaluated in a human *in vivo* setup whether implantation of an infrainguinal heparinized graft results in:

1) A measurable reduction of systemic markers of haemostasis activation as compared to control grafts.
2) Antibody formation against heparin, potentially responsible for HIT.

Recent data show that the short term one year primary patency of below-knee Propaten bypass grafts is approximately 80%, which is comparable to vein grafting [10]. The reason for this improved patency when compared to standard ePTFE grafts (66% primary patency) is unknown. From a prospective multi-center trial describing a 5 year follow up study using a heparinized Dacron graft compared with PTFE graft, we know that the beneficial effects of the heparin-graft were particularly evident in the early weeks after implantation and sustained thereafter [9]. This suggests that the thrombogenicity reduction due to the heparinization resulting in a decreased occurrence of acute graft thrombosis is –at least in part- responsible for the improved patency in favour of the heparin-bonded Dacron. Another explanation for the improved patency might be the reduction of the development of intimal hyperplasia (IH), as heparin is known to reduce the migration of smooth muscle cells, responsible for the formation of IH [12-16].

However, our research group recently concluded from a human *ex vivo* model comparing heparinized with untreated ePTFE that heparin bonding substantially reduces the thrombogenicity of ePTFE grafts [11]. But it was still unknown whether this translates in a systemic anticoagulant effect. It is also unknown whether antibodies can be formed against the locally bonded heparin. This might be of great clinical importance, not only because of the possible development of HIT(T), but also because of (clinically non relevant) antibody formation against heparin which might imply a life-time prohibition of heparin use.

We could not demonstrate an effect of heparin immobilization on systemic markers of haemostasis using both markers of platelet activation and fibrin generation. In contrast, our recent *ex vivo* study did show profound inhibitory effects of heparin immobilization on platelet deposition and fibrin formation [11]. The reason that the local anti-thrombotic effects of the immobilized heparin does not translate to a detectable reduction of markers of platelet activation or fibrin generation measured in the systemic circulation may be explained by a dilutional effect. The implanted heparinized grafts only constitute a minor fraction of the entire vasculature and this may explain why the local inhibition of platelet activation and fibrin generation by the heparin on the graft does not result in a detectable systemic anti-thrombotic effect.

We did not detect any antibodies against heparin/PF4 in all patients who received a Propaten graft, even after 6 weeks of implantation. We did observe a 20% decrease in platelet count in the Propaten group 1 day after surgery, but this decrease was not statistically significant, and resolved again at day 3, so we do not believe that this temporal drop in platelet count reflects the activity of very low titre (undetectable by our assay) heparin-PF4 antibodies. The fact that we do not detect antibodies against heparin/PF4 even at 6 weeks after surgery might imply that the immobilized heparin does not elicit an immune response such as frequently observed with systemic (i.v.) heparin. The apparent non-immunogenic nature of the Propaten grafts is in contrast with grafts to which heparin is coated non-covalently. With these grafts the heparin was observed to be immunogenic, as a result of leakage of heparin from the graft [17;18]. This leakage does not occur with Propaten grafts to which heparin is bonded covalently [7;8].

While systemic heparin infusion is only possible for a few days, because of the high risk of antibody development against heparin/PF4 potentially leading to HIT(T), implantation of Propaten grafts does not result in formation of these antibodies even after 6 weeks. The immobilized heparin is still active after 6 weeks as shown by animal experiments. To our knowledge, this is the first report of surveillance of antibody development after prolonged (6 weeks) *in vivo* exposure to (immobilized) heparin.

A single patient did receive i.v. heparin after implantation of an ePTFE graft and had a substantial drop (45%) in platelet count associated with the development of low-titre anti heparin/PF4 antibodies in the absence of overt HIT, indicating that clinically silent HIT can be detected with our assay methods.

In conclusion, the results of this study indicate that heparin coated vascular grafts do not result in a measurable systemic thromboresistant effect, but also do not result in development of HIT-inducing antibodies even after 6 weeks of implantation. The reason for the lack of immunogenicity of immobilized heparin is unclear, but is beneficial as this does not prohibit future use of intravenous heparin.

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Chapter 8 General Discussion and Summary

Background

It is well known that autologous grafts are superior to prosthetic grafts when comparing patency rates in peripheral bypass surgery. In a recent review comparing venous and polytetrafluoroethylene (PTFE) above-knee femoropopliteal bypasses, 5-year primary patency rates of 74% and 39% respectively, were reported [1]. However, almost a third of patients who undergo peripheral arterial reconstructive operations do not have suitable autologous veins available for grafting [2] For that reason, prosthetic grafts, such as PTFE grafts, are frequently used in arterial bypass procedures.

The patency of prosthetic vascular grafts is impaired not only by intimal hyperplasia (IH) near the anastomotic regions [3], but also by acute thrombotic occlusion. The absence of a functional endothelial monolayer on the prosthetic grafts is an important stimulus for IH. Also, prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade. Graft seeding with autologous endothelial cells (EC) has been shown to increase patency rates of prosthetic bypass-grafts in clinical trials [4-6]. The beneficial effects of endothelialized grafts are contributed to both a reduction in IH [7], and a reduction of thrombogenicity [8], as endothelial cells are known to influence both entities [9-11]. However, *in vitro* EC seeding is time consuming and therefore not suitable for (semi)acute vascular bypass grafting.

These shortcomings of the use of prosthetic grafts are the basis for the studies described in this thesis.

Outline

Two novel strategies to reduce thrombogenicity of vascular grafts are described in this thesis.

To improve the outcome of synthetic vascular bypass surgery, cell seeding is a promising concept that has extensively been investigated and is still evolving. To improve the short term effects due to acute thrombotic occlusion, thrombogenicity of prosthetic material could be reduced by both 'endothelialization' of the graft or heparin bonding to the endoluminal surface. Both strategies potentially also reduce IH, as both endothelial cells and heparin are known to influence the process of IH development [12-16].

Therefore, the emphases of the studies described were: 'auto-endothelialization' and 'thrombogenicity-reduction' of the vascular PTFE graft.

The central questions of this thesis were:

1. Can auto-endothelialization of grafts be realised using anti-CD34 coating?
2. Does this coating reduce intimal hyperplasia?

And

3. Does heparin immobilization reduce thrombogenicity of vascular grafts?

4. Is there a systemic effect of the locally immobilized heparin on the vascular grafts?

To answer these questions *in vitro*, *ex vivo* and *in vivo* studies were performed, which are described in this thesis.

The history of cell seeding

In **Chapter 2** a review describes the evaluation of the concept of cell seeding and other tissue engineering concepts to improve the outcome of non venous vascular bypass surgery [17]. The hypothesis of cell seeding using different types of cells as coverage of the endoluminal vascular graft, was explained in detail. The concept of endothelial cell seeding of vascular grafts is based on the assumption that these cells will constitute a biologically active lining that will not only attenuate activation of blood passing through the graft, but also as a result of inhibition of hemostasis activation will reduce the release of mitogens for vascular smooth muscle cells (VSMCs). The most promising cell type for seeding is the endothelial cell (EC) which must be harvested from veins or fat tissue, before it can be used as cell coverage on the endoluminal surface. An endothelialized vascular graft is – in theory- comparable to veins, as native ECs cover the inner surface of veins. Indeed, graft seeding with autologous ECs has been shown to increase patency rates of bypass grafts in clinical trials [5;18]. However, broad clinical application of *in vitro* seeding is hampered by the laborious procedures for harvesting and expansion of isolated ECs. For (semi)acute vascular bypass procedures, the ideal conduit is the one that the surgeon can simply pick off the shelf, implant in the patient, and which also resembles the patency of venous bypasses. Because of the time consuming drawback of the currently available techniques, a new concept of 'auto-endothelialization' was realised and described in **chapter 3.**

Anti-CD34 antibody coating: the concept of 'auto-endothelialization'

The *first strategy* described in this thesis to improve the patency of vascular prostheses is the use of *anti-CD34 antibody coating*.

- *In vivo* porcine study

This technique of auto-endothelialization, using an antibody against precursors of ECs, circumvents the laborious harvesting techniques to endothelialize prosthetic grafts. In theory, it offers the possibility to use prosthetic grafts off the shelf, since the graft will auto-endothelialize after implantation. Joris Rotmans et al. developed a porcine model for arterio-

venous (AV) graft failure [19] which was used for this study. This set-up was chosen because it offers the possibility to study both the new technique, i.e. *in vivo* auto-endothelialization, and focus on the development of IH, which, in theory should be reduced due to the presence of adhered ECs. The desired cells to attach to the anti-CD34 antibody coated grafts are Endothelial Progenitor Cells (EPCs).

EPCs are a subset of CD34+ cells with the potential to proliferate and differentiate into mature endothelial cells [20;21]. Recent studies have emphasized that circulating EPCs have the capacity to home to sites of vascular injury, thus promoting the process of reendothelialization [22;23]. Also, in recent animal studies, *in vitro* seeding of prosthetic vascular grafts using CD34+ progenitor cells increased graft endothelialization [24;25]. We hypothesized that *in vivo* auto-seeding using immobilized anti-CD34 antibodies establishes a confluent mature endothelial cell monolayer which may reduce IH formation at the AV anastomosis.

As described in this chapter, anti-CD34 coated grafts showed rapid and complete coverage with endothelial-like cells within 72 hours of implantation. The cell coverage of anti-CD34 coated grafts remained almost confluent up to 4 weeks after surgery. So, the concept of auto-seeding is proven valid, as the cells appeared to be permanently attached to the graft. In comparison with other seeding techniques that need *in vitro* preparation, this means a step ahead in the search for less laborious harvesting techniques- where surgeons can simply pick prepared grafts off the shelf. Unfortunately we observed a threefold increase in neo-intima formation in anti-CD34 coated grafts after 28 days of follow-up. Other observations have highlighted that EPCs can also have adverse effects on vascular integrity. First, in the MAGIC trial, infusion of progenitor cells in coronary arteries on stenting resulted in a significant increase of in-stent restenosis [26]. Second, a study where ePTFE grafts were pre-treated in rats with synthetic extra-cellular matrix and vascular endothelial growth factor resulted in better endothelialization, but also in increased intimal hyperplasia [27]. Both studies clearly identify potentially adverse effects of endothelial (progenitor) cells on neointimal formation under suboptimal conditions.

An explanation of this adverse, proliferative effect may relate to the capacity of CD34+ pluripotent progenitor cells to differentiate into various cell types. The CD34 positive progenitor cell is suggested not only to differentiate into ECs, but is also capable of differentiating into VSMCs [28;29] macrophages [30], granulocytes [31] and megakaryocytes [32]. Not only VSMCs but also platelets might be responsible for the increased IH, as they are known to induce neo-intima formation. It is also suggested that EPCs only differentiate into functional endothelial cells in an appropriate milieu. An adequate balance of CD34+ and CD14+CD34- cells is therefore recommended [33]. As this increased progression of IH was observed in this study,

a closer look to the adhered cells and/or the coating was sought-after in an *in vitro* setup to delineate this adverse effect. With this purpose the study described in **chapter 4** was performed.

- *In vitro* study

This study investigated the concept of auto-endothelialization with EPCs using immobilized anti-CD34 antibodies in an *in vitro* setup using human blood. To look at the adhered cells in detail, glass coverslips and pieces of ePTFE were used. This gave us the opportunity not only to study the adhered cells by scanning electronic microscopy on the ePTFE graft, but also by standard light microscopy and immunofluorescent staining on the glass coverslips. In this study we did not only look at non-coated and anti-CD34 coated surfaces, but also at base-coated and isotype coated surfaces. We observed that the amount of cells on the anti-CD34 coated surface was significantly higher when compared to both basecoat and isotype respectively. We did however observe a significant amount of cells on the base-coated and isotype coated glass coverslips, indicating that the surface on which the antibody is immobilized already attracts a certain unidentified celltype. Remarkably, the morphology of the cells on the base-coated, isotype and anti-CD34 coated coverslips was similar, which might suggest a-specific attraction of a certain celltype by the coating. For this reason, the cells were extensively characterized by immunostaining. From this part of the study it was concluded that a subset of the cells which were captured on the anti-CD34 coated surfaces, expressed established markers of endothelial cells (CD34, CD31, KDR, and VWF). The cells did not stain positive for CD133. Surprisingly, a substantial amount of CD34- cells adhered to the CD34-coated surface as well. These cells stained positive for monocytic (CD14) and granulocytic (CD66b) markers. Granulocytes can produce cytokines which directly function as a chemo-attractant for cells such as smooth muscle cells that lead to the development of IH [34;35]. This mechanism may explain the observed increase in IH in the anti-CD34 coated ePTFE grafts in our porcine model. From both the *in vivo* and *in vitro* studies we conclude that the concept of auto-endothelialization can be realized by this coating technique. We also conclude that the adherence of cells is – at least in part- mediated by the anti-CD34 antibody. At present it is unclear which mechanisms are responsible for cell adhesion to the base-coated or isotype coated grafts and glasses, and whether this a-specific attraction of cells also occurs *in vivo* and contributes to the increased IH observed in our animal model. We thus realize that not only the desired EPCs are attracted to the anti-CD34 coated surface, but also other types of cells which might contribute to a progressive IH. Future developments could be directed against improvement of the anti-CD34 antibody to reach better specificity and/or adjustment of the coating to

prevent a-specific adherence of cells. Therefore, the search for 'the perfect antibody' hasn't ended yet. As mentioned earlier, recent studies suggest that an adequate balance between CD34+ and CD14+CD34- cells is required for optimal endothelial cell differentiation and function of EPCs [33]. So, a carefully-designed mixture of anti-CD34 and anti-CD14 antibodies might result in a combination of trapped cells with a higher potential to improve vascular homeostasis. *In vitro* data suggest that monocytes are precursors of different subgroups of endothelial cells and that the formation of endothelial cells from CD34+ progenitor cells follows a similar pathway possibly via the monocyte and/or the immature dendritic cell [36]. Moreover, recent data show that injection of freshly isolated circulating CD14+ cells improves healing and vascular growth indicating their potential for use in acute clinical settings [37].

Does Endothelial Progenitor Cell (EPC) seeding have a future?

Despite of the promising concept and results of auto-endothelialization with respect to persisting cellular coverage, we of course realize that the perfect antibody hasn't been found yet, as both a progressive IH (*in vivo* study) and a-specific cell attraction (*in vitro* study) occurred. For this reason, an overview of the up to date techniques concerning graft cell seeding with emphasis on its newest era: seeding with endothelial progenitor cells, was described in **chapter 5**. In this review we conclude that although experimental studies on prosthetic graft seeding using EPCs showed excellent result on graft endothelialization, none of these studies reported favourable effects on the more important endpoints such as intimal hyperplasia or graft patency. As achieving an endothelial layer is the goal of auto-endothelialization, it is important to realize that the performance and amount of EPCs critically depends on environmental factors. For example, the maintenance of vascular homeostasis by EPCs may be attenuated with age, based on functional deficits [38]. Furthermore, classic risk factors for coronary artery disease, such as smoking, diabetes, hypercholesterolemia, and hypertension affect function and number of EPCs [39]. On the other hand, there are modalities to counteract the reduction of EPC number and the decreased functional activity in patients with atherosclerotic disease. From several studies it is concluded that HMG-CoA reductase inhibitors (statins), vascular endothelial growth factor (VEGF), estrogen, or exercise play a pivotal role in increasing the amount of circulating EPCs [40-42]. Even VEGF2 gene therapy is suggested to be successful for certain patients that are incapable of mobilizing sufficient EPCs [43].

The HEALING-FIM trial is a feasibility study, using the same anti- CD34 antibody as we did in our studies, on cardiac stents. In patients that had a diagnosis of stable or unstable angina or silent ischemia- no reduction in IH was found when compared with the usual late loss, seen after conven-

tional bare stent implantation. The results of this registry show that the EPC capture stent is safe and feasible: with no stent thrombosis (30 days or 6 months), and major adverse cardiac and cerebrovascular events (MACCE) occurred in only one patient (6.3%), despite only 30 days of clopidogrel therapy [44]. HEALING-II is a multi-center, prospective non randomized trial that included 63 patients. Analysis showed a MACCE of 7.9%, which was completely contributable to patients with a low (<6,5 EPCs/100µl) titre of EPCs (data presented at TCT meeting 2005). None of the patients with a low titre of EPCs were on HMG-CoA reductase inhibitors, once again suggesting that statins play an important role in the absolute amount of circulating EPCs.

So, not only developing the 'perfect vascular graft that can auto-endothelialize', but also stimulating patients in finding the right balance in atheroprotective factors, must be taken into account to improve vascular graft patency.

However, the true value of auto-endothelialization using EPC capture needs careful consideration since animal experiments are executed in an environment lacking risk factors that influence EPC number and function.

In conclusion, EPCs have emerged as a promising source for prosthetic graft seeding. The expanding knowledge of EPC function and differentiation should stimulate further research on the therapeutic application of EPCs to improve the poor patency rates of prostethic grafts.

Heparin bonding to reduce thrombogenicity of vascular grafts

The *second strategy* described in this thesis to improve the patency of vascular prostheses is the use of *heparin bonding*.

- *Ex vivo* human study

Prosthetic materials are thrombogenic and cause platelet adhesion and activation of the coagulation cascade. Heparin is a potent anticoagulant drug widely used to prevent and treat thrombosis [45]. A new ePTFE graft with long-term bonding of heparin is now commercially available in several European countries, but a basic analysis of its mechanism of action in humans has never been described. In **chapter 6** we performed a study to evaluate the thrombogenicity of heparin-bonded ePTFE grafts compared to standard ePTFE in our newly developed human *ex vivo* model [46]. We found a substantial reduction in thrombogenicity of the ePTFE grafts as a result of the heparin bonding. Platelet adhesion and fibrin deposition were only observed on the surface of the standard grafts. Moreover, a significant reduction in FPA production was observed on heparinized

grafts when compared to standard grafts. There was no increase in F_{1+2} in the perfusate over time on both types of vascular grafts, indicating that no measurable amount of thrombin was generated. P-selectin expression on the perfused platelets did not increase over time on either graft surface, indicating that no measurable amount of platelets was activated. Thus, heparin bonding reduces the thrombogenicity of ePTFE grafts. For the first time, we provided evidence that the mechanism of action of the heparin bonding is not only due to anti-coagulant, but also to anti-platelet effects. Heparin bonding may be an important improvement of ePTFE, expectantly resulting in better patency rates for vascular reconstructions.

- *In vivo* human study.

To study the *in vivo* thromboresistance of this heparin-bonded vascular graft, we examined the effects of heparin immobilization on systemic markers of haemostasis activation, as was described in **chapter 7**. Concluding from our human *ex vivo* model, heparin bonding substantially reduced the thrombogenicity of ePTFE grafts. Whether this could be translated in a systemic anticoagulant effect was unknown, and that was one of the focuses of this study.

Long term heparin administration is commonly associated with heparin-induced thrombocytopenia (HIT) as a consequence of development of antibodies against the heparin-platelet factor 4 complexes [47]. These antibodies lead to platelet activation, and formation of platelet aggregates, which both results in thrombocytopenia, and the development of thrombosis (HITT). These thromboses are clinically relevant in about 50% of the patients, and these patients with clinically relevant HITT have a poor prognosis; 20% will lose a limb, and 30% eventually dies [48].

We conducted a human *in vivo* study comparing heparinized ePTFE graft with standard ePTFE graft in patients undergoing femoro-popliteal bypass grafting. This study addressed whether heparinized graft implantation resulted in:

1) A measurable reduction of systemic markers of haemostasis activation as compared to control grafts?
2) Antibody formation against heparin, potentially responsible for HIT.

It was concluded that no measurable effect of heparin immobilization on systemic markers of haemostasis (F_{1+2}, FPA, sGPV, TAT and D-Dimers) was found using a heparin-bonded ePTFE graft *in vivo*. Moreover, no anti-PF4/heparin antibodies against heparin could be detected up to 6 weeks after implantation. The latter may be of clinical importance. We realize that this is concluded only on a small patient population. However, no human clinical study has looked at the long term effects of these heparinized grafts on the generation of HIT antibodies. Since it has been shown that the immobilized heparin remains active at least for months, it

is reassuring to find that even though active heparin is on the graft for prolonged time-periods, no antibodies were measured.
Development of HIT(T) after implanting heparinized conduits is not described frequently. In a recently published case report, a patient who received a heparinized cardiac stent developed HITT [49]. It is suggested by the authors that the local heparin might be responsible for this phenomena. However, i.v. heparin was given two weeks after implantation because of an ischemic cerebral stroke. Three days after heparin i.v. administration, the heparin was stopped because of proven antibodies against heparin. Although this case report and the accompanying editorial attributed the HITT episode to the heparin on the stent we believe the intravenous heparin to be mainly responsible [49;50].

Overall, we conclude that auto-endothelialization by antibody coating and heparin immobilization are techniques that have the potential to improve the patency of small diameter vascular grafts.

From both the *in vivo* and *in vitro* **anti-CD34 studies** we conclude that the concept of auto-endothelialization can be realized by this coating technique. The 'perfect antibody' however, is not found yet, as a threefold increase of neo-intima formation was the result of the anti-CD34 coating and a-specific cells attached to the coated surface.

From the *ex vivo* study we can conclude that **heparin immobilization** successfully reduces the thrombogenicity of ePTFE grafts. The major conclusion from the *in vivo* study, using the heparinized grafts is the lack of antibodies against the locally immobilized heparin, which is of great clinical importance.

As stated earlier, the ideal conduit for (semi)acute vascular bypass procedures is the one that the surgeon can simply pick off the shelf, implant in the patient and which also resembles the patency of venous bypasses. At the time of writing, on the basis of the studies described in this thesis and the literature available, this specific heparinized graft seems to be the best choice. Of course, longer follow up studies are needed to delineate whether heparin immobilized prostheses truly perform as good as venous bypass grafts do. In theory, both reduction of thrombogenicity and reduction of IH might benefit this clinical dilemma.

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Chapter 9 Summary in Dutch
Samenvatting

Nieuwe strategieën om de duurzaamheid van vaatprothesen te verbeteren

Achtergrond
Slagaderverkalking kan leiden tot het dichtslibben van een deel van het slagadersysteem. Het aanleggen van een omleiding of bypass, kan dan nodig zijn. Dit wordt gedaan om de klachten die ontstaan door een dichtgeslibd deel van het slagadersysteem te verminderen. Mensen die bijvoorbeeld 'etalagebenen' hebben kunnen in aanmerking komen voor een dergelijke bypass-operatie. Wanneer iemand last heeft van etalagebenen is de loopafstand beperkt. Het doel van de operatie is om de patiënt weer pijnvrij te laten lopen en uiteindelijk gaat het om behoud van het been.
Bij voorkeur wordt een ader uit hetzelfde been gebruikt om de dichtgeslibde slagader te vervangen. De bypass loopt dan vanaf de lies naar het niveau net boven de knie, of daaronder. Lang niet altijd is een ader beschikbaar. Dat geldt in ongeveer 1/3 van de patiënten die in aanmerking komen voor een dergelijke operatie. In dat geval wordt gebruik gemaakt van kunststofbloedvaten, ook vaatprothesen genoemd. De duurzaamheid van aderen is echter beter dan van vaatprothesen. In een recent verschenen publicatie is gekeken naar wat daarover in de literatuur bekend is. Deze analyse liet zien dat de 'primary patency', zeg duurzaamheid, na 5 jaar 74% is voor de bypass boven de knie met een eigen ader, en 39% voor het kunststof bloedvat. Het meest gebruikte kunststof daarvoor is PTFE, ofwel polytetrafluoroethyleen.

Dat de kunststof bypass het minder goed doet dan de eigen ader heeft onder andere te maken met twee problemen:

- Ter plaatse van de aanhechting van het kunststof bloedvat op de slagader, ontstaat een zogeheten 'intima-hyperplasie' (IH). Dat is een woekering van cellen op deze aanhechting, waardoor de bypass op die plek vernauwt en uiteindelijk dicht gaat zitten.
- Het andere probleem van de kunststof bypass is de verhoogde 'trombogeniciteit' of neiging tot stollen van het oppervlak waaruit de kunststof bestaat.

De ideale vaatprothese is die welke net zo duurzaam is als de eigen ader. Dit houdt onder andere in dat de chirurg een prothese van de plank pakt welke direct geïmplanteerd kan worden zonder dat bewerking van tevoren nodig is om een goede duurzaamheid te realiseren.
De hierboven beschreven tekortkoming van het gebruik van vaatprothesen is de verantwoording voor de studies zoals die zijn beschreven in dit proefschrift.

Opzet
Twee nieuwe strategieën om de duurzaamheid van vaatprothesen te verbeteren worden beschreven in dit proefschrift.
De eerste strategie gaat uit van het principe dat vaatprothesen duurzamer worden wanneer ze aan de binnenkant bekleed zijn met zogeheten endotheelcellen (ECs). Dit zijn cellen die in de normale architectuur van een bloedvat ook de binnenkant bekleden. Er zijn meerdere gunstige eigenschappen van ECs bekend. Zo weten we dat ECs juist de IH en trombogeniciteit kunnen verminderen. Het bedekken van vaatprothesen met EC wordt 'endothelialisatie' genoemd. Om deze endothelialisatie te bewerkstelligen hebben we gebruik gemaakt van zogeheten **anti-CD34 antilichamen** die als het ware 'geplakt' zaten op de binnenkant van de onderzochte vaatprothesen. Deze antilichamen zijn gericht tegen voorlopers van ECs. Die worden EPCs genoemd (=Endothelial Progenitor Cells). Deze EPCs kunnen worden 'omgezet' in ECs onder de juiste omstandigheden. Dit proces hebben we 'auto-endothelialisatie' genoemd.
De tweede strategie gaat uit van het principe dat de trombogeniciteit van vaatprothesen verminderd kan worden door het gebruik van heparine. Heparine is een medicijn dat frequent wordt toegepast ter voorkoming of behandeling van trombose (=stolling opgetreden in bloedvaten). Ook zijn er studies bekend die beschrijven dat heparine eveneens IH kan tegengaan. In het tweede deel van de hier beschreven studies hebben we dan ook gebruik gemaakt van **heparine gebonden vaatprothesen**.

Centraal in dit proefschrift staan 4 vragen:
1) Ontstaat auto-endothelialisatie bij gebruik van anti-CD34 gecoate vaatprothesen?
2) Vermindert deze auto-endothelialisatie het ontwikkelen van intima hyperplasie?

En
3) Vermindert heparine-binding de trombogeniciteit van vaatprothesen?
4) Is er een systemisch effect van de locaal aanwezige heparine op de vaatprothesen?

Om deze vragen te beantwoorden zijn zogeheten *in vitro*, *ex vivo* en *in vivo* studies verricht, welke zijn beschreven in dit proefschrift.

Het eerste deel van het proefschrift beschrijft onderzoeken met anti-CD34 antilichamen.

Hoofdstuk 2 geeft een overzicht van de ontstaansgeschiedenis van kunststofbloedvaten. Hier wordt in het bijzonder ingegaan op technieken om vaatprothesen te bekleden met verschillende typen lichaamseigen cellen.

Deze lichaamseigen cellen zouden ertoe moeten leiden dat de duurzaamheid van vaatprothesen verbetert. Niet elk celtype bleek daartoe geschikt. Vooral het zaaien van prothesen met ECs bleek succesvol. Jarenlange follow-up bij meer dan 100 patiënten, liet inderdaad een verbeterde duurzaamheid zien bij gezaaide prothesen. Het grote probleem echter is de arbeidsintensieve, tijdrovende en weinig toegankelijke methoden die nodig zijn om deze techniek toe te passen. Om die reden is het nooit op grote schaal toegepast.

Hoofdstuk 3 beschrijft een *in vivo* studie waarin we gebruik maken van een varkensmodel en **anti-CD34 gecoate vaatprothesen**. Het gebruikte model is ontwikkeld door Joris Rotmans et al. In dit varkensmodel wordt een vaatprothese (PTFE) in de hals ingehecht. Daarbij wordt een verbinding gemaakt tussen de halsslagader en de diepe halsader. Dit model is uitermate geschikt om IH te bestuderen. Binnen 4 weken kun je op die manier antwoord krijgen op de vraag of een toegepaste techniek op vaatprothesen de IH remt of juist niet. Bovendien is het in dit model mogelijk om te bestuderen of auto-endothelialisatie ontstaat als gevolg van het gebruik van de anti-CD34 antilichamen.
Binnen 72 uren bleken de anti-CD34 gecoate prothesen volledig bedekt te zijn met cellen die kenmerken vertoonden van ECs. Dit bleek na 4 weken ook nog steeds het geval te zijn. Dus het concept van auto-endothelialisatie is daarmee bewezen succesvol. In vergelijk met andere, tijdrovende technieken is dit een veelbelovend alternatief. Het gaat immers 'vanzelf'.
Helaas zagen we na 4 weken een drievoudige toename van de IH. Wellicht dat dit veroorzaakt wordt door het feit dat CD34+ voorloper cellen kunnen 'rijpen' tot meerdere typen cellen. Deze zouden dan juist verantwoordelijk zijn voor de toename van IH, waar een vermindering van IH nou juist het beoogde effect is.

Om de cellen die hechten aan het **anti-CD34 antilichaam** beter te kunnen bestuderen, hebben we een *in vitro* studie verricht met humaan bloed. Deze studie wordt beschreven in **hoofdstuk 4.** Hierin wordt onderzoek gedaan met anti-CD34 gecoate glaasjes en stukjes PTFE. In een gestandaardiseerd stroommodel stroomt bloed door heel kleine kamertjes en kunnen de gehechte cellen bestudeerd worden. Het voordeel van het gebruik van glas is dat we ook door de cel heen konden kijken om deze beter te kunnen karakteriseren. Dit werd gedaan met behulp van een speciale microscoop, de electronenmicroscoop.
We zagen dat een significant groter aantal cellen hecht aan de anti-CD34 gecoate glaasjes vergeleken met glaasjes waar het antilichaam niet op zat. Er hechten echter dezelfde soort cellen aan zowel de anti-CD34 gecoate glaasjes als aan de glaasjes waarop wel de coating maar niet het antilichaam zat.

Door de gehechte cellen aan te kleuren, konden we de cellen verder karakteriseren. Het bleek dat de gehechte cellen aan de anti-CD34 gecoate glaasjes eigenschappen bevatten welke beschouwd worden als 'endotheliaal'. Zo was een deel van de cellen positief voor CD34, CD31, KDR en vWf. Enkele gehechte cellen bleken echter ook niet-endotheliale eigenschappen te hebben. Zo waren deze ook positief voor CD14 en CD66b. Dit zijn kenmerken voor monocyten en granulocyten. Dat zijn cellen die verantwoordelijk zouden kunnen zijn voor de progressieve ontwikkeling van IH, welke we zagen in de varkensstudie.

Op basis van zowel de *in vivo* als de *in vitro* studie concluderen wij dat het concept van auto-endothelialisatie met antilichaam coating gerealiseerd kan worden. Een deel van de aangetrokken cellen is direct het gevolg van de aanwezigheid van dit specifieke antilichaam. Een deel van de aangetrokken cellen is echter zeer waarschijnlijk verantwoordelijk voor de progressieve ontwikkeling van de IH. Dit ongewenste effect maakt dat de zoektocht naar het 'perfecte antilichaam' nog niet voltooid is. Wellicht is een combinatie van 2 soorten antilichamen in staat om een meer specifieke celpopulatie aan te trekken met een remmend effect op de ontwikkeling van IH.

In **hoofdstuk 5** gaan we in op de studies die tot dan toe gedaan zijn naar het gebruik van EPCs als celtype voor de bedekking van vaatprothesen. We concluderen dat, hoewel meerdere experimentele studies op vaatprothesen laten zien dat endothelialisatie wordt bewerkstelligd, geen enkele studie laat zien dat er een reductie is in IH of een verbetering van de duurzaamheid van de prothesen. EPCs als celtype voor de bedekking van vaatprothesen is in ontwikkeling, maar heeft nog niet geleid het ontstaan van de 'ideale vaatprothese'.

Het tweede deel van het proefschrift beschrijft onderzoeken met heparine gebonden vaatprothesen.

Zoals eerder al gesteld, is het kunststofmateriaal waarvan vaatprothesen gemaakt zijn trombogeen. Ofwel de stolling wordt door het oppervlak geactiveerd. Dat kan ertoe leiden dat een vaatprothese dicht gaat zitten. Daarmee is de patiënt weer terug bij af.
Een nieuwe technologie heef het mogelijk gemaakt heparine te binden aan de binnenkant van PTFE vaatprothesen. Heparine is een veel gebruikt medicijn dat stolling tegengaat. Het is logisch te veronderstellen dat gebonden heparine ook daadwerkelijk leidt tot minder activatie van het stollingssysteem. Dit is in de humane situatie echter nooit aangetoond.
In **hoofdstuk 6** wordt dan ook gekeken naar die mogelijke vermindering

van de stollingsactivatie door de aanwezige heparine aan de binnenkant van de vaatprothese. Hiertoe hebben we bij 10 gezonde vrijwilligers bloed direct uit een ader door een vaatprothese laten stromen (zie figuur 1 in hoofdstuk 6). Dit duurde 2 keer 6 minuten. In de eerste afname van 6 minuten werd bloed uit de ene arm opgezogen dat stroomde door de 'gewone' vaatprothese. In de tweede afname werd de andere arm gebruikt en stroomde het bloed door de 'gehepariniseerde' prothese. Er werden tijdens de bloedafname bloedmonsters opgevangen en geanalyseerd. Tevens werd de vaatprothese in kleine stukjes gesneden om vervolgens te kunnen bestuderen onder de microscoop.
Uit de bloedmonsters konden we concluderen dat er inderdaad een vermindering van stollingsactivatie ontstaat in de gehepariniseerde prothese. Dit werd bevestigd door de opnamen die met de elektronenmicroscoop werden gemaakt. Op de gewone prothese zagen we dat bloedplaatjes en fibrine-draden zich hadden gehecht. Dit zijn beide tekenen van stollingsactivatie. Bij de gehepariniseerde prothese zagen we dit niet.

In **hoofdstuk 7** doen we onderzoek naar patiënten die een bypass operatie aan het been moeten ondergaan. In totaal hebben 17 patiënten meegedaan aan dit onderzoek. Acht patiënten kregen de gewone prothese en 8 patiënten de gehepariniseerde prothese. 1 patiënt kreeg een gewone prothese, maar had na de operatie heparine nodig en kon daarom niet worden meegenomen in de vergelijkende studie. Wel hebben we deze patiënt geanalyseerd, juist vanwege de toediening van heparine.
Bij deze patiënten werd 6 keer bloed afgenomen voor analyse. Ten eerste vóór en direct na operatie. Vervolgens op de eerste, derde en vijfde dag na de operatie. De laatste keer werd bloed afgenomen toen de patiënt op de polikliniek kwam, 6 weken na de operatie. Ook in deze bloedmonsters werd weer gekeken naar enkele stollingsparameters. Bovendien werd gekeken of er zich antilichamen ontwikkelden tegen de heparine. Bij gebruik van heparine kan dit fenomeen namelijk optreden. Dit wordt HIT genoemd. Dat staat voor heparine geinduceerde trombocytopenie. HIT ontwikkelt zich in ongeveer 3% van de patiënten waarbij heparine via het infuus wordt gegeven. Daarbij daalt het aantal bloedplaatjes fors en kan de stolling volledig ontspoord raken. Dat kan zelfs zo erg zijn dat mensen daaraan komen te overlijden. Gelukkig komt zoiets maar zelden voor. Mochten zich antilichamen ontwikkelen bij het gebruik van de gehepariniseerde prothese, dan is het zeer de vraag of dit klinisch relevant wordt. In ieder geval zou het onwenselijk zijn. Mocht een patiënt namelijk antilichamen hebben ontwikkeld, dan kan een eventuele volgende gift heparine in theorie leiden tot een klinisch relevante HIT.
In dit onderzoek vonden we geen significant verschil in de stollingsparameters tussen de twee soorten vaatprothesen. We zagen ook geen significant verschil de daling van de hoeveelheid bloedplaatjes. Verder

konden we geen antilichamen aantonen in deze periode van maximaal 6 weken. Zoals vermeld werd 1 patiënt wel geanalyseerd, maar niet meegenomen in de vergelijkende studie. Bij deze patiënt was te zien dat het aantal bloedplaatjes wel fors daalde (45%) na het gebruik van de heparine. Ook werden er bij deze patiënt antilichamen aangetoond tegen heparine.
Uit deze studie concluderen wij dat er geen systemisch effect is aan te tonen van de locaal aanwezige heparine. Er was wel dalende een tendens in het aantal bloedplaatjes op de eerste dag na de operatie bij de geheparinisseerde prothese. Dit herstelde zich echter al volledig op de derde dag.

Op basis van de studies met de **geheparinisseerde vaatprothesen** concluderen wij dat de locaal aanwezige heparine inderdaad leidt tot een vermindering van de trombogeniciteit. Dit effect is niet meer aan te tonen na implantatie. Antilichamen tegen de heparine zijn in deze tijdspanne niet aangetoond.

Zoals gesteld is de ideale vaatprothese die welke de vaatchirurg van de plank pakt zonder verdere bewerking, en een duurzaamheid heeft van een eigen ader. In dit proefschrift hebben wij twee nieuwe strategieën onderzocht die zouden kunnen leiden tot een verbeterde duurzaamheid van vaatprothesen. De **anti-CD34 coating** leidt tot een snelle bedekking met cellen die endotheliale kenmerken hebben. Helaas ontstaat er een progressieve IH, en worden er blijkbaar cellen aangetrokken die tot dit ongewenste resultaat leiden. De **geheparinisseerde vaatprothese** leidt tot een vermindering van de trombogeniciteit, welke na implantatie niet meer is aan te tonen. Mogelijk draagt het echter wel bij aan een verbeterde duurzaamheid van vaatprothesen.

Op basis van de studies welke in dit proefschrift zijn beschreven en op basis van wat er in de literatuur ten tijde van het schrijven van dit proefschrift bekend is over de duurzaamheid van vaatprothesen, is op dit moment de voorkeur te geven aan de geheparinisseerde vaatprothese.

Chapter 10 Dankwoord

Grâne, Saint Bardoux, Le Marronnier, zomer 2006

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Curriculum Vitae

Jan Heyligers is in december 1969 geboren in het Brabantse Asten waar hij opgroeide in een hecht gezin. In 1982 ging hij naar het Sint Willibrord Gymnasium te Deurne, waar hij in 1988 succesvol het eindexamen aflegde. Datzelfde jaar ging hij naar de Universiteit Utrecht waar hij startte met de opleiding tot arts. In 1994 rondde hij het doctoraal af en eind 1996 werd het artsexamen met succes afgelegd. Hij heeft als AGNIO chirurgie gewerkt in het Sint Lucas-Andreas Ziekenhuis Amsterdam en het Diakonessenhuis Utrecht. In datzelfde Diakonessenhuis deed hij onderzoek onder de paraplu van de SWODU (Stichting Wetenschappelijk Onderzoek Diakonessenhuis Utrecht) naar mesotheelcellen en handschoentypen (Begeleider Dr. P. Leguit).

In 1998 trouwde hij Pascalle Eygensteyn met wie hij drie kinderen heeft gekregen. Lotte (1999), Imke (2002) en Floris (2004).

In 1999 ving zijn opleiding tot chirurg aan in het Diakonessenhuis Utrecht (Opleider Dr. G.J. Clevers). De opleiding werd in 2002 voortgezet in het UMC Utrecht (Opleider Prof. Dr. I.H.M. Borel Rinkes). Gedurende een periode van 1 jaar (2003/2004) heeft hij zijn opleiding onderbroken om zich fulltime te wijden aan het onderzoek wat tot deze dissertatie heeft geleid (Begeleiders Prof. Dr. F.L. Moll, vaatchirurgie, en Prof. Dr. Ph.G. de Groot, hematologie). Sinds januari 2006 is hij chirurg. Hij is momenteel CHIVO (Chirurg In Vervolgopleiding) vaatchirurgie in het UMC Utrecht (Opleider Prof. Dr. F.L. Moll). Per januari 2007 zal hij deze opleiding voortzetten in het Sint Elisabeth Ziekenhuis Tilburg (Opleider Dr. D.P. van Berge Henegouwen). Deze opleiding verwacht hij medio 2007 af te ronden.

'Het is moeilijk te zeggen wat uit wat voortvloeit: een goed karakter van een kind uit de liefde die zijn ouders zonder maat en zonder berekening over hem uitstorten, of integendeel, dat een goed kind in de harten van zijn ouders al het beste oproept dat het heeft meegekregen'.

Reis naar de zevende hemel. Ljoedmila Oelitskaja.

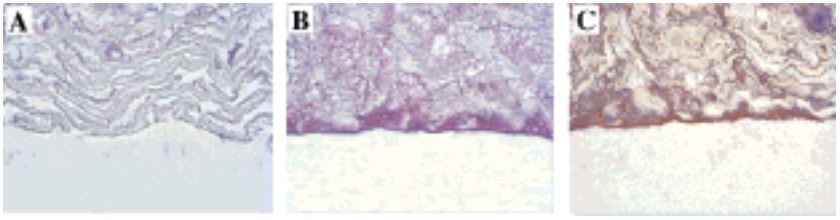

Figure 2. Representative Hematoxylin-eosin-stained sections of bare (A) and CD34-coated grafts (B) obtained from the center of the graft at 72 hours after implantation. The adhered cells on the CD34-coated grafts were identified as endothelial cells by lectin-staining (C).

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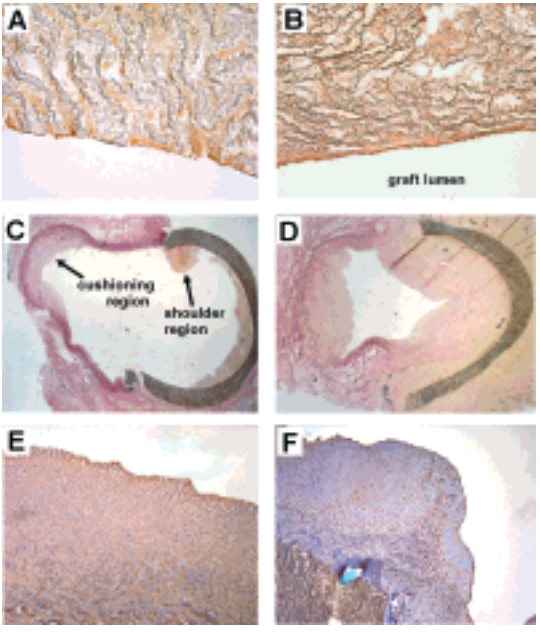

Figure 3. Representative sections obtained at 4 weeks after graft implantation. Lectin-stained sections of bare (A) and CD34-coated grafts (B) obtained from the center of the graft. EvG-stained sections of the venous anastomosis of bare-graft (C) and CD34-coated grafts (D). Detail of α-actin smooth muscle cell-stained section of the venous anastomosis (E). Detail of Ki67-stained section of the venous anastomosis. Extensive proliferation is observed at the shoulder region (F).

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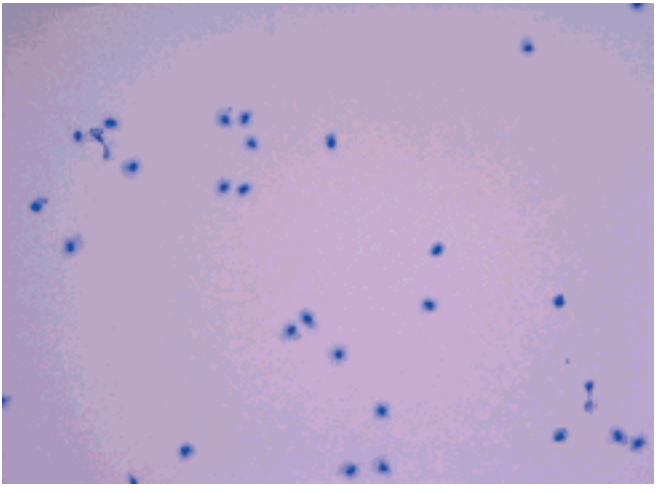
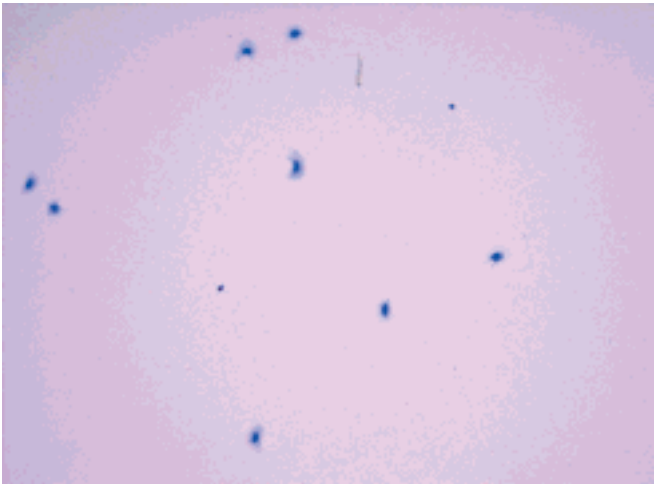
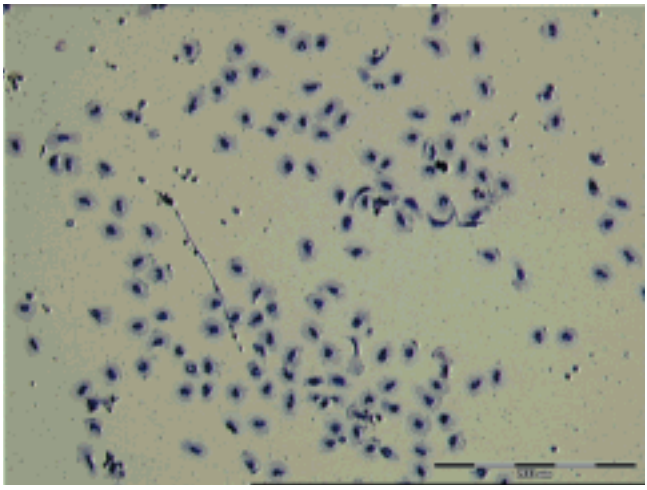


A Isotype coated coverslip



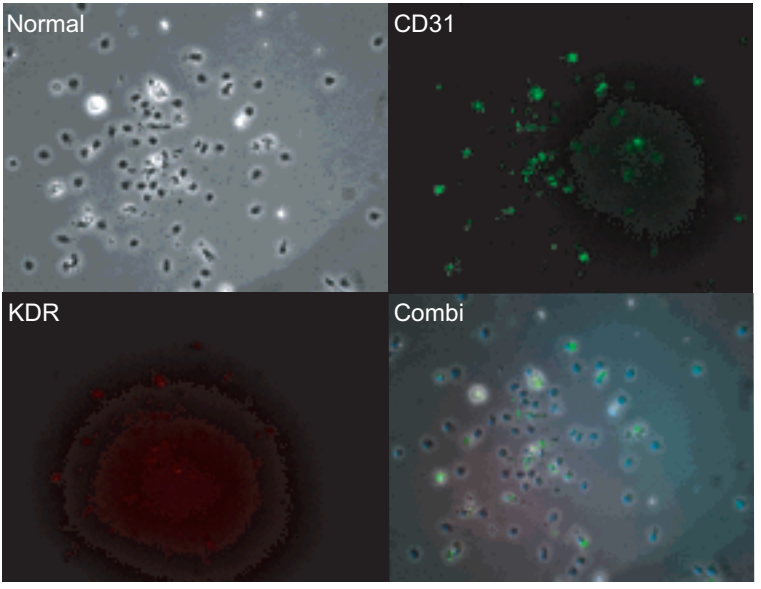
B Basecoat coverslip

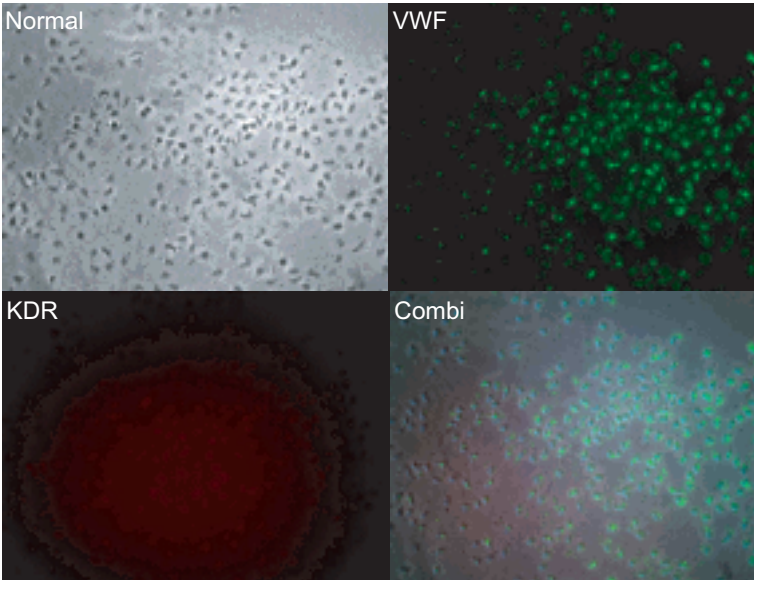


C anti-CD34 coated coverslip

Figure 2: Representative pictures of Light Microscopy after May–Grünwald Giemsa staining. LMWH-anticoagulated human blood was perfused for 90 minutes at a shear rate of 25/sec, after which the glass coverslips were prepared for May–Grünwald Giemsa staining. A is an example of isotype coated coverslip, B of basecoat coverslip and C of anti-CD34 coated coverslip. The cells show morphologic mononuclear and granulocytic features.

Chapter 4, page 60-61.


Normal
CD31
KDR
Combi


Normal
VWF
KDR
Combi

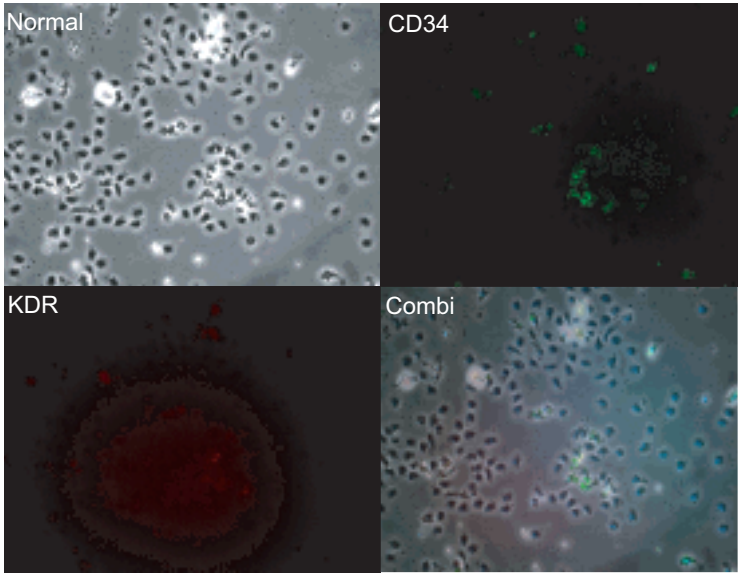


Figure 3: Characterisation of cells adhered to coverslips coated with an antibody against CD34. LMWH-anticoagulated human blood was perfused for 90 minutes at a shear rate of 25/sec, after which the coverslips were stained with antibodies against CD31, KDR, vWF and CD34. The blue colour indicates the nucleus (DAPI staining). Note that the cells stain positive for CD31, KDR, vWf and CD34. These are all endothelial characteristics.

Chapter 4, pages 62-63.

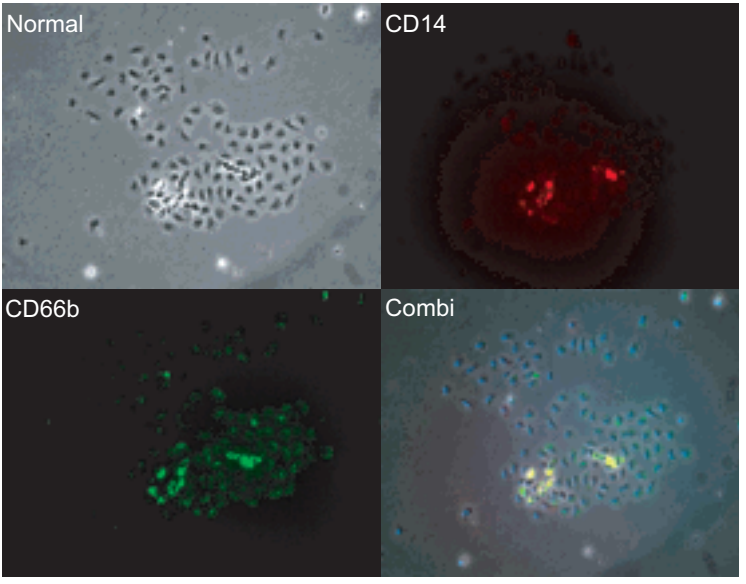


Figure 4: In concordance with the observation done in the May Grunwald Giemsa staining, the cells also show characteristics of mononuclear and granulocytic features, as they stained positive for CD14 and CD66b.

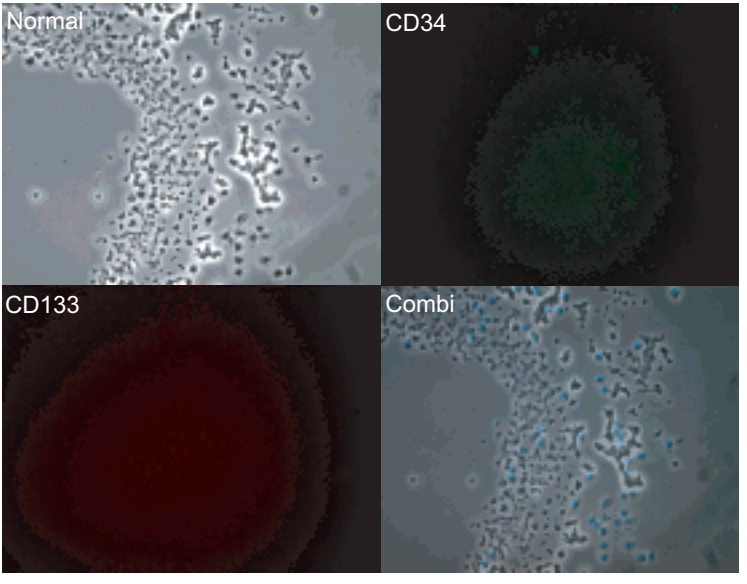


Figure 5: Example of adhered cells to the isotype of CD34 coated glass coverslip. Note that none of the adhered cells stain positive for either CD34 or CD133. Also note that only the blue DAPI stained dots, are cells. The structures around are probably lysed cells or debris.

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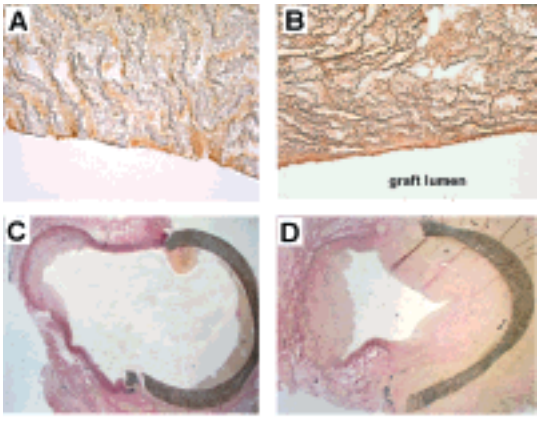

Figure 2: Representative sections obtained at 4 weeks after AV-graft implantation in pigs. Enhanced endothelialization in the anti-CD34 coated grafts coincided with profound increase in IH at the venous anastomosis. Lectin-stained sections of bare (A) and CD34-coated grafts (B) obtained from the center of the graft. Lectin is a marker for ECs. EvG-stained sections of the venous anastomosis of bare-graft (C) and CD34-coated grafts (D). Reproduced with permission. JI Rotmans et al. *Circulation*. 2005;112(1). Fig. 3, p15 (10).

Chapter 5, page 79.

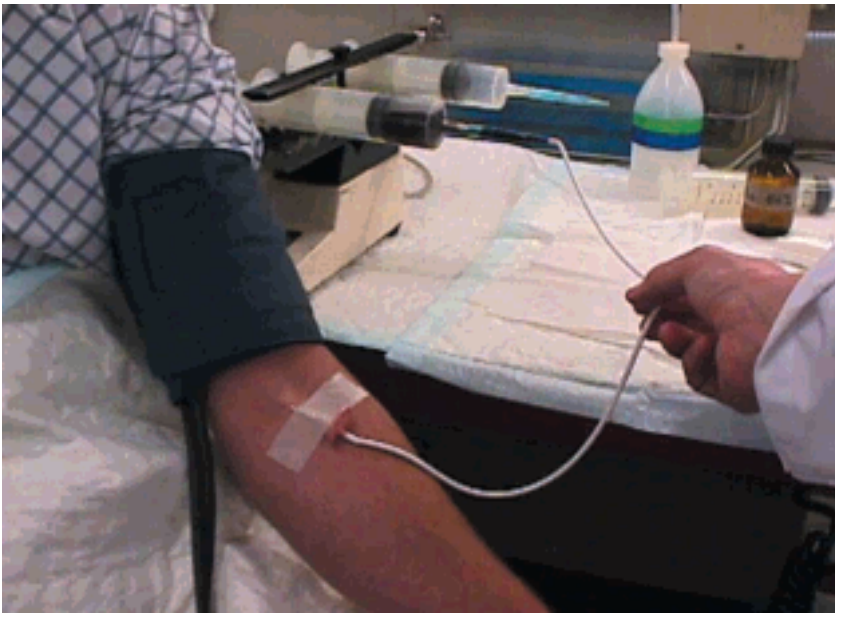

Figure 1: The newly developed *ex vivo* perfusion system using a cuff at a constant pressure of 45 mm Hg to ensure bloodflow. Blood is aspirated through the graft with a constant flow of 20ml/min, resulting in a shear rate of 74/sec.

Chapter 6, page 87.

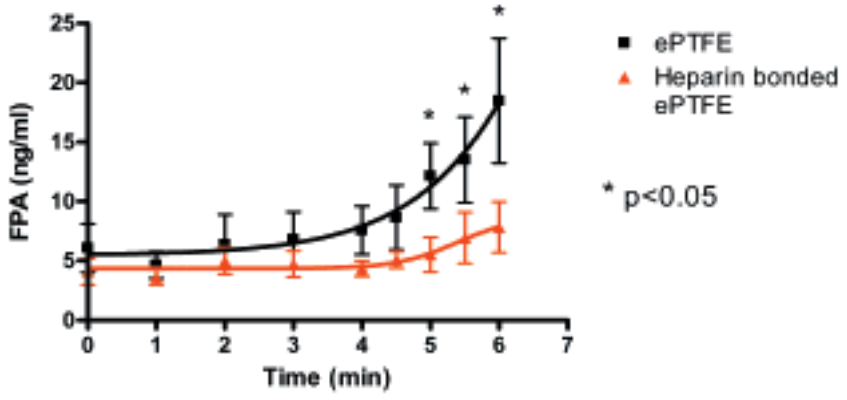

Figure 3: Heparin immobilization reduces fibrin formation during *ex vivo* perfusions. Perfusions were performed in 10 volunteers, using both a non-coated and heparin-bonded graft in each volunteer. Serial samples were taken during perfusion, in which levels of FPA were measured by ELISA. Error bars indicate standard error of mean.

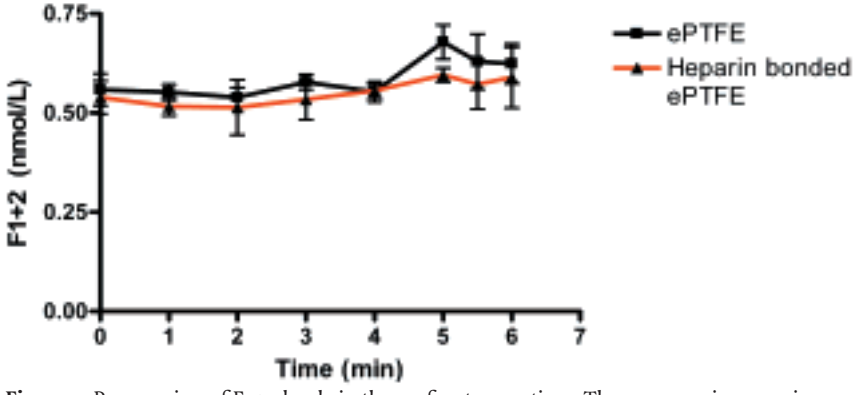

Figure 4: Progression of F1+2 levels in the perfusate over time. There was no increase in F_{1+2} in the perfusate over time on both types of vascular grafts, indicating that no measurable amount of thrombin was generated. Error bars indicate standard error of mean.

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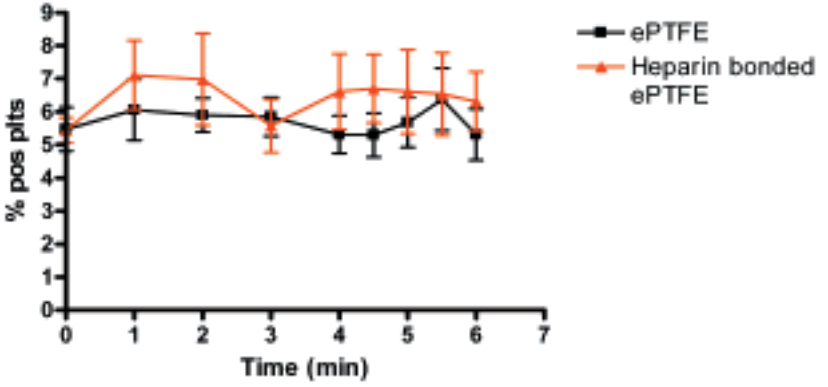

Figure 5: Progression of the percentage of P-selectin positive platelets in the perfusate over time. P-selectin expression on the perfused platelets did not increase over time on either graft surface, indicating that no measurable amount of platelets was activated. Error bars indicate standard error of mean.

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