ALBUMIN-HEPARIN MATRICES LOADED WITH GROWTH FACTOR AS SUBSTRATES FOR ENDOTHELIAL CELL SEEDING

PROEFSCHRIFT

ter verkrijging van
de graad van doctor aan de Universiteit Twente,
op gezag van de rector magnificus,
prof. dr. F.A. van Vught,
volgens besluit van het College voor Promoties
in het openbaar te verdedigen
op vrijdag 21 augustus te 13.15 uur

door

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geboren op 4 oktober 1968
te Lienden

Dit proefschrift is goedgekeurd door:

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Albumin-heparin matrices loaded with growth factor as substrates for endothelial cell seeding/
G.W. Bos
Thesis University of Twente, Enschede, The Netherlands.
With references -With summary in Dutch
ISBN 90-3651166-6

The research described in this thesis was financed by:
The Netherlands Heart Foundation

Press: FEBODRUK B.V., Enschede, The Netherlands
Cover: Stefan Bus, Meppel, The Netherlands
Background: LM image of HAMVEC culture
Left inset: vWF staining of HAMVECs
Right inset: Platelets deposited onto albumin-heparin conjugate

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Chapter 1 General Introduction

As a result of the life-style of many individuals in western society, vascular changes known as atherosclerosis frequently occur, resulting in progressive narrowing and eventually blocking of blood vessels. Also, thrombi narrowing the vascular lumen may be released from the vessel wall, blocking small or medium-sized blood vessels. Occlusion of these vessels will cause symptoms of hypoxia, as well as myocardial or cerebral infarction, and will eventually result in death. Although atherosclerotic lesions may occur at various stages of life, most life threatening situations will occur with elderly people. The increase of the average life-time in our society results in an increasing number of elderly people. Therefore, more atherosclerotic complications can be expected in the future, leading to an increased demand for vascular surgery.

Autologous veins and arteries have been extensively used to replace diseased blood vessels. Up till now, the saphenous vein is used most often for reconstructive vascular surgery of medium and small-diameter arteries.[1] Although saphenous veins remain the best choice in most cases, they are not ideal vascular grafts, because flow and pressure in the arterial circulation may cause deterioration of the veins.[2] Moreover, the number of non-essential veins is limited and atherosclerosis of these veins may render them unsuitable for use.

Alternatively, synthetic grafts may be used to replace atherosclerotic blood vessels. Vascular grafts of Dacron (polyethylene terephthalate) or Teflon (polytetrafluoroethylene), in combination with anticoagulants and platelet inhibitors, have been used successfully for decades.[1,3,4] Using this medication, the patency of large-diameter vascular grafts can be improved to such extent that it remains excellent for over 10 years.[4] In contrast, despite the use of medication, small-diameter (i.e. less than 5 mm) grafts of both synthetic materials occlude rapidly when placed in low-flow high-resistance locations.[5-7]

Animal studies have indicated that the haemocompatibility of these small-diameter vascular grafts may be improved by the seeding of endothelial cell (ECs) on the luminal side of the prosthesis prior to implantation.[8,9] Although hardly any ECs adhere to uncoated grafts of both Dacron and Teflon, EC adherence dramatically increases when the grafts are coated with plasma or extracellular matrix proteins like fibronectin.[10] Deposition of cellular fibronectin to the underlying substrate has been shown to be a prerequisite for proliferation of ECs.[11] Unfortunately, coating with fibronectin increases not only the adhesion of ECs to these surfaces, but of platelets as well.[12]

Although the results of animal studies were very promising, the first clinical trials in the mid 1980's demonstrated that endothelial cell seeding in humans does not improve the patency of vascular grafts.[1] From further clinical reports, it appeared that the ultimate success of endothelial cell seeding of vascular grafts depends on the confluency of the formed cellular layer.[13-15] Where seeding of ECs on ePTFE in low density failed to improve patency,[13] *in vitro* expansion of the cells prior to seeding of the grafts, followed by culture of the seeded grafts to allow maturation of the EC cytoskeleton, significantly improved the patency compared to unseeded grafts.[14,15]

An alternative for *in vitro* culture of the cells prior to implantation may be the acceleration of the proliferation of ECs seeded at relatively low density by means of a growth factor present on the graft surface, notably acidic or basic fibroblast growth factor (aFGF / bFGF). These growth factors bind to heparin, which is known to protect them from denaturation and enzymatic degradation.[16] Gray *et al.*[17] showed that aFGF and heparin immobilized to fibrin-glue-impregnated Teflon, implanted in the aorta of dogs, stimulate endothelialization of the graft without showing intimal hyperplasia. Doi *et al.*[18] recently demonstrated that co-immobilization of bFGF and heparin in a gelatin-impregnated porous polyurethane graft, implanted in

the aorta of rats, significantly enhanced the extent of endothelialization proceeding from the anastomotic sites, as well as transmural ingrowth of smooth muscle cells and fibroblasts.

Aim of this study

The aim of this study is to modify artificial surfaces in order to improve the adherence, spreading and proliferation of endothelial cells and to reduce blood coagulation and deposition of platelets. The strategy to realize this goal comprizes three aspects:

1. adherence and spreading of endothelial cells to artificial surfaces by means of covalently immobilized albumin-heparin conjugate (alb-hep conjugate), with or without added fibronectin;
2. inhibition of blood coagulation and platelet deposition through surface-immobilized alb-hep conjugates, during the period in which the layer of endothelial cells is not yet confluent;
3. enhancement of the proliferation of the cells by local release of a growth factor (bFGF), bound to the immobilized alb-hep conjugate.

Structure of this thesis

Chapter 2 gives an overview of the efforts made to improve vascular grafts used for replacement of small-diameter blood vessels, resulting in a view of future developments in this field.

In chapter 3, the immobilization of albumin and alb-hep conjugate to polystyrene discs and petridishes, treated with an argon or carbon dioxide gas plasma, is described, as well as the stability of the immobilized layers in contact with aqueous solutions at a physiological pH at 37°C.

In chapter 4, the adherence and proliferation of ECs derived from human umbilical veins to alb-hep conjugate immobilized to carbon dioxyde-treated polystyrene is described, as well as the effect of addition of fibronectin to the coating on the morphology and long-term adherence of the ECs.

Chapter 5 deals with the effect of basic fibroblast growth factor, loaded onto surface-immobilized alb-hep conjugate, on the proliferation of seeded ECs, and the stability of this growth factor in aqueous solutions at a physiological pH at 37°C.

In chapter 6, the blood compatibility of surfaces with immobilized alb-hep conjugate is described, as well as the effect of seeded ECs on platelet deposition to these substrates.

Chapter 7 deals with blood compatibility of crosslinked gels of albumin and heparin, the adhesion and proliferation of ECs on these substrates, as well as the effect of seeded ECs on platelet deposition.

In chapter 8, the isolation and characterization of human adipose microvascular endothelial cells (HAMVECs) are described, as well as their culture on surface-immobilized alb-hep conjugate.

Parts of this thesis have been published or will be submitted for publication.[19-23]

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Chapter 2 Small-Diameter Vascular Graft Prostheses: Current Status

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Introduction

Atherosclerosis is a prominent disease in western society, characterized by progressive narrowing and occlusion of blood vessels. These vascular changes are caused by smooth muscle cell proliferation, depositions of cholesterol, lipoproteins and calcium as well as thrombus formation which may occur at various stages of the disease. When thrombi narrowing the vascular lumen are released from the vessel wall, small or medium sized vessels may become blocked causing symptoms of hypoxia, organ damage (e.g. myocardial or cerebral infarction) and death. Although atherosclerotic lesions may occur in all large and medium-sized arteries, there are a number of predilection areas such as the abdominal aorta, iliac, carotid and coronary arteries as well as vascular bifurcations.

Surgical treatment of atherosclerotis began in 1952, when Voorhees *et al.*[1] postulated that diseased blood vessels, like aneurysmatic abdominal aorta, might be replaced by synthetic fabric. The feasibility of this strategy was confirmed by the same authors in 1954.[2] Thereafter, much effort has been devoted to develop artificial vascular prostheses with optimal biocompatible characteristics. This led to the widespread clinical use of Dacron (polyethylene terephthalate, PET) and Teflon (expanded polytetrafluoroethylene, ePTFE) grafts in vascular surgery.[3-5]

Long-term anticoagulant treatment and administration of platelet inhibitors have been shown to improve the patency of larger diameter vascular grafts,[5] for more than 10 years post-implantation. In contrast, small-diameter grafts (i.e. less than 5 mm) of both Dacron and Teflon, placed in low-flow vascular

segments, fail rapidly due to occlusion.[6,7] Since its development, ePTFE has become the most frequently used conduit for femoropopliteal reconstruction (when autologous saphenous veins cannot be used), because it is smooth, can be handled easily, and does not need preclotting. However, its preference over Dacron is not based on solid scientific evidence and recent reports demonstrate no difference in the patency of Dacron and ePTFE grafts implanted in femoropopliteal arteries.[7-9]

Besides synthetic grafts, autologous veins and arteries have been used extensively. Until today saphenous veins constitute the reference material for reconstructive vascular surgery of medium and small-diameter arteries.[3] For coronary bypass surgery however, the internal mammary artery has recently been shown to be a superior conduit to the saphenous vein.[10-12] Although saphenous veins remain the best option in most cases, especially when used as reversed grafts,[13] they have certain limitations such as detorioration when exposed to increased flow and pressure present in the arterial circulation.[14] Furthermore, the number of veins which can be transferred without causing circulatory (i.e. venous) disturbances is limited and varicous changes of autologous veins may render them unsuitable for use.

It is not yet established which graft is indicated in various situations.[7,8,12,15] Meanwhile, to improve grafts to bypass diseased medium and small-diameter blood vessels, three strategies have been persued (figure 1). Firstly, the development of synthetic grafts made of materials such as polyurethane, which are less reactive to plasma proteins and blood cells and provide mechanical properties similar to those of natural blood vessels (approach 1). A second approach has been to modify the surface of existing synthetic grafts, e.g. with regard to surface properties like hydrophilicity and presence of chemical groups (approach 2A), or alternatively by covalent attachment of bioactive substances (approaches 2B and 2C). A third strategy aims at the incorporation of autologous vessel wall cells into the

vascular graft. In this way, as for instance through the seeding of endothelial cells (ECs), various biological functions of the native vessel are mimicked (approaches 3A, 3B and 3C).

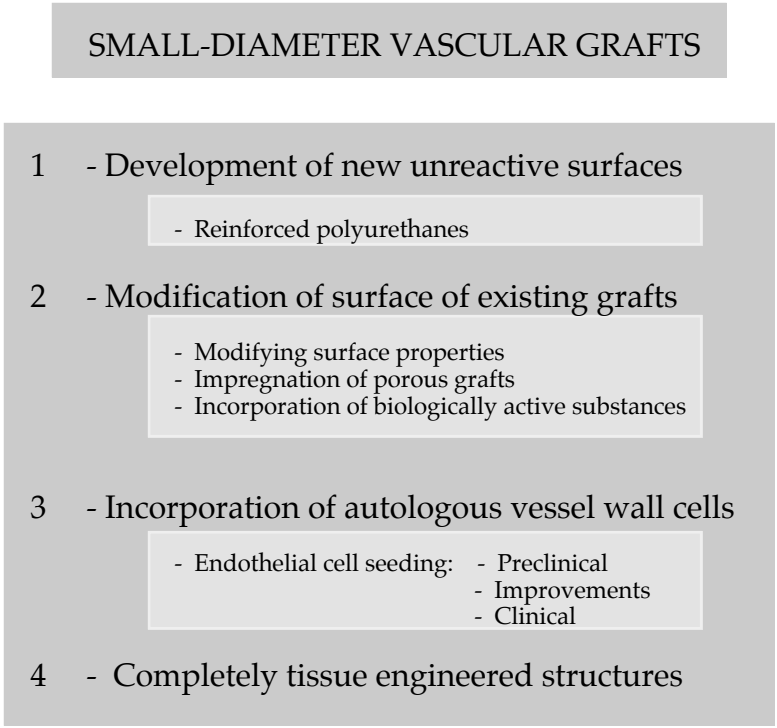

Figure 1. *Strategies persued in the field of small-diameter artificial vascular grafts.*

Approach 1: vascular grafts of new synthetic materials

The use of polyurethane as arterial substitute has been studied exhaustively in the past, but interest dropped when it appeared that thrombosis and embolism continued to present problems after such materials were implanted. Compounds designed to be less thrombogenic, were prone to biodegradation and failure

known as cracking, and release of toxic diamines.[16] However, research of polyurethanes has continued because *in vitro* blood compatibility and cellular adherence are favourable, and compliance can be controlled to match with the host artery. Special attention is paid to the *in vivo* stability of these grafts.[17]

Composites of polyurethanes and other more stable compounds appear to have improved stability characteristics. In the early nineties, Corvita Corporation introduced a compliant vascular graft composed of an inner layer of meshed polycarbonate urethane and an outer layer of meshed Dacron, which was sealed to prevent blood leakage.[18] Recently, these grafts were reported to have significantly improved patency, and decreased stenosis and intimal thickening in comparison with ePTFE-grafts when tested in a canine model.[19] Nakagawa *et al.*[20] developed a new polyurethane vascular access graft reinforced with knitted polyester fibers. When tested in a group of 52 patients, the graft showed several advantages over ePTFE grafts, like prompt hemostasis and absence of persistent edema. The cumulative patency rate however, was inferior compared to ePTFE-grafts. Promising results were reported by Gupta *et al.*,[21] with weaved grafts from prestretched polyurethane monofilament yarn and polyester multifilament yarn. After implantation in the carotid artery of dogs, a thin, stable neointima developed within 6 months.

Greisler *et al.*[22] studied the use of polypropylene woven grafts in dogs. One year patency (92%) was significantly better compared to Dacron grafts (70%), whereas intimal thickening of both grafts was comparable.

Approach 2A: modifying surface properties

A second strategy is to modify the properties of commonly used materials, avoiding modification of the bulk characteristics. This policy is likely to facilitate introduction of the modified vascular grafts to the market, as less problems with regulatory agencies and acceptance by surgeons are expected.

The simplest modification is to alter the morphology of the surface by changing the knitting procedure. Mary *et al*.[23] developed a gelatin-sealed polyester arterial prosthesis with a warp-knitted sharkskin structure as thoraco-abdominal bypass, which showed greater dimensional stability than the current commercial Dacron devices when implanted in dogs for one year.

Furthermore, the patency of ePTFE can be increased by denucleation of the graft prior to implantation. Removal of thrombogenic gas nuclei from 4 mm ePTFE grafts decreased the formation of thrombosis and increased the patency of grafts in the femoropopliteal position in dogs.[24]

Another approach is to modify chemical groups at the surface. Phaneuf *et al*.[25] applied mild hydrolyzation treatment of PET with NaOH, which created carboxyl groups at the surface, without significantly decreasing the tensile strength and the sample weight. The introduced groups serve as 'anchor sites' for covalent attachment of plasma albumin to the surface, thus creating a surface with improved biocompatibility.

A more generally used method is to modify the surface with a radiofrequency glow discharge process (RFGD), by which for instance PET can be modified to PTFE at the surface. Dacron vascular grafts, treated with tetrafluoroethylene during an RFGD process, resist thrombus deposition, embolization and thrombotic occlusion.[26] These surfaces, when compared with either PTFE or PET, show reduced elution of albumin by treatment with sodium dodecyl sulfate, whereas the adsorption of

albumin to the various materials is similar.[27] The immobilized albumin is supposed to be responsible for the improved behaviour of these grafts.

Another option is to introduce functional groups that can later be used to immobilize specific proteins. In this way, PTFE was modified using ammonia plasma or alkylamine plasma to covalently bind collagen, fibronectin or a mixture of these proteins, which improve adhesion of endothelial cells to the surface.[28,29] Since the chief advantage of ePTFE is good mechanical stability *in vivo*, any modification of the graft surface to improve healing characteristics or antithrombogenic properties should not be made at the expense of stability.[30]

Finally γ-radiation is used to modify graft surfaces. In the simultaneous grafting procedure, substrate and monomer are irradiated simultaneously. In the pre-irradiation procedure, the substrate is first irradiated, after which a monomer solution is added. Simultaneous radiation is the best option for biomedical applications, as it is independent of the substrate geometry and relatively low radiation doses can be applied. Kirkpatrick *et al.*,[31] for instance, demonstrated that growth of endothelial cells (ECs) on polyethylene, polyamide and ePTFE was significantly improved upon grafting a mixture of hydroxyethyl methacrylate and N-vinyl-pyrrolidone.

Approach 2B: impregnation of porous vascular grafts

Chemical modification is used to improve binding of specific biomolecules, such as collagen, its derivative gelatin and albumin, to the surface of vascular grafts.[32] These proteins can also be used to seal the pores of polyester vascular grafts, thereby making the pre-clotting of the grafts with autologous blood prior to implantation superfluous.

As collagen is intrinsically very thrombogenic, it has been postulated that crosslinking of collagen may decrease its thrombogenicity.[33] Several collagen and gelatin impregnated

vascular grafts are nowadays available, some of which have shown to be clinically well tolerated for medium-diameter grafts (5-6 mm). Examination of explants of the Omniflow collagen-polyester composite vascular prosthesis, for instance, demonstrated structural durability of the vessel wall, minimal smooth muscle cell proliferation and good haemocompatibility. Upon implantation as a 5 to 6 mm diameter vascular replacement at various sites, such grafts showed good long-term patency, without formation of aneurysms, thrombosis or hyperplasia.[34] The collagen-impregnated Hemashield graft was reported to be superior to ePTFE grafts when used as bilateral aorto-iliac vascular implant in a canine model.[35] Faster healing, improved endothelialization, and reduced adherence of thrombi was observed on the collagen-impregnated Hemashield grafts compared to ePTFE grafts. However, in most of the collagen- and gelatin-coated vascular grafts, glutaraldehyde or formaldehyde is used to crosslink the protein matrix. These crosslinking agents, which are incorporated into the coating, are released during degradation and may evoke cytotoxic reactions.[36] Recently, the use of urazole (1,2,4-triazole-3,5-dione) to detoxify valved porcine aortic roots, which were crosslinked by glutaraldehyde, was reported. This resulted in a significant reduction of calcification.[37] The detoxified surface is a suitable substrate for ECs.[37]

Alternatively, non-toxic carbodiimide can be used to crosslink gelatin in vascular grafts.[38] In contrast to collagen, which to some extent remains present in the Omniflow graft for years upon implantation,[39] biodegradation of gelatin occurs rapidly. Marois *et al*.[38] reported that the resorption of carbodiimide crosslinked gelatin in a polyester graft (Uni-graft) upon implantation in a canine model was complete within a month. Possibly, increasing the crosslink density may retard the gelatin degradation. A recent development is the use of myristoyl gelatin[40] which retains its gel structure above body temperature without any crosslinking. Myristoyl gelatin-impregnated Dacron

grafts, implanted in canine aortas, showed no thrombus formation, immune reactions, nor excessive foreign body reactions.[40] The sealant had degraded after 8 weeks.

Sealing of vascular grafts with albumin has been shown to improve biocompatibility. Guidoin *et al*.[41] reported retarded healing characteristics for albuminated polyester grafts compared with pre-clotted grafts implanted in the thoracic aorta of dogs. The healing followed the same sequence of events as pre-clotted control grafts.[41] In a later study, the same authors reported a negative influence of the glutaraldehyde crosslinker, whereas improved healing of the graft in the canine system was reported upon crosslinking albumin with carbodiimide.[42] Recently, authors from the same institute reported excellent biocompatibility of albumin-coated grafts in terms of T-lymphocyte behaviour and acid phosphatase activity at the implant site in a rat model: the cellular reactivity towards the albumin-coated and uncoated grafts was similar.[43] Good biofunctionality in thoracoabdominal bypass in dogs was reported as well.[43] The albumin resorption was complete within 2 months.

Furthermore, fibrin glues or sealants were developed based on their likeness with a hemostatic clot. In most studies reported, these sealants contained a number of additives,[44,45] such as growth factors, which are added to promote wound healing, endothelialization of vascular grafts, and the *in vitro* proliferation and differentiation of seeded cells.

Approach 2C: incorporation of biologically active substances in the sealant

Since crosslinked proteins started to be used to fill the pores of vascular grafts, pre-clotting of the grafts became superfluous. Besides this elementary function, other biological activities have been added to sealants to make them even more useful. The

most frequently used additives are anticoagulants and growth factors. More recently, incorporation of antibiotics in sealants has been proposed.

To decrease the thrombogenicity of collagen, the anticoagulant heparin has been immobilized to collagen-coated and gelatin-coated vascular grafts.[46,47] This combination has been subject of many patents in the past as well as presently.[48] To a lesser extent, combinations of albumin and heparin have been explored as vascular graft sealants. Kurumatani *et al*.[49] patented a system in which, after impregnation of the pores of vascular grafts with heat denatured bovine albumin, the inner surface is coated with a heparin-gelatin solution.

Recently, heparin immobilized on protein coating of arterial prosthesis was shown to influence certain cell functions.[50] Laemmel *et al*.[50] described that heparin immobilized to crosslinked gelatin and/or albumin exhibits anti-proliferative activity towards smooth muscle cells seeded on these gels. This is of particular relevance for the inhibition of a growth factor, secreted by activated platelets *in vivo*, which triggers smooth muscle cells to migrate from the media of the blood vessel wall into the intima and to the anastomoses of the graft. Upon arrival, smooth muscle cells start to produce fibrous connective tissue which accounts for the bulk of the intimal thickening, that ultimately leads to occlusion of the prosthesis.

Besides heparin, the more potent, specific antithrombin agent hirudin has been immobilized on a basecoat of albumin.[51,52] Phaneuf *et al.*[52] demonstrated that recombinant hirudin retained its ability to bind and inhibit thrombin when immobilized to albumin-impregnated Dacron vascular grafts.

Heparinization of biomaterial surfaces is not only applied to reduce fibrin formation, but to minimize adhesion and activation of platelets as well. It was shown, however, that in a canine chronic AV shunt heparin did not inhibit biomaterial-associated platelet activation *in vitro* and *ex vivo*.[53] However, platelet adhesion to biomaterials may be reduced by a number

of synthetic compounds known to inhibit complement activation, such as benzamidine, pentamidine, pyridoxal-5-phosphate, cysteine, and N-acetyl-aspartyl glutamic acid.[53] These compounds may be added to sealants instead of heparin.

The effectiveness of immobilized heparin in maintaining nonthrombogenic activity during *in vivo* long-term implantation continues to be the subject of much debate. Nojiri *et al*.[54] reported that immobilization of heparin to the polyurethane surface of a ventricular assist device prevented the formation of thrombi for 3 months in a sheep model, whereas formation of thrombi was observed on the non-heparinized controls. However, it was demonstrated that the non-thrombogenicity of the heparinized surfaces was caused by the predominant presence of albumin on these surfaces, instead of by anticoagulant activity of heparin, which had completely disappeared after 15 days.[54]

A second group of additives employed in vascular graft sealants are growth factors, notably acidic and basic fibroblast growth factor (aFGF / bFGF). Growth factors are mostly added to the sealant in combination with heparin,[55-57] which is known to bind and protect them from denaturation and enzymatic degradation.[58] bFGF is a potent mitogen which stimulates proliferation, migration, and differentiation of cells of mesenchymal and neuroectodermal origin, such as ECs.[58] Combined with heparin, aFGF shows equipotent activity as bFGF.[88] To explain the mechanism by which bFGF stimulates cell proliferation, three models, based on a different stoichiometry between bFGF and cell membrane receptors (FGFR) have been proposed (see figure 2).

The first model presumes that bFGF requires cell surface heparan sulfate in order to bind to classical high affinity receptors (FGFR) and subsequently to be internalized and translocated to the nuclei of the cells, after which stimulation of DNA synthesis becomes initiated (1 bFGF/ 1 FGFR).[58,59] The second model is based on the ability of heparan sulfate and heparin to

induce bFGF oligomerization. Subsequently, two molecules of bFGF have the potential to juxtapose two FGF receptors, facilitating receptor dimerization (2 bFGF/ 2FGFR).[60] More recent findings demonstrate that bFGF has two binding sites for cell membrane receptors on opposite sites of the same growth factor molecule, one of which binds to the cell membrane receptor with high affinity, whereas the other has a 250-fold lower affinity.[61] The third model states that bFGF binding to these two receptors, acting in concert after receptor dimerization, is required for growth factor mediated cell proliferation (1 bFGF/ 2 FGFR).[61] Binding of bFGF to the second cell membrane receptor molecule occurs only when growth factor is bound to cell surface heparan sulfate, or when exogenously added heparin is present, like in vascular graft sealants.[58]

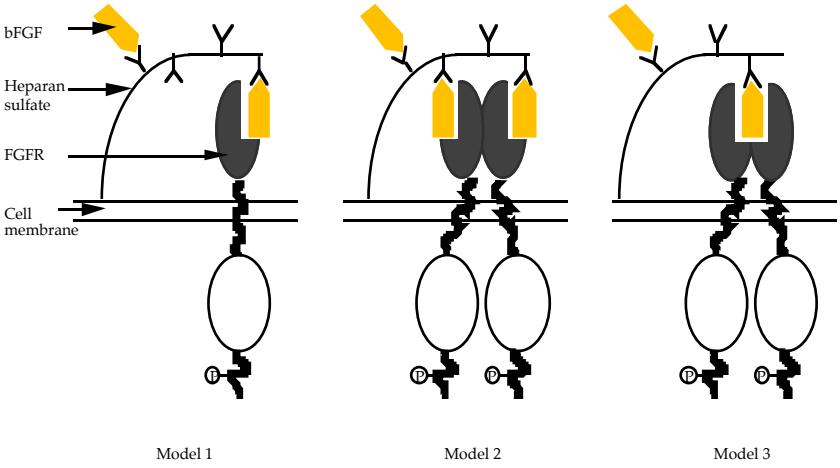

Figure 2. *Three models used to explain the mechanism by which bFGF stimulates cell proliferation. The models are based on a different stoichiometry between bFGF and cell membrane receptors (FGFR).*

Soldani *et al.*[62] incorporated albumin and bFGF in a porous, distensible, tubular membrane made of Biomer (polyesterurethane urea) using a combined spraying, phase-inversion technique. They showed that *in vitro*, during at least two weeks, biologi-

cally active bFGF was released from the polymer. Gray *et al.*[56] showed that aFGF and heparin immobilized to fibrin-glue-impregnated ePTFE, implanted in the aorta in dogs, stimulated endothelialization of the graft, without inducing intimal hyperplasia. After 140 days, all grafts were patent, and 87 percent of the surface was endothelialized, compared with 46-48 percent in control grafts. Doi *et al.*[57] recently demonstrated that co-immobilization of bFGF and heparin in a gelatin-impregnated porous polyurethane graft, implanted in the aorta of rats, significantly accelerated neoarterial regeneration via perianastomotic and - to a lesser extent - transmural tissue ingrowth.

In order to reduce the risk of vascular graft infection, a disastrous complication in vascular surgery, antibiotics may be added to graft sealants. This additive aims to reduce the systemic effects of antibiotics administered to the blood, while providing local resistance to graft infection. Graft-related infection is often caused by common skin microorganisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. Special attention is paid to the use of rifampicin, because its elution from albumin- and gelatin-sealed grafts is much slower compared to the elution of other antibiotics like perfloxacin and vancomycin.[63] Release of rifampicin from collagen-sealant is faster than from albumin- or gelatin-sealant.[63] Upon implantation of a collagen- or gelatin-sealed graft impregnated with rifampicin, release of antibiotic shows a burst of loosely bound molecules during the first hours, followed by a much slower elution of the material from the sealant during the next days.[64] Gahtan *et al.*,[65] using a canine model, demonstrated that rifampicin-bonding to gelatin-impregnated Dacron grafts showed antistaphylococcal activity. When used as an *in situ* replacement to treat established aortic graft infection caused by *Staphylococcus epidermidis*, persistent biofilm colonization was observed in only 29 percent of the rifampicin-treated grafts versus 80 percent of the untreated control grafts.[65]

Approach 3A: endothelial cell seeding - preclinical studies

Besides serving to eliminate the need to pre-clot porous polyester grafts prior to implantation and acting as carrier-vehicle for various additives, vascular sealants have been applied as matrix to allow seeding of ECs on the luminal surface of the graft. Especially collagen, which is a candidate substrate for cell adhesion and proliferation, has been studied with respect to endothelialization of vascular grafts.

The endothelial cell seeding technique was initiated in 1978 by Herring *et al.*,[66] who isolated canine venous ECs, and after mixing these cells with whole blood used the cell suspension to preclot porous 6 mm internal diameter Dacron prostheses, which were then implanted in the infrarenal aorta of dogs. The mean thrombus-free area at 4 weeks was 76% for seeded versus 22% for non-seeded grafts. In a later study,[67] these authors compared the proliferation of ECs seeded on 14 different prosthetic grafts, including Dacron and Teflon grafts. They concluded that weft-knit Dacron grafts were most suitable for endothelial cell seeding, whereas it was difficult for cells to adhere to Teflon.[67] However, a few years later the successful seeding of ECs on Teflon prostheses in a canine model was reported.[68]

The next development in endothelial cell seeding was introduced by Graham *et al*..[69] ECs, isolated enzymatically from canine external jugular veins, were cultured *in vitro* for 14 days prior to seeding on a 6 mm internal diameter Dacron graft, which was subsequently implanted as a thoracoabdominal bypass graft in dogs. In this way, more cells could be seeded onto the graft surface.

It should be emphasized that the significance of endothelial cell seeding techniques is not primarily to improve the patency rate in high-flow and high pressure situations, because in those circumstances non-seeded grafts will remain patent as well. However, significant improvements are expected for prostheses

in areas of low blood-flow.[70,71] Using a canine model in these vascular trajects, both the patency rate and the mean thrombus-free area increased significantly as a result of endothelial cell seeding. Within 4 weeks a confluent layer of ECs was created.

With regard to the mechanisms by which endothelial cell seeding improves vascular graft performance,[3] it was observed that when endothelial cell seeding was technically successful, the degree of platelet interaction with artificial surfaces was reduced and platelet survival became restored. This effect was attributed to both increased prostacyclin production by ECs and decreased thromboxane A_2 production by platelets.[3]

The use of the canine model to evaluate endothelial cell seeding has been controversial. It was observed that the patency rate of vascular grafts in these animals varies considerably. Dogs can be categorized as low or high responders, according to their thrombotic potential.[3] Patency rates in these groups at 3 weeks after seeding of ECs were 100 respectively 10 %. Even without endothelial cell seeding, vascular grafts implanted in dogs endothelialize spontaneously, in contrast to vascular grafts in humans.[4] Spontaneous endothelialization of vascular grafts in dogs may occur via three mechanisms: pannus ingrowth from the host intima across the anastomoses,[69] transinterstitial ingrowth of microvessels from the perigraft tissue through the graft to the vascular lumen,[72] and deposition of circulating ECs onto the surface.[73] Despite these limitations, dogs, and to some extent baboons and pigs, continue to be used to investigate endothelial cell seeding at the preclinical stage.

Approach 3B: improvement of endothelial cell seeding procedures

To allow routine clinical application of the endothelial cell seeding technique, various limitations needed to be resolved. Besides the limited number of potentially available cells in autologous veins, both the efficiency of harvest and the degree of adherence were low, whereas during restoration of the blood flow a significant number of seeded cells were lost.[3,74] The detachment of ECs from the surface, which essentially occurs during the first 20 min after restoration of flow, is greater at increasing levels of shear stress.[75] To increase the number of available ECs, other isolation techniques and other tissues from which ECs can be isolated have been explored. Furthermore, studies were performed to improve the adherence of seeded cells to materials for grafting, as well as to change the anticoagulant activity of seeded ECs.

The harvesting efficiency of ECs was improved by selecting specific lots of collagenase with a high level of basement membrane lysis activity.[76] Furthermore, the possibility of culturing ECs *in vitro* prior to implantation of the grafts was explored.[77] The successful long-term use of seeded grafts that were cultured *in vitro* prior to implantation was recently reported.[78] It should be emphasized that the efficiency of cell harvesting and *in vitro* culturing depends on factors such as the age and health status of the patient, and the lipid content of autologous serum used for cell culture.[76-79]

As alternative for ECs, mesothelial cells (MCs) have been studied for vascular graft seeding. In contrast to ECs, a large number of MCs can readily be made available at the time of surgery. It was shown that Dacron grafts seeded with MCs at the time of graft implantation in dogs released more prostacyclin than unseeded grafts,[80] but thereafter tissue factor expression increased during culture of MCs *in vitro*.[81] During perfusion with whole blood, fibrin fibers were generated in close contact

with seeded cells, and fibrinopeptide-A generation in the perfusate was observed. It was concluded recently, that seeding with MCs does not improve the patency of vascular grafts.[82]

Another source that may provide sufficient cells to cover the graft surface are microvessels present in human fat tissue. Over the years several sources of fat tissue have been used to obtain ECs, with omental fat being the most studied tissue. Controversy started when Visser *et al*.[83] reported that cells derived from omental tissue were predominantly of mesothelial instead of endothelial origin. In later studies, it was clarified that the terms omentum and omental fat have been used interchangably. Cells derived from omentum are mainly MCs, whereas cells isolated from adipose tissue associated with the omentum (omental fat) contain mostly adipocytes and microvascular ECs.[84,85] A significant methodological improvement was made by the use of liposuction derived subcutaneous fat.[86] Patients undergoing vascular procedures do not need to undergo a laparotomy to remove omental or perirenal fat. Instead, removal of fat can be achieved with a syringe, via a small skin incision.

A major problem in the isolation of human microvascular endothelium is the presence and rapid overgrowth of contaminating cells, such as fibroblasts.[87-89] Although various techniques have been developed to eliminate such contaminating cells, most of these methods are time consuming and/or do not give a reproducible yield of early passage endothelial cells.[88] The most promising technique to remove contaminating cells appears to be the repetitive positive selection of ECs with *Ulex Europaeus Agglutinin I*-coated paramagnetic beads.[88]

The adherence of adult human ECs to both Dacron and Teflon was studied *in vitro* by Williams *et al*..[90] They concluded that hardly any ECs adhere to uncoated grafts, whereas EC adherence dramatically increases when the grafts are coated with extracellular matrix, plasma or fibronectin. Fibronectin, to which ECs adhere *in vitro* and *in vivo*, is an adhesive glycoprotein that

is one of the constituents of the basement membrane. Deposition of cellular fibronectin to the underlying substrate is a prerequisite for proliferation of ECs.[91] It was demonstrated that fibronectin coating of ePTFE grafts not only improves adhesion of ECs *in vitro*,[92,93] but also augments the retention of cells to the graft surface after restoration of the blood flow.[94,95] The strength of endothelial cell adhesion to fibronectin-coated surfaces depends both on the fibronectin surface concentration and on the conformation of the adsorbed protein.[96] Schmidt *et al*.[3] reported no advantage of coating of PTFE grafts with fibronectin versus coating with autologous serum. Endothelial cell retention was significantly increased when fibronectin was bound to ePTFE grafts coated with the cationic surfactant tridodecylmethylammonium chloride (80 % retention) instead of to unmodified ePTFE (40 % retention).[97] Unfortunately for graft compatibility, coating with fibronectin increases not only the adhesion of ECs to these surfaces, but of platelets as well.[92,98,99] In animal experiments, pretreatment with anti-platelet agents proved to be essential to maintain graft patencies.[94]

Besides fibronectin, the synthetic tripeptide arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD), an amino acid sequence found in many adhesive plasma and extracellular matrix proteins, including fibronectin, has been used to enhance cell adherence. Binding of cells, including endothelial cells, fibroblasts, smooth muscle cells, and activated platelets, to the RGD-sequence (and thereby to fibronectin) occurs via so-called integrins on the cell membrane, which are able to recognize RGD-sequences.[100,101] When the RGD-tripeptide was immobilized to the surface of Dacron and Teflon,[102] or to poly(vinyl alcohol), polyacrylamide, and poly(carbonate)-urethanes,[103] attachment and spreading of ECs was improved. However, since RGD-peptides are involved in the spreading of adherent platelets as well,[105] it is expected that surfaces to which RGD-sequences are immobilized will enhance platelet deposition. The concept of using specific peptide sequences has been taken one step further by Mazzucotelli *et al.*,[106] who constructed a recom-

binant fibronectin-like engineered adhesion factor from the specific Arg-Gly-Asp cell-adhesion repeats. This synthetic protein supported short-term adhesion of ECs to PTFE, but did not promote proliferation and long-term survival of these cells.[106]

Synthetic proteins based on other cell binding sequences have been covalently immobilized to vascular graft surfaces as well. The cell-binding sequence Tyr-Ile-Gly-Ser-Arg (YIGRS) of laminin was shown to improve endothelial cell adhesion.[107] Of special interest is the oligopeptide Arg-Glu-Asp-Val (REDV), found in the type III connecting segment region of fibronectin. Hubbell *et al.*[101] immobilized this sequence (GREDVY) to glycerolpropylsilane bonded glass and to polyethylene glycol-modified PET. The immobilized oligopeptide promoted adherence and spreading of ECs onto these surfaces. In contrast, fibroblasts, smooth muscle cells, and activated platelets did not spread on surfaces with immobilized REDV.[101] The specific binding of ECs to this peptide, which occurs via the integrin $\alpha_4\beta_1$, makes it of particular interest for selectively enhancing the adherence of ECs to vascular graft surfaces.

Several authors have emphasized the importance of the porosity of vascular prostheses on neointima formation in seeded grafts. Boyd *et al*.[108] developed three ePTFE grafts with internodal distances of 28, 40 and 52 μm seeded with venous ECs. Upon implantation in the carotid arteries of dogs, it was shown that the prostheses with internodal distance of 40 μm were most successful in maintaining patency and thrombus-free surface area. Hirabayashi *et al.*[109] demonstrated that in rats abdominal aorta neointimal healing was rapid and organization (extensive tissue ingrowth through the micropores) was excellent on the grafts with large (60 - 90 μm) internodal pores compared to grafts with 20 - 40 μm pores. When inserted into the arterial system of baboons, optimal porosity of ePTFE was shown to be 60 μm, since 10 and 30 μm grafts failed to achieve luminal endothelial cell coverage, and 90 μm grafts exhibited instability of the intima with focal endothelial cell loss.[110] Using unseeded 60 μm ePTFE grafts in dogs, it was shown that en-

dothelialization of the mid-portion of the graft occured via microvessel ingrowth.[72] Thus, increasing the internodal distance from 30 μm (standard), may improve neointimal healing, while the optimal internodal distance varies between species. When the internodal distance is increased, the grafts need to be impregnated prior to use, to prevent leakage upon restoration of the blood flow.[110]

Cell attachment and growth have been improved by modification of the surface using radio frequency glow discharge (RFGD) in the presence of various organic vapours.[111,112] This treatment creates surfaces with specific oxygen or nitrogen-containing groups. When using such surfaces, the growth of ECs was found to increase with surface carbonyl or amide concentration, but did not correlate with the hydroxyl, carboxyl or amine concentrations.[111,112] Yet another effect created by RFGD treatments is the change in surface hydrophylicity, which may improve the surface adherence of ECs. Optimal adherence has been reported for gas plasma-treated surfaces with hydrophilicity in the range of 40-60°. This effect was attributed to specific protein adsorption favourable for adhesion, spreading and proliferation of ECs, and improved deposition of endothelial matrix proteins.[113,114]

Another way to improve endothelial cell seeding is to restrict cell detachment upon restoration of the blood flow.[74,75] It was demonstrated that endothelial cell adhesion and retention on vascular grafts *in vitro* is markedly enhanced by preconditioning the seeded EC monolayer with long-term shear stress.[115] Preconditioning by shear stress of endothelial cell seeded vascular grafts may improve retention of ECs *in vivo*, thereby contributing to graft patency.

Finally, seeding of vascular grafts with genetically modified ECs, which secrete higher amounts of anticoagulant and fibrinolytic agents than unmodified ECs, offers a potential means of improving patency rates. Shayani *et al.*[116] demonstrated tissue plasminogen activator (t-PA) secretion from small-diameter synthetic graft segments of collagen-impregnated Dacron

seeded with sheep venous ECs that were genetically engineered to secrete human t-PA. These results demand further *in vivo* studies to verify if genetically engineered ECs are capable of providing a local thrombolytic effect.

Approach 3C: endothelial cell seeding in humans

Herring et al.[117] were the pioneers of endothelial cell seeded Dacron vascular grafts implanted in the lower extremities in humans. Endothelial cell seeding improved the patency rate during the first year, although not significantly. A detrimental effect of smoking on graft patency was reported. A second clinical trial by the same authors showed that two-year patency of endothelial cell seeded PTFE grafts was not significantly different from that of vein grafts.[118] Based on the first four clinical trials, it was concluded in 1987 that in humans endothelial cell seeding does not improve the patency of vascular grafts. [117,119-121] Furthermore, it was shown that the incidence of platelet activation of both seeded and unseeded PTFE grafts implanted in the femoropopliteal or femorocrural artery was significantly higher compared to reversed saphenous vein grafts.[122] With respect to platelet activation, no difference was observed between seeded and unseeded grafts.[122]

Further clinical reports have since been published, but comparison of the results is difficult due to the large number of variables such as the internal diameter (4-10 mm), the graft material (Dacron, Teflon), the placement of the graft (above/below knee femoropopliteal, aorto bifemoral), the type of ECs used (venous, subcutaneous fat, omental fat), the method of seeding and the use of anticoagulants.

One important observation is, that the ultimate success of endothelial cell seeding of vascular grafts depends on the confluency of the formed cellular layer.[78,123,124] Herring *et al*.[123] reported failure of low-density endothelial cell seeding on ePTFE to improve patency. Compared to the patency of vein grafts after 30 months (92 ± 4 %), the patency of the seeded artificial

grafts (38 ± 9 %) was extremely low. In contrast, Zilla *et al.*[78,125] cultured autologous ECs, obtained from the external jugular vein under local anesthesia using *in situ* cannulation, prior to seeding of the grafts. Before implantation, the seeded grafts were cultured for 12 ± 3 days to allow maturation of the endothelial cell cytoskeleton. Seeding the grafts with ECs in this manner led to a confluent endothelial cell layer prior to implantation, and significantly improved the patency compared to unseeded grafts at 3 years.[125] The patencies of these seeded grafts were similar to those of autologous vein grafts after 7 years.[78] Laube *et al.*[124] employed a similar procedure, using 4 mm internal diameter PTFE grafts coated with fibrin-glue and human recombinant bFGF as bypass graft for coronary artery revascularisation, and found that the patency rate of the seeded grafts was 91 percent after a mean period of 522 days.

An alternative for *in vitro* culture of the cells prior to implantation may be the acceleration of the proliferation of ECs seeded at relatively low density by means of a growth factor present on the graft surface, such as basic fibroblast growth factor (bFGF). Bos *et al*.[126] reported enhanced cell proliferation of ECs which were seeded on an albumin-heparin coating to which bFGF was immobilized. Similar results were reported for endothelial cell proliferation on carbodiimide crosslinked films of albumin and heparin[127] or collagen I and heparin.[128] (figure 3)

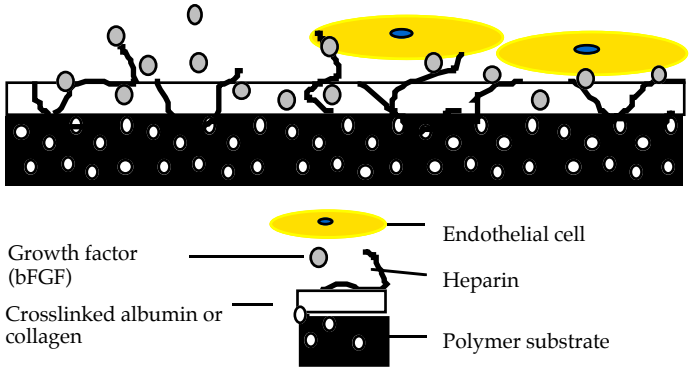

Figure 3. *Schematic representation of endothelial cells seeded onto heparin containing sealants to which bFGF is loaded.*

Novel approaches in cardiovascular tissue engineering

A novel approach to improve the patency of small-diameter vascular grafts is to promote the migration of ECs from tissue to establish an endothelial lining on the graft lumen. According to this concept, ECs recruited from the microcirculation in the surrounding tissue, migrate within the porous graft wall. Subsequently, these cells may form capillaries that emerge at the luminal surface and provide ECs to colonize the luminal surface. Two strategies have been used so far. The first method uses biosynthetic Omniflow™ vascular prostheses which are perforated with an excimer laser, resulting in holes of 50-100 μm.[129] Upon implantation in the carotid artery of sheep, ECs were found on the mid-portion of the perforated grafts after 3 months. Spontaneous endothelialization was shown to be achieved by transmural capillary ingrowth through perforations made in the graft wall.[129] The second method is more specific, as selected proteins are used to promote transmural neovascularization of 4 mm internal diameter ePTFE vascular grafts (30 μm internodal distance).[130] Several laminin/collagen IV mixtures, immobilized using SurModics Photolink® technology, were found to greatly enhance the capillary ingrowth into ePTFE implanted in subcutaneous and epididymal fat pads of rats. Upon implantation of a 1 mm internal diameter graft in the rat aorta, several coatings showed high capillary density in the graft walls, and grafts coated with laminin also exhibited extensive endothelial cell coverage, consistent with transmural endothelialization.[130]

Advances in tissue engineering have provided opportunities for new concepts in vascular surgery. Unlike traditional devices, tissue constructs are engineered to remain entirely biointeractive after implantation. In this way, they do not only offer the needed structure like artificial grafts, but the physiologic functions of the replaced tissue as well. The tissue engineering approach mostly involves the use of completely closed bioreactor systems to maintain sterility while aiming to mimick a physio-

logical environment. In this environment, cells seeded onto biocompatible three-dimensional scaffolds proliferate and secrete growth factors and matrix proteins to form a functional tissue. Yue *et al*.[131] seeded cultured smooth muscle cells on microporous, compliant, biodegradable vascular grafts composed of polyurethane (95 w%) and poly-L-lactic acid (5 w%). After implantation into the abdominal aorta of rats, 65 percent of the seeded grafts showed fast and uniform neomedia development. In contrast, non-seeded control grafts showed only limited smooth muscle cell ingrowth. Clearly, smooth muscle cell seeding enhanced the process of arterial waal regeneration in these grafts.[131] Naughton[132] reported the manufacture of small-diameter vascular grafts based on polyglycolic acid, utilizing a bioreactor which applies pulsed physiological stresses onto the tissue during growth. These forces induce a more rapid and denser matrix deposition by the fibroblasts or smooth muscle cells used. Initial studies of these grafts in a lamb model demonstrated long-term patency without neointimal hyperplasia. A similar approach, using fibroblasts and endothelial cells, was recently reported by Zünd *et al*..[133] As an alternative to the closed bioreactor systems, L'Heureux *et al.*[134] used seperately cultured smooth muscle cells and fibroblasts. The smooth muscle cell layer was wrapped around a tubular support to produce the 'media' of the vessel, and subsequently the fibroblast sheet was wrapped around this 'media' to provide the 'adventitia'. After maturation, the tubular support was removed and ECs were seeded on the lumen. The viability of these cells after constructing the blood vessel was demonstrated.[134]

Conclusions

The future of small-diameter vascular prosthesis looks promising. An important advantage of completely tissue engineered vascular grafts compared to traditional polymer based implants is the ability to grow when implanted in young patients. Limited willingness of surgeons to use tissue engineered grafts without polymeric reinforcement, and time and effort consuming regulatory issues may delay the wide-spread use of this promising approach. Meanwhile, seeding of endothelial cells on vascular graft materials, impregnated or coated with biologically relevant proteins (e.g. collagen, albumin, fibronectin, and laminin) and additives such as anticoagulants, growth factors and antibiotics, remains the method of choice to improve the patency of small-diameter vascular grafts.

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Chapter 3 Immobilization of Albumin and Albumin-heparin Conjugate on Gas Plasma Modified Polystyrene[1]

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Abstract

Polystyrene (PS) discs and petridishes were treated with an argon or carbon dioxide gas plasma, which resulted in the introduction of hydroxyl, epoxide, carbonyl, and carboxylic acid groups at the surface. Some of these groups were used to immobilize monolayers of albumin or albumin-heparin conjugate (alb-hep conjugate), either by contacting the surface directly with an aqueous solution of the protein or conjugate at pH 8.2, or by preactivating surface carboxylic acid groups with N-(3-dimethylamino-propyl)-N'-ethylcarbodiimide (EDC) at pH 4.6, followed by coupling at pH 8.2.

Using ^{14}C-labeled alb-hep conjugate, it was shown that formation of a monolayer, which is relatively stable in contact with aqueous solutions at a physiologic pH, required preactivation of carboxylic acid groups at the surface of argon-treated PS discs (PS-Ar-d), whereas for carbon dioxide-treated PS discs (PS-CO_2-d) binding can occur through other groups than carboxylic acid groups, and therefore preactivation of carboxylic acid groups was not necessary. The surface concentration of alb-hep conjugate for PS-CO_2-d (0.41 ± 0.01 $\mu g/cm^2$) was higher than for PS-Ar-d (0.26 ± 0.01 $\mu g/cm^2$). Small quantities (7-15%) of alb-hep conjugate were released during one day from either surface at 37°C in PBS, in 3% (w/v) sodium dodecyl sulfate (SDS) solution in PBS, and in serum-containing cell culture

medium (CMS), whereafter only very minor release was observed in CMS during the next two weeks. Subsequent investigations were carried out using PS-CO_2-petridishes (PS-CO_2-p), without preactivation.

The surface concentration of alb-hep conjugate and albumin, immobilized onto PS-CO_2-p and tissue culture PS petridishes (TCPS-p) were compared, as well as the stability of these coatings. The surface concentrations of alb-hep conjugate on PS-CO_2-p (0.77 ± 0.13 $\mu g/cm^2$) and TCPS-p (0.71 ± 0.11 $\mu g/cm^2$) were similar, whereas that on PS-p (0.37 ± 0.05 $\mu g/cm^2$) was much lower. For albumin similar differences were found between the substrates, but the surface concentrations were lower: 0.43 ± 0.02, 0.41 ± 0.02 and 0.28 ± 0.04 $\mu g/cm^2$ for PS-CO_2-p, TCPS-p and PS-p, respectively. Release of albumin and alb-hep conjugate bound either to PS-CO_2-p or TCPS-p was comparable to release of alb-hep conjugate from PS-CO_2-d. The striking similarity between TCPS-p and PS-CO_2-p with regard to surface concentrations of albumin and alb-hep conjugate, and the subsequent stability of these coatings, indicate that TCPS may also have groups at the surface, which are able to covalently bind proteins.

The surface concentration of alb-hep conjugate for PS-CO_2-petridishes was higher than for PS-CO_2 discs, which may be caused by differences in alb-hep conjugate lots used.

Coupling of ^{14}C-labeled methylamine to PS-CO_2 petridishes suggests that epoxide and/or aldehyde groups at the surface play a role in the covalent attachment of albumin and alb-hep conjugate to this surface.

In conclusion, a relatively stable monolayer of albumin or alb-hep conjugate on polystyrene can be obtained by first pretreating the PS-surface with a CO_2 gas plasma, followed by contacting this surface with an aqueous solution of the protein (conjugate) at pH 8.2.

Introduction

To study the interaction between proteins or protein-containing conjugates immobilized on polymeric surfaces and blood constituents or cultured cells, well defined and stable coatings are required. Stable coatings can be obtained by covalent coupling of either the proteins or the conjugates to the polymer surface.

Gas plasma treatment using oxidizing gases is often applied to introduce functional groups at polymeric surfaces and to adjust the wetting properties of such surfaces.[2-11] These functional groups permit covalent attachment of bioactive molecules for biomedical or biotechnological applications.[12-14] Furthermore, gas plasma treatment modifies the outermost surface of polymers without changing the bulk characteristics of the material. The chemical composition of gas-plasma modified surfaces depends on both the gas used and the experimental conditions. Carbon dioxide- and argon-plasma treatments are unspecific and result in the introduction of a series of oxygen-containing groups, including hydroxyl, hydroperoxide, epoxide, aldehyde, ketone, ester, and carboxylic acid groups.[15-17]

The use of Ar- or CO_2-plasma to modify polystyrene (PS) surfaces has been reported by several authors. Van Delden *et al.*[12] treated spincoated PS with an Ar-plasma. This treatment resulted in the introduction of a limited number of carboxylic acid groups, which were used in combination with a water-soluble carbodiimide to immobilize alb-hep conjugates to the surface. Takens *et al.*[17] showed that spincoated PS is oxidized in a CO_2-plasma, resulting in high concentrations of oxygen at the outermost surface and a high wettability of the surface. By the CO_2-plasma treatment, hydroperoxide, epoxide, carbonyl and carboxylic acid groups were introduced at the surface.[17]

The introduced functional groups can be used to covalently immobilize biomolecules, for example via their amine groups.[12] In contrast to the reaction of an amine group with a carboxylic acid group, no coupling agent is required for the reaction between amine and epoxide or aldehyde groups. The reaction be-

tween amine and epoxide groups will result in a covalent linkage, whereas the reaction between amine and aldehyde results in the formation of a Schiff base, which may decompose into the starting compounds.[18] Reduction of the double bond of this compound leads to the formation of a secondary amine, providing a stable linkage.[19]

Previous research in our group[20,21] demonstrated, that coating of biomaterials with conjugates consisting of albumin and heparin improves their blood compatibility. Coating of polyurethane catheters and poly(tetrafluoroethylene) sheets with alb-hep conjugates decreases the *in vitro* deposition, aggregation and activation of platelets.[20,21] Alb-hep conjugate coating is also effective in reducing complement activation.[20] The conjugate is a heterogeneous mixture of molecules with different molar ratios of albumin and heparin, such as alb_1-hep_1, alb_2-hep_1, and alb_1-hep_2.[21]

The aim of the present study was to develop suitable plasma-treatment methods for the introduction of functional groups at polystyrene surfaces, which can be used for coupling of albumin or alb-hep conjugate. Since in subsequent studies the use of these surfaces as substrates for endothelial cell seeding will be evaluated, the stability of the immobilized layers in SDS-solution, in PBS and in serum-containing culture medium was determined as well.

Materials and Methods

Materials

Alb-hep conjugates (11.6% and 15.2% (w/w) heparin) were obtained from Holland Biomaterials Group (Enschede, The Netherlands). ^{14}C-Formaldehyde (55 mCi/mmol), ^{14}C-methylamine (50 mCi/mmol), and ^{14}C-acetic acid (12.4 mCi/mmol) were purchased from Amersham (Amersham, UK). $NaCNBH_3$ was from Janssen Chimica (Beerse, Belgium). Optiphase HiSafe-3 was from Wallac (Milton Keynes, UK). Phosphate-buffered

saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene discs (22 mm diameter) were obtained from W.S.V. Kunststoffen (Utrecht, The Netherlands). Polystyrene (PS) and tissue culture polystyrene (TCPS) petridishes (35 mm diameter) were from Greiner GmbH (Frickenhausen, Germany). Argon (Ar) and carbon dioxide (CO_2) were obtained from Hoekloos (Schiedam, The Netherlands). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, and trypsin-EDTA were purchased from Life Technologies (Paisley, UK). Bovine serum albumin (fraction V) was from Sigma Chemical Company (St. Louis, Missouri, USA). Dialysis tubing (size 9) was obtained from Medicell International Ltd. (London, UK). Human serum was pooled from 12 healthy volunteers. All other chemicals were of the highest purity available and obtained from Merck (Darmstadt/Hohenbrunn, Germany).

Gas plasma treatment of polystyrene discs and petridishes

Polystyrene surfaces used for gas plasma treatment were rinsed ultrasonically with hexane, ethanol and demineralized water (4 times 15 min for each solvent) and subsequently dried *in vacuo*.

The plasma reactor consisted of a glass tube with an internal diameter of 6.5 cm and a length of 80 cm (figure 1). The reactor was equipped with 3 externally placed, capacitively coupled electrodes. The hot electrode was placed in the centre of the reactor and the cold electrodes at 10 cm (Ar treatment) or 30 cm (CO_2 treatment) distance at both sides. One side of the reactor was connected to a gas inlet system with flow control. The reactor was evacuated by a two stage rotary vane pump. The electrodes were powered through a matching network by a 13.56 MHz radio frequency generator. The plasma treatment time was computer controlled. The plasma treatment procedure was as follows: 12 polystyrene discs or 6 polystyrene petridishes were placed between the powered and grounded electrodes on glass substrate holders (see figure 1).

The reactor was evacuated until a pressure of 0.01 mbar, before an Ar flow of 10 cm^3/min (STP) or a CO_2 flow of 20 cm^3/min (STP) was established through the reactor. After 5 min equilibration, the plasma was initiated (41 W). After 1 min, the plasma treatment was terminated and the gas flow was maintained for 2 min. The reactor was brought to atmospheric pressure with air. The discs were turned and the procedure was repeated. To prevent surface rearrangement processes after treatment, surfaces were stored at a temperature of -20 °C until characterization or use.[17]

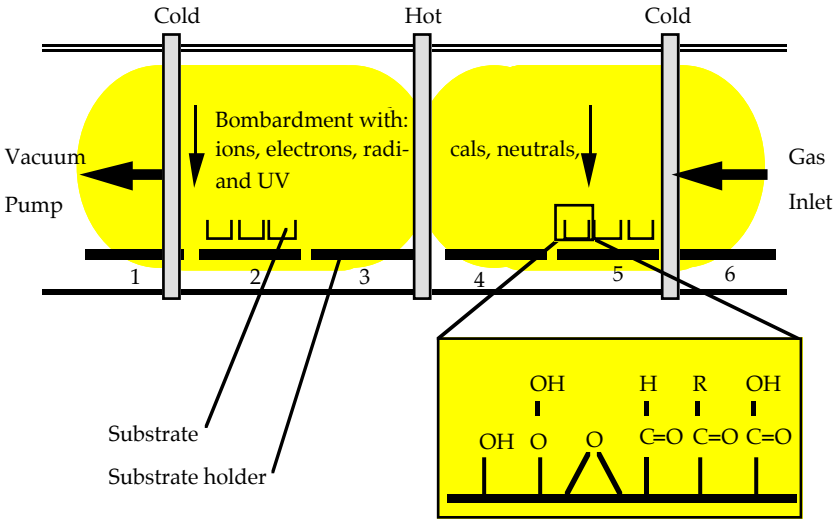

Figure 1 *Schematic representation of the gas plasma reactor. Substrates were placed close to the cold electrodes on substrate holders 2 and 5.*

XPS analysis

Atomic percentages of incorporated oxygen were determined for PS-Ar-d, PS-CO_2 -d, PS-CO_2-p, TCPS-p and PS-p. The atomic percentages were measured by X-ray photoelectron spectroscopy (XPS) using a Kratos XSAM-800 (Kratos, Manchester, United Kingdom), equipped with a Mg Kα X-ray source

(15 kV, 10 mA).The analyzer was placed perpendicular to the sample surface. Survey scans (0-1100 eV) were recorded to qualitatively determine the elemental composition of the samples and detail scans were recorded for quantitative analyses. The relative peak areas for the different elements were calculated and corrected using empirically derived sensitivity factors. After normalization, the elemental composition in atomic percentages was obtained. Calculations were performed using the multi-user DS 800 software system (Kratos).

^{14}C-labeling of albumin and alb-hep conjugate

Bovine albumin and alb-hep conjugates were labeled by reductive methylation using ^{14}C-formaldehyde in the presence of $NaCNBH_3$.[22] ^{14}C-formaldehyde (6.1 μmol, 55 mCi/mmol) was added to albumin (500 mg, 7.2 μmol) or alb-hep conjugate (500 mg, ca. 7 μmol) in 10 ml PBS. Subsequently, 6 freshly prepared aliquots of $NaCNBH_3$ in PBS (100 μl, 100 μmol/ml) were added with 15 min intervals. After 3 hours at room temperature, the labeled products were dialyzed for 48 hours against 5 l demineralized water, which was replaced every 12 hours. Finally the products were lyophilized. After addition of 8 ml HiSafe3, ^{14}C-activity of quantities of albumin or alb-hep conjugate with known weight was determined using a β-counter (Wallac Winspectral 1414, Wallac, Turku, Finland). The specific activity was determined by dividing the ^{14}C-activity of the sample by the weight of the sample. The specific activities of ^{14}C-albumin, ^{14}C-alb-hep conjugate (11.6% (w/w) heparin) and ^{14}C-alb-hep conjugate (15.2% (w/w) heparin) were respectively 1013 ± 10, 1502 ± 22 and 1325 ± 14 DPM/μg.

Immobilization of alb-hep conjugate to gas plasma-treated PS-discs; alb-hep coating of PS-discs

^{14}C-Alb-hep conjugate (11.6% (w/w) heparin) was bound to PS-Ar (PS-Ar-d) or PS-CO_2 (PS-CO_2-d) discs (9.0 cm^2) by adding conjugate solution (1.5 ml, 15 mg alb-hep/ml) in borate buffer (5 mM $K_2B_4O_7$, pH 8.2) to the surface either directly or after preactivation of carboxylic acid groups at the surface using N-(3-dimethylamino-propyl)-N′-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS).[23] Preactivation of carboxylic acid groups was achieved by immersing the discs for 30 min in 2-morpholino-ethane sulfonic acid (MES) buffer (5 ml, 5 mM, pH 4.6) to which EDC (1 mM) and NHS (0.2 mM) were added. Discs that were not preactivated were stored in MES buffer. After rinsing with cold MES buffer, followed by rinsing with borate buffer (5 ml, 5 mM $K_2B_4O_7$, pH 8.2), preactivated discs as well as discs that were not preactivated were placed in borate buffer containing alb-hep conjugate (1.5 ml, 15 mg/ml). After one hour of incubation with alb-hep conjugate, the discs were rinsed using PBS (3 times), PBS to which NaCl was added (final concentration1M) which was acidified to pH 3.5 using 1 M HCl (3 times), followed by PBS to which NaCl was added (final concentration1M) which was brought to pH 3.5 using 1 M NaOH (3 times). Finally, the surfaces were rinsed twice with PBS.

PS-discs was coated with ^{14}C-alb-hep conjugate (11.6% (w/w) heparin) using a procedure similar to the non-preactivated immobilization of alb-hep to PS-Ar-d or PS-CO_2-d described above.

After immobilization of ^{14}C-alb-hep conjugate to PS-Ar-d, PS-CO_2-d or PS-d, the surfaces were incubated twice with NaOH (2 ml, 1M) for 24 h. By this procedure, the majority of the immobilized conjugate was removed from the surface. Subsequently, after the addition of 18 ml HiSafe3 (18 ml, scintillation cocktail), the radioactivity of NaOH-solutions was determined. After the addition of 18 ml HiSafe3 to the NaOH-

treated petridish, the radioactivity remaining at the surface was determined. This latter value was multiplied by 1.4 to roughly account for the radioactivity adsorbed by the polystyrene. Surface concentrations of alb-hep conjugate were calculated by dividing the surface-bound radioactivity (sum of radioactivity of the NaOH-solutions and remaining at the surface) by the specific activity of the conjugate and surface area.

Stability of alb-hep conjugate coatings on PS, PS-Ar and PS-CO_2 discs

^{14}C-Alb-hep (11.6% (w/w) heparin)-coated discs were kept at 37 °C in vials containing 8 ml of complete cell culture medium (CM) supplemented with 20% (v/v) pooled human serum (CMS). CM consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). At selected times, the supernatant was replaced by fresh CMS. After addition of 13 ml HiSafe-3, the radioactivity of the supernatant was measured. At day 17, the radioactivity present at the surfaces was determined as described above.

The stability of alb-hep conjugate-coated surfaces in SDS-solution was determined by measuring the radioactivity of the supernatant and at the surface after vigorous shaking of the discs in a SDS solution in PBS (8 ml, 3% (w/v)) at 37 °C for 48 hours.

Immobilization of albumin or alb-hep conjugate on PS, TCPS and PS-CO_2 petridishes

^{14}C-Albumin or ^{14}C-alb-hep conjugate (15.2% (w/w) heparin) was bound to PS (PS-p), tissue culture polystyrene (TCPS-p) or PS-CO_2 (PS-CO_2-p) petridishes (10.9 cm^2) by adding a solution of albumin or conjugate (1 ml, 15 mg alb or alb-hep/ml) in borate buffer (5 mM $K_2B_4O_7$, pH 8.2) to the surface. After 45 min incubation, the petridishes were rinsed using PBS (3 times), a 12

mM phosphate buffer of pH 3.5 containing 1M NaCl (3 times), followed by a similar solution of pH 10.5 (3 times). Finally, the surfaces were rinsed twice with PBS. Determination of surface concentrations of albumin or alb-hep conjugate was carried out according to the procedure given for alb-hep conjugate immobilization on discs.

Stability of albumin and alb-hep conjugate coatings on PS, TCPS and PS-CO_2 petridishes

To ^{14}C-albumin and ^{14}C-alb-hep (15.2% (w/w) heparin) conjugate-coated PS-p, TCPS-p or PS-CO_2-p, 2 ml of CMS or PBS was added and the petridishes were kept at 37 °C. At selected times, the supernatant was replaced by fresh CMS or PBS. After addition of 14 ml HiSafe-3, the radioactivity of the supernatants was measured. At day 14, the radioactivity of the surfaces was determined as described above. Surface concentrations were calculated as described above.

Albumin and alb-hep-coated surfaces were also incubated with 2 ml of an SDS solution in PBS (3% (w/v) at 20 °C or at 37 °C for 48 hours, after which the radioactivity in the supernatant and at the surface was determined.

Binding of methylamine and acetic acid to PS-CO_2

^{14}C-Methylamine (20 nmol/ml) or ^{14}C-acetic acid (20 nmol/ml) in 1 ml buffered solution was added to a PS-CO_2 petridish. The buffer solutions used were MES-buffer (5 mM MES) at pH 3 or 5, and borate buffer (5 mM $K_2B_4O_7$) at pH 8.2, 9 or 10. After 1 hour incubation, the petridishes were rinsed using PBS (3 times), a 12 mM phosphate buffer of pH 3.5 containing 1M NaCl (3 times), followed by a similar solution of pH 10.5 (3 times). Finally, the surfaces were rinsed twice with PBS. Determination of surface concentrations of methylamine or acetic acid was carried out according to the procedure given for alb-hep conjugate immobilization on discs.

Surface concentrations of albumin and alb-hep conjugate and stability of albumin and alb-hep conjugate coatings on aged PS-CO_2-petridishes

PS-CO_2-petridishes were stored at -20 °C for 6 months after plasma treatment. The atomic composition of the surface of aged PS-CO_2-petridishes was determined using XPS. ^{14}C-Albumin and ^{14}C-alb-hep conjugate were immobilized onto the aged PS-CO_2-petridishes according to the procedures described before. The stability of the coatings during incubation with an SDS-solution in PBS (2 ml, 3% (w/v)) or with CMS was determined as described above. The surface concentrations of albumin and of alb-hep conjugate on aged PS-CO_2-p and the stability of the coatings on the aged petridishes were compared with those of freshly gas plasma modified petridishes.

Results

Gas plasma treatment of polystyrene discs and petridishes

The results of XPS analyses of Ar- and CO_2-treated surfaces (table 1) demonstrate that the oxygen content of the surfaces increased by the plasma treatment. The oxygen content of CO_2-treated surfaces was significantly higher than of Ar-treated surfaces and of the reference surface TCPS. One atomic percent of nitrogen was incorporated in Ar-treated surfaces, whereas nitrogen was hardly detectable on CO_2-treated surfaces. The atomic percentage of oxygen was low for untreated polystyrene.

surface	n	C (atomic%)	O (atomic%)	N (atomic%)
PS-d	6	99.6 ± 0.2	0.4 ± 0.2	0.0 ± 0.0
PS-Ar-d	4	87.3 ± 0.9	11.6 ± 0.7	1.0 ± 0.2
PS-CO_2-d	2	79.2 ± 2.0	20.7 ± 2.0	0.1 ± 0.0
PS-CO_2-p	4	79.0 ± 2.0	20.9 ± 1.9	0.1 ± 0.1
TCPS-p	2	86.3 ± 0.1	13.7± 0.1	0.0 ± 0.0

Table 1. *XPS analysis of unmodified, gas plasma-treated polystyrene surfaces and tissue culture polystyrene (TCPS, reference). n is the number of samples measured.*

Selection of gas plasma treatment

^{14}C-Alb-hep conjugate (11.6% w/w heparin) was immobilized onto native PS discs as well as onto Ar and CO_2 gas plasma-treated polystyrene discs, with or without preactivation of carboxylic acid groups at the surface. Selection of the optimal gas plasma treatment was based on the surface concentrations and stabilities of ^{14}C-alb-hep conjugate, immobilized onto the various gas plasma-treated polystyrene discs.

Surface concentrations of alb-hep conjugate on PS-d, PS-Ar-d and PS-CO_2-d without preactivation were respectively 0.27 ± 0.01, 0.10 ± 0.03 and 0.41 ± 0.03 μg/cm^2 (figure 2). Preactivation of carboxylic acid groups increased the surface concentration of alb-hep conjugate on PS-Ar-d significantly, whereas preactivation of PS-CO_2-d decreased the surface concentration of alb-hep conjugate.

In contrast to the various gas plasma-modified discs, most of the bound alb-hep conjugate was released from PS-d upon incubation with SDS.

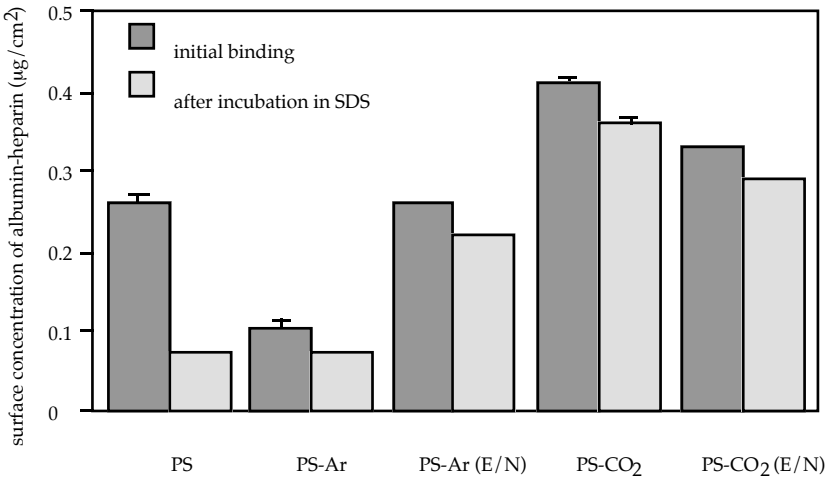

Figure 2. *Surface concentration of ^{14}C-alb-hep conjugate (11.6% (w/w) heparin) immobilized on polystyrene (PS), Ar plasma-treated polystyrene (PS-Ar) and CO_2 plasma-treated polystyrene (PS-CO_2) discs without or with (E/N) preactivation of surface carboxylic acid groups by EDC and NHS. Solid bars represent surface concentrations before, and striped bars represent surface concentrations after exposure to 3% SDS solution for 48 hours at 37°C. (n=3, ± sd)*

A small amount of alb-hep conjugate was released from the gas plasma-treated surfaces in CMS within one day, after which the immobilized layers on the surfaces were stable (figure 3). In contrast, almost 50 percent of the initially bound alb-hep conjugate was released from native PS-d during the first 24 hours, after which the remainder of the coating was stable during more than two weeks.

The surface concentration of alb-hep conjugate on PS-CO_2-d was much higher than on preactivated PS-Ar-d, whereas the stability of the coatings did not differ significantly.

Subsequent investigations were carried out using PS-CO_2-petridishes (PS-CO_2-p), without preactivation with EDC/NHS. Furthermore a different lot of alb-hep conjugate had to be used since the previous lot could not be supplied any more.

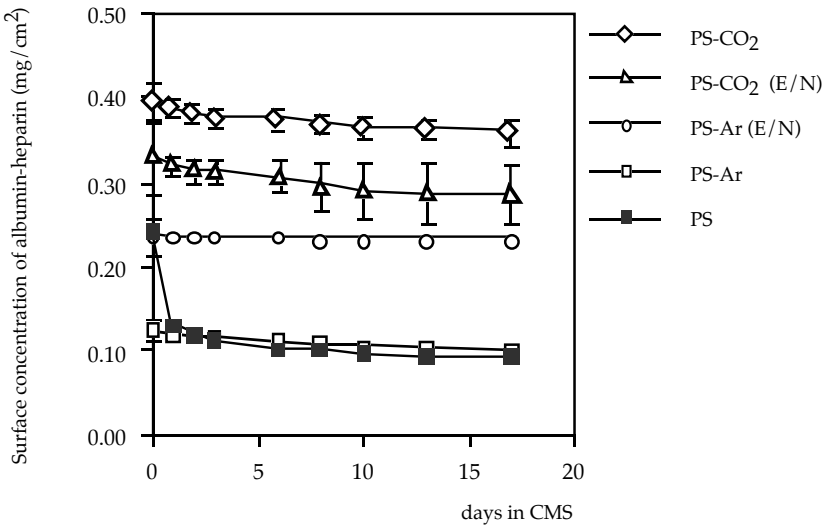

Figure 3. *Surface concentration of ^{14}C-alb-hep conjugate (11.6% (w/w) heparin) immobilized on polystyrene (PS), Ar plasma-treated polystyrene (PS-Ar) and CO_2 plasma-treated polystyrene (PS-CO_2) discs with or without preactivation of surface carboxylic acid groups by EDC and NHS (E/N), as a function of storage time in cell culture medium (CMS) at 37 °C. (n=3, ± sd)*

Comparison of surface concentration and stability of albumin and alb-hep conjugate on PS, TCPS and PS-CO_2 petridishes

^{14}C-Albumin and ^{14}C-alb-hep conjugate (15.2% (w/w) heparin) were immobilized onto petridishes with different surface characteristics. After adding an alb-hep conjugate solution (15 mg/ml) to PS-CO_2-p, the maximum surface concentration was reached within 5 min (data not shown). After 45 min, surface concentrations of alb-hep conjugate on PS-CO_2-p (0.77 ± 0.13 μg/cm^2) and on TCPS-p (0.71 ± 0.11 μg/cm^2) were higher than on PS-p (0.37 ± 0.05 μg/cm^2) (figure 4A). For albumin similar differences were found between the substrates, but the surface concentrations were lower: 0.43 ± 0.02, 0.41 ± 0.02 and 0.28 ± 0.04 μg/cm^2 for PS-CO_2-p, TCPS-p and PS-p, respectively (figure 4B).

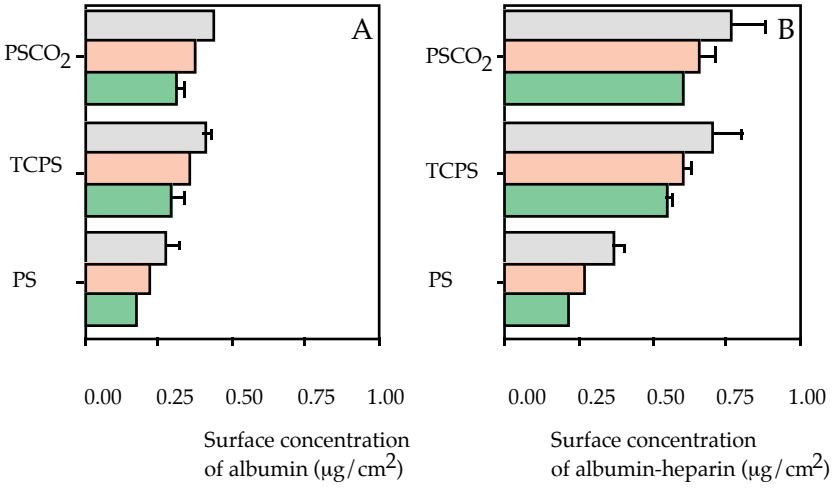

Figure 4. *Surface concentration of ^{14}C-albumin (A) and ^{14}C-alb-hep conjugate {15.2% (w/w) heparin} (B) immobilized on polystyrene (PS), tissue culture polystyrene (TCPS) and CO_2 plasma-treated polystyrene (PS-CO_2) petridishes. Bars represent surface concentrations before ( ) and after exposure to 3% SDS solution for 48 hours at 20°C ( ) or 37°C ( ). (n=3, ± sd)*

Upon incubation of the coated dishes for 48 h in SDS solution at room temperature, respectively 14, 14 and 24 percent of surface-bound conjugate and 14, 15 and 21 percent of surface-bound albumin were removed from PS-CO_2-p, TCPS-p and untreated PS-p. Treatment of these petridishes with SDS at 37°C for 48 h resulted in more release of the coatings: about 21, 23 and 41 percent conjugate and about 28, 27 and 36 percent albumin, respectively (figures 4A and 4B).

Between 20 and 30 percent of the coating was removed from all petridishes during 14 days in CMS at 37°C (figure 5). Most of the material was released during the first day of incubation, but the release of alb-hep conjugate from PS-CO_2-p and TCPS-p continued for about a week. The immobilized layers were slightly more stable in PBS at 37°C than in CMS (data not shown).

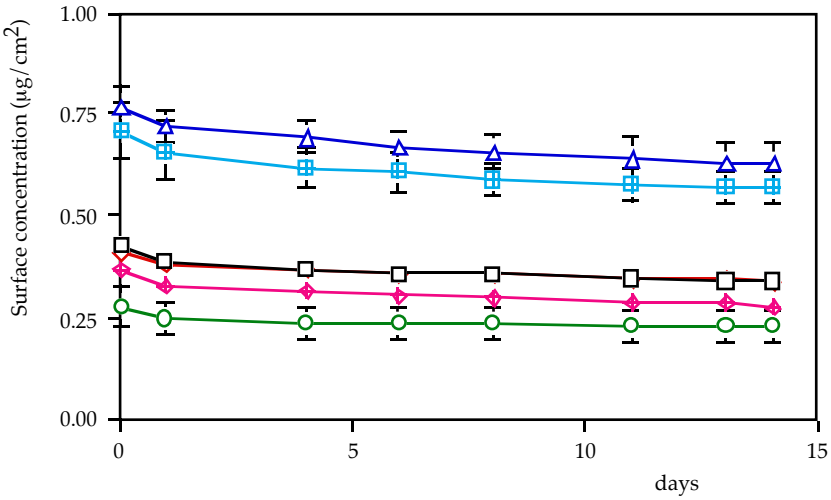

Figure 5. *Surface concentration of ^{14}C-albumin and of ^{14}C-alb-hep conjugate (15.2% (w/w) heparin) immobilized on polystyrene (○ alb, ◈ alb-hep), tissue culture polystyrene (◇ alb, ⊞ alb-hep) and CO_2 plasma-treated polystyrene (□ alb, △ alb-hep) petridishes as a function of storage time in cell culture medium (CMS) at 37 °C. Note that ◇ and □ overlap one another. (n=3, ± sd)*

Binding of methylamine and acetic acid to PS-CO_2

To obtain more insight in the coupling mechanism taking place after addition of albumin or alb-hep conjugate to non-preactivated PS-CO_2-petridishes, the binding of ^{14}C-methylamine and ^{14}C-acetic acid to this surface was investigated as a function of pH during coupling. In the range of pH values from 4 to 10 almost no acetic acid was detected at the surface after washing. For coupling of methylamine a distinct effect of pH was observed. The amount of immobilized methylamine increased with increasing pH, especially above pH 8.

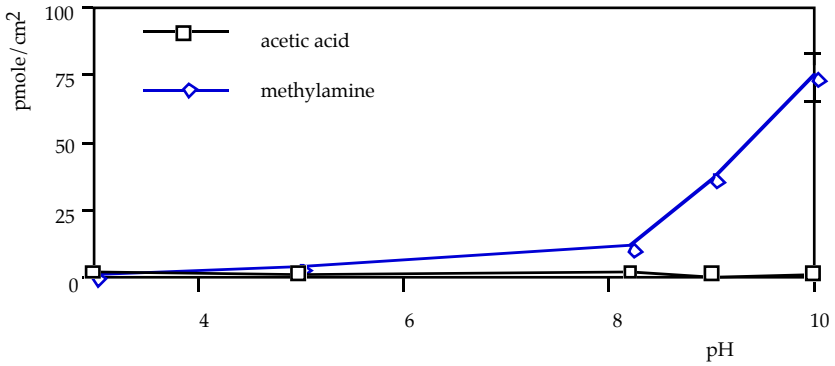

Figure 6 *Surface concentration of methylamine and acetic acid after treatment of PS-CO_2 petridishes with methylamine- or acetic acid-containing buffer solutions of varying pH. (n=3, ± sd)*

The effect of storage of PS-CO_2-petridishes on immobilization of albumin and alb-hep conjugate and stability of the coatings

The average atomic composition of the surface was not altered upon storage (data not shown). Surface concentration of both albumin and alb-hep conjugate were not significantly different for PS-CO_2-petridishes aged for 6 months at -20°C and for

petridishes used directly after plasma-treatment (n = 3, P = 0.05, student-*t* test). Also the stability of albumin as well as alb-hep coatings were not significantly different for aged and fresh surfaces (n = 3, P = 0.05, student-*t* test).

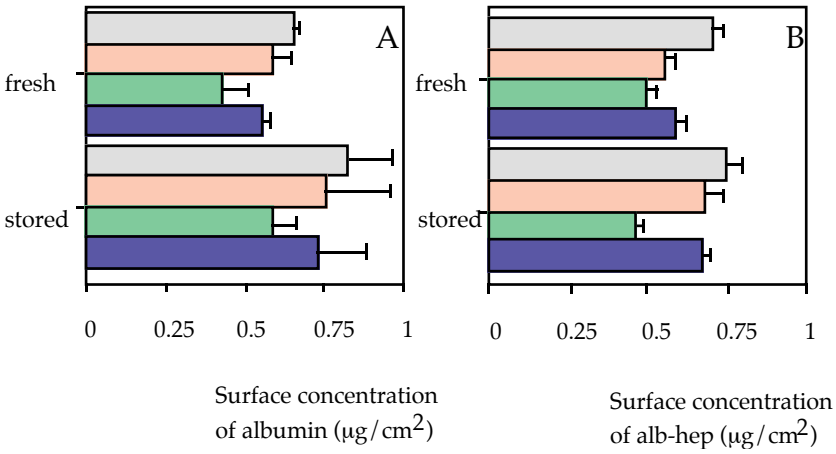

Figure 7. *Surface concentration of ^{14}C-albumin (A) and ^{14}C-alb-hep conjugate {15.2% (w/w) heparin} (B), immobilized on CO_2 plasma-treated polystyrene (PS-CO_2) petridishes from a borate solution at pH 8.2. Either freshly treated surfaces or surfaces that were stored for 6 months at -20°C were used for the immobilization. Bars represent surface concentrations before ( ) and after exposure to 3% SDS solution for 48 hours at 20°C ( ) or 37°C ( ), or after incubation in CMS for 14 days ( ). (n=3, ± sd)*

Discussion

The surfaces of polystyrene discs and petridishes were provided with functional groups by means of gas plasma treatment. Some of these groups were subsequently used for immobilization of albumin or alb-hep conjugate. Both Ar and CO_2-plasma treatment of polystyrene discs resulted in the introduction of oxygen-containing groups at the surface (table 1). Ar-plasma treatment of polystyrene discs resulted in less oxygen-containing groups at the surface than treatment with CO_2,

which agrees with data reported by Van Delden *et al.*[12] for PS-Ar and by Takens[17] for PS-CO_2. Van Delden *et al*.[12] reported an oxygen content of 11.9 atomic percent in the analyzed top layer of 100 Å of argon-treated PS, while 8 ± 2 percent of these oxygen atoms was present in carboxylic acid groups. Takens[17] used a CO_2-plasma to incorporate 22 atomic percent oxygen on solution-casted polystyrene films, and showed that this plasma introduced hydroperoxide, epoxide, carbonyl, and carboxylic acid groups at the surface of polystyrene. It is assumed that these findings are valid for the gas-plasma treatments presented in this study, since these surfaces were treated using a similar experimental setup.

A small amount of alb-hep conjugate (0.10 ± 0.03 $\mu g/cm^2$) was bound to PS-Ar-d (figure 2). When the carboxylic acid groups present at the surface were preactivated with EDC and NHS, significantly more alb-hep was immobilized (0.27 ± 0.01 $\mu g/cm^2$). Assuming that the conjugate consists of one albumin and one heparin molecule, and binds to the surface via the albumin moiety, this surface concentration is in the order of a theoretical side-on monolayer.[12,21]

Without preactivation of PS-CO_2 discs with EDC and NHS, more alb-hep conjugate was immobilized (0.41 ± 0.03 $\mu g/cm^2$) than on either preactivated or non-preactivated Ar-treated polystyrene discs (figure 2). This may be due to differences in chemical composition of the surfaces, such as the presence of more epoxide or aldehyde groups on PS-CO_2 compared to PS-Ar. Preactivation of surface carboxylic acid groups of PS-CO_2-d by EDC and NHS prior to coupling with alb-hep conjugate yielded lower surface concentrations of alb-hep conjugate than without preactivation. Using the above mentioned assumptions, layers of alb-hep conjugate obtained with or without preactivation, are in-between those of a side-on and an end-on monolayer.

The stability of the alb-hep conjugate coatings, immobilized on PS-Ar-d and PS-CO_2-d with or without preactivation, was determined by measuring surface concentrations after contact

with an SDS solution (figure 2) or cell culture medium (CMS, figure 3). In CMS, some alb-hep conjugate was initially released from the various disc surfaces, after which almost no further release took place. The final surface concentrations of alb-hep conjugate after 14 days of contact with CMS were similar to that after SDS desorption, for all gas plasma-treated PS discs.

A high percentage of the alb-hep conjugate, immobilized to PS-Ar-d and PS-CO_2-d without EDC and NHS, was not desorbed from these surfaces in SDS solution or CMS, which suggests that at least part of the alb-hep conjugate was immobilized via aldehyde and/or epoxide groups which were introduced at the surface by the gas-plasma treatments.

Because the surface concentration of alb-hep conjugate on CO_2 plasma-treated polystyrene was significantly higher than on Ar-treated polystyrene and the stability of the coating on these surfaces were similar, PS-CO_2 was selected as substrate for future cell culture experiments.[1]

In the next part of this study, albumin and alb-hep conjugate were immobilized to PS-CO_2 petridishes instead of discs, and another conjugate was applied, which had a different heparin content (15.2 % instead of 11.6 % (w/w) heparin), and possibly also a different composition in terms of molecules with different molar ratios of albumin and heparin, such as alb_1-hep_1, alb_2-hep_1, and alb_1-hep_2.[21,24]

The surface concentration of alb-hep conjugate on PS-CO_2 petridishes was 0.77 ± 0.13 μg/cm^2, which corresponds to a tight monolayer (with albumin-moieties of the conjugate in an end-on position) (figure 4B). The surface concentration of albumin on PS-CO_2 petridishes was about 0.43 ± 0.02 μg/cm^2, which corresponds to a theoretical monolayer in-between side-on and end-on orientation (figure 4A). Likewise, on TCPS-p and PS-p, surface concentrations of albumin were lower than of the alb-hep conjugate. Surface concentrations of albumin and alb-hep conjugate, as well as the stabilities of these coatings,

were strikingly similar for PS-CO_2-p and TCPS-p. Moreover, on these hydrophylic surfaces more albumin and alb-hep conjugate were immobilized than on hydrophobic PS-p. This appears to be in disagreement with previously reported results found for alb-hep conjugate adsorption on a number of other polymeric surfaces such as poly(ester-urethane),[25] PVC, Biomer® and Silastic®.[24] Hennink *et al.*[24] found higher surface concentrations of adsorbed alb-hep conjugate for hydrophobic polymer substrates than for hydrophilic ones. Maximum surface concentrations of adsorbed plasma proteins are in general higher on hydrophobic than on hydrophilic materials.[26] Contrary to the substrates used by Hennink *et al.*,[24] the TCPS-p and PS-CO_2-p surfaces used in the present study contain reactive chemical groups.

The surface concentration of alb-hep conjugate on PS-CO_2-p and on TCPS petridishes was very high (0.77 ± 0.13 and 0.71 ± 0.11 μg/cm^2 respectively), which probably corresponds to tight monolayers. The surface concentration of albumin on PS-CO_2-p and on TCPS petridishes were lower and almost equal (0.43 ± 0.05 and 0.41 ± 0.05 μg/cm^2 respectively), corresponding to monolayers with an average albumin conformation in-between end-on and side-on. From the results of the desorption experiments (figures 4A, 4B, and 5), it appeared that most of the bound albumin and alb-hep conjugate was not removed from the surfaces of PS-CO_2-p and TCPS-p by SDS or by exchange with plasma proteins present in the cell culture medium. Similar results were reported by Pettit *et al.*[27] for fibronectin-coated TCPS. These authors found that only about 22 percent of TCPS-bound fibronectin was released in a 3% (w/v) SDS-solution during 24 hrs incubation. The striking similarity between TCPS-p and PS-CO_2 petridishes with regard to surface concentrations of albumin and alb-hep conjugate, and the subsequent stability of these coatings, indicate that TCPS may also have reactive groups for covalent binding of proteins.

Engbers *et al.*[25] demonstrated that about 50 percent of alb-hep conjugate adsorbed onto polyurethane (0.17 ± 0.01 $\mu g/cm^2$) is released from the surface upon incubation in a protein solution containing physiologic concentrations of albumin, fibrinogen and γ-globulin at room temperature for 24 hrs. Under these conditions, no significant decrease in surface concentration was observed for alb-hep conjugate which was covalently immobilized to decanedioyl activated polyurethane (0.75 ± 0.14 $\mu g/cm^2$).[25] The layers of alb-hep conjugate immobilized to PS-CO_2-p and TCPS-p, as reported in this study, are more stable than the adsorbed monolayers on poly(esterurethane) reported by Engbers *et al.*.[25] This indicates that a high percentage of the alb-hep conjugate may have been covalently immobilized to PS-CO_2-p and TCPS-p, whereas a low percentage of the alb-hep conjugate is adsorbed to these surfaces, possibly in a partial double layer.

Methylamine is bound to PS-CO_2-p when added to the surface (figure 6). At pH 5 or higher, the amount of methylamine bound to the surface increases with increasing pH. At higher pH the equilibrium between protonated (NH_3^+) and unprotonated (NH_2) groups shifts towards the latter, making more amine-groups available for reaction.[18] The amine-group can either react with epoxide or aldehyde groups introduced at the surface by the gas plasma treatment. Reactions of the amine with an epoxide group can either be base- or acid catalyzed. The base-catalyzed reaction would lead to increased amounts of immobilized methylamine at higher pH, as was observed. The acid-catalyzed reaction would lead to increased amounts of immobilized methylamine at a pH below 4, but this reaction evidently does not take place. Reaction between amine groups and aldehyde groups occurs via the formation of an imminium bond,[19] which is a pH-dependent equilibrium reaction. The formed double bond can be reduced to yield a stable bond.

Under the conditions used in this study, however, no reducing agent was present. Therefore the equilibrium binding remains.

From derivatization-XPS data reported by Takens *et al.*[17] it can be estimated that about 0.05 nmol/cm^2 epoxide groups and about 0.5 nmol/cm^2 aldehyde groups are present at the surface of PS-CO_2 petridishes. This estimation, together with the calculation that a surface area of about 220 Å^2 is present per methylamine group, leads to the conclusion that sufficient reactive groups are available to account for the surface-immobilized methylamine (ca. 0.07 nmol/cm^2).

Based on these observations, together with the absence of acetic acid immobilization to PS-CO_2-p (figure 6), it is concluded that albumin and alb-hep conjugate are most probably covalently bound to PS-CO_2-p by reactions between amine groups of protein or conjugate and aldehyde and/or epoxide groups at the surface. The high surface concentrations and the relatively high stability of the coatings result from these covalent bonds. The initial release of a small percentage of albumin or alb-hep conjugate bound to PS-CO_2-p and the subsequent very slow release in PBS and CMS (figure 5) indicate that not all albumin or alb-hep conjugate was covalently bound.

Rearrangement phenomena of plasma-oxidized surfaces, indicated as ageing, are known to occur upon storage. These phenomena are due to diffusion, migration and rotation of polar groups away from the surface.[17] To prevent rearrangement, treated surfaces were stored at -20 °C. It was shown that the oxygen content of the outer 100 Å layer, as determined with XPS, is not affected by prolonged storage. Furthermore, the surface concentration of albumin and alb-hep conjugate as well as the stability of the coatings were not significantly changed when PS-CO_2-samples were stored at -20 °C before immobilization (figure 7). It is concluded that CO_2 plasma-treated polystyrene surfaces can be stored at -20 °C without effecting the reactivity of the surface towards albumin and alb-hep conjugate.

Conclusions

Gas plasma treatment of polystyrene with argon or carbon dioxide results in the introduction of oxygen-containing groups at the surface. These groups can be used to immobilize albumin or alb-hep conjugate. Surface concentrations of albumin and alb-hep conjugate immobilized to argon- or CO_2-treated polystyrene discs correspond to theoretical monolayers in-between side-on and end-on orientation. Upon contact with an SDS solution or a serum-containing cell culture medium, only a small part of the conjugate coating is removed from the surfaces.
The method of choice to immobilize a monolayer of albumin or alb-hep conjugate on gas plasma-treated polystyrene consists of contacting the surface of CO_2 gas plasma-treated PS with an albumin or conjugate solution in an aqueous solution of pH 8.2, without preactivation of the carboxylic acid groups at the surface.

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Chapter 4 Adherence and Proliferation of Endothelial Cells on Surface-Immobilized Albumin-heparin Conjugate[1]

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Abstract

In principle the patency of vascular grafts can be improved using a combination of a blood-compatible coating and seeded endothelial cells (ECs). When proliferating ECs have not reached confluency, uncovered areas of the graft should not induce thrombus formation. Conjugates of albumin and heparin (alb-hep conjugates) can be used to create blood compatible coatings. Moreover, heparin can bind proteins with cell adhesive properties like fibronectin, thus facilitating adherence and possibly also proliferation of ECs.

Addition of an alb-hep conjugate in an aqueous solution (pH 8.2) to CO_2 gas plasma-treated polystyrene (PS-CO_2) resulted in a relatively stable monolayer of covalently bound alb-hep conjugate. Human umbilical vein endothelial cells (HUVECs) were cultured on this surface and on fibronectin-coated tissue culture polystyrene (TCPS). The number of HUVECs adhering and proliferating on these surfaces were comparable. However, the structure and size of the HUVECs proliferating on surface-immobilized alb-hep conjugate were more irregular than on a fibronectin coating. Long-term adherence of HUVECs was improved by adding a small amount of fibronectin to the alb-hep conjugate-coated surface. With respect to the anticoagulant properties of the alb-hep conjugate,

which are needed when the endothelial lining is still subconfluent, the amount of fibronectin added will have to be minimized.

HUVECs cultured on fibronectin-coated TCPS and on alb-hep conjugate-coated $PSCO_2$ showed secretion of both von Willebrand factor and prostacyclin. The secretion of both compounds could be stimulated using Ca^{2+}-ionophore A23187. It is concluded that alb-hep conjugate may be applied for coating of vascular grafts after adequate preactivation of the graft surface to improve the blood-compatibility of these grafts. The coating also allows seeding and proliferation of endothelial cells when small amounts of fibronectin are present.

Introduction

Synthetic vascular grafts of Dacron or expanded Teflon with a diameter exceeding 5 mm, placed in high-flow and low-resistance locations such as the aortoiliac position, have been successfully used in vascular surgery for some decades.[2-4] However, small diameter grafts replacing low-flow blood vessels rapidly occlude due to thrombosis and fibrotic hyperplasia.[5-7] Although anticoagulant treatment and administration of platelet inhibitors may improve the patency of large-diameter vascular grafts,[8] a similar effect has not been reported for small-diameter grafts.

Endothelial cells (ECs), which form the inner lining of the natural vessel wall, perform a regulatory role in haemostasis.[9,10] In contrast to a number of animal species, spontaneous outgrowth from the anastomoses of endothelium on vascular prostheses implanted in humans does generally not occur.[3,11] Seeding of ECs on the luminal surface of the vascular graft therefore is a promising method to avoid occlusion of small-diameter prostheses in humans.[12-15]

There are several requirements to be fulfilled for the successful seeding of prostheses. ECs used for seeding need to be autologous and, depending on the size of the vascular graft, numerous ECs are required to allow the formation of a confluent layer. In many cases the number of ECs available for seeding will be limited. Because the layer of seeded ECs will in most cases be incomplete immediately after seeding, the prosthetic material itself should display antithrombogenic properties. Furthermore, the material used for vascular grafts must be able to promote both adherence and proliferation of ECs.

A coating consisting of albumin and the anticoagulant heparin (alb-hep conjugate) may fulfill these requirements. Coating of polyurethane catheters and poly(tetrafluoroethylene) sheets with alb-hep conjugates decreases the *in vitro* deposition, aggregation and activation of platelets.[16,17] Alb-hep conjugate, which represents a heterogeneous mixture of conjugates with different compositions, such as alb_1hep_1, alb_2hep_1, and alb_1hep_2,[17] is also effective in reducing complement activation.[17] Moreover, a number of adhesive proteins such as fibronectin, excreted by ECs, have binding sites for heparin, and can therefore mediate the binding of ECs to the alb-hep conjugate coating. Conjugates of albumin and heparin could, therefore, constitute a suitable coating for vascular grafts, by combining anticoagulant properties with support of endothelialization.

In the present model study, polystyrene was modified by gas plasma treatment to generate chemical groups at the surface which were used to covalently immobilize the alb-hep conjugate. The coupling chemistry was previously described.[18] This substrate was subsequently tested for its capability to allow adherence and proliferation of ECs (figure 1).

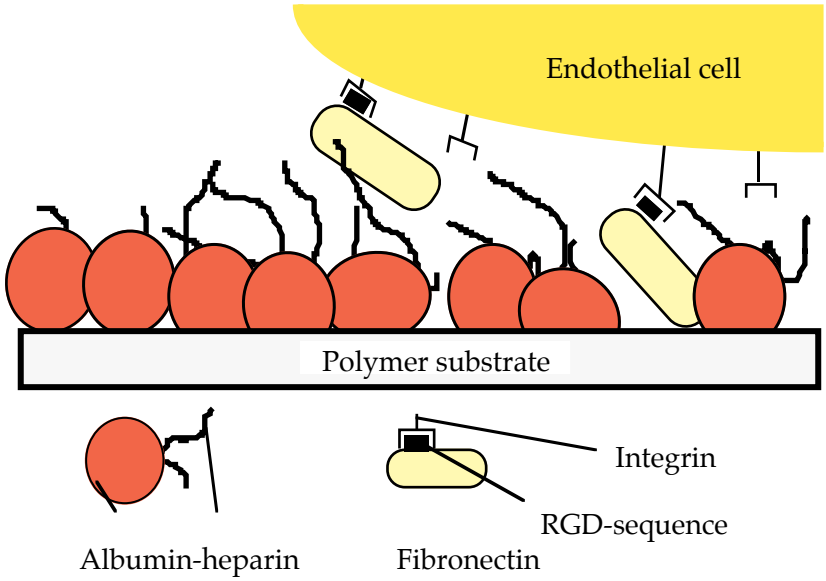

Figure 1. *Schematic representation of endothelial cells using heparin-binding adhesive proteins (e.g. fibronectin) to adhere to alb-hep conjugate immobilized on a polymeric substrate. In the case of endothelial cell binding to fibronectin, the integrins can be either VLA-5 ($\alpha_5\beta_1$) or vitronectin receptor ($\alpha_v\beta_3$).*

Materials and Methods

Materials

Alb-hep conjugate (15.2% (w/w) heparin) was obtained from Holland Biomaterials Group (Enschede, The Netherlands). ^{14}C-formaldehyde (55 mCi/mmol), Na^{125}I (17.4 mCi/µg) and Biotrak 6-keto-prostaglandin $F_{1\alpha}$ EIA were bought from Amersham (Amersham, UK). $NaCNBH_3$ was from Janssen Chimica (Beerse, Belgium). Iodobeads were obtained from Pierce (Rockford, Illinois, USA). Optiphase HiSafe3 (scintillation cocktail) was from Wallac (Milton Keynes, UK). PD10 columns and gelatin-Sepharose were from Pharmacia (Uppsala, Sweden). Phosphate-buffered saline (PBS, pH 7.4) was purchased

from NPBI (Emmercompascuum, The Netherlands). Polystyrene (PS) and tissue culture polystyrene (TCPS) petridishes (35 mm diameter) were from Greiner GmbH (Frickenhausen, Germany). TCPS 12-wells plates and 162 cm^2 TCPS-flasks were obtained from Costar (Cambridge, UK). Carbon dioxide (CO_2, purity > 99.995%) was from Hoekloos (Schiedam, The Netherlands). Pig mucosal heparin sodium (195 U/mg) was from Bufa Chemie (Castricum, The Netherlands). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, trypsin-EDTA, and basic fibroblast growth factor (bFGF) were purchased from Life Technologies (Paisley, UK). Ca^{2+}-ionophore A23187 was obtained from Boehringer Mannheim (Mannheim, Germany). Bovine serum albumin (BSA, fraction V) was from Sigma Chemical Company (St. Louis, Missouri, USA). vWF ELISA was obtained from Gradipore (North Ryde, NSW, Australia). Human serum was pooled from 12 healthy volunteers. A plasma fraction containing human fibronectin (FNc) was a gift from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/ Hohenbrunn, Germany).

Gas plasma treatment of polystyrene petridishes

Polystyrene petridishes were cleaned and CO_2 gas plasma treated as previously described.[1] The dishes were stored at a temperature of -20°C after treatment, and used within 5 days.

^{14}C labeling of alb-hep conjugate

Alb-hep was labeled by reductive methylation using ^{14}C-fomaldehyde in the presence of $NaCNBH_3$,[19] as previously described.[1] ^{14}C-activity was determined using a β-counter (Wallac Winspectral 1414, Wallac, Turku, Finland). The specific activity of ^{14}C-alb-hep conjugate was 1502 ± 22 DPM/μg.

Purification of fibronectin

Human fibronectin (FN) was purified from FNc by affinity chromatography on gelatin-Sepharose at room temperature. Phenylmethylsulfonylfluoride (0.1 mM) was added as protease inhibitor to all buffers. FNc (200 ml, 2.5 mg/ml in 50 mM Tris-HCl, pH 7.5) was applied to a 125 ml column of gelatin-Sepharose. The column was washed with several volumes of Tris-HCl-buffer (50 mM, pH 7.5), 2 volumes of NaCl-solution (1 M NaCl in 50 mM Tris-HCl, pH 7.5), and again with Tris-HCl-buffer (50 mM, pH 7.5). Elution of fibronectin was carried out with 4 M urea in Tris-HCl-buffer (50 mM, pH 7.5). The absorption of the eluate was monitored at 280 nm (Pharmacia Single Path Monitor UV-1, Pharmacia, Uppsala, Sweden), and the fibronectin-containing fractions were pooled. After dialysis against PBS, aliquots (± 0.50 mg FN/ml) were stored at -30°C.

^{125}I labeling of fibronectin

Fibronectin was labeled with ^{125}I using Iodobeads according to the method of Markwell.[20] Na^{125}I (500 μCi, 15.5 mCi/μg) was added to 3 Iodobeads, that had been rinsed with PBS. After 5 min of incubation at room temperature, 5 mg FN in PBS was added. After 12 min, free iodine was removed from the reaction mixture using a series of 3 PD10 columns and PBS as eluent. ^{125}I-activity was determined using a

γ-counter (Compugamma 1282, LKB, Stockholm, Sweden). The specific activity of ^{125}I-FN was 30.5 ± 0.4 DPM/ng, with less than 1 % unbound ^{125}I, as determined by elution using a PD10-column.

Immobilization of albumin, alb-hep conjugate and fibronectin

Albumin (alb) or alb-hep conjugate (alb-hep) was immobilized on PS-CO_2 petridishes (10.9 cm^2) by incubating the dishes with the protein or conjugate (1 ml, 15 mg/ml) in borate buffer (5mM $K_2B_4O_7$, pH 8.2) for 45 min at room temperature. Subsequently, the petridishes were rinsed using PBS (3 times), PBS to which NaCl was added (final concentration1M) which was acidified to pH 3.5 using 1 M HCl (3 times), followed by PBS to which NaCl was added (final concentration1M) which was brought to pH 3.5 using 1 M NaOH (3 times). Finally, the surfaces were rinsed twice with PBS.

In a similar way, FN was immobilized on PS-CO_2 petridishes by incubating the dishes with FN (1 ml, 0.025 mg/ml) in borate buffer (5mM $K_2B_4O_7$, pH 8.2), followed by the same washing procedures.

Surfaces onto which alb-hep was immobilized, were also incubated with fibronectin (1 ml, 0.05 mg/ml in PBS) for 30 min, after which the surfaces were rinsed three times with PBS (PS-CO_2 alb-hep, FN added in second incubation). A mixture of conjugate (15 mg/ml) and fibronectin (0.05 mg/ml) in borate buffer was immobilized as well (PS-CO_2 alb-hep, FN present in mixture), as described above.

Analogous procedures were used to coat tissue culture polystyrene (TCPS) and polystyrene (PS) petridishes with albumin, alb-hep conjugate and fibronectin.

Surface concentration of albumin, alb-hep conjugate and fibronectin

After immobilization of ^{14}C-alb-hep conjugate on PS-CO_2 petridishes, the surfaces were incubated twice with NaOH (2 ml, 1M) for 24 h. By this procedure, the majority of the immobilized conjugate was removed from the surface. Subsequently, after the addition of 18 ml HiSafe3 (18 ml, scintillation cocktail), the radioactivity of NaOH-solutions was determined. After the addition of 18 ml HiSafe3 to the NaOH-treated

petridish, the radioactivity remaining at the surface was determined. This latter value was multiplied by 1.4 to roughly account for the radioactivity adsorbed by the polystyrene. Surface concentrations were calculated by dividing the surface-bound radioactivity (sum of radioactivity of the NaOH-solutions and remaining at the surface) by the specific activity of the conjugate and surface area.

The surface concentration of alb-hep conjugate bound to TCPS and PS were previously shown to be 0.71 ± 0.11 and 0.37 ± 0.05 μg cm^2 respectively.[18] The surface concentration of albumin immobilized to PS-CO_2 respectively bound to PS were previously shown to be 0.43 ± 0.02 and 0.28 ± 0.04 μg/cm^2.[18]

^{125}I-fibronectin was immobilized on uncoated and on alb-hep conjugate-coated PS-CO_2 petridishes as described above. ^{125}I-radioactivity bound at the surface and surface concentrations were determined as described for ^{14}C-alb-hep conjugate, except for the addition of HiSafe3.

The surface concentration of fibronectin bound to uncoated TCPS was determined using an analogous procedure, whereas the surface concentration of fibronectin bound to PS was not determined.

Stability of alb-hep conjugate and fibronectin coatings

Coated petridishes were placed overnight at room temperature in PBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Next, the stability of the coatings was determined by incubating the petridishes at 37°C with 2 ml of complete cell culture medium (CM) supplemented with 20% (v/v) pooled human serum (CMS). CM consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). At selected times, the supernatant was replaced by fresh CMS and ^{14}C-radioactivity in the supernatant was measured after addition of 13 ml HiSafe3, while ^{125}I-radioactivity

was measured directly. Surface concentrations at a specific day were calculated from the initial surface concentration and the released amount of alb-hep conjugate or fibronectin.

The stability in CMS of a layer of alb-hep conjugate bound to either PS-CO_2, TCPS or PS was previously shown to be similar; between 20 and 30 percent of the coating was removed from all petridishes during 14 days in this medium at 37°C.

Isolation of endothelial cells

HUVECs were isolated from umbilical veins according to the method of Jaffe *et al.*,[21] modified by Van Wachem *et al.*.[22] In short, the umbilical vein was rinsed with PBS and incubated for 20 min at 37 °C with trypsin-EDTA solution in PBS (0.05% and 0.02% (w/v)). After harvesting HUVECs, CMS was added to inactivate trypsin and after exchange of the medium by fresh medium, cells were plated in fibronectin-coated TCPS flasks. HUVECs were grown at 37 °C in 95% air/5% CO_2, saturated with water vapour, until the third passage. Before passage or seeding, cells were detached from the TCPS surface by incubation with trypsin-EDTA solution in PBS (0.05% and 0.02% (w/v)), centrifuged and resuspended in CMS.

Initial adherence of endothelial cells on various substrates

Before cell seeding, surfaces (9.6 cm^2) were placed overnight at room temperature in 3 ml PBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Third passage HUVECs were seeded in CMS at a density of 40,000 cells per square centimeter and cultured as described above. At selected times (30, 60 and 240 min), cells were trypsinized and cell numbers were determined using a Bürker counting device.

Proliferation of endothelial cells on various substrates

Before cell seeding, surfaces (9.6 cm^2) were placed overnight at room temperature in 3 ml PBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Third passage HUVECs were seeded in CMS at a density of 10,000 cells per square centimeter and cultured as described above. Culture medium supplemented with 5U/ml heparin and 0.3 ng/ml bFGF (0.20 ml/cm^2) was replaced every other day. At selected times, cells were trypsinized and cell numbers were determined.

Secretion of Von Willebrand factor and prostacyclin

Before cell seeding, surfaces (9.6 cm^2) were placed overnight at room temperature in 3 ml PBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Third passage HUVECs were seeded in CMS at a density of 10,000 cells per square centimeter and cultured in CMS for 3 or 10 days on fibronectin-coated TCPS respectively alb-hep conjugate-coated PS-CO_2 as described above.

After washing once with CM supplemented with fatty acid-free BSA (CM/BSA, 10 mg/ml), the petridishes with HUVECs were incubated for 30 min at 37°C with 1 ml of either CM/BSA (basal secretion) or CM/BSA containing 10 μM Ca^{2+}-ionophore A23187 (activated secretion). Subsequently, the medium was removed, centrifuged for 7 min at 400 g, and aliquots of the supernatant were stored at -20°C until assayed. Simultaneously, cells of corresponding surfaces were trypsinized and cell numbers were determined.

Von Willebrand factor concentration in the medium was determined with the aid of a sandwich ELISA, while prostacyclin concentration was determined using a competitive EIA for 6-keto-prostaglandin $F_{1\alpha}$, the product of spontaneous hydroly-

sis of PGI_2 (half-life of 3 min). 6-Keto-prostaglandin $F_{1\alpha}$ is generally accepted as a measure of PGI_2 formation.[23] The ELISA and EIA were used according to instructions supplied by the manufacturers.

Results

Surface concentration and stability of alb-hep conjugate and fibronectin coatings

The amount of alb-hep conjugate immobilized onto PS-CO_2 (0.57 ± 0.03 $\mu g/cm^2$, figure 2A) corresponds to a conjugate monolayer. A small percentage of the initially bound alb-hep conjugate (5%) was released during overnight incubation in a solution of penicillin and streptomycin in PBS. About 20 percent of bound alb-hep conjugate was released from PS-CO_2-surfaces during overnight incubation in cell culture medium (figure 2A). The quantities of radiolabeled fibronectin immobilized onto respectively PS-CO_2 and TCPS were comparable (figure 2B). As observed for the alb-hep conjugate, a small percentage of the immobilized fibronectin (6-7%) was released from both surfaces overnight in a solution of penicillin and streptomycin in PBS, as well as in CMS.

A relatively large amount of fibronectin was bound to alb-hep conjugate coated PS-CO_2, during a second incubation step (PS-CO_2 alb-hep, FN added in second incubation), while only a small amount of alb-hep conjugate was released from the surface during this incubation and subsequent washing (figures 2A and 2B). In contrast to fibronectin immobilized onto PS-CO_2 or TCPS, fibronectin adherent to alb-hep conjugate was almost completely released in CMS.

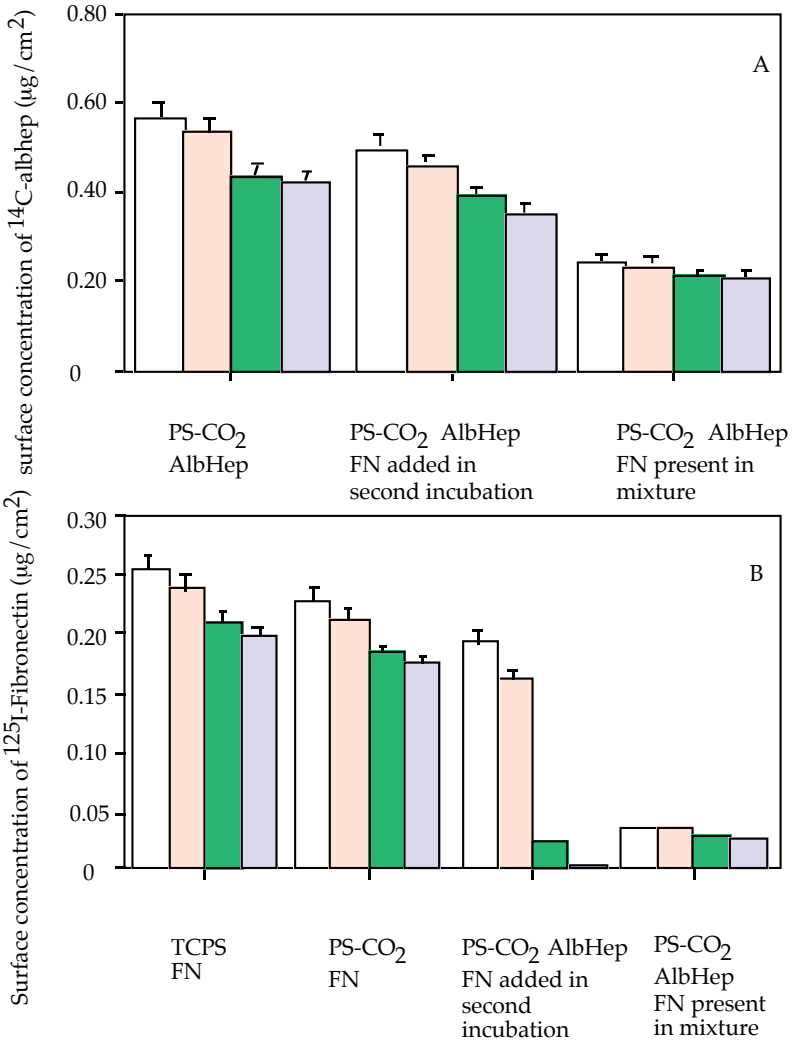

Figure 2 *Surface concentration of alb-hep conjugate (A) and fibronectin (B) on a series of surfaces, immediately after coating and rinsing* □, *after overnight incubation in penicillin-streptomycin solution* □, *and after one* ■ *and 4 days* □ *incubation in CMS at 37 °C. 'PS-CO$_2$ AlbHep/FN added in second incubation' represents an alb-hep conjugate coating onto which additional fibronectin was bound during a second incubation step and 'PS-CO$_2$ AlbHep/FN present in mixture' represents a coating of a mixture of alb-hep conjugate and fibronectin. (n=3, ± sd)*

When a mixture of alb-hep conjugate and fibronectin was immobilized (PS-CO_2 alb-hep, FN present in mixture), only a small quantity of fibronectin was bound, whereas the quantity of alb-hep conjugate immobilized in this case was significantly lower than in the absence of fibronectin.

The surface concentration of albumin immobilized onto PS-CO_2 was previously shown to be 0.43 ± 0.01 μg/cm^2, whereas the stability of the albumin coating was similar to that of an alb-hep conjugate coating on this surface.[18]

Initial adherence of endothelial cells on alb-hep conjugate and albumin coated surfaces

The number of HUVECs which adhered within 30 min to the reference surface fibronectin-coated TCPS, was about 20 percent (7600 ± 2000 ECs/cm^2) of the number seeded (figure 3).

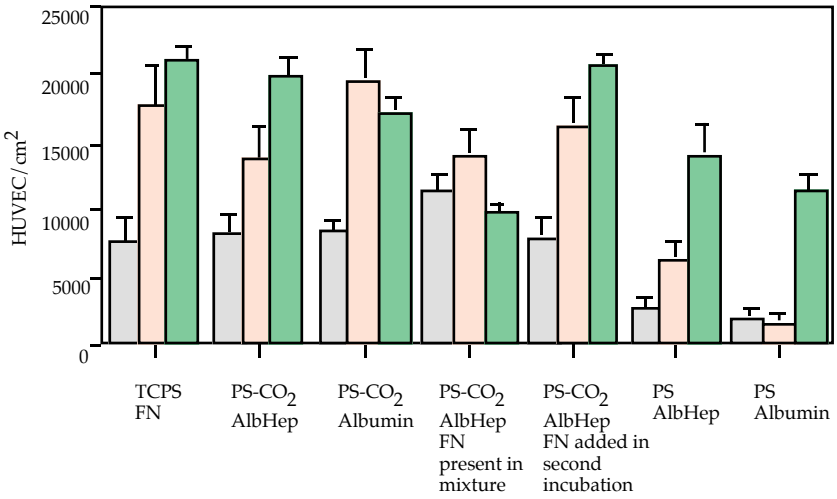

Figure 3. *Number of endothelial cells on PS-CO_2 with immobilized albumin or alb-hep conjugate, with or without fibronectin present in the coating mixture or added during a second incubation step, on fibronectin coated TCPS and on PS coated with albumin or alb-hep conjugate, at* ☐ *30 min,* ☐ *60 min,* ☐ *4 h. (n=3, ± sd)*

After adhesion, cells showed microscopically progressive flattening. Four h after seeding, approximately 50 percent (20800 ± 1600 ECs/cm^2) of the seeded cells had adhered and spread.

Adherence of HUVECs to PS-CO_2, coated with alb-hep conjugate, progressed in an identical way (figure 3), also when fibronectin was added in a second incubation step, although cell numbers 1 h after seeding were higher. When fibronectin was added to the surface together with alb-hep conjugate, the number of adhered cells initially (30 min) increased, but after 4 h less cells had adhered compared to alb-hep conjugate coating without fibronectin. Initial adherence of HUVECs to PS-CO_2 coated with albumin was higher than adherence to PS-CO_2 coated with alb-hep conjugate (60 min), whereas after 4 h less cells were adhered compared to the latter surface. A relatively low number (30%) of cells had adhered to albumin-coated polystyrene 4 h after seeding. Coating of polystyrene with alb-hep conjugate accelerated cell adhesion. Spreading and progressive flattening of ECs occurred on all surfaces.

Proliferation of endothelial cells on various substrates

HUVECs adhered to and proliferated on both uncoated PS-CO_2 and TCPS, but hardly on native PS (figure 4A). On PS-CO_2 and TCPS, coated with alb-hep conjugate (figure 4B), without or with fibronectin coated in a separate incubation step (figure 4D), cells adhered and proliferated as well. Cell proliferation was only transient on PS coated with alb-hep conjugate, even when fibronectin was added in a second incubation step.

A confluent cellular monolayer was formed at day 7 on all surfaces except on native and alb-hep conjugate-coated PS. At day 13, cell numbers on TCPS and on fibronectin-coated TCPS were higher than on the other surfaces. After coating of surfaces with fibronectin (figure 4C), cell proliferation on the various substrates was similar, with the exception of TCPS during the second week of culturing, when cell numbers increased more than on the other surfaces.

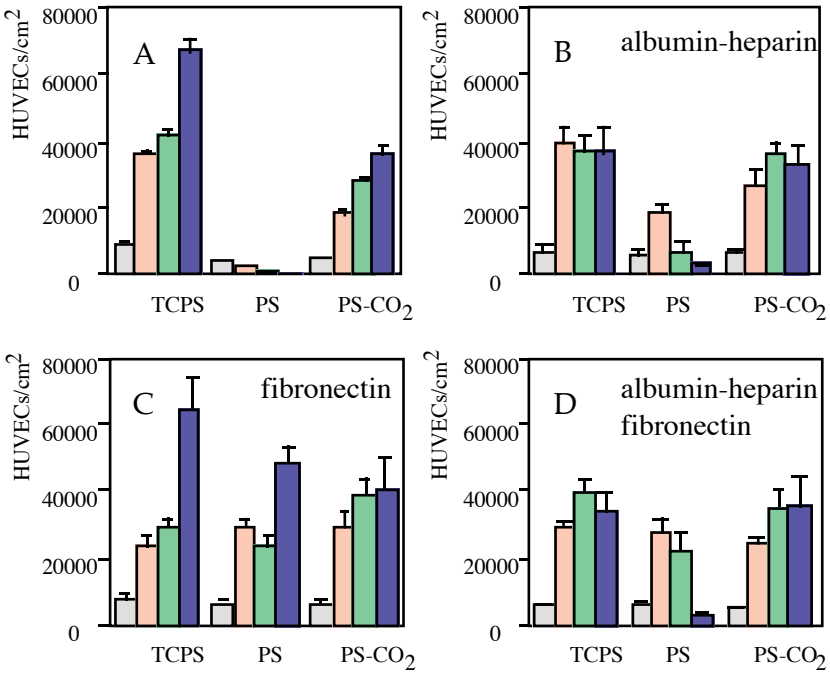

Figure 4. *Number of endothelial cells on TCPS, PS and PS-CO_2, either without (A) or with immobilized alb-hep conjugate (B), fibronectin (C) or alb-hep conjugate with fibronectin added during a second incubation step (D), at different culture times:* ☐ *day 1,* ☐ *day 4,* ☐ *day 7,* ☐ *day 13. (n=3, ± sd)*

During the second week of culturing, some detachment of cells from all alb-hep conjugate-coated surfaces was observed, and non-cell-covered areas of the substrates became visible. However, proliferation continued and total cell numbers remained constant or increased slightly on TCPS and PSCO_2, coated with alb-hep conjugate.

Although HUVECs initially adhered to albumin-coated PS-CO_2 surfaces (figure 3), proliferation was not observed in most experiments. Still, proliferation of HUVECs on this substrate occurred occasionally (data not shown).

HUVECs showed a cobblestone structure, typical for endothelial cells in culture, when seeded on fibronectin-coated surfaces and on uncoated PS-CO_2 and TCPS (figure 5). The structure and size of ECs were more irregular on uncoated and alb-hep conjugate-coated surfaces (figures 5 B and 5C) than on fibronectin-coated surfaces (figures 5A and 5B).

Secretion of von Willebrand factor and prostacyclin

Cultured HUVECs were treated with Ca^{2+}-ionophore A23187 to release von Willebrand factor (vWF) from intracellular storage sites and to enhance production of prostacyclin (PGI_2). Basal secretion of these substances was measured as well.

Basal secretion of vWF from HUVECs at day 3 was higher in the case of alb-hep conjugate-coated $PSCO_2$ compared to fibronectin-coated TCPS. Differences were much smaller at day 10 (figure 6).

Stimulation of cell cultures with Ca^{2+}-ionophore A23187 led to an increase of the release of vWF from HUVECs at day 3 and 10 in the case of both surfaces (figure 6). Cell numbers per square centimeter on alb-hep conjugate-coated $PSCO_2$ and on fibronectin-coated TCPS were 13500 ±1000 and 22000 ± 1900 respectively at day 3, and 44500 ± 4900 and 39200 ± 4200 respectively at day 10. At day 10 the cell layer was confluent on both surfaces. The stimulatory effect of the ionophore on HUVECs, cultured on fibronectin-coated TCPS or on alb-hep conjugate-coated $PSCO_2$, was small at day 3 and large at day 10.

Figure 5. *Light-microscopic images of HUVECs grown for 7 days on TCPS-FN (A), PS-CO_2 (B), and on alb-hep conjugate immobilized on PS-CO_2 without (C) or with (D) fibronectin added during a second incubation step. (original magnification: 128X)*

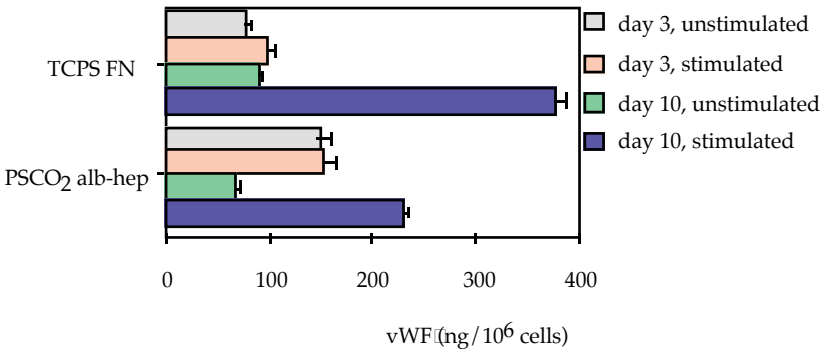

Figure 6 *Basal- and Ca^{2+}-ionophore A23187-stimulated secretion of vWF by HUVECs on fibronectin-coated TCPS and on alb-hep conjugate-coated $PSCO_2$ at 3 and 10 days culture. (n=3 ± sd.)*

Basal secretion of PGI_2 from HUVECs at day 3 was higher in the case of alb-hep conjugate-coated $PSCO_2$ compared to fibronectin-coated TCPS (figure 7). Differences were much smaller at day 10.

Stimulation with Ca^{2+}-ionophore A23187 significantly increased the release of PGI_2 of cultured cells both at day 3 and 10, irrespective of the surface (figure 7). As observed for basal secretion, stimulated release from cells using alb-hep conjugate-coated PS-CO_2 was higher at day 3 compared to fibronectin-coated TCPS, whereas at day 10 the difference was small.

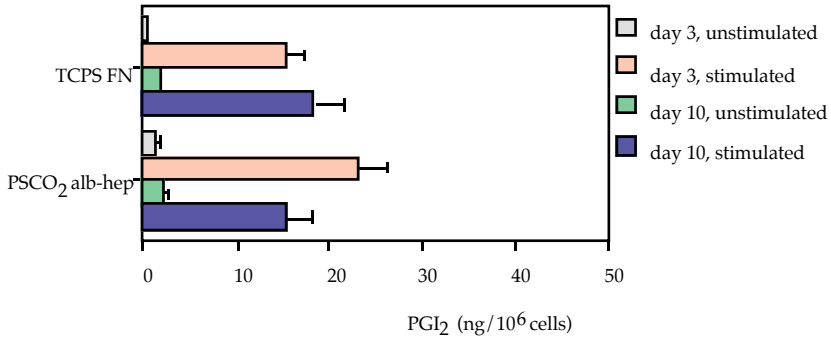

Figure 7 *Basal- and Ca^{2+}-ionophore A23187-stimulated secretion of PGI_2 by HUVECs on fibronectin-coated TCPS and on alb-hep conjugate-coated $PSCO_2$ at 3 and 10 days culture. (n=3 ± sd.)*

Discussion

In humans, the luminal surface of vascular prostheses is usually not spontaneously overgrown by endothelium. Therefore, seeding of ECs has been proposed to promote endothelialization of vascular grafts, thus preventing thrombotic complications.

A prerequisite for successful cell seeding is the adherence of ECs to the graft surface. In addition, the graft surface should have antithrombogenic properties because it is unlikely that the layer of seeded ECs will be confluent immediately after seeding. An alternative is to culture the ECs *ex vivo* prior to implantation till confluency is reached. However, it is more practical when isolation and seeding of cells are done during the surgical procedure of blood vessel replacement. In the present study it was shown that coating of polymeric surfaces with alb-hep conjugate, which displays anticoagulant activity, provides a suitable substrate for seeding of ECs.

In order to prevent displacement of alb-hep conjugate from the surface by seeded cells and plasma proteins, CO_2 gas plasma treatment was used to generate oxygen containing groups at a PS surface, which serve to immobilize the alb-hep conjugate. Following immobilization of a monolayer of alb-hep conjugate on PS-CO_2 surfaces, some release of alb-hep conjugate in cell culture medium (CMS) initially occurred, after which almost no further release took place.[1]

Our studies demonstrate that ECs adhere to and proliferate on alb-hep conjugate which is immobilized onto PS-CO_2. Several authors have postulated that deposition of fibronectin by ECs is essential for adherence and proliferation of these cells.[24-27] Adhesion of ECs to alb-hep conjugate-coated surfaces therefore is expected to occur via adhesive proteins (e.g. fibronectin, vitronectin, laminin) bound either directly or via heparin to the surface. Adherence of ECs to alb-hep conjugate-coated PS-CO_2 and to fibronectin-coated TCPS was similar. Cells proliferated

not only on a coating of alb-hep conjugate, but also on uncoated PS-CO_2, indicating that sufficient adhesive proteins adsorbed from serum-containing medium, or that fibronectin released by seeded cells displaced proteins that were already present at the surface. Besides deposition of adhesive proteins on top of the alb-hep conjugate layer, part of the immobilized alb-hep conjugate may be displaced during cell culture by cellular proteins which promote adherence of HUVECs to the substrate.

When alb-hep conjugate-coated PS-CO_2 was incubated with fibronectin, a quantity of fibronectin similar to that bound to uncoated PS-CO_2 was immobilized, but only a small portion of the alb-hep conjugate monolayer was displaced. Therefore, it is postulated that fibronectin binds predominantly to the alb-hep conjugate via the heparin moiety. This is supported by the observation that when fibronectin was added together with alb-hep conjugate, significantly less fibronectin and alb-hep conjugate bound to the surface. During overnight incubation in penicillin/streptomycin solution part of the fibronectin and alb-hep conjugate was released from the surfaces. Upon incubation in CMS, most labeled fibronectin was removed from a layer of alb-hep conjugate, possibly through exchange with other proteins or with unlabeled fibronectin present in the CMS. The conjugate itself was not released from the surface.

Differences in morphology rather than in numbers were observed during cell proliferation. Cells were more spread out on all substrates after coating with fibronectin than on alb-hep conjugate-coated surfaces. Moreover, addition of fibronectin to alb-hep conjugate-coatings clearly improved adherence and spreading of ECs to these surfaces during proliferation. Fibronectin, however, is expected to enhance adhesion of circulating platelets to the surface following implantation.[29-31] Therefore, the quantity of fibronectin added to the coating to improve endothelial cell adherence needs to be minimized to prevent platelet-surface interaction.

Rather unexpected, HUVECs were found to adhere to PS-CO_2 coated with albumin. In previous reports,[e.g.32] coating of surfaces with albumin was suggested to prevent adhesion of HUVECs. However, transient adherence of HUVECs to albumin-coated poly(ethylene terephthalate), as found in this study for albumin-coated PS, has been reported before.[32] Cell adherence after 24 h to albumin-coated PS-CO_2 was generally lower than on alb-hep conjugate-coated PS-CO_2. Probably cells are not adhering to albumin itself, but to (cellular) proteins which adsorb from the medium to the surface. These (cellular) proteins may bind to the surface either by displacing albumin which was not covalently bound to the surface or via hydrophobic interaction with immobilized possibly denatured albumin. In the case of PS, alb-hep conjugate coating improved adhesion and proliferation of HUVECs compared to albumin coating. These findings strongly suggest that the heparin moiety of the alb-hep conjugate is involved in the adherence of ECs to the substrate.

HUVECs cultured on alb-hep conjugate-coated PSCO_2 and on fibronectin-coated TCPS secreted von Willebrand factor (vWF), which provides a bridge between adhering platelets and the (sub-)endothelium. The cells also released prostacyclin (PGI_2), a vasodilator and inhibitor of platelet aggregation. Basal expression of vWF on both surfaces was equal and comparable to data reported for fibronectin-coated TCPS and for crosslinked collagen type I.[21,33] Stimulation of the cultured endothelial cells by Ca^{2+}-iono-phore A23187 increased the secretion of vWF, as reported by other authors.[21,33-35]

Production of PGI_2, synthesized and secreted by ECs, provides an important mechanism for the prevention of platelet deposition by inhibition of platelet function.[36] Basal production of PGI_2 was low on both substrates, which is in agreement with data of van Wachem *et al*.[21] and Wissink.[33] According to Chesterman[37] this is caused by the high turnover of ECs in culture compared to *in vivo*, resulting in less transcription of the biosynthetic enzymes. The presence of heparin at the surface had

no large influence on basal expression of PGI_2, which corresponds to findings reported by others.[38-40] Stimulation with Ca^{2+}-ionophore A23187 reflects the functional capability of the prostacyclin biosynthetic enzymes cyclooxygenase and prostacyclin synthetase. Stimulation of cultured cells by Ca^{2+}-ionophore A23187 significantly increased the secretion of PGI_2, which indicates retention of prostacylin biosynthetic capacity.

The secretion of vWF and PGI_2 by HUVECs cultured on alb-hep conjugate-coated PS-CO_2 demonstrates the viability of these cells. Alb-hep conjugate coating therefore forms a good substrate for seeded ECs. As endothelial cell functions like vWF-release and PGI_2-production and -secretion are influenced by factors such as shear[41] and cyclic stretching,[42] care should be taken when data obtained from stationary *in vitro* experiments are extrapolated to the *in vivo* situation.

Since polystyrene cannot be used in vascular graft surgery, the present model system needs to be tested using Dacron or Teflon. Unpublished results of Klomp *et al.*[43] demonstrate that proteins in an aqueous solution (pH 8.2) may be immobilized on CO_2 gas plasma-modified polyethylene terephthalate, the base material of Dacron. Similarly, a layer of alb-hep conjugate may be immobilized on CO_2-treated Dacron fibers. Furthermore, the alb-hep conjugate may be used to fill the pores of Dacron grafts. After crosslinking to increase the stability of the sealant, ECs may be seeded on the crosslinked alb-hep conjugate matrix. It is therefore suggested that alb-hep conjugate might be useful as a vascular graft coating/sealant to prevent thrombus formation and to promote endothelialization of the graft.

In conclusion, coating of vascular grafts with alb-hep conjugate in combination with seeding of ECs may improve graft patency. However, since cell adherence is not optimal on alb-hep conjugate-coated PS-CO_2, addition of a small amount of an adhesive protein like fibronectin is probably required. Endothelial cell adhesion versus blood compatibility of such a coating needs to be further evaluated.

Conclusions

HUVECs adhere to and subsequently proliferate on CO_2 plasma-modified polystyrene. A confluent monolayer is formed, although the number of adherent cells is lower than on TCPS. Immobilization of alb-hep conjugate onto CO_2-treated polystyrene results in surfaces with slightly increased cell numbers during the first days of culturing compared to uncoated PS-CO_2, after which similar cell densities are found on both surfaces. During the first week of culture, the same number of cells is found on alb-hep conjugate-coated PS-CO_2 compared to fibronectin-coated TCPS. It is strongly suggested that the heparin moiety of the alb-hep conjugate is involved in the binding of ECs to the substrate during proliferation, via adhesive proteins which bind to the heparin moiety.

Since cell binding during proliferation is not optimal on alb-hep conjugate-coated PS-CO_2, the presence of a small amount of an adhesive protein like fibronectin in the coating may improve cell binding to the substrate under flow conditions. Endothelial cell adhesion versus blood compatibility of such a coating needs to be further evaluated.
HUVECs cultured on fibronectin-coated TCPS and on alb-hep conjugate-coated PSCO_2 show comparable levels of basally expressed von Willebrand factor and prostacyclin. These levels can be increased using Ca^{2+}-ionophore A23187.

It is concluded that a coating of alb-hep conjugate forms a good substrate for seeded ECs.

Acknowledgement

We wish to thank the staff of the departement of obstetrics (Medisch Spectrum Twente, Enschede, The Netherlands) for providing umbilical cords.

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Chapter 5 Proliferation of Endothelial Cells on Surface-Immobilized Albumin-Heparin Conjugate loaded with basic Fibroblast Growth Factor

Gert W. Bos, Nicole M. Scharenborg, André A. Poot, Gerard H.M. Engbers, Tom Beugeling, Willem G. van Aken, and Jan Feijen

Abstract

Seeding of endothelial cells (ECs) on the luminal surface of small-diameter vascular grafts is a promising method to avoid occlusion of these prostheses. Immobilization of basic fibroblast growth factor (bFGF) to substrates used to coat or fill porous prostheses may enhance the formation of a confluent monolayer of ECs.

Human umbilical vein endothelial cells (HUVECs) were grown in cell culture medium with 5 percent human serum on a bFGF-loaded substrate of albumin-heparin conjugate (alb-hep conjugate) bound to CO_2 gas-plasma treated polystyrene (PS-CO_2). In the order of 2-3 ng/cm^2 bFGF had to be immobilized to form a confluent monolayer of HUVECs. The most prominent effect of surface-immobilized bFGF was stimulation of the proliferation of HUVECs shortly after seeding, resulting in confluent cell monolayers with high density within 3 days. In contrast, in cultures with 0.3 ng/ml bFGF in the medium instead of bFGF bound to the surface, it took almost a week before the cell layers reached confluency.

Binding of bFGF to heparin, which was shown to be specific, and the biological activity of bFGF towards ECs were not influenced by (radio-)labeling of bFGF with iodine. However, only a minor part of the bFGF used in this study displayed heparin affinity. Furthermore, degradation and multimerization

of labeled bFGF in time occurred when the growth factor was stored at 20-37°C in PBS containing 20 mg/ml albumin, either with or without heparin. This limits the use of labeled bFGF to short term (hours) experiments, like for instance the determination of the amount of bFGF bound to a matrix.
In conclusion, bFGF-loading of vascular graft surfaces through complexation of bFGF with a heparin-containing matrix will probably lead to more rapid formation of a confluent monolayer of endothelial cells on the graft surfaces upon seeding of the cells.

Introduction

Synthetic vascular grafts of Dacron or expanded Teflon (ePTFE) with a diameter exceeding 5 mm, have been used successfully in vascular surgery.[1-3] However, small-diameter grafts rapidly occlude.[4-6] In contrast to large-diameter vascular prostheses, anticoagulant treatment and administration of platelet inhibitors to the patient do not improve the patency of small-diameter vascular grafts.[7]

Endothelial cells (ECs), which form the inner lining of the natural vessel wall, perform a regulatory role in haemostasis.[8,9] Unlike in animal species, spontaneous outgrowth of endothelium on implanted vascular prostheses does generally not occur in humans.[3,10] Seeding of ECs on the luminal surface of vascular grafts, therefore, is a promising method to improve the patency of small-diameter prostheses.[11-14]

Since the availability of autologous ECs needed for seeding is limited, the graft surface will generally not be completely endothelialized immediately after seeding. When the incompletely endothelialized vascular graft is implanted, a blood compatible surface of the uncovered graft is essential to avoid occlusion.

Previously we have shown that a coating consisting of albumin and the anticoagulant heparin, is a suitable substrate for ECs.[15] HUVECs which adhere to and subsequently proliferate on albumin-heparin conjugate, which is covalently immobilized on CO_2 plasma-modified polystyrene, express normal amounts of von Willebrand factor and prostacyclin.[16] Precoating of small amounts of fibronectin onto albumin-heparin-coated PS-CO_2 promotes the binding of HUVECs to the substrate. Thus, coating of vascular grafts with albumin-heparin conjugate in combination with seeding of ECs may improve the graft patency.

However, it takes a long period of time to reach confluency of the formed cell layer when cells are seeded in low cell densities,[15,16] and presumably even longer *in vivo*. Possibly, an improvement is found in the local release of growth factors, which may accelerate the formation of a confluent cell layer. ECs could then be seeded sub-confluently in a vascular graft and subsequently be stimulated by specific exogenous growth factors to rapidly form a confluent monolayer *in vivo.*

Proteins known to stimulate proliferation of ECs are the acidic and basic forms of fibroblast growth factor (aFGF and bFGF)[17-19] and vascular endothelial growth factor (VEGF), of which the most potent -although not the most specific- is bFGF.[19,20] bFGF has a high-affinity binding-site for heparin,[21] cell surface heparan sulphate[22] and glycosaminoglycans present in the extracellular matrix,[22] which functions as a storage depot for FGF.[23] Furthermore, binding to heparin or heparan sulphate protects bFGF against denaturation.[22,24,25]

The possible use of FGF in vascular graft coatings has been reported before. Soldani *et al*.[26] demonstrated that bFGF remained active for two weeks when incorporated in a polyurethane prosthesis in combination with albumin. Greisler *et al*.[27-29] combined fibrin glue, heparin and either aFGF or bFGF to promote endothelialization of ePTFE vascular grafts. Recently, it was demonstrated that co-immobilization of bFGF and heparin in a gelatin-impregnated porous polyurethane graft, implanted in aortas of rats for 4 weeks, significantly enhanced the extent

of endothelialization proceeding from the anastomotic sites, as well as transmural ingrowth of smooth muscle cells and fibroblasts.[30] Furthermore, application of a heparin-based bFGF release system for wound healing,[31] for artificial liver development,[32] and for enhanced endothelialization of cardiac valve bioprostheses[33] has been reported.

The goal of the present study was to evaluate whether the *in vitro* proliferation of seeded ECs is stimulated by bFGF,which is bound to a surface of gas plasma-modified polystyrene provided with a monolayer of albumin-heparin conjugate (figure 1).

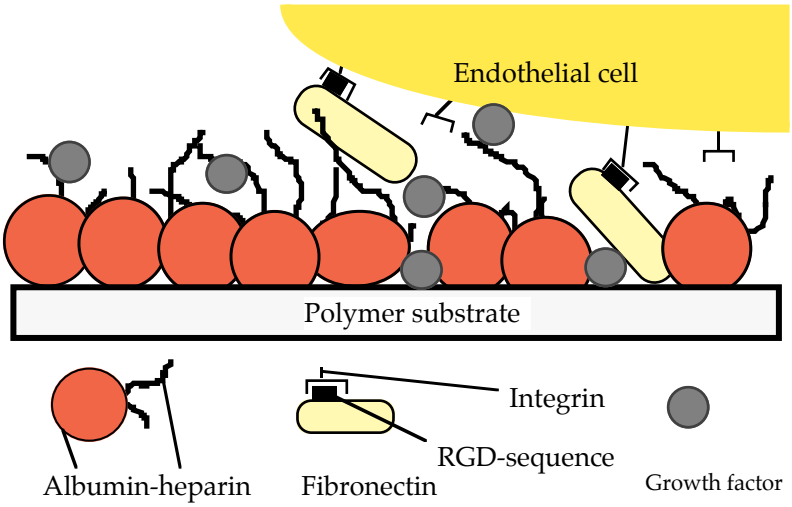

Figure 1. *Schematic representation of endothelial cells using heparin-binding adhesive proteins to adhere to albumin-heparin conjugate immobilized on a polymeric substrate. bFGF is bound to the matrix to stimulate endothelial cell proliferation. In the case of endothelial cell binding to fibronectin, the integrins can either be VLA-5 ($\alpha_5\beta_1$) or the vitronectin receptor ($\alpha_v\beta_3$).*

Materials and Methods

Materials

Albumin-heparin conjugate (11.6% (w/w) heparin) was obtained from Holland Biomaterials Group (Enschede, The Netherlands). $Na^{125}I$ (17.4 mCi/μg) and Sulphur Labeling Reagent were bought from Amersham (Amersham, UK). Iodobeads were obtained from Pierce (Rockford, Illinois, USA). Optiphase HiSafe3 (scintillation cocktail) was purchased from Wallac (Milton Keynes, UK) PD10 columns, HiTrap-heparin columns, gelatin-Sepharose, ExcelGel® SDS gradient 8-18 precast gels, Protein Silverstaining Kit and LMW-markers were from Pharmacia (Uppsala, Sweden). Phosphate-buffered saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene (PS) and tissue culture polystyrene (TCPS) petridishes (35 mm diameter) were from Greiner GmbH (Frickenhausen, Germany). TCPS 12-wells plates were obtained from Costar (Cambridge, UK). Carbon dioxide (CO_2, purity > 99.995%) was from Hoekloos (Schiedam, The Netherlands). Pig mucosal heparin sodium (195 U/mg) was from Bufa Chemie (Castricum, The Netherlands). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, trypsin-EDTA, and basic fibroblast growth factor (bFGF) were purchased from Life Technologies (Paisley, UK). Quantikine human bFGF ELISA was from R&D systems (Minneapolis, USA). Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris), lysine, bovine serum albumin (BSA, fraction V) and agarose-heparin were from Sigma Chemical Company (St. Louis, Missouri, USA). A plasma fraction containing human fibronectin (FNc) was a gift from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Human serum was pooled from 12 healthy volunteers. Human umbilical cords were a gift of Medisch Spectrum Twente (MST, Enschede, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/Hohenbrunn, Germany).

Human recombinant basic fibroblast growth factor (bFGF) was labeled with ^{125}Iodine using Iodobeads according to the method of Markwell.[34] In this way, ^{125}I is incorporated in tyrosine residues. Na^{125}I (500 μCi, 15.5 mCi/μg) was added to 3 Iodobeads that had been rinsed with PBS. After 5 min of incubation at room temperature, bFGF (50 μg) in PBS was added. Subsequently, after 12 min of incubation, free iodine was removed from the reaction mixture using a series of 3 PD10 columns with a bovine serum albumin solution in PBS (20 mg/ml) as eluent. Alternatively, in one experiment (bFGF binding to agarose-heparin as a function of concentration) labeled bFGF was purified using a Sepharose-HiTrap heparin column. After loading of the column with labeled bFGF, bFGF was eluted from the column using a NaCl solution in PBS containing bovine serum albumin (20 mg BSA/ml, final NaCl concentration of 2.5 M). After 1 : 4 dilution with PBS containing 20 mg BSA/ml, labeled bFGF was stored at -20°C until use. The bFGF concentration in the solution after labeling was determined using an ELISA, according to the instructions supplied by the manufacturer. The specific activity of ^{125}I-bFGF varied between 12 and 48 DPM/ng, with 3-15 % unbound ^{125}I (determined using a PD10-column) after thawing. The quantities of immobilized bFGF presented in this study were corrected for free label, assuming that free label does not bind to heparin or to surfaces. ^{125}I-activity was determined using a γ-counter (Compugamma 1282, LKB, Stockholm, Sweden). Different lots of ^{125}I-bFGF were used for stability experiments, for binding to agarose-heparin beads, and for immobilization of bFGF to coated petridishes.

^{35}S-labeling of basic fibroblast growth factor

Human recombinant bFGF was labeled with ^{35}S employing t-butoxy-carbonyl-L-[^{35}S] methionine N-hydroxy succinimidyl ester (Sulphur Labeling Reagent, SLR), which reacts with amine groups present in bFGF.

After removing toluene from the SLR (250 μCi) at room temperature by directing a gentle stream of dry nitrogen through the vial, the reaction vessel was cooled on ice. bFGF (40 μg) in borate buffer (1 ml, 0.1M, pH 8.5) was added to the SLR together with HiTrap heparin beads (250 mg, added to prevent labeling of the heparin binding-site). After 45 min, during which the suspension was gently shaken, the reaction was terminated with lysine in borate buffer (100 μl, 0.2M, pH 8.5). NaCl solution in PBS (900 μl, 3.33M) was added to the mixture and subsequently the labeled bFGF was isolated using a series of PD10 columns with PBS as eluent. Protein-containing fractions were pooled and BSA was added (20 mg/ml). Labeled bFGF was stored at -20°C until use. The bFGF concentration in the solution after labeling was determined using an ELISA, as mentioned above. The specific activity of ^{35}S-bFGF was 466 ± 4 DPM/ng, with about 5 % unbound ^{35}S (PD10-column) after thawing. ^{35}S-activity was determined using a β-counter (Wallac Winspectral 1414, Wallac, Turku, Finland).

Stability of ^{125}I- and ^{35}S-labeled bFGF

Solutions of ^{125}I- or ^{35}S-labeled bFGF in PBS containing BSA (250 μl, 20 mg BSA/ml), with or without heparin (50 μg), were stored at 37°C. At days 0, 1, 2, 3, 6, 8 and 10 the percentage free label was determined by PD10 column separation. For comparison, the amount of free label of ^{35}S-albumin (labeled with SLR in a similar procedure as bFGF without addition of heparin beads) and ^{3}H-heparin (labeled as previously described[35]) was determined as well. Free label contents of the

samples before storage at 37°C were subtracted from the determined values (^{35}S-bFGF: 5.0%, ^{125}I-bFGF: 11.2%, ^{35}S-albumin: 2.5%, ^{3}H-heparin: 3%). ^{3}H-activity was determined using a β-counter.

^{125}I-bFGF (28 ng/ml) in PBS/BSA (1 μg/ml), either with or without heparin (1 μg/ml), was stored at 37°C for 10 days. Every other day a sample was examined using reducing and non-reducing polyacrylamide SDS gel-electrophoresis. Equivolume samples of either bFGF solution or molecular weight markers and SDS in Tris-buffer (10 mg/ml SDS ,0.5M Tris, pH 7.5), with or without dithiotreitol (1.5 mg/ml), were treated at 95°C for 5 min. After cooling, 40 μl aliquots of the SDS-treated samples were applied to ExcelGel® SDS gradient 8-18 precast gels using a 26 sample SDS applicator strip. Electrophoresis was carried out for 80 min with a water-cooled flat-bed electrophoresis apparatus (LKB 2117 Multiphore II, LKB, Bromma, Sweden), using 600 V, 50 mA and 30W as running condition limits. Gels were stained using a Protein Silverstaining Kit and preserved in 10% (v/v) glycerol.

bFGF binding to agarose-heparin beads: effect of incubation time, concentration and ^{125}I-labeling

^{125}I-bFGF in PBS (100 μl) was added to agarose-heparin beads (50 μl packed gel in 1.9 ml PBS).

In the first experiment, the incubation time at room temperature was varied between 15 and 240 min and a concentration of 77.5 ng bFGF/ml was used. After incubation, the beads were washed with PBS and bound radioactivity was measured. The experiment was carried out with and without a 100-fold excess of heparin present in the incubation medium, compared to the heparin present on the beads.
In a second experiment, ^{125}I-bFGF was diluted with unlabeled bFGF, resulting in solutions containing a range of 0 to 100 percent labeled bFGF with identical overall bFGF-concentration (115 ± 5 ng/ml) as determined using the ELISA. These solutions

were added to agarose-heparin beads, incubated for 30 min at room temperature, after which the beads were washed with PBS and bound radioactivity was measured.

In a third experiment, using an incubation time of 30 min at room temperature, the bFGF concentration was varied between 10 and 114.5 ng/ml. Following the incubation, the beads were washed with PBS and bound radioactivity was measured. The supernatant of the highest concentration used during the incubation was diluted 1.75-fold with PBS and added to an aliquot of fresh agarose-heparin beads (50 µl packed gel, final volume 2 ml), in order to verify if bFGF, that did not bind during the first incubation, would bind during the second incubation. Binding of bFGF to agarose-heparin as a function of the bFGF concentration was repeated using bFGF purified with the aid of a Sepharose-HiTrap-heparin column.

Purification of fibronectin

Human fibronectin (FN) was purified from FNc by affinity chromatography on gelatin-Sepharose at room temperature. Phenylmethylsulfonylfluoride (0.1 mM) was added as protease inhibitor to all buffers. FNc (200 ml, 2.5 mg/ml in 50 mM Tris-HCl, pH 7.5) was applied to a 125 ml column of gelatin-Sepharose. The column was washed with several volumes of Tris-HCl-buffer (50 mM, pH 7.5), 2 volumes of NaCl-solution (1 M NaCl in 50 mM Tris-HCl, pH 7.5), and again with Tris-HCl-buffer (50 mM, pH 7.5). Elution of fibronectin was carried out with 4 M urea in Tris-HCl-buffer (50 mM, pH 7.5). The absorption of the eluate was monitored at 280 nm (Pharmacia Single Path Monitor UV-1, Pharmacia, Uppsala, Sweden), and the fibronectin-containing fractions were pooled. After dialysis against PBS, aliquots (± 0.50 mg FN/ml) were stored at -30°C.

Isolation of endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffe *et al.*,[36] with slight modifications as previously described.[15] In short, the umbilical vein was rinsed with PBS and filled for 20 minutes at 37 °C with trypsin-EDTA solution in PBS (0.05% and 0.02% (w/v)). After harvesting HUVECs, cell culture medium supplemented with 20% (v/v) human serum (CMS 20, see below) was added to inactivate trypsin and after exchange of the medium by fresh medium, cells were plated in fibronectin-coated TCPS flasks and grown until the third passage at 37 °C in 95% air/5% CO_2, saturated with water vapour. Before passage or seeding, cells were detached from the TCPS surface by incubation with trypsin-EDTA solution in PBS (0.05% and 0.02% (w/v)), centrifuged and resuspended in CMS 20.

Proliferation of endothelial cells

Third passage HUVECs were seeded at a density of 10,000 cells per square centimeter and cultured at 37 °C in 5% CO_2/95% air saturated with water vapour.

Culture media used were complete cell culture medium (CM) supplemented with 5 or 20% (v/v) pooled human serum (CMS 5 respectively CMS 20), and CMS 5 containing heparin (5 U/ml) and bFGF (0.3 ng/ml) (CMS 5/HF). CM consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). Medium (0.20 ml/cm^2) was replaced every other day. At selected times, cells were trypsinized and cell numbers were determined, using a Bürker counting device.

Effect of iodine-labeled bFGF, serum concentration and presence of heparin on the proliferation of HUVECs on fibronectin-coated TCPS

bFGF labeled with non-radioactive iodine (I-bFGF) or unlabeled bFGF in PBS/BSA (20 mg BSA/ml) were added to CMS 5 (final bFGF-concentration 0.3 ng/ml) either with or without heparin (5 U/ml). These media, together with CMS 5 and CMS 20, were used in a 10-day HUVEC proliferation experiment as described above, employing a substrate of fibronectin-coated TCPS.

The effect of bFGF (0.3 ng/ml) addition to CMS with varying serum concentrations (1, 2, 3.5 and 5%) and the effect of heparin (5 U/ml) addition to CMS 5 and CMS 20 were investigated in separate experiments.

Gas plasma treatment and coating of polystyrene petridishes

Polystyrene petridishes were cleaned and CO_2 gas plasma-treated as previously described.[15] Surfaces were stored at a temperature of -20°C after treatment, and used within 5 days.

Albumin (Alb) or alb-hep conjugate (AH) were immobilized on PS-CO_2 petridishes (10.9 cm^2) by incubating the dishes with the protein or alb-hep conjugate (1 ml, 15 mg/ml) in borate buffer (5mM, pH 8.2) for 45 minutes. Subsequently, the petridishes were rinsed using PBS (3 times), PBS to which NaCl was added (final concentration1M) which was acidified to pH 3.5 using 1 M HCl (3 times), followed by PBS to which NaCl was added (final concentration1M) which was brought to pH 3.5 using 1 M NaOH (3 times). Finally, the surfaces were rinsed twice with PBS.

Some of the surfaces onto which alb-hep conjugate had been immobilized, were also incubated with fibronectin (1 ml, 0.05 mg/ml in PBS) for 30 minutes, after which the surfaces were rinsed three times with PBS (surfaces coded as PS-CO_2/AH+FN). A mixture of alb-hep conjugate (15 mg/ml) and

fibronectin (0.05 mg/ml) in borate buffer was immobilized as well (surfaces coded as PS-CO_2/AH/FN), as described above.

The surface concentrations of the different constituents of the coatings are presented in table 1. Initial surface concentrations were determined using radiolabeled compounds (albumin, alb-hep conjugate and fibronectin).[16] Small quantities of the immobilized compounds were released during one day incubation in serum-containing cell culture medium, whereas almost all FN was released from the surface of PS-CO_2/alb-hep+FN.[16]

coating	^{14}C-alb ($\mu g/cm^2$)	^{14}C-alb-hep ($\mu g/cm^2$)	^{125}I-FN ($\mu g/cm^2$)
PS-CO_2/Alb	0.43 ± 0.01		
PS-CO_2/AH		0.57 ± 0.03	
PS-CO_2/AH/FN		0.51 ± 0.03	0.19 ± 0.01
PS-CO_2/AH+FN		0.24 ± 0.02	0.03 ± 0.00

Table 1. *Surface concentrations of albumin, alb-hep conjugate (AH) and fibronectin (FN) after immobilization on PS-CO_2.*[16]

^{125}I-bFGF-loading of surfaces

PS-CO_2-surfaces either uncoated or coated with albumin, alb-hep conjugate or a combination of alb-hep conjugate and fibronectin were incubated with ^{125}I-bFGF (1 ml, 137 ng/ml) in PBS containing 20 mg BSA/ml (PBS/BSA) at room temperature for 90 min. Subsequently, the wells were washed with PBS (5 ml, 3 times for 15 min), and radioactivity bound to the surface was determined.

Effect of surface-immobilized bFGF on cell proliferation

Proliferation of HUVECs was investigated using petridishes of PS-CO_2, either with or without a coating of albumin or alb-hep conjugate. Some surfaces were loaded with unlabeled bFGF analogous to the procedure given in *^{125}I-bFGF-loading of surfaces.* Before cell seeding, surfaces (9.6 cm^2) were placed overnight at room temperature in PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Proliferation experiments were carried out as described above.

In the first experiment, bFGF (1 ml, 28 or 280 ng/ml PBS/BSA) was bound to albumin-heparin coated PS-CO_2. In the second experiment, bFGF (1 ml, 205 ng/ml PBS/BSA) was applied to albumin or albumin-heparin coated PS-CO_2. Surfaces to which bFGF was bound were cultured in CMS 5, whereas surfaces to which no bFGF was immobilized were cultured in CMS 5/HF.

Results

Stability of ^{125}I- and ^{35}S-labeled bFGF

The amount of free radioactivity released from ^{125}I-labeled bFGF was found to increase with time upon storage at 37°C (figure 2). Although the stability of ^{125}I-bFGF was slightly increased by the addition of heparin, this effect was not significant.

Release of free ^{125}I-label increased with increasing storage temperature (-20, 4, 20, 37 °C, not illustrated). During the first 48 hours, the release of free label from ^{35}S-bFGF was similar to that from ^{125}I-bFGF (figure 2). Subsequently, release of ^{125}I levelled off, whereas release of ^{35}S-label continued. In contrast to the labeled bFGF, ^{35}S-labeled albumin and ^{3}H-labeled heparin were stable under these conditions.

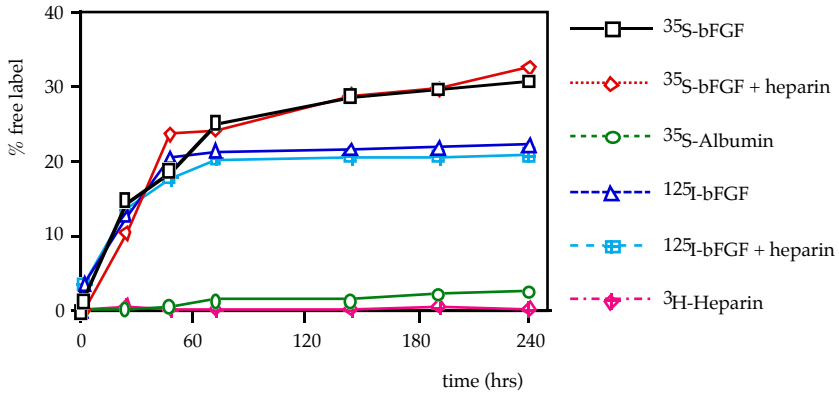

Figure 2 *Percentage free label of ^{125}I- and ^{35}S-labeled bFGF with or without heparin as a function of storage time in PBS/BSA (20 mg BSA/ml) at 37°C, determined by PD10 column separation. For comparison free label of ^{35}S-albumin and ^{3}H-heparin are presented. Free label present prior to storage at37°C was subtracted from the determined values. (n=2)*

Dimerization or even multimerization of bFGF as a result of labeling with ^{125}I-was not observed directly after labeling (figure 3, lane 2). However, during storage of ^{125}I-bFGF at 37°C tetra- and trimers were formed (figure 3, lanes 4 and 8). Still, a band at 17 kD remained present. Addition of albumin stabilized the growth factor to some extent (figure 3, lanes 3 and 7). Reduction with dithiotreitol resulted in a single band of the growth factor after electrophoresis, and no difference in this band could be observed between samples with or without albumin present.

bFGF binding to agarose-heparin

Binding of ^{125}I-bFGF (77.5 ng/ml) from PBS/BSA (20 mg BSA/ml) to agarose-heparin increased with incubation time until a plateau was reached after 30 min (data not shown). Binding was almost completely inhibited by a 100-fold excess of heparin compared to the heparin present on the beads (data not shown).

Figure 3 *Electrophoresis gels of ^{125}I-bFGF and ^{125}I-bFGF/albumin as a function of storage time at 37℃. Lane 1: LMW markers (14.4, 20.1, 30, 43, 67, and 94 kD); lane 2: ^{125}I-bFGF prior to storage; lanes 3 and 4: ^{125}I-bFGF in the presence (lane 3) or absence (lane 4) of albumin, non-reduced after 2 days of storage at 37℃; lanes 5 and 6: as lanes 3 and 4, but reduced; lanes 7 to 10: as lanes 3-6, but at day 6. Gels were silver stained.*

Labeling of bFGF with ^{125}I did not significantly affect the binding of the growth factor to agarose-heparin. The quantity of ^{125}I-bFGF bound to agarose-heparin beads decreased linearly upon dilution of ^{125}I-bFGF with unlabeled bFGF, while the overall bFGF concentration was kept constant (figure 4). Irrespective of the ratio between radioactive and unlabeled bFGF, the bFGF concentrations, measured using ELISA, were not significantly different. The amount of bFGF depleted from solutions with 0% and 100% unlabeled bFGF, as determined by ELISA, were similar (10.1 ± 1.5 ng/ml).

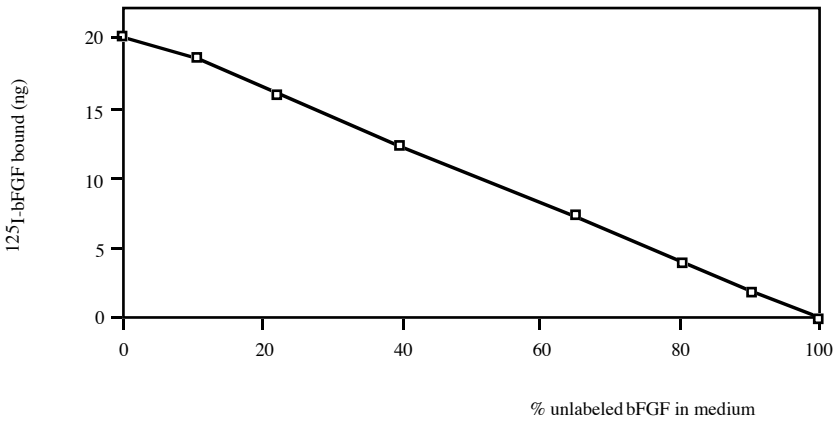

Figure 4 *Binding of ^{125}I-bFGF to agarose-heparin beads upon serial dilution with unlabeled bFGF. bFGF concentrations determined by ELISA were identical for all mixtures (115 ± 5 ng/ml). (n=5, ± sd)*

In the range of 10 to 114.5 ng/ml bFGF, only about 10 percent of ^{125}I-bFGF was bound to agarose-heparin beads (figure 5). To elucidate if this low binding resulted from the bFGF itself or was caused by the experimental conditions used, two control experiments were carried out. In the first experiment, the supernatant of the solution with the highest bFGF concentration was diluted to 54 ng/ml after the incubation and this solution was added to fresh agarose-heparin beads. This time, only 1 percent of the added bFGF bound to the beads (diamond-sign, figure 5). In the second control experiment, bFGF capable of binding to heparin was obtained by means of Sepharose-HiTrap heparin. Using this bFGF solution, the percentage of bFGF which bound to agarose-heparin was about 75 percent (data not shown).

In a separate experiment, in which a concentration range of 10 to 100 ng/ml bFGF was used, it was shown that the affinity of ^{35}S-bFGF and ^{125}I-bFGF to bind to agarose-heparin was similar (data not shown).

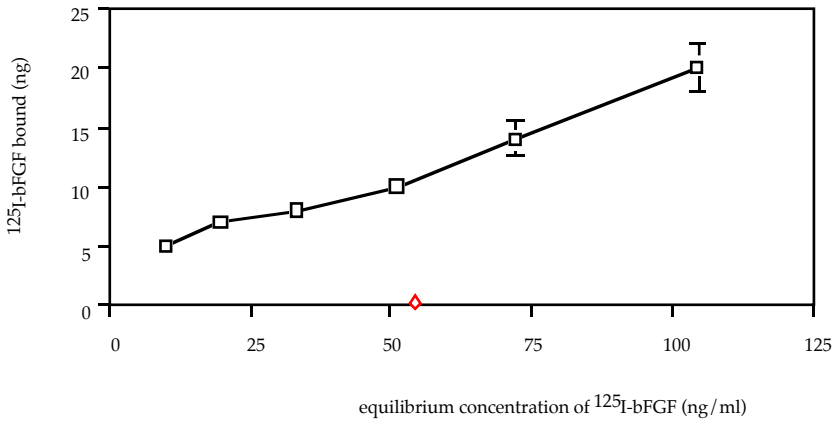

Figure 5 *Concentration-dependence of bFGF binding to agarose-heparin beads (50 µl packed gel, 2 ml total volume) after 30 min at room temperature Supernatant of the experiment in which the highest bFGF concentration was used, was diluted after incubation to a concentration of 54 ng/ml and incubated again with fresh agarose-heparin beads (◇). (n=5, ± sd)*

Effect of iodine-labeled bFGF, serum concentration and presence of heparin on HUVEC proliferation on fibronectin-coated TCPS

To study the effect of iodine-labeling of bFGF on its biological activity with respect to the stimulation of endothelial cell growth, bFGF was labeled with non-radioactive iodine (I-bFGF). Addition of I-bFGF or unlabeled bFGF to CMS5 (without heparin present) resulted in a significant increase of cell numbers throughout 10 days of culture (figure 6). Cell density was slightly lower when I-bFGF was used as compared to unlabeled bFGF. In both cases (CMS5/bFGF and CMS5/I-bFGF) cell numbers were lower than when CMS 20 was used as culture medium. When besides the growth factor 5U/ml heparin was present in the medium (CMS5/bFGF/hep and CMS5/I-bFGF

/hep), cell numbers increased to a level higher than found for CMS 20 as culture medium. When unlabeled bFGF was used (CMS5/bFGF /hep), cell numbers increased to a larger extent than with labeled bFGF (CMS5/I-bFGF/hep), especially at day 7.

A positive effect of bFGF (0.3 ng/ml) and heparin (5 U/ml) on cell proliferation was observed using medium with 1, 2, 3.5 or 5 percent human serum (not illustrated). Because the effect was most clearly seen with 5 percent serum, CMS 5 was used throughout this study to determine the effect of bFGF loading of the surface. Addition of only heparin to CMS 5 or CMS 20 did not influence the proliferation of HUVECs, seeded on fibronectin-coated TCPS (data not shown).

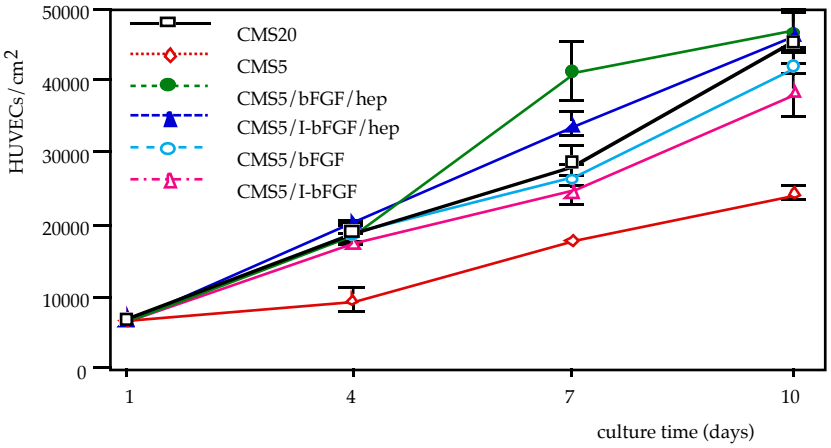

Figure 6 *Comparison of the effects of non-radioactive I-labeled bFGF (I-bFGF) and unlabeled bFGF on the proliferation of HUVECs plated on fibronectin-coated TCPS in the presence of CMS 5 with or without 5 U/ml heparin, or CMS 20. (n=3, ± sd)*

^{125}I-bFGF-loading of surfaces

^{125}I-bFGF was bound from PBS/BSA (20 mg BSA/ml) to PS-CO_2-surfaces that were uncoated or coated with albumin, alb-hep conjugate or a combination of alb-hep conjugate and fibronectin (figure 7). More bFGF was immobilized on surfaces containing heparin than on surfaces without heparin. When besides alb-hep conjugate also fibronectin was included in the coating, the amount of surface-immobilized bFGF was not reduced. Slightly more bFGF was bound to albumin-coated PS-CO_2 compared to uncoated PS-CO_2. Only a small percentage (about 11 %) of the added bFGF was bound to surface-immobilized heparin.

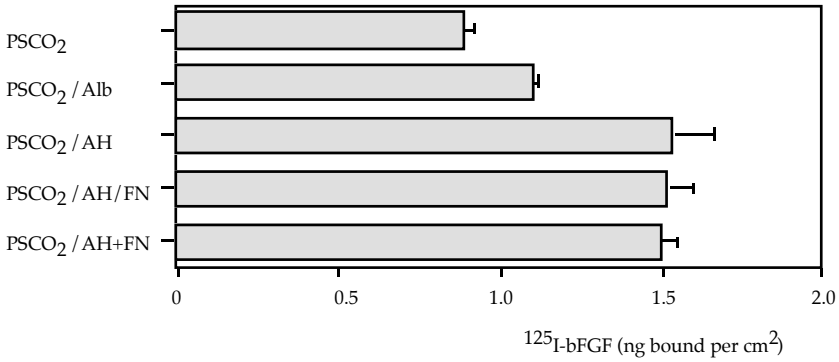

Figure 7 *Quantity of ^{125}I-bFGF immobilized after incubation for 90 min at ambient temperature using 1 ml 137 ng/ml bFGF per well on uncoated PS-CO_2 or on PS-CO_2 coated with albumin, alb-hep conjugate (AH), or alb-hep conjugate in combination with fibronectin, either added in a separate second incubation (AH+FN) or added during coating from a mixture of alb-hep conjugate and fibronectin (AH/FN). (n=3, ± sd)*

For the first proliferation experiment (figure 8), alb-hep conjugate-coated surfaces were pretreated using solutions with two distinct bFGF concentrations (28 and 280 ng/ml). On alb-hep conjugate-coated PS-CO_2 incubated with the high bFGF-concentration, a very rapid proliferation of seeded HUVECs was observed. Cell numbers increased faster than in the presence of CMS 20 or CMS 5 with heparin and growth factor (CMS5/HF). After 4 days of proliferation, confluency was reached and cell numbers did not increase further. Likewise, when a low concentration of bFGF was used during immobilization of bFGF, cell numbers did not increase further after 4 days of proliferation. Cell densities, however, corresponded to sub-confluent monolayers. All other surfaces showed confluent monolayers after 8 days of proliferation.

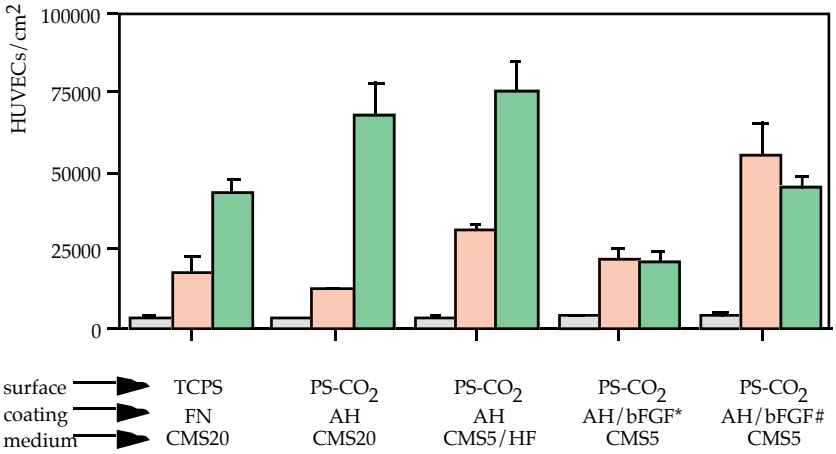

Figure 8 *Number of endothelial cells on alb-hep conjugate (AH)-coated PS-CO_2 with or without immobilized bFGF and on fibronectin-coated TCPS at different proliferation times: day 1, day 4, day 8. Two bFGF concentrations were used during immobilization: 28 ng/ml (bFGF*) and 280 ng/ml (bFGF#). Media used are presented in the graph. CMS 5 and CMS 20 are cell culture media with 5 respectively 20 percent human serum; CMS 5/HF is CMS 5 with 5 U/ml heparin and 0.3 ng/ml bFGF. (n=3, ±sd)*

In the second experiment (figure 9), besides alb-hep conjugate-coated PS-CO_2, albumin-coated PS-CO_2 was used as well, applying a solution with a high bFGF concentration (205 ng/ml). On both surfaces loaded with bFGF, proliferation proceeded rapidly to confluency at day 3, in contrast to the surfaces where bFGF was present in the medium (0.3 ng/ml). At 10 days of culturing, however, no significant differences in cell numbers were observed between loading of surfaces with bFGF or addition of bFGF to the culture medium, using either albumin- or alb-hep conjugate-coated PS-CO_2. Cell layers were confluent on all surfaces at day 10.

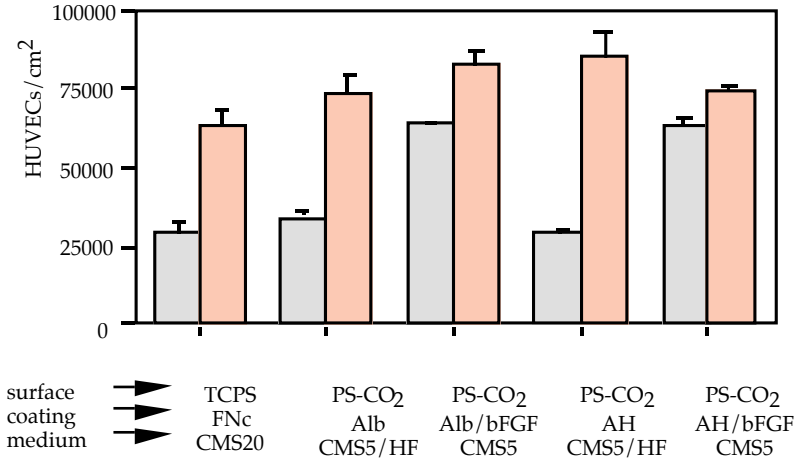

Figure 9 *Number of ECs on fibronectin-coated TCPS and on albumin- or alb-hep conjugate-coated PS-CO_2, with or without immobilized bFGF, at different proliferation times:* ☐ *day 3,* ☐ *day 10. bFGF was immobilized using a concentration of 205 ng/ml. Media used are presented in the graph. CMS 5 and CMS 20 are cell culture media with 5 respectively 20 percent human serum; CMS 5/HF is CMS 5 with 5 U/ml heparin and 0.3 ng/ml bFGF. (n=3, ± sd)*

The morphology of HUVECs cultured on alb-hep conjugate-coated PS-CO_2 loaded with bFGF did not differ from the morphology of HUVECs on this surface with bFGF in the medium (figure 10). Moreover, no difference was found in morphology of HUVECs grown on albumin-coated PS-CO_2, alb-hep conjugate-coated PS-CO_2 or fibronectin-coated TCPS (not shown).

A B

C

Figure 10. *Light-microscopic images of HUVECs grown for 3 days on fibronectin-coated TCPS (A, CMS20), and on alb-hep conjugate immobilized on PS-CO_2 with bFGF either in the medium (B, CMS5/HF) or immobilized to the surface (C, CMS5). (original magnification: 128x)*

Discussion

In this study it was shown that the formation of a confluent endothelial cell monolayer upon a coating of alb-hep conjugate on PS-CO_2 was accelerated when basic fibroblast growth factor (bFGF) was bound to the substrate. bFGF is known to be a very potent growth factor. DNA-synthesis and other cellular functions are stimulated by very low concentrations of bFGF, resulting in enhanced cell proliferation.[23,37,38] This characteristic of bFGF may be of interest for clinical applications such as endothelial cell seeded vascular grafts, as can be deduced from animal studies.[39,40] However, to minimize the possibility of tumour formation or enhancement, the mitogen needs to be directed to the appropriate site of action.[41] Therefore, local release of bFGF is preferred over systemic administration of the growth factor.

To study the release profile of bFGF from bFGF-loaded substrates, the growth factor was labeled with ^{125}I. It was shown, however, that the ^{125}I-label was released during storage at room temperature and even faster at 37°C. Addition of heparin, which is reported to stabilize bFGF,[23-25] hardly decreased the release of free ^{125}I-label. Therefore, it was expected that the increase of ^{125}I-label during storage was the result of dissociation of the label from intact bFGF molecules, a problem more often encountered with ^{125}I-labeled proteins.[42] When bFGF was labeled with the N-hydroxysuccinimide ester of ^{35}S-methionine, which theoretically results in stable incorporation of the label, a similar release of free label (or low molecular weight ^{35}S-containing compounds) from bFGF, as in the case of ^{125}I-labeled bFGF, was observed. After 10 days of storage at 37°C, release of ^{35}S was higher than release of ^{125}I from bFGF, but taking into account the initial free label which was present in the solutions prior to storage at elevated temperature, the total release of either ^{125}I or ^{35}S was comparable. It cannot be excluded that free label release (both ^{125}I and ^{35}S) is due to degradation of bFGF. This degradation could only to a certain extent be inhibited by the addition

of heparin. Since heparin is known to stabilize bFGF,[23-25] it is postulated that the tendency of labeled bFGF towards degradation is a result of the labeling itself, and not merely caused by elevated temperatures. The increased stability of bFGF in the presence of heparin, reported in literature, is caused by a conformational change of the growth factor upon binding to heparin.[24] In the case of labeled bFGF reported in this study, this change of conformation may be hindered by the label, resulting in only a moderate increase in stability.

In addition to these findings, SDS polyacrylamide gel-electrophoreses (SDS-PAGE) of ^{125}I-bFGF showed that tri- and tetramers were formed upon storage at 37°C. Without the presence of albumin, added to stabilize the growth factor, the electrophoresis band of monomeric bFGF disappeared completely after 6 days of storage at 37°C, whereas in the presence of albumin a 17 kD protein band remained present. The multimers are formed by disulfide-bridges, since reduction with dithiotreitol resulted in a single 17 kD protein band. On the electrophoresis gels, the intensity of the reduced bands appears to be similar at day 2 and 6, but this does not mean that no degradation took place, since staining of a gel is only semi-quantitative. Low molecular weight degradation products (< 10 kD) may have been run from the gels. However, no protein bands between 10 and 17 kD were observed.

Thus, degradation and multimerization of labeled bFGF in time occurred when the growth factor was stored at 20-37°C, limiting the use of labeled bFGF to short term (hours) experiments, like for instance determination of the amount of bFGF bound to a matrix. The degradation and multimerization of part of the bFGF should be taken into account while evaluating the experiments discussed here.

Upon serial dilution of ^{125}I-bFGF with unlabeled bFGF, the amount of heparin-bound ^{125}I-bFGF decreased linearly when the percentage of unlabeled bFGF was increased, while total bFGF-binding to the heparin-beads was constant. Therefore, labeling of bFGF with ^{125}I does not influence the quantity of bFGF bound to heparin. ^{125}I-bFGF binding to heparin, immobilized to agarose-beads, reached equilibrium within 30 min and increased linearly over the range of 10 to 115 ng/ml bFGF. Binding of ^{125}I-bFGF was almost completely inhibited by addition of a 100-fold excess of heparin compared to the heparin present at the beads, indicating that ^{125}I-bFGF binding to agarose-heparin occurred via specific binding to immobilized heparin. The amount of bound bFGF, however, was only about 10 percent of the bFGF added to the heparin-beads.Almost no bFGF remaining in the supernatant was capable of binding to fresh agarose-heparin beads. Low binding capability of bFGF to surface-immobilized heparin was found by Wissink[43] as well. Based on the affinity of bFGF for heparin (K_d = 470±20 nM)[21] and the huge excess of heparin compared to bFGF, it was expected that a high percentage of bFGF would bind to the heparin-beads. Apparently the bFGF used in the present study was either impure or partly denaturated. Moreover, since recombinant bFGF was used in this study, the low binding of bFGF to immobilized heparin could be the result of incomplete folding as well as incomplete post-translational modifications of the protein, as suggested by Linemayer *et al*..[44]

I-labeled bFGF (non-radioactive) showed only slightly reduced biological activity compared to unlabeled bFGF. As far as biological activity of bFGF is concerned, the use of labeled bFGF in cell culture experiments raises no problems.

Using ^{125}I-bFGF it was shown that more bFGF bound to a monolayer of alb-hep conjugate on PS-CO_2 than to a monolayer of albumin on this surface or to uncoated PS-CO_2. This result strongly suggests that surface-immobilized heparin is involved

in the binding of bFGF. The difference in bFGF binding to albumin-coated and uncoated PS-CO_2 may result from a decrease in hydrophylicity upon albumin coating of the PS-CO_2-surface. In general, higher amounts of proteins adsorb to materials that are less hydrophylic.[45] Alternatively, since in the electrophoresis experiment albumin was shown to stabilize bFGF to some extent, the increased bFGF surface concentration upon coating PS-CO_2 with albumin may result from a carrier function for bFGF displayed by albumin. Addition of fibronectin, either separately in a second incubation step or present in a mixture with alb-hep conjugate, did not influence the binding of bFGF to the coating, probably because a large excess of heparin compared to bFGF is present at the surface even after part of the heparin has been covered by fibronectin.

HUVECs were capable of growing in CMS 5 culture medium on a substrate of bFGF-loaded alb-hep conjugate immobilized to the surface of PS-CO_2. It was shown that when bFGF concentrations of 200 - 280 ng/ml were used for coating, sufficient bFGF was bound to form a confluent monolayer of HUVECs. Concentrations of bFGF used in this study were very low, compared to concentrations used by others.[39,41] Since about 10 percent of added bFGF bound to the surface, the surface concentration of bFGF was estimated to be 2 respectively 3 ng/cm^2 for albumin and alb-hep conjugate. In the case of alb-hep conjugate, this corresponds to about 1 bFGF molecule being bound per 33 heparin molecules present at the surface. Theoretically, a much higher amount of bFGF can be bound to the surface, with a maximum of about 4 bFGF molecules per heparin moiety. However, the amount of bFGF that was bound to the surface was sufficient to enhance cell proliferation. In contrast, when 28 ng bFGF/ml was used for loading of the surface, the cell layers remained subconfluent. The most prominent effect of surface-immobilized bFGF (in the case of bFGF concentrations of 200 - 280 ng/ml) was the very rapid cell proliferation shortly after seeding, resulting in high density confluent monolayers within three days. These monolayers were

found not only on alb-hep conjugate-coated PS-CO_2, but on albumin-coated PS-CO_2 as well. When bFGF was present in the culture medium instead of surface-bound, cell numbers increased at a slower rate, but eventually similar cell numbers were reached on both albumin- and alb-hep conjugate-coated PS-CO_2 surfaces. These differences may be the result of the initial high bFGF concentrations present when bFGF was bound to the surface, causing more rapid proliferation of the HUVECs in this case. On the other hand, the biological activity of bFGF present in freshly prepared culture medium used for replacement, is more or less constant, whereas that of the surface-bound bFGF may decrease in time.

In conclusion, bFGF bound to surface-immobilized alb-hep conjugate facilitates the rapid formation of a confluent layer of HUVECs, provided that enough bFGF had been bound to the surface. Therefore, bFGF-loading of vascular graft surfaces through complexation of bFGF with a heparin-containing matrix may accelerate the formation of a confluent endothelial cell layer upon seeding of these cells to the graft surfaces.

Conclusions

Basic fibroblast growth factor (bFGF) can be labeled with ^{125}I using Iodobeads without significantly decreasing its biological effect on HUVEC proliferation. However, ^{125}I-bFGF is unstable in PBS/BSA with or without added heparin, especially at elevated temperatures. Besides degradation, multimerization of labeled bFGF occurs during storage at 37°C. Therefore, the use of radiolabeled bFGF is limited to short-term experiments, e.g. determination of the amount of immobilized bFGF.

bFGF bound to immobilized albumin or albumin-heparin on PS-CO_2, is capable of reducing the time nescessary for the formation of a confluent monolayer of HUVECs on these surfaces, provided that enough bFGF (2-3 ng/cm^2) is bound to the surface.

Therefore, bFGF-loading of vascular graft surfaces may provide more rapid endothelialization, resulting in improved patency of small-diameter vascular grafts seeded with endothelial cells.

Acknowledgement

We wish to thank the staff of the departement of obstetrics of Medisch Spectrum Twente (MST, Enschede, The Netherlands) for providing umbilical cords.

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Chapter 6 Blood Compatibility of Surfaces with Immobilized Albumin-heparin Conjugate and the Effect of Endothelial Cell Seeding on Platelet Adhesion

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Abstract

Seeding of endothelial cells (ECs) onto the luminal surface of small-diameter vascular prostheses is a promising method to avoid graft occlusion. A coating consisting of albumin and heparin is a suitable substrate for the seeded ECs. However, binding of ECs to the substrate improves when small amounts of fibronectin are present in the coating. In the present study, the blood compatibility was investigated of a surface-immobilized monolayer of albumin-heparin conjugate (alb-hep conjugate) with or without added fibronectin, as well as the effect of seeded ECs on platelet deposition onto these surfaces.

Alb-hep conjugate immobilized on carbon dioxyde gas plasma-treated polystyrene (PS-CO_2), significantly increased the recalcification time of blood plasma exposed to this surface. Furthermore, surface-immobilized alb-hep conjugate was able to inhibit thrombin. Heparin activity was reduced by adding fibronectin on top of a monolayer of alb-hep conjugate, but not by simultaneous coating of fibronectin and alb-hep conjugate. Coating of PS-CO_2 with alb-hep conjugate significantly decreased contact activation (FXII activation).

The number of platelets deposited on different alb-hep conjugates under stationary conditions showed a positive correlation with the heparin activity of the conjugates. Numbers of platelets deposited from blood plasma on PS-CO_2 coated with

albumin and alb-hep conjugate with relatively low anticoagulant activity were similar, whereas on alb-hep conjugate with higher activity the number of platelets increased two-fold. Addition of fibronectin to alb-hep conjugate-coated PS-CO_2 had no significant effect on the number of adhered platelets.

Seeding of the substrates with ECs significantly reduced the number of adhered platelets under stationary conditions. However, on surfaces seeded with ECs the number of deposited platelets slightly increased with endothelial cell density. Platelets deposited onto endothelialized surfaces were primarily found on endothelial cell edges, and sparingly on areas between ECs.

In conclusion, alb-hep conjugate-coated surfaces display anticoagulant activity. ECs adhering to and proliferating on these coatings significantly decrease the number of platelets which adhere to the surface. Therefore, alb-hep conjugate-coated surfaces form a suitable substrate for seeding of ECs in low density. Although application of fibronectin on top of the coating decreases the anticoagulant activity to some extent, it might be useful in view of the improved adherence of ECs to the coating.

Introduction

Synthetic vascular grafts of Dacron or expanded Teflon with an inner diameter exceeding 5 mm, have been successfully used in vascular surgery for decades.[1-3]. Small-diameter vascular prostheses, however, occlude rapidly.[4-6] In contrast to large-diameter vascular prostheses, anticoagulant treatment and administration of platelet inhibitors do not improve the patency of small-diameter grafts.[7]

Unlike in animal species, spontaneous outgrowth of endothelium on the inner surface of implanted vascular prostheses generally does not occur in humans.[3,8] Since ECs perform a key regulatory role in haemostasis,[9,10] seeding of ECs on the luminal

surface of vascular grafts is a promising method to avoid occlusion of small-diameter prostheses implanted in humans.[11-14] However, as ECs for seeding need to be autologous and the efficiency of endothelial cell harvesting from vascular tissue is generally poor, the number of ECs available is usually too small to allow immediate formation of a confluent cell layer. During the period in which the layer of ECs is not yet confluent, activation of the blood coagulation system and platelet deposition must be inhibited. This goal may be reached by using an albumin-heparin conjugate (alb-hep conjugate) coating as substrate for the seeded ECs.

As shown previously, an alb-hep conjugate coating, which comprises a heterogeneous mixture of conjugates with different compositions, such as alb_1-hep_1, alb_1-hep_2, alb_2-hep_1,[15] may be used as a substrate for ECs.[16] Human umbilical vein endothelial cells (HUVECs) were grown to confluency on alb-hep conjugate, that was covalently immobilized on CO_2 plasma-modified polystyrene. HUVECs cultured on this surface released amounts of von Willebrand factor (vWF) and prostacyclin (PGI_2) comparable to those of HUVECs grown on fibronectin-coated TCPS, which demonstrated the viability of these cells.[17]

For many years, immobilization of heparin has been applied to enhance thromboresistance of artificial prostheses exposed to blood or plasma.[18] Binding of antithrombin III (ATIII) to a specific pentasaccharide sequence in heparin, results in a marked increase of the inactivation of serine proteases like factor Xa and thrombin by ATIII.[19] When heparin is immobilized to a surface in the form of alb-hep conjugate, 27 percent of its anticoagulant activity is retained.[20]

Contact of artificial surfaces with blood inevitably leads to contact activation, which initiates intrinsic coagulation, fibrinolysis and activation of the kinin system. The first step in the contact activation process is the activation of the plasma glycoprotein Factor XII (FXII) at the surface of the biomaterial,

which results in a conformational change of the molecule. Activated factor XII (FXIIa) can be cleaved into two polypeptide chains of 52 (αFXIIa) and 28 kD (βFXIIa), of which the latter diffuses away from the surface.[21] Surface-bound αFXIIa is involved in the activation of the intrinsic coagulation, the fibrinolytic system, and the kinin system.[19,22,23] βFXIIa is involved only in the activation of the kinin system.[23]

On foreign surfaces, deposition of platelets, and to a lesser extent leukocytes, generally occurs. Coating of surfaces with von Willebrand factor, fibrinogen or fibronectin increases adhesion of platelets, whereas coating with albumin or plasma decreases adhesion of platelets.[24-28] Seeding of ECs on artificial surfaces may result in decreased platelet deposition in dogs,[29] baboons,[30] as well as in humans,[11,14,31] although at least one study failed to show this effect.[32] Decreased numbers of platelets adhering to endothelialized surfaces, may be explained by the production of PGI_2 and nitric oxide by ECs.[33]
The purpose of the present study was to investigate the blood compatibility of a monolayer of alb-hep conjugate, which was immobilized on gas plasma modified polystyrene, and to study the effect of endothelial cell seeding on platelet deposition onto this surface.

Materials and Methods

Materials

Unfractionated alb-hep conjugates were obtained from Holland Biomaterials Group (Enschede, The Netherlands). alb-hep 1 (16.5% (w/w) heparin) was based on porcine albumin and alb-hep 2 and 3 (11.6% and 12.3% (w/w) heparin respectively) were based on human albumin. Phosphate-buffered saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene (PS) and tissue culture polystyrene

(TCPS) petridishes (35 mm diameter, 9.6 cm^2) were from Greiner GmbH (Frickenhausen, Germany). TCPS 12-wells plates were obtained from Costar (Cambridge, UK). Carbon dioxide (CO_2) was from Hoekloos (Schiedam, The Netherlands). Pig mucosal heparin sodium (195 U/mg) was from Bufa Chemie (Castricum, The Netherlands). Gelatin-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Kallikrein/FXIIa chromogenic substrate (S2203) was obtained from Chromogenix (Möhndal, Sweden). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, and trypsin-EDTA were purchased from Life Technologies (Paisley, UK). ^{111}In-oxine was bought from Amersham (Amersham, UK). Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris) and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Company (St. Louis, Missouri, USA). Dialysis tubing (size 9) was obtained from Medicell International Ltd. (London, UK). Human serum was pooled from 12 healthy volunteers. Fresh buffy coats, obtained from healthy volunteers, were a gift of Bloedbank IJssellanden (Enschede, The Netherlands). A plasma fraction containing human fibronectin (FNc) was a gift from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/Hohenbrunn, Germany).

Purification of fibronectin

Human fibronectin (FN) was purified from FNc by affinity chromatography on gelatin-Sepharose at room temperature. Phenylmethylsulfonylfluoride (0.1 mM) was added as protease inhibitor to all buffers. FNc (200 ml, 2.5 mg/ml in 50 mM Tris-HCl, pH 7.5) was applied to a 125 ml column of gelatin-Sepharose. The column was washed with several volumes of Tris-HCl-buffer (50 mM, pH 7.5), 2 volumes of NaCl-solution (1 M NaCl in 50 mM Tris-HCl, pH 7.5), and again with Tris-HCl-buffer (50 mM, pH 7.5). Elution of fibronectin was carried

out with 4 M urea in Tris-HCl-buffer (50 mM, pH 7.5). The absorption of the eluate was monitored at 280 nm (Pharmacia Single Path Monitor UV-1, Pharmacia, Uppsala, Sweden), and the fibronectin-containing fractions were pooled. After dialysis against PBS, aliquots (± 0.50 mg FN/ml) were stored at -30°C.

Gas plasma treatment and coating of polystyrene petridishes

Polystyrene petridishes were cleaned and CO_2 gas plasma-treated as previously described.[16] After treatment, surfaces were stored at a temperature of -20°C and used within 5 days.

Albumin or alb-hep conjugate (alb-hep) were immobilized on PS-CO_2 petridishes (10.9 cm^2) by incubating the dishes with the protein or conjugate (1 ml, 15 mg/ml) in borate buffer (5mM, pH 8.2) for 45 minutes. Subsequently, the petridishes were rinsed, using PBS (3 times), a low pH (3.5) phosphate buffered (NaH_2PO_4/Na_2HPO_4, 12 mM) saline (1 M) solution (3 times), and a similar solution with a pH of 10.5 (3 times). Finally, the surfaces were rinsed twice with PBS.

A number of the surfaces onto which alb-hep conjugate had been immobilized, were incubated with fibronectin (1 ml, 0.05 mg FN/ml in PBS) for 30 minutes, after which the surfaces were rinsed with PBS three times (surfaces coded as PS-CO_2/alb-hep+FN). Analogous to the procedure with alb-hep conjugate, a mixture of alb-hep conjugate (15 mg/ml) and fibronectin (0.05 mg/ml) in borate buffer was added to PS-CO_2 (surfaces coded as PS-CO_2/alb-hep/FN).

The surface concentrations of the different constituents are presented in table 1. Initial surface concentrations were determined using radiolabeled compounds (albumin, alb-hep conjugate and fibronectin).[17] Small quantities of the immobilized compounds were released during one day incubation in serum-containing cell culture medium, whereas almost all FN was released from the surface of PS-CO_2/alb-hep+FN.[17]

coating	^{14}C-alb ($\mu g/cm^2$)	^{14}C-alb-hep ($\mu g/cm^2$)	^{125}I-FN ($\mu g/cm^2$)
PS-CO_2/Alb	0.43 ± 0.01		
PS-CO_2/AH		0.57 ± 0.03	
PS-CO_2/AH/FN		0.51 ± 0.03	0.19 ± 0.01
PS-CO_2/AH+FN		0.24 ± 0.02	0.03 ± 0.00

Table 1. *Surface concentrations of albumin, alb-hep conjugate and fibronectin after immobilization on PS-CO_2.*[17]

Recalcification assay

Activation of the intrinsic coagulation pathway was studied by determining the coagulation time of recalcified human blood plasma in contact with albumin and alb-hep conjugate immobilized on PS-CO_2 petridishes. Fresh frozen human plasma was thawed at 37°C and stored on ice until further use. Human plasma (900 µl), PBS (100 µl) and rabbit brain cephalin (100 µl) were pipetted into the petridishes and warmed to 37°C in 3 min, after which $CaCl_2$ (100 µl 0.2M, prewarmed to 37°C) was added. The recalcification time was determined by the time needed to detect the first clot with a stainless steel hook. As a positive control, recalcification time was also determined for a petridish to which a glass coverslip was added.

Thrombin inactivation assay

The activity of surface-immobilized heparin with respect to neutralization of added thrombin was determined using a chromogenic substrate for thrombin in an endpoint assay as described by Chandler *et al.*.[34] The assay was carried out using Tris buffer (50 mM, pH 8.4) containing poly(ethylene glycol) 6000 (1.0 g/l), bovine serum albumin (1.0 g/l) and NaCl (150 mM) (assay buffer). To a petridish with a coating of albumin, alb-hep conjugate, and/or fibronectin, assay buffer (600µl) was added and warmed to 37°C in 10 min. Next, assay buffer (300

μl) containing chromogenic substrate S2238 (0.2 mg/ml) and ATIII (70 mU/ml), which had been kept at 0°C until use, was added and allowed to warm to 37°C for 5 min. The reaction was started by addition of thrombin (100 μl, 1.2 U/ml) and stopped after 10 min of shaking at 37°C by the addition of an acetic acid solution (100 μl, 40% (v/v) in demineralized water). The absorbance at 405 nm of the supernatant, caused by p-nitroanilin cleaved from the substrate by thrombin, was determined with an ELISA reader (340 ATTC, SLT, Austria). The absorbances at 405 nm were converted to mU/cm^2 using a reference curve obtained with heparin samples of known activity.

Contact activation assay

Activation of the contact system was determined using chromogenic substrate S2302, as described by Van Delden *et al.*.[20] This chromogenic substrate can be cleaved by several proteases, of which FXIIa (both αFXIIa and βFXIIa) and kallikrein are the most important.[35]

A plasma solution (1 ml, 25% (v/v) in PBS, stored on ice until use) was added to a PS-CO_2-petridish, after which the dish was placed on an orbital shaker at 37°C for 15 min. Uncoated PS-CO_2-petridishes were used, as well as PS-CO_2-petridishes coated with albumin, alb-hep conjugate or fibronectin. A sample of the supernatant (70 μl) was incubated at 37°C with substrate S2302 solution (110 μl, 1.12 mg/ml in PBS) and the increase in optical density at 405 nm was determined during 30 min using a kinetic ELISA reader (340 ATTC, SLT, Austria). A plasma solution in PBS (25% (v/v)) that was not exposed to the petridishes, was used as control.
After washing the surfaces twice with 3 ml PBS, the enzymatic activity present on the incubated surfaces was measured by incubating the surface with S2302 (1 ml, 0.25 mg/ml in PBS) for 60 min at 37°C. The absorbance of the supernatant at 405 nm was determined using an ELISA reader. A PS petridish, which was not incubated with plasma, was used as control.

Isolation of endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated using the method of Jaffe *et al.*,[36] with slight modifications. The umbilical vein was rinsed with PBS and filled for 20 minutes at 37 °C with trypsin-EDTA solution (0.05% respectively 0.02% in PBS). After harvesting HUVECs, cell culture medium supplemented with 20% (v/v) pooled human serum (CMS), was added to inactivate trypsin. After exchange of the medium by fresh medium, cells were plated in fibronectin-coated TCPS flasks and grown until the third passage at 37 °C in 95% air/5% CO_2, saturated with water vapour. Cell culture medium (CM) consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). Before passage or seeding, cells were detached from the TCPS surface by incubation with trypsin-EDTA solution, centrifuged and resuspended in CMS.

Proliferation of endothelial cells

Before cell seeding, surfaces (9.6 cm^2) were placed overnight at room temperature in PBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Third passage HUVECs were seeded at a density of 10,000 cells per square centimeter and cultured at 37 °C in 5% CO_2/95% air saturated with water vapour. Medium (0.20 ml/cm^2, CMS) was replaced every other day. At selected times, cells were trypsinized, after which cell numbers were determined using a Bürker counting device.

Preparation of platelet suspension and ^{111}In-labeling of platelets

Fresh buffy coats from healthy volunteers were diluted 2.5 times with Krebs-Ringer buffer (107 mM NaCl, 4 mM KCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4, pH 7.3), containing trisodiumcitrate (19 mM) and D (+)-glucose (27 mM), after which platelet-

rich plasma (PRP) was obtained by centrifugation (1700 g, 4 min, 20°C). Subsequently, PRP was diluted 1:1 with Krebs-Ringer buffer (pH 5.0) containing 19 mM trisodiumcitrate and 27 mM D (+)-glucose (final pH 6.1). Platelets were isolated according to the method of Cazenave *et al.*[37] and labeled with ^{111}Indium oxine.[24] Finally, platelets were resuspended at a concentration of 1.0 x 10^5/μl in ABO-compatible plasma or in CM with 3.5 mg/ml albumin (CM/alb).

Deposition of ^{111}In-labeled platelets

Stationary deposition of ^{111}In-labeled human platelets from human plasma or CM/alb was determined using a series of substrates. Test surfaces (petridishes, 9.6 cm^2) were incubated for 4 h with PBS (2 ml) and after removal of the PBS, a suspension of 1.0x10^5 platelets/μl in plasma or CM/alb (1 ml) was added. After 1 h at 37°C the platelet suspension was removed. Next, some of the surfaces were used to determine the number of adhered platelets, whereas others were used to investigate the morphology of the adhered platelets.

To determine the number of adhered platelets, surfaces were washed three times with PBS (3 ml) at 10 min intervals, and subsequently incubated overnight with NaOH (2 ml, 1 M). The radioactivity in this solution was determined using a γ-counter (Compugamma 1282, LKB, Stockholm, Sweden). The number of deposited platelets was determined by dividing the radioactivity found in the NaOH solution by the radioactivity incorporated per platelet.

To study the morphology of adhered platelets, surfaces were washed three times with PBS (3 ml) at 1 min intervals, and subsequently the platelets were fixed with glutaraldehyde (2 ml, 2% (v/v) in PBS). Samples were kept in this medium at 4°C for at least 52 days, 20 times the half-life of ^{111}In. After washing three times with water (4 ml, 2 min per step), samples were dehydrated by incubation with a graded series of ethanol solutions in water (4 ml, 25, 50, 75, 90, 98 % (v/v) of ethanol, 2

times 5 min per step) and finally in ethanol (4 ml, 4 times 5 min). After drying *in vacuo*, the samples were sputtered with gold and examined by scanning electron microscopy using 7 kV accelerating voltage (S-800 field emission SEM, Hitachi, Japan).

Stationary deposition of ^{111}In-labeled human platelets suspended in CM/alb was also determined using a series of substrates onto which HUVECs had been seeded 4 h before the experiment, after which the surfaces were rinsed with PBS at 37 °C. In one series used for SEM evaluation the HUVECs were allowed to proliferate for 48 h prior to incubation with platelets. The control surfaces without HUVECs used in this experiment were incubated with cell culture medium for 4 h before the experiment and rinsed with PBS at 37 °C.

Results

Recalcification time

All tested surfaces showed longer coagulation times for recalcified blood plasma than glass. Furthermore, for alb-hep conjugate-coated surfaces the coagulation time was significantly longer than for albumin coated PS-CO_2 ($P<0.05$, figure 1). Differences in coagulation times were observed between the three different alb-hep conjugate batches. A significant difference between recalcification time was found for alb-hep 1 and alb-hep 3 ($P<0.05$). The alb-hep conjugate batches, as supplied by the manufacturer, differed with respect to heparin content (16.5, 11.6, 12.3% (w/w) heparin respectively for alb-hep 1, alb-hep 2, and alb-hep 3), and the origin of the albumin used (porcine for alb-hep 1 and human for alb-hep 2 and alb-hep 3). The heterogeneity of the alb-hep conjugates may differ as well. The highest coagulation time was found for the alb-hep conjugate with the highest heparin content (alb-hep 1).

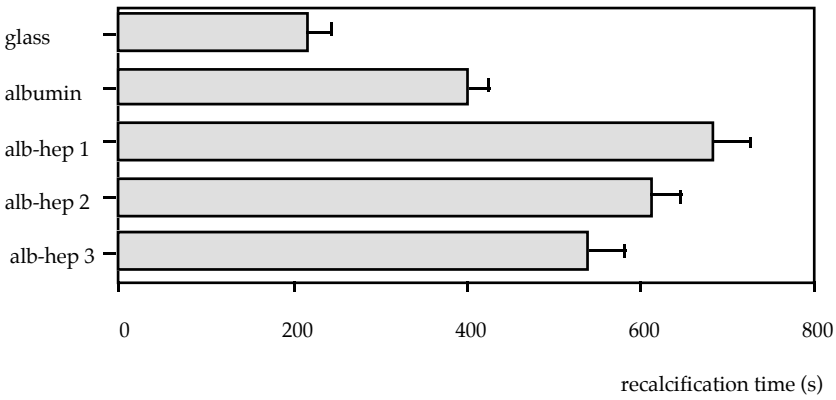

Figure 1. *Recalcification times of plasma contacted with various surfaces at 37°C. The recalcification time of glass (215 ± 29 s) served as positive control. Heparin content of alb-hep 1, 2 and 3 was 16.5, 11.6, 12.3% (w/w) respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3. The heterogeneity of the alb-hep conjugates may differ. (n=5, ± sd)*

Thrombin inactivation assay

Anticoagulant activity, defined as the inactivation of added thrombin, was found for all heparinized surfaces (figure 2), but not for control surfaces without heparin (legend of figure 2). The activity of alb-hep 3 was lower compared to the other two conjugates ($P<0.01$). Application of fibronectin to the PS-CO_2 surface together with alb-hep conjugate (alb-hep 3 /FN) did not influence the anticoagulant activity of the immobilized alb-hep conjugate, whereas application of fibronectin on top of the alb-hep conjugate layer (alb-hep 1, 2 or 3 +FN), reduced the anticoagulant activity.

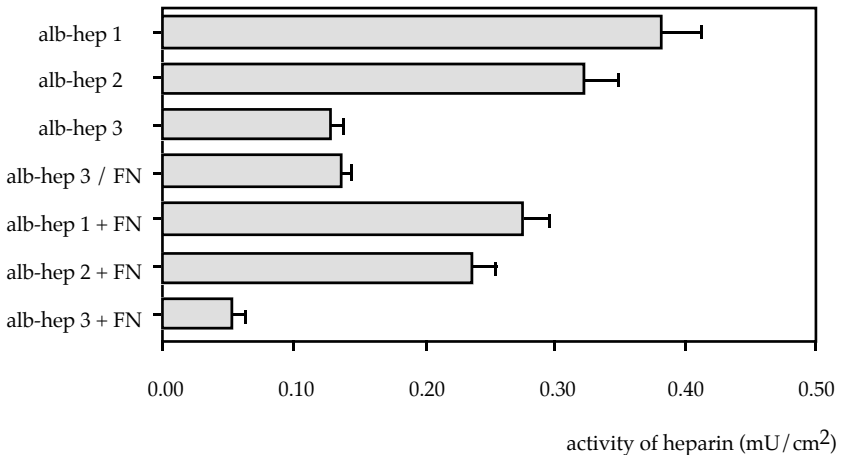

Figure 2. *Anticoagulant activity determined by a thrombin inhibition assay. Activity found for control surfaces PS, PS-CO_2, PS-CO_2 albumin and PS-CO_2 FN was -0.007 ± 0.004, -0.005 ± 0.004, 0.001 ± 0.002, and 0.005 ± 0.004 mU/cm^2 respectively. Heparin content of alb-hep 1, 2 and 3 was 16.5, 11.6, 12.3% (w/w) respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3). The heterogeneity of the alb-hep conjugates may differ. (n=5, ± sd)*

Contact activation

The cleavage rate of the chromogenic substrate in plasma solutions upon contact with alb-hep conjugate monolayers was 2 - 6 times higher compared to the blank (non-activated plasma, 1.6 ± 0.4 mOD/min). The enzymatic activity of plasma solutions which had been in contact with alb-hep conjugate or albumin were similar, and significantly lower compared to fibronectin coating and the reference surface glass (table 2). In contrast, the contact activation observed for uncoated PS-CO_2 was significantly higher than for glass.

The enzymatic activity of contact-activated proteins which were adsorbed to the surfaces was determined after the diluted plasma had been removed (table 2). The surfaces with immobi-

lized alb-hep 1 and alb-hep 2 showed less cleavage of the chromogenic substrate by contact activation proteases than the other surfaces. Surface-bound enzymatic activity of all surfaces was significantly higher than the blank (47 ± 6 mOD).

Surface	Proteolytic activity in solution (mOD/min)	Proteolytic activity on the surface (mOD)
glass	59 ± 1	297 ± 15
PS-CO_2	110 ± 3	306 ± 5
PS-CO_2 alb	5.4 ± 0.9	270 ± 19
PS-CO_2 alb-hep 1	7.0 ± 0.7	129 ± 15
PS-CO_2 alb-hep 2	3.9 ± 1.2	106 ± 8
PS-CO_2 alb-hep 3	4.1 ± 0.2	190 ± 11
PS-CO_2 FN	32 ± 1	346 ± 5

Table 2. *Contact activation measured as the activity in solution and of the surface after incubation of the surfaces with 1 ml of 25% human plasma in PBS for 15 min. at 37℃. The rate of cleavage of the chromogenic substrate of non-activated plasma (blank activity in solution) was 1.6 ± 0.4 mOD/min. The absorbance of chromogenic substrate incubated in PS that had not been in contact with plasma (blank activity on surface) was 47 ± 6 mOD. Heparin content of alb-hep 1, 2 and 3 was 16.5, 11.6, 12.3% (w/w) respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3). The heterogeneity of the alb-hep conjugates may differ. (n=5, ± sd)*

Stationary platelet deposition in the absence and presence of seeded HUVECs

The number of platelets deposited from CM/alb onto various coatings present on PS-CO_2 and onto uncoated PS-CO_2 did not significantly differ, except for the coatings with alb-hep 1 and alb-hep 2 ($P<0.01$, figure 3).

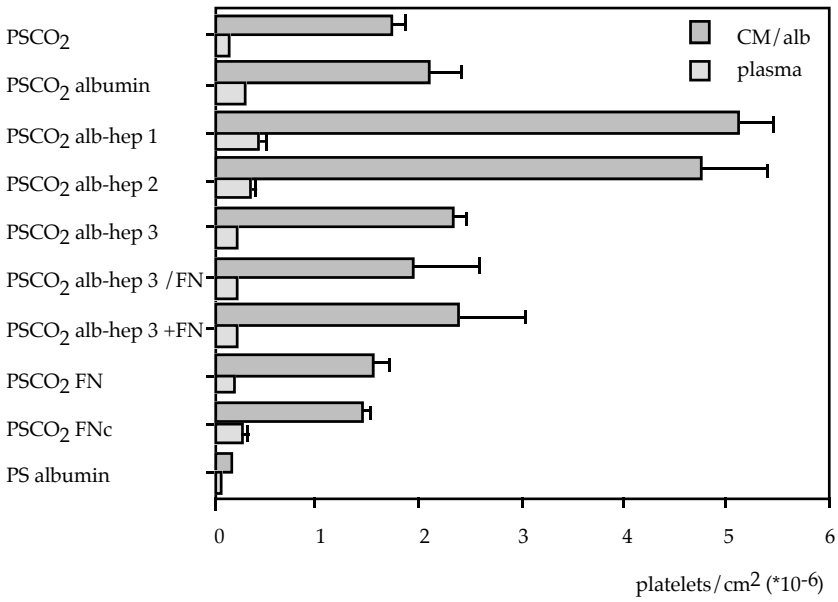

Figure 3. *Deposition of ^{111}In-labeled platelets on PS-CO_2, covered with various coatings, and on albumin-coated PS after incubation for 1 h at 37°C with 1.0x10^5 platelets/µl resuspended in different media: ■ CM/alb (cell culture medium with 3.5% albumin), □ human plasma. Heparin content of alb-hep 1, 2 and 3 was 16.5, 11.6, 12.3% (w/w) respectively. The albumin was of porcine origin in alb-hep 1 and of human origin in alb-hep 2 and alb-hep 3). The heterogeneity of the alb-hep conjugates may differ. (n=3, ± sd)*

On the latter coatings, a two-fold increase of the number of adhered platelets compared to uncoated PS-CO_2 was observed. Significantly less platelets adhered to albumin-coated PS compared to coated and uncoated PS-CO_2. The same trend was observed using plasma instead of CM/alb, but the absolute numbers found for CM/alb were much higher than the numbers found for plasma (figure 3).

Deposition of platelets from CM/alb was determined on surfaces seeded with HUVECs, using different cell seeding densities (figure 4). The number of adhered platelets was significantly reduced on all surfaces where ECs were present. Surfaces seeded with higher cell densities, however, showed a slightly higher number of adhered platelets than surfaces seeded with a low cell density. The presence of fibronectin on alb-hep conjugate-coated surfaces seeded with ECs, did not influence the number of adhered platelets.

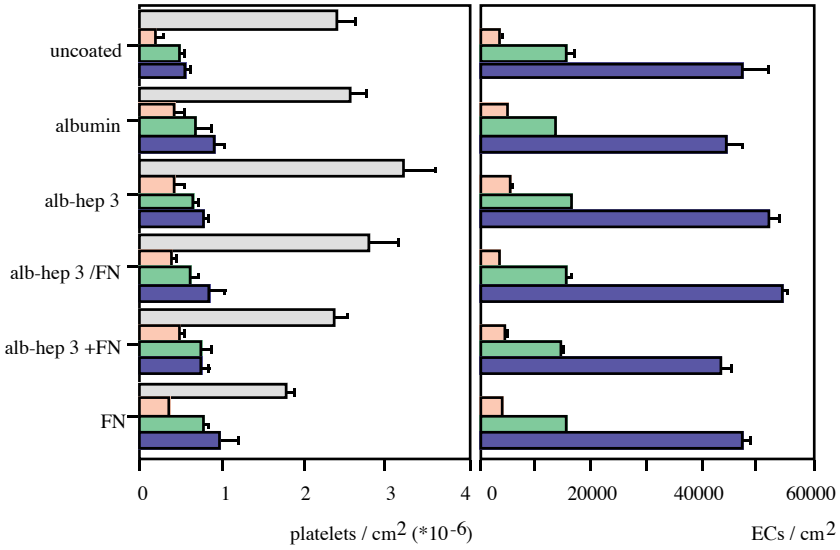

Figure 4. *Deposition of ^{111}In-labeled platelets on PS-CO_2, covered with various coatings, after incubation for 1 h at 37°C with 1.0x10^5 platelets/µl resuspended in CM/alb (cell culture medium with 3.5% albumin): surfaces without HUVECs, surfaces with HUVECs seeded at a density of 10.000/cm^2, 40.000/cm^2, or 100.000/cm^2 4 h prior to the addition of platelets. Heparin content of alb-hep 3 was 12.3% (w/w), whereas the albumin was of human origin. (n=3, ± sd)*

Morphology of deposited platelets

Samples used for scanning electron microscopy (SEM) were washed three times for 1 min instead of three times for 10 min as used for determining the number of adhered platelets. In a separate experiment it was shown that both the number of platelets determined at the surface and the morphology of adhered platelets had not changed significantly by this modification of the washing procedure ($P < 0.05$, data not shown).

SEM examination of the surfaces demonstrated that deposited platelets were distributed homogeneously on non-seeded surfaces. On HUVEC-seeded surfaces, adhered platelets were almost exclusively localized near endothelial cell edges; hardly any platelets were present on uncovered areas between HUVECs.

When the morphology of platelets adherent to the surfaces was studied with SEM, it appeared that platelets adhering to alb-hep conjugate- or albumin-coated PS-CO_2 demonstrated various stages of spreading. Some platelets were globular with several pseudopodia, whereas others were almost completely spread (figures 5A, 5B, 5C and 5D). SEM observations were in agreement with the differences between numbers of adhered platelets on the conjugates determined by using radiolabeled platelets. Platelets adhering to albumin-coated PS showed less pseudopodia compared to albumin-coated PS-CO_2 and none of the platelets on albumin-coated PS were completely spread (figures 5D and 5E). Extensive spreading of platelets was observed in the case of platelets adhering to fibronectin-coated surfaces as well as to uncoated PS-CO_2 (figures 5F, 5G and 5H). Addition of fibronectin to the alb-hep coating (alb-hep 3 +FN and alb-hep 3 /FN) appeared to have slightly increased the percentage of fully spread platelets compared to alb-hep 3 coated PS-CO_2 (figures 5C, 5I and 5J). Platelets were distributed more or less regularly on all surfaces; platelet aggregates were not observed.

A B

C D

Figures 5A-5D. *Representative SEM micrographs of platelets deposited on PS-CO_2, with various coatings, after incubation for 1 h at 37°C with 1.0x10^5 platelets/μl suspended in CM/alb. A: PS-CO_2 alb-hep 1, B: PS-CO_2 alb-hep 2, C: PS-CO_2 alb-hep 3, D: PS-CO_2 albumin.*

E F

G H

Figures 5E-5H. *Representative SEM micrographs of platelets deposited on PS-CO_2, with various coatings, after incubation for 1 h at 37℃ with 1.0x10^5 platelets/μl suspended in CM/alb. E: PS albumin, F: PS-CO_2 FN, G:PS-CO_2 FNc, H: PS-CO_2.*

I J

Figures 5I-5J. *Representative SEM micrographs of platelets deposited on PS-CO_2, with various coatings, after incubation for 1 h at 37°C with 1.0x10^5 platelets/µl suspended in CM/alb. I: PS-CO_2 alb-hep 3 +FN, J: PS-CO_2 alb-hep 3 /FN.*

The remarkable reduction in the numbers of adhered platelets by seeding of HUVECs onto the various substrates illustrated in figure 6 was also observed with SEM. Moreover, platelets were observed almost exclusively at the borders of ECs and not on uncovered areas between the cells. Similar results were found when seeded cells were allowed to adhere for 4 or 48 h (figures 6B and 6C). Figure 6D shows discoid platelets attached to the border of the endothelial cells with bridging pseudopod-like structures. No spread platelets were observed on surfaces seeded with HUVECs.

A B

C D

Figure 6. *Representative SEM micrographs of platelets deposited onto endothelial cell-seeded coatings present on PS-CO_2, after incubation for 1 h at 37℃ with 1.0x10^5 platelets/μl suspended in CM/alb. A: PS-CO_2 alb-hep 3, B: PS-CO_2 alb-hep 3 ECs cultured for 48 h, C and D: PS-CO_2 alb-hep 3 ECs cultured for 4 h. Cells were seeded at a density of 10.000/cm^2.*

Discussion

Albumin-heparin conjugate, covalently immobilized to PS-CO_2, displayed anticoagulant activity as demonstrated by the significant prolongation of the recalcification time of plasma and the inactivation of added thrombin compared to uncoated PS-CO_2 (figures 1 and 2). The conjugate with the highest heparin content (16.5% (w/w) heparin, alb-hep 1) showed the largest increase of recalcification time and the highest activity in the thrombin inhibition assay. While the heparin contents of alb-hep 2 and alb-hep 3 (11.6 respectively 12.3 % (w/w) heparin) were not significantly different, the heparin activity determined by the thrombin inhibition assay was significantly lower on surfaces coated with alb-hep 3 than on surfaces coated with alb-hep 2. This indicates that the heparin content of the alb-hep conjugate is not necessarily a measure of the anticoagulant activity displayed by a surface coated with an alb-hep conjugate. The differences between anticoagulant activity of conjugates with similar heparin content are probably caused by differences in the number of covalent bonds per heparin molecule, or the position of covalent bonds on the heparin-chain (modification of the ATIII binding site). The relatively low anticoagulant activity of alb-hep 3 may therefore result from multiple bonds between the heparin and albumin molecules.

Addition of fibronectin on top of monolayers of alb-hep conjugate (alb-hep +FN) significantly reduced heparin activity of all three conjugates, whereas addition of fibronectin during the coating with alb-hep conjugate (alb-hep /FN) did not affect the activity of the immobilized heparin. In the latter case, however, the quantity of fibronectin immobilized on the surface was significantly lower (table 1). Moreover, in this case a large part of the fibronectin was probably bound directly to the surface instead of via heparin,[17] and therefore hardly influenced the activity of the surface-immobilized heparin. Apparantly, the effect of FN on the activity of heparin, which is presumed to be caused by blocking of the ATIII binding sequence of heparin, is

concentration-dependant. This assumption is affirmed by Byun *et al.*,[38] who showed that addition of fibronectin to surface-immobilized heparin reduced the binding of ATIII to the surface in a concentration-dependent manner.

Carbon dioxide-treated PS strongly activated the contact system, even more than the positive control surface glass. This high activation may be caused by the negatively charged groups present at both surfaces (table 2). Coating of PS-CO_2 with either albumin or alb-hep conjugate resulted in a ten- to thirtyfold lower rate of cleavage of the chromogenic substrate. Alb-hep conjugate-coated surfaces activate the contact system only minimally. Although surface-immobilized heparin is known to inhibit activation of FXII and kallikrein via binding of ATIII,[20,38] the low levels found indicate that small quantities of FXIIa and/or kallikrein were present. Furthermore, contact activation caused by alb-hep conjugate coatings, both in solution and at the surface, was significantly lower than the contact activation by fibronectin coatings. This may be a result of the low surface concentration of FN (0.23 ± 0.02 μg/cm^2, ref. 16), which indicates the presence of uncovered areas. The PS-CO_2-surface at these uncovered areas may account for the high contact activation, and possibly not the FN itself. A surface-coating with alb-hep conjugate instead of fibronectin therefore may improve the blood compatibility of surfaces used for seeding of endothelial cells.

Significantly more platelets were deposited under stationary conditions onto the substrates when the platelets were suspended in cell culture medium with 3.5% (w/v) albumin (CM/alb) instead of citrated plasma (figure 3). CM/alb was used in this experiment because it was found that the spreading of ECs decreased in citrated plasma, probably as a result of the absence of Ca^{2+}. In CM/alb this phenomena was not observed. Comparable trends in the numbers of adhered platelets onto the various substrates were found using both suspension media, but

when the platelets were deposited from CM/alb the absolute numbers of adhered platelets were higher. Increased deposition of platelets suspended in CM/alb instead of plasma, may be caused by the presence of higher concentrations of calcium and magnesium in CM/alb, which stimulate platelet function.[39,40] Immobilization of fibronectin to the surface resulted in extensive spreading of platelets (figure 5). This effect of fibronectin was also reported by Beumer *et al.*,[41] who demonstrated that platelets bind to fibronectin via the so-called RGD-sequence (Arg-Gly-Asp) present in fibronectin molecules.

A striking difference was observed between the numbers of platelets adhering under stationary conditions to the various conjugates used in this study. On alb-hep 3 the same number of platelets was deposited as on albumin, and the state of spreading of the platelets was also similar. Significantly more platelets were deposited onto surfaces coated with alb-hep 1 or 2. Furthermore, the average degree of spreading was further progressed on these latter surfaces. The number of platelets on the alb-hep conjugates showed a positive correlation with the heparin activity as determined by the recalcification and the thrombin-inhibition assay. Heparin fractions with high molecular weight are known to be more reactive towards platelets than fractions with lower molecular weight.[42-44] Besides the molecular weight, the degree of sulfation plays an important role, and it has been postulated that heparin-platelet binding is principally mediated by an ionic interaction between the negatively charged sulfate groups on heparin and positively charged regions exposed on the platelet surface.[44-46] As suggested earlier, heparin in alb-hep 3 may be coupled to albumin by multiple bonds and therefore smaller chains of heparin may be exposed from the albumin-moiety of the conjugate. This results not only in less heparin-activity with respect to inactivation of blood coagulation, but also to reduced interaction with platelets. However, the cause of these effects may be different. Whereas the reduced ability of alb-hep 3 to bind ATIII is probably caused by a decrease in the number of available ATIII binding pentasac-

charides,[19] the reduced binding of platelets to this conjugate may be the result of decreased charge interactions between heterogeneous cationic sites on the platelet membrane and electronegative charge present in the heparin chain.[45,46]

Increased *in vitro* platelet binding to heparinized biomaterial surfaces, as described in this study, as well as decreased binding have been described in literature.[43-45,48-50] These controversies may be due to the different effects of heparin on platelet activation, induced by various agonists.[47] Heparin inhibits the activation of platelets by thrombin in the presence of plasma (or probably ATIII), whereas it is uneffective, or sometimes stimulates activation, in the absence of plasma.[47] Therefore, it may not be the direct interaction of heparin with platelets that matters, but the interaction of heparin with proteins, such as ATIII and fibronectin, present at the surface and in the medium.

Seeding of substrates with endothelial cells resulted in a significantly reduced number of adhered platelets on all surfaces under stationary conditions (figure 4). This is in agreement with data reported previously.[11,14,28-32] On seeded substrates however, the number of platelets deposited on the substrates under stationary conditions slightly increased with endothelial cell density. It was observed with SEM that platelets deposited onto endothelialized surfaces, were primarily found at the edges of ECs, and not on uncovered spots between the cells (figure 6). Possibly, ECs exhibit two opposite functions: on the one hand they prevent adhesion of platelets to the surface, probably caused by PGI_2 and/or nitric oxide secreted by the cells,[33] whereas on the other hand an interaction of the ECs with platelets occurs. This binding of platelets to the border of ECs may result from the binding of platelets to the platelet-endothelial cell adhesion molecule (PECAM-1), a homotypic cell adhesion molecule present on endothelial cells, mainly at cell junctions (see figure 2 of chapter 8). The PECAM-1 molecules are also present on platelets, T cells and monocytes.[51]

Apparently, this interaction is not inhibited by PGI_2 and/or nitric oxide released by the ECs. Binding of platelets to HUVECs via PECAM-1 may result from the stationary conditions used in this experiment. The adhesion of platelets to fibronectin-coated polyethylene under flow conditions was previously shown to decrease with increasing endothelial cell density.[52] This difference may be explained by differences in contact time between the platelets and the surface. Under flow conditions this time is much smaller, and perturbation occurs, which may prevent the interaction between platelets and PECAM-1 present on the ECs. Similarly, alb-hep conjugate coating of polyurethane vascular catheters was previously shown to significantly reduce the number of platelets deposited onto the surface under flow conditions.[53] Therefore, less interaction between platelets and alb-hep conjugate coatings seeded with ECs is expected under flow.

Conclusions

Albumin-heparin conjugate immobilized on PS-CO_2 retains sufficient anticoagulant activity to increase the recalcification time of blood plasma and to significantly decrease contact activation by the surface. However, more platelets adhere to immobilized albumin-heparin conjugate than to immobilized albumin. Addition of a small amount of fibronectin during coating of PS-CO_2 with albumin-heparin conjugate, which was previously shown to improve endothelial cell adherence, decreases anticoagulant activity only slightly, while platelet interaction with the surface does not increase. Seeding of the substrates with endothelial cells significantly reduces the number of adhering platelets, which are primarily found on endothelial cell edges.

Therefore, coating of vascular graft surfaces with albumin-heparin conjugate supplied with a small amount of fibronectin results in a promising substrate for low density seeding of endothelial cells.

Acknowledgement

We wish to thank the staff of the department of obstetrics of the Medisch Spectrum Twente (MST, Enschede, The Netherlands) for supplying the human umbilical cords, Bloedbank Ijssellanden (Enschede, The Netherlands) for their gift of buffy coats, and Ing. M.A. Smithers for carrying out the SEM experiments.

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Chapter 7 Endothelialization of Crosslinked Albumin-Heparin Gels

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Abstract

Crosslinked gels of albumin and heparin (Alb/hep-gel) and crosslinked gels of albumin to which heparin is immobilized (Alb-gel with hep), potential sealants of prosthetic vascular grafts as well as substrates for endothelial cell seeding, were studied with regard to *in vitro* stability, binding of basic fibroblast growth factor (bFGF) and cellular interactions. Between 5 and 10 percent of the heparin present in these gels, was released during storage for 48 h in sodium dodecyl sulfate (SDS)-solution at 37°C. During storage for 14 days in serum-containing cell culture medium at 37°C, heparin release was 21 - 25 percent. Release of albumin did not occur.

Human umbilical vein endothelial cells (HUVECs) rapidly adhered and subsequently spread on these crosslinked gels as well as to crosslinked albumin-gels without heparin (Alb-gel), but proliferation was only observed if heparin was present in the gel. HUVECs cultured on either substrate secreted both von Willebrand factor (vWF) and prostacyclin (PGI_2). The expression of both endothelial cell products could be stimulated by Ca^{2+}-ionophore A23187. Per cell, HUVECs secreted more vWF and PGI_2 when grown on crosslinked gels than when cultured on fibronectin-coated tissue culture polystyrene (TCPS). On the other hand, as cell numbers were much higher on fibronectin-coated TCPS, the release of vWF and PGI_2 per cm^2 was higher on this surface compared to the gels, notably after 10 days of culture. An inverse correlation between secreted amounts per

cell and the cell density on various gels and on fibronectin-coated TCPS was observed for both endothelial cell products.

Binding of ^{125}I-bFGF to Alb-gel with hep was 35 percent higher than to crosslinked Alb-gel. Growth of HUVECs in cell culture medium supplemented with 5 percent serum occurred on Alb-gel with hep loaded with bFGF, but not on Alb-gel loaded with bFGF.

The number of platelets deposited under stationary conditions onto heparinized gels was more than twice the number found on Alb-gels without heparin. Seeding of endothelial cells (ECs) on Alb-gel with hep, significantly reduced the number of platelets adhering to this surface. On Alb-gel the decrease of platelet numbers caused by endothelial cell seeding was less pronounced. Moreover, no spreading of platelets was observed on substrates seeded with ECs. Most platelets were deposited near the edges of HUVECs, and sparingly on areas between ECs.

It can be concluded that crosslinked gels of albumin to which heparin is immobilized, are candidate sealants for prosthetic vascular grafts and suitable substrates for endothelial cell seeding.

Introduction

In contrast to small-diameter grafts which occlude rapidly,[1-3] large-diameter vascular grafts of Dacron or expanded Teflon in combination with anticoagulant treatment and administration of platelet inhibitors have been successfully applied in vascular surgery for decades.[4-6] Unlike in animal species, spontaneous outgrowth of endothelium on the inner side of implanted vascular prostheses does not occur in humans.[6,7] Since ECs play a key regulatory role in haemostasis,[8,9] seeding of vascular grafts with ECs is a promising method to avoid occlusion of small-diameter prostheses.[10-13] However, as ECs for seeding need to be autologous and the harvesting efficiency from vascular tissue is

generally poor, the number of ECs available is usually too small to allow immediate formation of a confluent cell layer.

Previously we have shown that a coating consisting of albumin and the anticoagulant heparin is a suitable substrate for ECs.[14] HUVECs adhere to and subsequently proliferate on surface-immobilized albumin-heparin conjugate.Cell viability was illustrated by the observation that ECs grown on this substrate released von Willebrand factor and prostacyclin.[15] Basic fibroblast growth factor (bFGF) was shown to stimulate proliferation of ECs seeded at low density on these surfaces.[16] Binding to heparin protects bFGF from inactivation by acid, heat, cold, proteolysis and fosforylation by protein kinase C,[17-19] and facilitates binding to high-affinity cell-membrane receptors.[20] bFGF, that was released from surface-immobilized heparin,mediated the rapid formation of a confluent layer of ECs seeded at low density.[16]

For the impregnation of vascular grafts, mixtures of anticoagulants and collagen, albumin, or proteins with an RGD-sequence, have been the subject of many investigations.[21] Guidoin *et al*.[22,23] for instance, who compared different sealants for vascular prostheses, observed that the healing process of albuminated grafts was comparable to preclotted controls. However, contrary to collagen-impregnated grafts, tissue ingrowth of albuminated Dacron prostheses in pigs was limited.[24]

Crosslinking of sealants (such as albumin) to prevent early hydrolytic and enzymatic degradation *in vivo* may be achieved using carbodiimide which, in contrast to glutaraldehyde, has been shown to be non-toxic.[25]

In the present study, bFGF was bound to carbodiimide crosslinked gels of albumin as well as albumin and heparin. Subsequently, EC proliferation on these materials was studied. Furthermore, interaction of human platelets with the gels was investigated.

Materials and Methods

Materials

^{14}C-formaldehyde (55 mCi/mmol), Na^{125}I (17.4 mCi/μg), sodium ^{3}H-borohydride (NaB^{3}H$_4$, 359 mCi/mmol) and Biotrak 6-keto-prostaglandin $F_{1\alpha}$ EIA were obtained from Amersham (Amersham, UK). $NaCNBH_3$ was from Janssen Chimica (Beerse, Belgium). Iodobeads were obtained from Pierce (Rockford, Illinois, USA). Optiphase HiSafe3 (scintillation cocktail) was from Wallac (Milton Keynes, UK). PD10 columns were bought from Pharmacia (Uppsala, Sweden). Phosphate-buffered saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene (PS) and tissue culture polystyrene (TCPS) petridishes (35 mm diameter, 9.6 cm^2) were from Greiner GmbH (Frickenhausen, Germany). TCPS 12-well plates and TCPS-flasks (162 cm^2) were obtained from Costar (Cambridge, UK). Carbon dioxide (CO_2) was from Hoekloos (Schiedam, The Netherlands). Pig mucosal heparin sodium (195 U/mg) was from Bufa Chemie (Castricum, The Netherlands). Bovine serum albumin (BSA, fraction V) was obtained from Sigma Chemical Company (St. Louis, Missouri, USA). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, trypsin-EDTA, and basic fibroblast growth factor (bFGF) were purchased from Life Technologies (Paisley, UK). Quantikine human bFGF ELISA was from R&D systems (Minneapolis, USA). Ca^{2+}-Ionophore A23187 was obtained from Boehringer Mannheim (Mannheim, Germany). vWF ELISA was obtained from Gradipore (North Ryde, NSW, Australia). Human serum was pooled from 12 healthy volunteers. A plasma fraction containing human fibronectin (± 0.50 mg/ml) was a gift from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/Hohenbrunn, Germany).

Gas plasma treatment and coating of polystyrene petridishes

Polystyrene petridishes were cleaned and CO_2 gas plasma treated as previously described.[14] Treated petridishes (PS-CO_2) were stored at a temperature of -20°C.

^{14}C-labeling of albumin and ^{3}H-labeling of heparin

Bovine albumin was labeled by reductive methylation using ^{14}C-formaldehyde in the presence of $NaCNBH_3$, as previously described.[14] The specific activity of the labeled albumin was 81.9 dpm/μg. Before use, the radiolabeled albumin was mixed with unlabeled albumin, using a ratio of 1:20 (w/w).

Heparin was labeled with tritium according to the procedure described by Hatton *et al*..[26] The specific activity of ^{3}H-heparin was 7,6 dpm/ng. Before use the radiolabeled heparin was mixed with unlabeled heparin, using a ratio of 1:20 (w/w).

Crosslinking of albumin and albumin/heparin

To albumin (200 mg) or to a mixture of albumin (180 mg) and heparin (20 mg), both in 2-morpholinoethane sulfonic acid buffer (MES, 900 μl, 0.05 M, pH 5.3), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC, 16.6 mg) and N-hydroxysuccinimide (NHS, 2.0 mg) in MES buffer (100 μl) were added, after which the solution was pipetted into a PS-CO_2 petridish. After 4 hours of crosslinking at room temperature, the crosslinked gels (coded as Alb-gel respectively Alb/hep-gel) were washed 11 times with buffered solution (3 ml, twice with PBS, twice with PBS/1M NaCl/pH3.5, twice with PBS/1M NaCl/pH10.5 and twice with PBS, 15 min per step, and 3 times with PBS at 37°C, once for 20 h and twice for 1 h). For quantification purposes, gels were prepared with radiolabeled albumin or heparin as well.

Heparin immobilization on Alb-gel and Alb/hep-gel

To heparin in MES buffer (300 μl, 100 mg/ml, 0.05 M MES, pH 5.3), EDC and NHS were added in MES buffer (1200 μl), using a molar ratio of EDC : NHS : heparin of 5 : 2 : 1. After 10 minutes pre-incubation, the solution was pipetted onto an air-dried crosslinked gel (gels coded as Alb-gel with hep or Alb/hep-gel with hep). Alternatively, 300 μl of 200 mg/ml heparin was used in one experiment (coded as Alb-gel with more hep). After 4 hours crosslinking at room temperature, surfaces were washed 11 times with buffered solutions (3 ml) as described above. For quantification purposes, gels were prepared with radiolabeled heparin as well.

Stability of crosslinked Alb-gel and Alb/hep-gel with or without hep

Crosslinked gels were placed overnight at room temperature in PBS (4 ml), containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Next, the stability of the gels was determined by incubation at 37°C with sodium dodecyl sulfate (SDS) solution in PBS (2 ml, 3% (w/v)), urea in PBS (2 ml, 6M), or complete cell culture medium (CM) supplemented with 20% (v/v) pooled human serum (CMS). CM consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml). At selected times, the supernatant was replaced. ^{3}H- and ^{14}C-radioactivity in the supernatant were measured after addition of HiSafe3 (13 ml, scintillation cocktail) using a β-counter (Wallac Winspectral 1414, Wallac, Turku, Finland). At the same time, the amount of free label, emanating from ^{14}C-albumin and ^{3}H-heparin during incubation in CMS at 37°C, was determined separately by means of gel filtration using PD10 columns.

Isolation and proliferation of endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffe *et al.*,[27] as previously described.[14] Before passage and seeding, cells were detached from the fibronectin-coated TCPS surface by incubation with trypsin-EDTA solution in PBS (0.05% respectively 0.02% (w/v)).

Before cell seeding, surfaces were placed overnight at room temperature in PBS (3 ml), containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Third passage HUVECs were seeded at a density of 10,000 cells per square centimeter and cultured at 37 °C in 5% CO_2/95% air, that was saturated with water vapour.

Culture media used were CM supplemented with 5 or 20% (v/v) pooled human serum (CMS 5 respectively CMS 20) and CMS 5, containing heparin (5 U/ml) and bFGF (0.3 ng/ml, CMS5/HF). At this bFGF-concentration, cell numbers after three days of culturing ECs seeded at a density of 10.000/cm^2 on fibronectin-coated TCPS were identical when either CMS5/HF or CMS 20 was used as medium. Medium (0.20 ml/cm^2) was replaced every other day. At selected times, cells were trypsinized and cell numbers were determined using a Bürker counting device.

Secretion of Von Willebrand factor and prostacyclin

The gels used in this experiment were washed 11 times at room temperature with buffered solution (3 ml, three times with PBS, three times with PBS/1M NaCl/pH3.5, three times with PBS/1M NaCl/pH10.5 and twice with PBS, 45 min per step) after crosslinking. HUVECs were cultured on the various gels as described above.

After washing once with CM that was supplemented with fatty acid-free BSA (1% (w/v), CM/BSA), the petridishes with HUVECs were incubated for 30 min at 37°C with 1 ml of either CM/BSA (basal excretion) or CM/BSA containing Ca^{2+}-iono-

phore A23187 (10 μM, activated excretion). Subsequently, the medium was removed and centrifuged for 7 min at 400 g. Aliquots of the supernatant were stored at -20°C until assayed. Simultaneously, the cells were trypsinized and cell numbers were determined.

Von Willebrand factor concentrations in the media were determined with the aid of a sandwich ELISA. Prostacyclin concentrations were determined using a competitive EIA for 6-keto-prostaglandin $F_{1\omega}$, the product of spontaneous hydrolysis of PGI_2 (half-life of 3 min). 6-keto-prostaglandin $F_{1\alpha}$ is generally accepted as a measure of PGI_2 formation.[28] The ELISA and EIA were performed according to the instructions of the manufacturers.

^{125}I-labeling of basic fibroblast growth factor

Human recombinant basic fibroblast growth factor (bFGF) was labeled with ^{125}Iodine using Iodobeads according to the method of Markwell.[29] Na^{125}I (500 μCi, 15.5 mCi/μg) was added to 3 Iodobeads that had been rinsed with PBS. After 5 min of incubation at room temperature, bFGF in PBS (1 ml, 50 μg/ml) was added. After 12 min, free iodine was removed from the reaction mixture using a series of 3 PD10 columns with PBS/BSA (20 mg BSA/ml) as eluent. Labeled bFGF was stored at -20°C until use. The bFGF concentration in the solution after labeling was determined using an ELISA, according to the instructions supplied by the manufacturer. The specific activity of ^{125}I-bFGF was 24.6 dpm/pg, with 5.7 % unbound ^{125}I (PD10-column). ^{125}I-activity was determined using a γ-counter (Compugamma 1282, LKB, Stockholm, Sweden).

^{125}I-bFGF-loading of surfaces and effect of surface-immobilized bFGF on cell proliferation

Alb-gels with hep were incubated for 7.5 to 120 min with ^{125}I-bFGF (1 ml, 183 ng/ml) at room temperature. Subsequently, the gels were washed with PBS (5 ml, 3 times 15 min) and with NaCl-solution in PBS (2 ml, 2 M final NaCl-concentration, 30 min). The radioactivity in the solutions as well as that remaining on the surface were determined. The quantity of bound bFGF was determined from the summation of radioactivity present in the 2M NaCl-solutions and remaining at the surface, and corrected for free ^{125}I-label, assuming that free label did not bind to the surface. ^{125}I-bFGF-loading of Alb-gels and Alb/hep-gels after 120 min of incubation was determined as well. Proliferation of HUVECs on bFGF-loaded Alb-gel, Alb-gel with hep, and Alb/hep-gel was investigated. The gels were loaded during 120 min with unlabeled bFGF (1 ml, 200 ng/ml) and subsequently washed with PBS (5 ml, 3 times 15 min).

Preparation of platelet suspension and ^{111}In-labeling of platelets

Fresh buffy coats from healthy volunteers were diluted 2.5 times with Krebs-Ringer buffer (107 mM NaCl, 4 mM KCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4, pH 7.3), containing trisodiumcitrate (19 mM) and D (+)-glucose (27 mM), after which platelet-rich plasma (PRP) was obtained by centrifugation (1700 g, 4 min, 20°C). Subsequently, PRP was diluted 1:1 with Krebs-Ringer buffer (pH 5.0) containing 19 mM trisodiumcitrate and 27 mM D (+)-glucose (final pH 6.1). Platelets were isolated and labeled with ^{111}Indium oxine as previously described.[30] Finally, platelets were resuspended at a concentration of $1.0 \times 10^5/\mu l$ in ABO-compatible plasma or in CM with 3.5 mg/ml albumin (CM/alb).

Deposition of ^{111}In-labeled platelets in absence and presence of HUVECs

Stationary deposition of ^{111}In-labeled human platelets from human plasma or CM/alb was determined using a series of substrates. Furthermore, stationary deposition of ^{111}In-labeled human platelets from CM/alb was studied, using a series of substrates that were seeded with HUVECs 4 h before the experiment.

Test surfaces (crosslinked gels, 9.6 cm^2) were incubated for 4 h with PBS (3 ml), after which a platelet suspension of $1.0x10^5$ platelets/μl in plasma or CM/alb (1 ml) was added to the well. After 1 h at 37°C the platelet suspension was removed and each sample was washed three times with 3 ml PBS at 10 min intervals. The samples were incubated overnight with NaOH-solution (2 ml, 1M). Radioactivity in the supernatant was determined using a γ-counter. The number of deposited platelets was determined by dividing the radioactivity found in the NaOH solution by the specific activity per platelet.

Scanning electron microscopy (SEM)

For some of the samples, immediately after the final wash step of the stationary platelet deposition experiment, adherent platelets and HUVECs were fixed with glutaraldehyde in PBS (2 ml, 2% v/v) at 4°C. Samples were kept in this medium at 4°C for at least 52 days, which is 20 times the half-life of ^{111}In. After washing three times with water, samples were dehydrated by subsequent incubation with a graded series of ethanol solutions in water (5 ml; 25, 50, 75, 90, 98 % (v/v) of ethanol; 2 times 5 min per step) and finally in ethanol (5 ml; 4 times 5 min). After drying *in vacuo*, the samples were sputtered with gold and examined with scanning electron microscopy using 7 kV accelerating voltage (S-800 field emission SEM, Hitachi, Japan).

Results

Preparation and stability of crosslinked gels

Gelation of a solution of albumin or a mixture of albumin and heparin occurred within 5 minutes after addition of EDC and NHS. The crosslinked gels were slightly opaque and had a smooth surface. With SEM, occasionally holes of a few micrometers in diameter at the surface were observed, which may be caused by air bubbles trapped during crosslinking.

The quantity of heparin incorporated in the gels was significantly higher when a mixture of albumin and heparin was used (Alb/hep-gel) than when heparin was immobilized in a separate step after crosslinking of albumin gels (Alb-gel with immobilized heparin). Applying twice as much EDC, NHS and heparin to Alb-gels (Alb-gel with more hep) resulted in only a modest increase in the amount of immobilized heparin. Immobilization of heparin to Alb/hep-gels hardly increased the heparin content of these gels.

Substrate	^{14}C-Albumin mg	^{3}H-Heparin mg
Alb-gel	200.4 ± 4.1	-
Alb-gel with hep	nd	7.2 ± 0.1
Alb-gel with more hep	nd	9.9 ± 0.5
Alb/hep-gel	178.8 ± 3.9	19.3 ± 0.4
Alb/hep-gel with hep	nd	20.9 ± 0.7

Table 1. *Composition of crosslinked gels. (n=3 ± sd) Per gel 30 mg heparin was used, except for Alb-gel with more hep where 60 mg was used.* nd*: the quantity of albumin present in a gel was assumed not to be influenced by immobilization of heparin in a second step.*

The gels proved to be unstable with regard to the heparin incorporated in the gels (table 2). After 14 days in CMS 20, between 20 and 25 percent of the immobilized heparin had been released from the gels. The quantity of heparin that was released in CMS 20 at 37°C rapidly decreased in time, and after 7 days hardly any heparin was released (data not shown).
Incubation of the crosslinked gels for 48 h in SDS-solution resulted in about 10 percent release of heparin. In contrast, the gels were very stable during 48 h incubation in 6M urea at 37°C; 1,0 ± 0.1 % of the albumin was released from Alb-gel, while 1.9 ±0.2 % of the heparin coupled to Alb-gel with hep was released by this denaturating agent. (data not shown)

Substrates	Release of ^{14}C-Albumin (%)		Release of ^{3}H-Heparin (%)	
	3% SDS 48 h	CMS 20 14 days	3% SDS 48 h	CMS 20 14 days
Alb-gel	0.5 ± 0.1	1.8 ± 0.1	-	-
Alb-gel with hep	0.6 ± 0.1	1.8 ± 0.1	8.9 ± 1.4	21.4 ± 0.4
Alb-gel with more hep	nd	nd	nd	24.8 ± 0.6
Alb/hep-gel	1.6 ± 0.3	6.3 ± 0.4	4.5 ± 1.1	21.0 ± 0.2

Table 2. *Stability of crosslinked gels in 3% SDS solution (48 h) or CMS20 (14 days), at 37 °C. (n=3, ± sd) Per gel 30 mg heparin was used, except for Alb-gel with more hep where 60 mg was used. nd = not determined.*

Free label content of ^{14}C-albumin after thawing was 1.3 ± 0.3 %, which increased to 3.7 ± 1.2 % upon incubation at 37°C during 1 day, after which it did not increase further. Free label content of ^{3}H-heparin was 2.8 ± 0.4 %, which did not increase upon storage at 37°C for 14 days.

Adherence and proliferation of endothelial cells on crosslinked gels of albumin and heparin

HUVECs did adhere to and proliferate on fibronectin-coated TCPS as well as on Alb-gel with immobilized heparin and Alb/hep-gel (figure 1). A small number of cells initially adhered on Alb-gels without incorporated heparin, but proliferation was not observed.

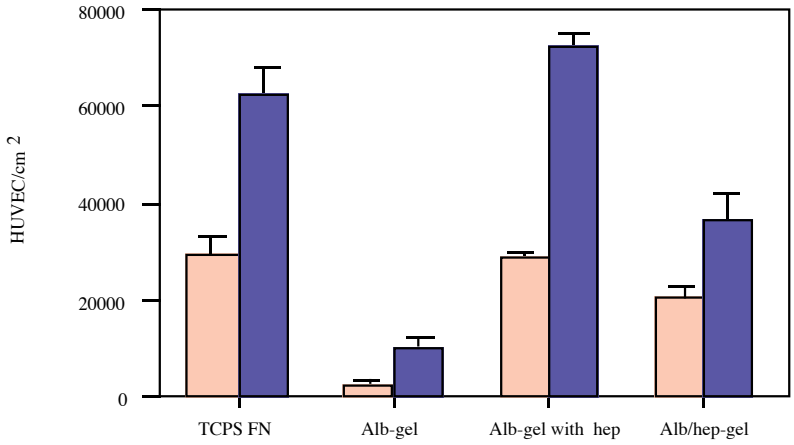

Figure 1. *Number of endothelial cells on fibronectin-coated TCPS and on crosslinked gels of albumin or albumin and heparin at different periods of culture using CMS 20 as medium: day 4, and day 10. (n=3, ± sd)*

Confluent cellular monolayers had formed on Alb-gel with hep by day 10, while a sub-confluent layer was present on Alb/hep-gels at that time. A confluent cell layer was observed at day 10 on fibronectin-coated TCPS as well. The cell layer on Alb-gel was not confluent. On all surfaces except Alb-gel, HUVECs showed a cobblestone structure typical for endothelial cells in culture (figure 2).

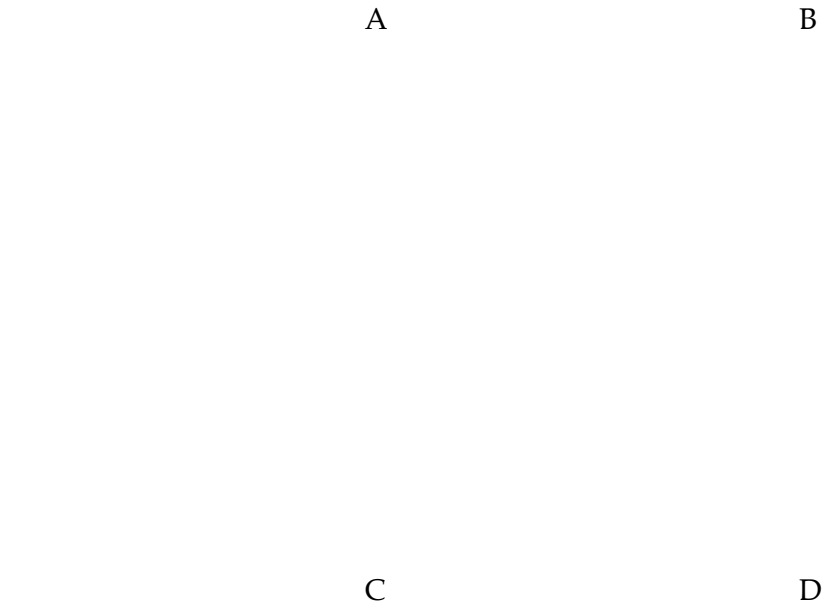

Figure 2. *Light-microscopic images of HUVECs grown for 10 days on (A) fibronectin-coated TCPS, (B) Alb-gel, (C) Alb-gel with immobilized heparin, and (D) Alb/hep-gel. (Original magnification: 128x)*

In contrast to all other experiments presented, the gels used in this experiment were rinsed less extensively (not in PBS at 37°C). The heparin release during the first week was approximately twice as high as reported in table 2 (data not shown). Release of heparin had a negative influence on endothelial cell proliferation, as can be seen by comparison of cell numbers presented in figure 1 and tables 3 and 4.

Secretion of von Willebrand factor and prostacyclin

Basal secretion of von Willebrand factor (vWF) per 10^6 ECs by cells grown on Alb-gel was relatively high, approximately a factor 10 higher compared to cells grown on fibronectin-coated TCPS (table 3). Basal secretion per 10^6 cells was 2 to 3 times higher on Alb-gel with hep and Alb/hep-gel compared to

	Day 3		
Substrate	ECs/cm^2	basal vWF	stimulated vWF
TCPS FN	22000 ± 1900	78 ± 7	99 ± 8
Alb-gel	1300 ± 300	860 ± 107	1012 ± 111
Alb-gel with hep	5300 ± 600	189 ± 32	285 ± 35
Alb/hep-gel	5700 ± 1400	149 ± 28	298 ± 37
	Day 10		
substrate	ECs/cm^2	basal vWF	stimulated vWF
TCPS FN	39200 ± 4200	89 ± 5	377 ± 14
Alb-gel	180 ± 160	8800 ± 847	10061 ± 883
Alb-gel with hep	4900 ± 700	280 ± 37	312 ± 38
Alb/hep-gel	5000 ± 1500	171 ± 29	408 ± 41

Table 3. *Basal and Ca^{2+}-ionophore A23187-stimulated secretion of vWF (ng/10^6 ECs) by HUVECs on fibronectin-coated TCPS, Alb-gel, Alb-gel with hep, and on Alb/hep-gel after 3 and 10 days of culture using CMS 20 as culture medium. (n=3 ± sd)*

fibronectin-coated TCPS. Basal vWF secretion per 10^6 cells at day 10 was slightly higher than at day 3 for all surfaces, with the exception of Alb-gel.

With all surfaces, stimulation with Ca^{2+}-ionophore A23187 increased the release of vWF by cells cultured for 3 and 10 days, with the exception of Alb-gel (table 3). The stimulatory effect of the ionophore on HUVECs cultured on fibronectin-coated TCPS was initially small. Stimulation of cells cultured on the Alb/hep- gel at least doubled the secretion of vWF, whereas for the Alb-gel with hep only a small increase in vWF secretion was observed upon stimulation.

Basal secretion of prostacyclin (PGI_2) at day 3 was relatively high for cells grown on Alb-gel, intermediate for heparin-containing gels and low for fibronectin-coated TCPS. Except for cells cultured on Alb-gel, unstimulated PGI_2 secretion per cell was higher at day 10 than at day 3 (table 4).

Stimulation by Ca^{2+}-ionophore A23187 significantly increased the release of PGI_2 from cells cultured on all surfaces, with the exception of Alb-gel at day 3 and Alb/hep-gel at both days (table 4). Stimulated release was highest for cells grown on albumin gel and lowest when cells were cultured on fibronectin-coated TCPS.

^{125}I-bFGF-loading of surfaces

The quantity of ^{125}I-bFGF bound to Alb-gel with hep increased with incubation time. At 120 min maximal binding was not yet reached (figure 3A). Significantly more bFGF was immobilized after 120 min on Alb-gel with hep than on Alb-gel, whereas the difference between Alb/hep-gel and Alb-gel was not significant (figure 3B).

	Day 3		
Substrate	ECs/cm^2	basal PGI_2	stimulated PGI_2
TCPS FN	22000 ± 1900	0.71 ± 0.13	15.4 ± 1.9
Alb-gel	1300 ± 300	114 ± 12	113 ± 12
Alb-gel with hep	5300 ± 600	22.9 ± 2.8	39.6 ± 5.2
Alb/hep-gel	5700 ± 1400	31.8 ± 4.1	36.7 ± 6.9

	Day 10		
substrate	ECs/cm^2	basal PGI_2	stimulated PGI_2
TCPS FN	39200 ± 4200	2.08 ± 0.21	18.4 ± 3.2
Alb-gel	180 ± 160	147 ± 12	639 ± 64
Alb-gel with hep	4900 ± 700	48.3 ± 6.6	75.8 ± 11.9
Alb/hep-gel	5000 ± 1500	102 ± 18	108 ± 20

Table 4. *Basal and Ca^{2+}-ionophore A23187-stimulated secretion of PGI_2 (ng/10^6 ECs) by HUVECs on fibronectin-coated TCPS, Alb-gel, Alb-gel with hep, and on Alb/hep-gel after 3 and 10 days of culture using CMS 20 as culture medium. (n=3 ± sd)*

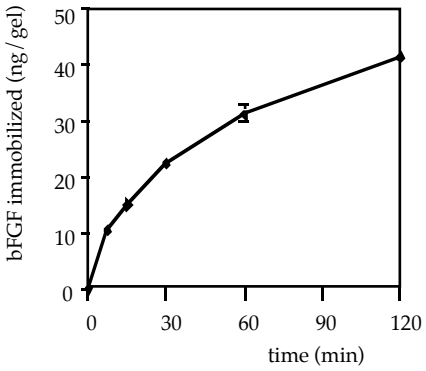

Figure 3A

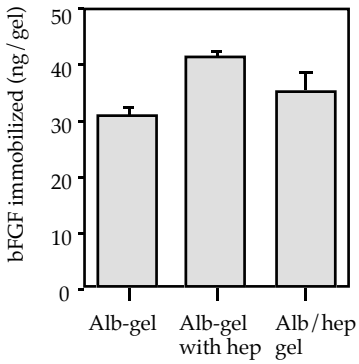

Figure 3B

Figure 3 *Quantity of ^{125}I-bFGF bound after varying incubation times at room temperature using 1 ml of 183 ng/ml bFGF per well (9.6 cm^2) on Alb-gel with hep (figure A), or after 120 min on Alb-gel, Alb-gel with hep and Alb/hep-gel (figure B). (n=3, ± sd)*

Immobilization of bFGF on heparin-containing gels resulted in rapid cell proliferation compared to similar gels with bFGF (0.3 ng/ml) and heparin (5U/ml) added to the culture medium (figure 4). Cell numbers increased on the bFGF-loaded heparin-containing gels between day 3 and day 10. Sub-confluent cell layers were formed on Alb/hep-gel, whereas on Alb-gel with hep confluent cell layers were observed. Some cells did adhere on Alb-gel, but they failed to proliferate, regardless whether bFGF was applied to the surface prior to seeding of the cells or added to the medium.

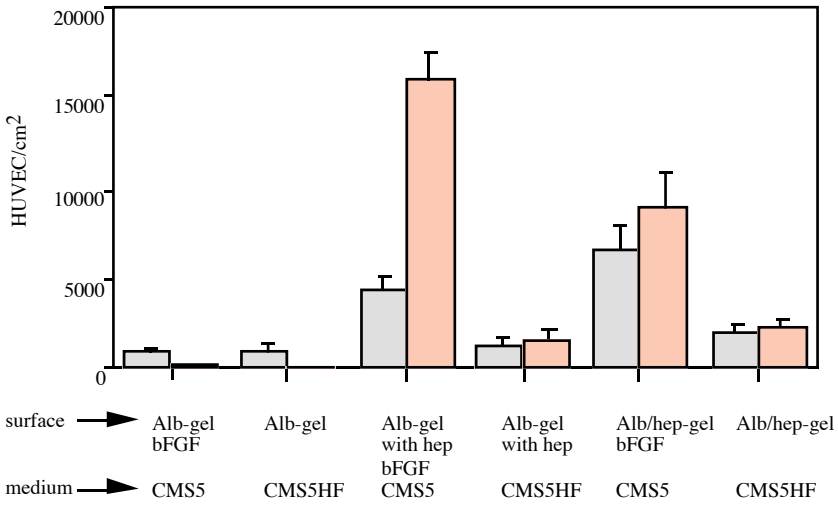

Figure 4 *Number of endothelial cells on Alb-gels, Alb-gels with hep, and Alb/hep-gels, either with or without loaded bFGF at 3 ( ) respectively 10 ( ) days of culture. Concentration of bFGF used during loading with bFGF was 200 ng/ml. Media used are presented in the graph (n=3, ±sd)*

Platelet deposition in absence or presence of HUVECs

The deposition of ^{111}In-labeled human blood platelets onto crosslinked gels of albumin or albumin and heparin under static conditions was studied by incubating the gels with washed platelets in complete medium, supplemented with 3.5 mg/ml albumin (CM/alb) or in human plasma (figure 5).

Using CM/alb, the number of platelets deposited onto the surfaces of Alb-gel with hep and Alb/hep-gel was similar, whereas the number of platelets found on Alb-gel was significantly lower. Similar differences between the substrates were found using plasma, although the absolute numbers found were a factor 10 - 30 lower compared to numbers found using CM/alb.

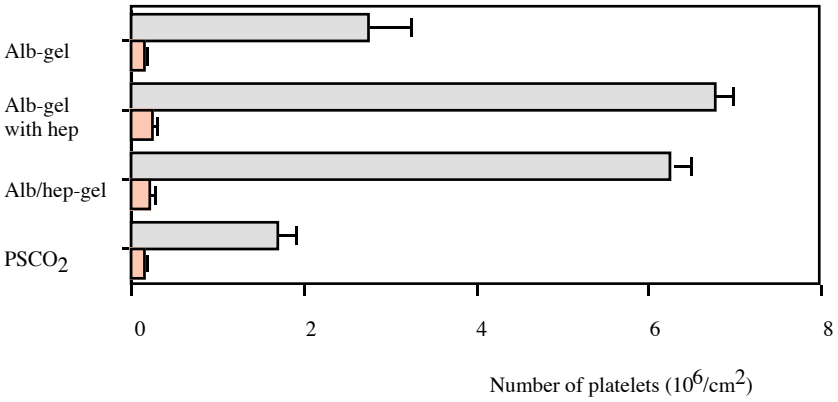

Figure 5. *Deposition of ^{111}In-labeled platelets onto PS-CO_2 and onto Alb-gels with or without immobilized heparin after incubation for 1 h at 37°C with 1.0x10^5 platelets/µl suspended in different media: CM/alb (cell culture medium with 0.35% albumin), human plasma. (n=3, ± sd)*

Deposition of platelets on gels seeded with 40.000 HUVECs/cm^2 was determined using CM/alb as suspension medium for platelets (figure 6). Cell numbers on the seeded surfaces at the time of incubation with platelets were 9300 ± 300, 12100 ± 500 and 15900 ± 1300 for Alb-gel, Alb-gel with

hep, and PS-CO_2 respectively. The number of platelets was significantly reduced by the ECs present at the surface, both on Alb-gels and on Alb-gel with hep. The reduction of the number of deposited platelets was higher when ECs were seeded on Alb-gel with hep.

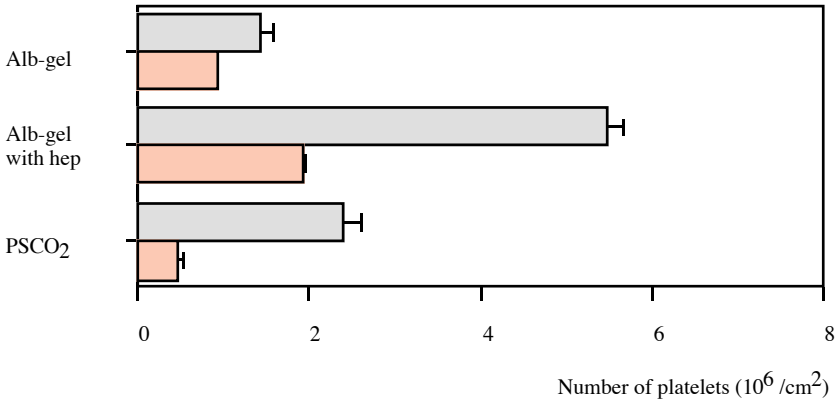

Figure 6. *Deposition of ^{111}In-labeled platelets onto PS-CO_2 and onto Alb-gels with or without immobilized heparin after incubation for 1 h at 37°C with 1.0x10^5 platelets/µl suspended CM/alb: ☐ surfaces without HUVECs, ☐ surfaces with HUVECs seeded at 40.000/cm^2 in CMS 20 4 h prior to the addition of platelets. (n=3, ± sd)*

Scanning electron microscopy

A relatively high percentage of the platelets adhered on Alb-gel was spread. The distribution of these platelets was irregular. The extent of pseudopod formation and spreading of platelets on Alb-gel with hep was less compared to pseudopod formation and spreading on Alb-gel. Moreover, the distribution of platelets was more regular when immobilized heparin was present.

As can be seen in figure 7C, the distribution of the platelets on Alb-gel seeded with HUVECs was very irregular, with only a few platelets adhering to the edges of ECs, and a large number of platelets clustering together on a spot where no ECs were

present. However, the close-up in the inset reveals that the extent of pseudopod formation and spreading of the platelets on this surface was minimal. Large surface areas contained no ECs and occasionally some platelets were observed that had not developed pseudopodia. On Alb-gel with hep seeded with HUVECs, platelets were found more often near the ECs and not on uncovered areas between cells (figure 7D). Most platelets had developed some pseudopodia, but no spread platelets were observed.

Discussion

The results of this study suggest that crosslinked gels of albumin and heparin provide a surface coating for artificial vascular grafts which allows endothelial cell adhesion and proliferation after seeding, while only minimal platelet deposition may occur.

Previously we have shown that HUVECs, seeded on a monolayer coating of a conjugate consisting of albumin and heparin which had been covalently immobilized on CO_2 plasma-modified polystyrene, adhered, proliferated and released von Willebrand factor and prostacyclin.[14,15] Surface-immobilized albumin-heparin conjugate inhibited thrombin and prolongated the recalcification time of plasma.[30] Significantly more platelets adhered to albumin-heparin conjugate-coated surfaces compared to albumin-coated surfaces. Seeding of ECs on surfaces coated with a monolayer of albumin-heparin conjugate, however, significantly reduced the number of adherent platelets.[30]

The amount of heparin coupled to Alb-gel and Alb/hep-gel in the present study, was much higher than would have been possible for a monolayer (table 1). Apparantly, the heparin diffused into the gel and reacted with the matrix. The homogeneity of the heparin throughout the matrix was not determined.

Figure 7. *Representative SEM micrographs of platelets deposited onto endothelial cell-seeded (C, D) and unseeded (A, B) crosslinked Alb-gel with(B, D) or without (A, C) immobilized heparin. Static incubation was carried out for 1 h at 37°C using 1.0x10^5 platelets/μl CM/alb.*

Crosslinked gels of albumin and of a mixture of albumin and heparin were stable in SDS for 48 h at 37°C with regard to the albumin, whereas some heparin was released under these conditions (table 2). Release of albumin from the crosslinked gels was not observed during storage for 2 weeks in CMS at 37°C. However, a substantial part of the immobilized heparin (20-25 %) was released from the gels during this period. To obtain gels which do not release heparin, more rigorous washing after crosslinking is essential to release loosely bound heparin from the gels. Improved washing can be done with solutions having high salt concentrations (> 1M),[31] by storage for several weeks in PBS at room temperature,[32] or for several days at 37°C.

Although HUVECs adhered to all crosslinked gels, proliferation was only observed in the case of crosslinked albumin gels containing heparin (figure 1). The release of heparin from the gels appeared not to hinder the formation of a confluent layer of ECs (figure 2) However, the ECs used in the vWF and PGI_2 release experiments were cultured on gels that were washed less rigorously. Significantly more heparin was released from these gels, and proliferation was inhibited. After about 10 days, cell numbers on these gels started to increase. Apparantly, the amount of heparin that is released from the gels should be limited, in order to allow proliferation of seeded endothelial cells.

HUVECs cultured on crosslinked gels of albumin with or without immobilized heparin, secreted both vWF, which provides a bridge between adhering platelets and the subendothelium, and PGI_2, a vasodilator and inhibitor of platelet function (tables 3 and 4). Increased release of vWF by cultured cells is indicative for activation or damage of ECs which may be due to the cytotoxicity of medium or substrate.[33] Stimulation of cultured cells with Ca^{2+}-ionophore A23187 has been found to increase the secretion of vWF.[31,24,35] Since release of vWF by HUVECs grown on albumin, was initially significantly higher

compared to release from cells cultured on heparin-containing Alb-gels, it is suggested that the latter gels are more suitable as a substrate for ECs than Alb-gels.

The secretion of PGI_2 by seeded ECs provides an important mechanism for preventing platelet deposition onto vascular grafts.[36] Basal production of PGI_2 per cell by ECs seeded on crosslinked gels was high in comparison with that by HUVECs grown on either albumin-heparin conjugate monolayers[15] or crosslinked collagen.[31] Stimulation by Ca^{2+}-ionophore A23187 reflects the functional capability of the prostacyclin biosynthetic enzymes cyclo-oxygenase and prostacyclin synthetase. As was also found by others,[31,34,35] Ca^{2+}-ionophore A23187 significantly increased the secretion of PGI_2, which indicates that the prostacyclin biosynthetic capacity of the cells was intact.

High basal secretion per cell of both vWF and PGI_2 on crosslinked gels, especially on Alb-gel without heparin, may be due to low cell numbers on these substrates. An inverse correlation between the release of vWF and PGI_2 and cell density has previously been reported. Notably with cell densities below 10.000 ECs/cm^2, increased secretion per cell was observed.[37] Therefore, the cell densities should be regarded when comparing the absolute quantities determined on the various surfaces.

Slightly more ^{125}I-bFGF bound to Alb-gel with hep than to Alb-gels without immobilized heparin (figure 3B). Only a limited part of the bFGF bound to the matrix during incubation, as was previously observed for bFGF binding to monolayers of albumin-heparin conjugate.[16]

HUVECs were capable of growing on Alb-gels with hep that were loaded with bFGF, but not on bFGF-loaded Alb-gels without hep (figure 4). Compared to data from literature, bFGF concentrations used in this study were very low.[38,39] The surface concentration of bFGF was estimated to vary between 3 and 5 ng/cm^2, compared to approximately 5 μg/cm^2 used by Yamamura *et al.*.[39] Still, these low amounts were capable of enhancing endothelial cell proliferation. The most prominent effect of

bFGF bound to Alb-gels with hep was the initially very rapid cell proliferation, which led to high-density confluent monolayers within three days. When bFGF was present in the culture medium (containing 5% serum) instead of bound to the surface, cell numbers remained low when crosslinked gels were used. In contrast, with monolayers of albumin-heparin conjugate, HUVECs were found to proliferate well in cell culture medium with 5% serum and bFGF.[16] Possibly, some of the bFGF diffuses into the crosslinked gels, resulting in a decreased availability of bFGF to the cells.

The difference between proliferation of HUVECs on Alb-gels with hep and monolayers of albumin-heparin, may also be caused by the underlying polymer substrate. When the monolayer coating is incomplete, binding of cell adhesive proteins such as fibronectin to the underlying polymeric surface instead of binding to heparin, may provide optimal adherence for the ECs. In the case of crosslinked gels which are about 1 mm thick, such binding is impossible.

These results, combined with the observation that proliferation of ECs on crosslinked Alb-gels only occurs if the gels contain heparin, support our previous postulation that (cellular) fibronectin and other heparin-binding adhesive proteins mediate the binding of ECs to surface-immobilized heparin.[14,15]

Significantly more platelets were deposited under stationary conditions onto the substrates when cell culture medium with 0.35% (w/v) albumin (CM/alb) instead of citrated plasma was used to suspend the platelets (figure 5). In both media significantly more platelets adhered to surfaces containing heparin than to surfaces without heparin, as was observed previously for platelet adhesion to albumin-heparin conjugate and albumin-coated PS-CO_2.[30] In that study, it was postulated that it may not be the direct interaction of heparin with platelets that

causes the increased platelet deposition, but the interaction of heparin with proteins, such as ATIII and fibronectin, present at the surface and in the medium.[30]

Using CM/alb as suspension medium for platelets, absolute numbers of deposited platelets were significantly higher and the differences between substrates more pronounced. Increased deposition of platelets is probably caused by the presence of higher concentrations of calcium and magnesium in CM/alb, which is essential for platelet adhesion.[40]

Seeding of ECs on Alb-gels did not affect the adherence of platelets to these surfaces (figure 6). In contrast, the number of adherent platelets on Alb-gels with hep was significantly reduced, which is in agreement with observations reported by others.[11-13,48-50] The limited effect on platelet deposition of endothelial cell seeding on Alb-gels is probably the result of the very low EC density on this surface, and the fact that the gel itself is relatively non-reactive towards platelets.

Platelets deposited onto endothelialized Alb-gel with hep, were primarily found near and on the edges of the ECs, and not on uncovered areas between ECs (figure 7), as was observed previously for platelets adhering to albumin-heparin conjugate-coated PS-CO_2 seeded with ECs.[30] As postulated,[30] binding of platelets to the border of ECs under stationary conditions may result from binding of the platelets to the platelet-endothelial cell adhesion molecule (PECAM-1), a homotypic cell adhesion molecule present on endothelial cells, mainly at cell junctions (see figure 2 of chapter 8). Under flow conditions, contact time between the platelets and the surface is much smaller, which may prevent the interaction between platelets and PECAM-1 present on the ECs. Adhesion of platelets to fibronectin-coated polyethylene under flow conditions was previously shown to decrease with increasing endothelial cell density.[53] Therefore, less interaction is expected under flow conditions between platelets and albumin-heparin matrices seeded with ECs.

Unpublished results of Klomp *et al.*[54] demonstrate that albumin in an aqueous solution (pH 8.2) may be immobilized on CO_2 gas plasma-modified polyethylene terephthalate (Dacron). A layer of albumin immobilized on modified Dacron fibers could form the basis of a stable, crosslinked albumin-heparin matrix that fills the pores of a Dacron graft. This matrix can subsequently be loaded with bFGF. After seeding of autologous ECs on this substrate, rapid endothelial cell proliferation may lead to the formation of a confluent endothelial cell layer, either *in vitro* prior to implantation or *in vivo* after implantation. Moreover, it has recently been demonstrated that heparin, immobilized to a crosslinked gel of albumin and gelatin,
inhibits the growth of smooth-muscle cells, and thereby intimal hyperplasia.[32]

Conclusions

HUVECs adhere to and spread on crosslinked gels of albumin with or without heparin, but proliferate only if heparin is present at the surface. HUVECs secrete both vWF and PGI_2, which can be stimulated by Ca^{2+}-ionophore A23187. Unlike on bFGF-loaded albumin gels, HUVECs are capable of growing in a medium containing 5% serum on bFGF-loaded albumin-gels with immobilized heparin.

Significantly more platelets deposit onto heparinized gels compared to gels without heparin, but seeding with ECs significantly reduces the number of adherent platelets on heparin-containing gels.

It can be concluded that crosslinked gels of albumin and heparin are candidate sealants for prosthetic vascular grafts and suitable substrates for endothelial cell seeding.

Acknowledgement

We wish to thank the staff of the department of obstetrics of the Medisch Spectrum Twente (MST, Enschede, The Netherlands) for supplying the human umbilical cords, and Ing. M.A. Smithers for carrying out the SEM experiments.

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Chapter 8 Isolation and Characterization of Human Adipose Microvascular Endothelial Cells and subsequent Culture on Surface-Immobilized Albumin-Heparin Conjugate

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Abstract

Endothelial cell seeding of vascular prostheses may improve graft patency. One way to collect sufficient endothelial cells (ECs) to completely cover the graft surface, is the use of adipose tissue as a source of ECs. The aim of the present study was to develop a method for the isolation of human ECs from adipose microvessels without contamination by fibroblasts, and to evaluate the proliferation of these cells on surface-immobilized albumin-heparin (alb-hep) conjugate. The contaminating cells were eliminated from the primary and subsequent cultures by repeated positive selection of ECs, using *Ulex Europaeus Agglutinin-I* lectin (*UEA*-I) coated para-magnetic beads.

The endothelial origin of the cells was demonstrated by positive immunofluorescent staining of the isolated cells for von Willebrand factor (vWF), endoglin and platelet endothelial cell adhesion molecule-1 (PECAM-1). Microvascular ECs obtained from human adipose tissue (HAMVECs) grew to confluency on CO_2 gas plasma-treated polystyrene (PS-CO_2), coated with a monolayer of alb-hep conjugate. Although release of vWF by HAMVECs under stationary conditions was comparable to

release by human umbilical vein ECs (HUVECs), stationary release of prostacyclin (PGI_2) by HAMVECs was significantly lower.

Since isolated HAMVECs need to be purified several times, immediate seeding onto vascular grafts after isolation is not feasible, which restricts the use of these cells to non-emergency vascular replacements.

It is concluded that adipose tissue is a candidate source to obtain ECs for seeding of vascular prostheses, provided that these cells release sufficient PGI_2 under flow conditions.

Introduction

Synthetic vascular grafts of Dacron or expanded Teflon with a diameter exceeding 5 mm, placed in high-flow and low-resistance locations such as the aortoiliac position, are successfully used in vascular surgery.[1-3] However, small-diameter grafts inserted in low-flow blood vessels fail rapidly due to occlusion caused by thrombosis and fibrotic hyperplasia.[4-6] Although anticoagulant treatment and administration of platelet inhibitors improves the patency of larger diameter vascular grafts,[7] a similar effect has not been reported for small-diameter grafts.

In contrast to a number of animal species, spontaneous outgrowth of endothelium on vascular prostheses implanted in humans does generally not occur.[2] Since ECs perform a key regulatory role in haemostasis,[8,9] seeding of these cells on the luminal surface of the vascular graft is a promising method to avoid occlusion of small-diameter prostheses.[10-13]

There are several requirements to be fulfilled in order to prepare endothelial cell seeded prostheses. ECs used for seeding need to be autologous and, depending on the size of the vascular graft, relatively numerous ECs are required to allow the formation of a confluent monolayer. Furthermore, the material used for vascular grafting must be able to support both

adhesion and proliferation of ECs. Also, because the layer of seeded ECs will in most cases be incomplete immediately after seeding, the prosthetic material itself should display antithrombogenic properties.

In previous studies[14,15] it was shown that coating of polyurethane catheters and poly(tetrafluoroethylene) sheets with conjugates of albumin and heparin decreases the *in vitro* deposition, aggregation and activation of platelets.[14,15] Alb-hep conjugate is also effective in reducing complement activation.[15] Moreover, it was shown that HUVECs can adhere to and proliferate on CO_2 gas plasma-treated polystyrene (PS-CO_2) coated with alb-hep conjugate.[16,17] This coating displays antithrombin activity and reduces contact activation by the surface.[18] Although platelet deposition increases by immobilization of alb-hep conjugate on PS-CO_2, seeding of ECs on this coating significantly reduces the number of adherent platelets.[18]

The present study describes the isolation of ECs from microvessels present in human adipose tissue. The major problem with regard to the isolation of human microvascular endothelium is the presence and rapid overgrowth of contaminating cells, such as fibroblasts.[19,20] Although various techniques have been developed to eliminate these contaminating cells, most of them are time consuming and / or do not give a reproducible yield of early passage ECs.[20] In this study, we have purified the HAMVEC populations according to a modification of the methods of Van Hinsbergh *et al.*[19] and Jackson *et al.*,[20] using *Ulex Europaeus Agglutinin* (*UEA-I*) coated Dynabeads. The cells were characterized using immunofluorescent staining. The capability for adhesion and proliferation of HAMVECs was tested, using PS-CO_2 coated with albumin or alb-hep conjugate, as well as crosslinked gels of albumin and of albumin and heparin. Release of von Willebrand factor and prostacyclin by HAMVECs was also investigated.

Materials and Methods

Materials

Alb-hep conjugate (15.2% (w/w) heparin) was obtained from Holland Biomaterials Group (Enschede, The Netherlands). Phosphate-buffered saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene (PS) petridishes (35 mm diameter) were from Greiner GmbH (Frickenhausen, Germany). TCPS flasks and TCPS 12-well plates were obtained from Costar (Cambridge, UK). Carbon dioxide (CO_2, purity > 99.995%) was from Hoekloos (Schiedam, The Netherlands). Pig mucosal heparin sodium (195 U/mg) was obtained from Bufa Chemie (Castricum, The Netherlands). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, trypsin-EDTA, 8-Chamber slides, and basic fibroblast growth factor (bFGF) were purchased from Life Technologies (Paisley, UK). Quantikine human bFGF ELISA was from R&D systems (Minneapolis, USA). Bovine serum albumin (BSA), L-fucose, Triton X-100, Dabco (1,4-diazobicyclo [2,2,2] octane) and *Ulex Europaeus Agglutinin-I* lectin (*UEA-I*) were from Sigma Chemical Company (St. Louis, Missouri, USA). Collagenase/dispase from *vibrio alginolyticus/bacillus polymyxa* (>0.1 U/mg / >0.8 U/mg), collagenase P from *clostridium histolyticum* (1.67 U/mg, clostripain: 12.7 U/mg, neutral proteases: 138.2 E/mg, trypsin-like proteases: 0.58 U/mg), and Ca^{2+}-ionophore A23187 were obtained from Boehringer Mannheim (Mannheim, Germany). Tosyl activated Dynabeads M450 were from Dynal A.S. (Oslo, Norway). vWF ELISA was obtained from Gradipore (North Ryde, NSW, Australia). Biotrak 6-keto-prostaglandin $F_{1\alpha}$ EIA was from Amersham (Amersham, UK). A plasma fraction containing human fibronectin (FN), and the monoclonal antibodies (MoAbs) HEC-19, HEC-65, Rag-35, Rag-38 and GαM-FITC were obtained from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Mono-

clonal antibodies (MoAbs) CK-8, CK-18 were purchased form ITK Diagnostics (Uithoorn, The Netherlands). Mesothelial cells (second passage) were a gift from Dr. A. Pronk (Diaconessenhuis, Utrecht, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/Hohenbrunn, Germany).

Isolation of HUVECs and culture of HUVECs and mesothelial cells

Human umbilical vein ECs (HUVECs) were isolated according to the method of Jaffe *et al.*,[21] modified by Van Wachem *et al.*.[22]

HUVECs and mesothelial cells (MCs) derived from human omentum were cultured for three passages in fibronectin-coated TCPS flasks at 37°C in 95% air/5% CO_2 saturated with water vapour, using complete cell culture medium (CM) containing 20% (v/v) human serum (CMS20). CM consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml).
Before passage or seeding, cells were detached from the surface by incubation with trypsin-EDTA solution in PBS (0.05% and 0.02% (w/v)), centrifuged and resuspended in CMS20.

Isolation and culture of HAMVECs

HAMVECs were isolated from fat tissue according to a modification of the methods of Van Hinsbergh *et al.*[19] and Jackson *et al.*.[20] Adipose tissue was collected through a surgical procedure and kept in calcium- and magnesium-free Hank's balanced salt solution (pH 7.3, osmolality ± 290 mOsm) with 5% (w/v) fatty acid free BSA ($HBSS^{--}$ / BSA) until further use. After determining the dry weight, the tissue was minced with scalpels. For fat tissue obtained by liposuction this mincing procedure was omitted. To the slurry 2 mg collagenase/dispase and 2 mg collagenase P in $HBSS^{--}$ / BSA per gram tissue were added, and the volume was adjusted with $HBSS^{--}$ / BSA to 2 ml per gram

tissue. The tissue was incubated with these enzymes at 37°C for about 1.5 hours while gently shaken. The resulting digest was centrifuged at room temperature for 10 min at 200 g. When small quantities of adipose tissue were used, $HBSS^{--}$ / BSA was added to facilitate separation. Using a large bore needle, which was immersed in ethanol before use to avoid adsorption of fat and oil, the microvessel pellet with some fluid was separated from the fat and oil layers, filtered through a sterile 94 μm filter, and centrifuged at room temperature for 10 min at 200 g. The pellet was carefully resuspended in 0.5 ml CMS20 to which heparin (5U/ml) and basic fibroblast growth factor (bFGF, 0.3 ng/ml) were added (CMS20/HF). After cooling the cell suspension to 4°C, ECs were selected from the mixture using *UEA-I*-coated Dynabeads (see below). Next, the cells were cultured in CMS20/HF on fibronectin-coated TCPS flasks.

Before passage or seeding, cells were detached from the surface by incubation with trypsin-EDTA solution in PBS (0.05% and 0.02% (w/v)), centrifuged and resuspended in CMS20/HF.

The selection procedure with *UEA-I*-coated Dynabeads was repeated during most passages until the cell culture was free of contaminating cells like fibroblasts. In the other cases, the purity of the cultures was improved by using short incubation times when detaching cells with trypsin/EDTA during passage.

Coating Dynabeads with UEA-I lectin

Ulex Europaeus Agglutinin-I lectin (0.2 mg/ml) was dissolved in sodium borate buffer (0.5 M, pH 9.5), divided in 0.5 ml aliquots and kept at -20°C until further use. Before usage, the solution was thawed and resuspended.

After washing of 500 μl homogenized tosyl-activated Dynabeads (4×10^8 beads/ml) twice with sodium borate buffer (7 ml, 0.5 M, pH 9.5), using a magnetic particle concentrator, beads were resuspended in borate buffer (500 μl, 0.5 M,

pH 9.5) and added to *UEA-I* lectin solution (500 µl). After 24 h incubation at room temperature, during which the suspension was gently turned over, coated beads were washed three times for 10 min with BSA in PBS (7 ml, 1 mg/ml) at room temperature. Alternatively, the incubation was at 37°C followed by washing once for 4 h at 37°C and once for 5 min at 4°C. Beads were separated from the supernatant using a magnetic particle concentrator. After resuspension of the beads in BSA in PBS (0.5 ml, 1 mg/ml), the beads were kept at 4°C in 50 µl aliquots (2×10^7 beads). Before use, binding capacity was tested using HUVECs (4 beads per cell).

Endothelial cell selection procedure using UEA-I-coated Dynabeads and fucose[20]

A heterogeneous cell suspension in CMS20/HF (7 ml, 4°C), containing HAMVECs, fibroblasts, and possibly other cell types, was incubated with freshly washed *UEA-I*-coated Dynabeads on a roller-bank for 20 min at 4°C, after which the cell suspension was placed for 1 min in a magnetic particle concentrator. After careful removal of the supernatant, the beads were gently mixed for 5 min with CMS20/HF (7 ml, 4°C). Next, the bead-suspension was placed again in the magnetic particle concentrator. This washing procedure was carried out four times. The supernatants, containing cells which did not bind to *UEA*-I-coated beads, were pooled and centrifuged at room temperature for 10 min at 200 g. The pellet was resuspended in 7 ml CMS20/HF, after which the cells were plated in fibronectin-coated TCPS-flasks (25 cm^2) and cultured as described above.

In order to detach cells from the *UEA-I*-coated Dynabeads, 7 ml 100 mM fucose in CMS20 was added, and after 5 min gentle shaking at room temperature, the bead-suspension was placed in the magnetic particle concentrator for 1 min and subsequently the supernatant was removed. This procedure was repeated three times. After pooling of the supernatants, the cells that were removed from the beads by fucose were plated and cultured as described above. The beads with remaining

cells were resuspended in 7 ml of CMS20/HF, plated and cultured as well. After 2 h adhesion, cell cultures were washed three times with PBS (37°C) after which fresh CMS20/HF was added.

Characterization of cells by means of immunofluorescence

Cells, seeded on 8-chamber slides at a density of $10^5/cm^2$ and cultured overnight, were washed twice with 1% BSA in PBS, fixed by incubation for 15 min with either para-formaldehyde in PBS (400 µl, 3.7% (w/v)) or methanol (400 µl, -20°C), and washed two times with PBS. When para-formaldehyde was used, cells were permeabilized for 5 min with 400 µl 1% Triton X-100 in PBS, followed by washing twice with PBS.

Fixed cells were washed twice with 1% BSA in PBS, incubated for 1 h at 37°C with the first monoclonal antibody (mouse-anti-human, table 1), followed by washing twice with 1% BSA in PBS, and incubation for 1 h at 37°C with FITC-labeled second antibody (goat-anti-mouse). After washing twice with 1% BSA in PBS and twice with PBS, the incubation chambers were removed, leaving flat slides with stained cells. The slides were dried and Dabco (2 drops, anti-bleach) was added. Thereafter, glass coverslips were mounted on the slides and sealed with nail-varnish.

MoAb	directed against	specific for	references
Rag-35	Von Willebrand factor	ECs	23
Rag-38	Von Willebrand factor	ECs	23, 24, 25
HEC-19	endoglin	ECs	25, 26, 27
HEC-65	PECAM1/CD31	ECs	25, 26, 28
CK-8	cytokeratin	MCs	19, 29, 30
CK-18	cytokeratin	MCs	19, 29, 30

Table 1. *Antibodies used for immunofluorescence assay. Specificity of monoclonal antibodies according to quoted references.*

Gas plasma treatment of PS and immobilization of albumin and alb-hep conjugate

After cleaning, polystyrene petridishes were CO_2 gas plasma-treated as previously described,[16] and stored at -20°C before usage. Alb-hep conjugate and albumin were immobilized on PS-CO_2 petridishes (9.6 cm^2) as previously described.[16]

Preparation of crosslinked gels of albumin and of albumin/heparin

To albumin (200 mg) or to a mixture of albumin (180 mg) and heparin (20 mg), both in 2-morpholinoethane sulfonic acid buffer (MES, 900 μl, 0.05 M, pH 5.3), N-(3-dimethylamino-propyl)-N'-ethylcarbodiimide (EDC, 16.6 mg) and N-hydroxy-succinimide (NHS, 2.0 mg) in MES buffer (100 μl) were added, after which the solution was pipetted into a PS-CO_2 petridish. After 4 hours of crosslinking at room temperature, the crosslinked gels (coded as alb-gel respectively alb/hep-gel) were washed 11 times with buffered solutions (3 ml, twice with PBS, twice with PBS/1M NaCl/pH3.5, twice with PBS/1M NaCl/pH10.5 and twice with PBS, 15 min per step, and 3 times with PBS at 37°C, once for 20 h and twice for 1 h).

To immobilize heparin to a crosslinked alb-gel, heparin in MES buffer (300 μl, 100 mg heparin/ml, 0.05 M MES, pH 5.3) was mixed with EDC and NHS in MES buffer (1200 μl), using a molar ratio of EDC : NHS : heparin of 5 : 2 : 1. After10 minutes pre-incubation, the solution was pipetted onto an alb-gel. After 4 hours crosslinking at room temperature, surfaces (coded as alb-gel with hep) were washed 11 times with buffered solutions (3 ml) as described above.

Proliferation of HAMVECs on various substrates

Proliferation of HAMVECs was investigated, using petridishes of PS-CO_2, coated with albumin or alb-hep conjugate, fibronectin-coated TCPS, as well as alb-gel, alb/hep-gel and alb-gel with hep.

Before cell seeding, surfaces were placed overnight at room temperature in PBS, containing penicillin (100 U/ml) and streptomycin (100 μg/ml). HAMVECs were seeded at a density of 10,000 cells/cm^2 and cultured at 37 °C in 95% air/ 5% CO_2 saturated with water vapour. Culture medium CMS20/HF was replaced every other day. After 3 and 10 days, cells were trypsinized and cell numbers were determined using a Bürker counting device.

Secretion of Von Willebrand factor and prostacyclin

After washing once with CM supplemented with fatty acid-free BSA (CM/BSA, 10 mg/ml), the petridishes with HAMVECs were incubated for 30 min at 37°C with 1 ml of either CM/BSA (basal secretion) or CM/BSA containing 10 μM Ca^{2+}-ionophore A23187 (activated secretion). Subsequently, the medium was removed, centrifuged for 7 min at 400 g, and aliquots of the supernatant were stored at -20°C until assayed. Adherent cells were trypsinized and cell numbers were determined.

Von Willebrand factor concentration in the medium was determined with the aid of a sandwich ELISA, while prostacyclin concentration was determined using a competitive EIA for 6-keto-prostaglandin $F_{1\omega}$, the product of spontaneous hydrolysis of PGI_2 (half-life of 3 min). 6-Keto-prostaglandin $F_{1\alpha}$ is generally accepted as a measure of PGI_2 formation.[31] The ELISA and EIA were used according to the instructions of the manufacturers.

Results

Endothelial cell selection procedure using UEA-I-coated Dynabeads and fucose

Positive selection of ECs from a mixture of ECs and fibroblasts was made using *UEA-I*-coated Dynabeads and a magnetic particle concentrator. Part of the ECs bound to the beads were detached by competitive binding with L-fucose.

It was found that the endothelial cell selection procedure was not completely specific. In cultures with a small percentage of contaminating cells such as fibroblasts, the procedure was more efficient than in cultures with a large percentage of contaminating cells.

The number of beads bound per endothelial cell varied between one to over fifty. The detachment procedure with L-fucose was not always successful. In some cases, many cells remained bound to the beads after incubation with L-fucose. In most of these cases, beads were phagocytosed by the cells and could therefore not be removed from the cell membrane by L-fucose. A similar behaviour was also observed using HUVECs. ECs with one to four beads at the outer membrane proliferated and after a few passages almost no cells with beads were left in the culture. ECs which had incorporated beads did not or hardly proliferate.

Endothelial cell selection procedure using short incubation with trypsin

A positive selection of ECs with regard to fibroblasts was obtained by shortening the incubation time with trypsin. Thereby, most ECs detached whereas almost all fibroblasts remained adherent. Cell cultures consisting of a mixture of ECs and fibroblasts were partly purified by this method.

Eighteen HAMVEC isolations were carried out. The average age of the donors was 50 ± 15 years (28- 67 years). Five were male, eleven were female and from two donors information about gender was not available. In most cases a piece of superficial subcutaneous abdominal fat was used, but tissue from the knee joint and tissue obtained by liposuction was also used successfully.

In 17 out of 18 isolations, a mixture of ECs and fibroblasts was obtained, while in one experiment no cells were found at all. Six cultures were purified by repeating the positive selection of ECs with *UEA-I*-coated magnetic beads, whereas the other eleven cultures were partially purified with these beads. Purified HAMVECs were cultured until fourth to fifteenth passage. Average time between passage of the cultures was 5.9 ± 1.3 days, estimated population doubling time was 1.8 days. Senescence, i.e. loss of proliferative activity and progressive enlargement of cell bodies, was observed in two of the HAMVEC cultures after eight respectively twelve passages.

HAMVECs present in contact-inhibited monolayers, exhibited the cobblestone morphology typical for cultured ECs,[32] as illustrated in figure 1. However, in some cultures less regular cell shapes were observed. Low numbers of isolated HAMVECs and failure to remove all contaminating cells in one purification step resulted in prolonged culture times necessary to establish confluent homogeneous HAMVEC cultures. Purification steps had to be repeated 3 to 6 times; besides removing a large percentage of the contaminating cells, each step resulted in a loss of ECs.

Because the number of isolations was limited, the effects of gender and age of the donor and origin of the tissue on the results of the isolation were not significant.

Figure 1. *Light-microscopy of different isolations of HAMVECs cultured on TCPS-FN. (Original magnification: 128x)*

Characterization of cultured cells by means of immunofluorescence

Four different homogeneous HAMVEC cultures were characterized by fluorescent staining and compared with ECs from umbilical veins (HUVECs) and mesothelial cells (MCs). Results are shown in table 2; characteristic fluorescent stainings are illustrated in figure 2.

MoAb	Antigen	HAMVECs	HUVECs	MCs
Rag-35	vWF	positive	positive	weak pos.
Rag-38	vWF	positive	positive	weak pos.
HEC19	endoglin	positive	positive	negative
HEC65	PECAM1	positive	positive	negative
CK-8	cytokeratin	negative	negative	positive
CK-18	cytokeratin	weak pos.	weak pos.	strong pos.

Table 2. *Immunofluorescent staining of HAMVECs, HUVECs, and MCs (4 HAMVECs, 6 HUVECs, and 1 MCs cultures were tested) with endothelial and mesothelial cell specific antigens. (n = 2)*

Granular staining of von Willebrand factor (vWF) was present in both HAMVECs and HUVECs, whereas MCs showed diffuse staining of vWF. PECAM-1 of ECs showed intense fluorescence, which increased markedly at sites of cell-to-cell contact. MCs demonstrated immunoreactivity to both epithelial cytokeratin 8 antigen and intermediate filament cytokeratin 18, whereas ECs only showed weak immunoreactivity towards the latter cytokeratin.

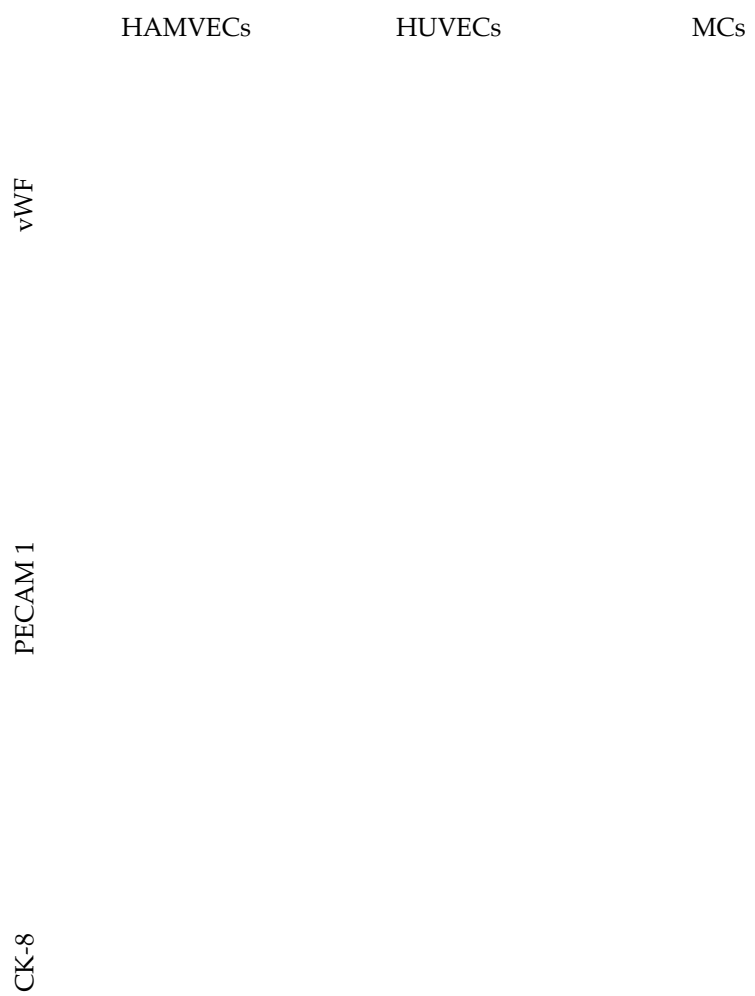

Figure 2. *Fluorescent staining of HAMVECs, HUVECs, and MCs, using MoAbs directed against vWF (Rag-35), PECAM1 / CD31 (HEC-65) and cytokeratin (CK-8). Staining was carried out with Goat-anti-Mouse-FITC. (Original magnification = 128x)*

HAMVECs adhered to and proliferated on fibronectin-coated TCPS (TCPS FN) and on albumin and alb-hep conjugate immobilized onto PS-CO_2 (figure 3). On these surfaces, confluent monolayers were present at day 10, but cell density on TCPS-FN was significantly higher compared to albumin and alb-hep conjugate coated surfaces.

HAMVECs did not adhere to crosslinked alb-gel, and hardly to alb-gel with hep. However, HAMVECs did adhere and proliferate to some extent on alb/hep gel.

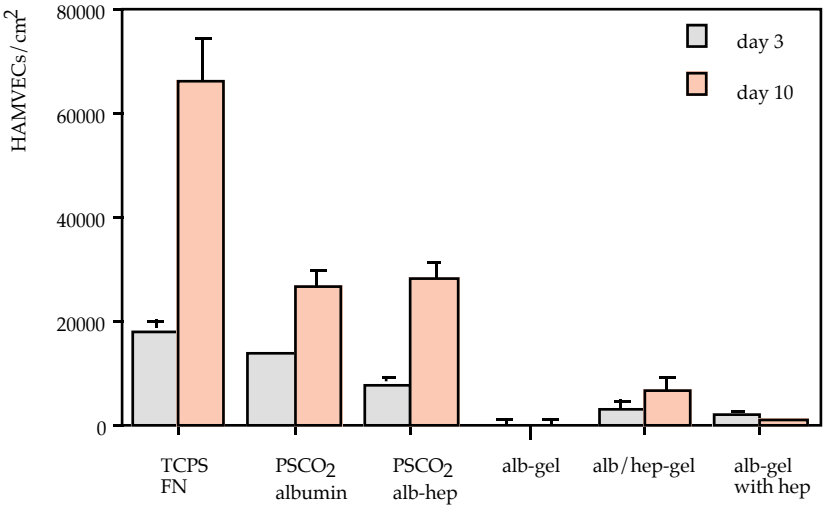

Figure 3. *Cell density of HAMVECs on fibronectin-coated TCPS, on albumin and alb-hep conjugate monolayers immobilized onto PS-CO_2, and on crosslinked alb-gel, alb/hep-gel, and alb-gel with hep, after 3 and 10 days of culture. (n=3, ± st.dev.)*

Cultured HAMVECs from one isolation were stimulated with Ca^{2+}-ionophore A23187 to release von Willebrand factor (vWF) from cellular storage sites and to enhance secretion of prostacyclin (PGI_2). Besides stimulated secretion, basal secretion of these metabolites was determined as well.
Compared to cells seeded on TCPS-FN, PS-CO_2-albumin and PS-CO_2-alb-hep conjugate, basal release of vWF from cells plated on crosslinked gels was high, notably when alb-gel with hep was used as substrate (figure 4). On fibronectin-coated TCPS, alb-hep conjugate coated PS-CO_2 and on alb/hep gel, basal secretion of vWF by cells was higher at day 3 than at day 10. Irrespective of the surface used, stimulation of the culture with Ca^{2+}-ionophore A23187 had no effect on the release of vWF at day 3. On day 10, however, an increase of vWF secretion was measured.

Secretion of PGI_2 by cells grown on TCPS-FN and albumin- and alb-hep conjugate-coated PS-CO_2, was very low (figure 5, inset); stimulation with Ca^{2+}-ionophore A23187 had no effect on the amount of PGI_2 secreted by the HAMVECs. Secretion of PGI_2 by cells grown on crosslinked gels was higher (figure 5), but again no significant stimulation occurred after incubation with Ca^{2+}-ionophore A23187.

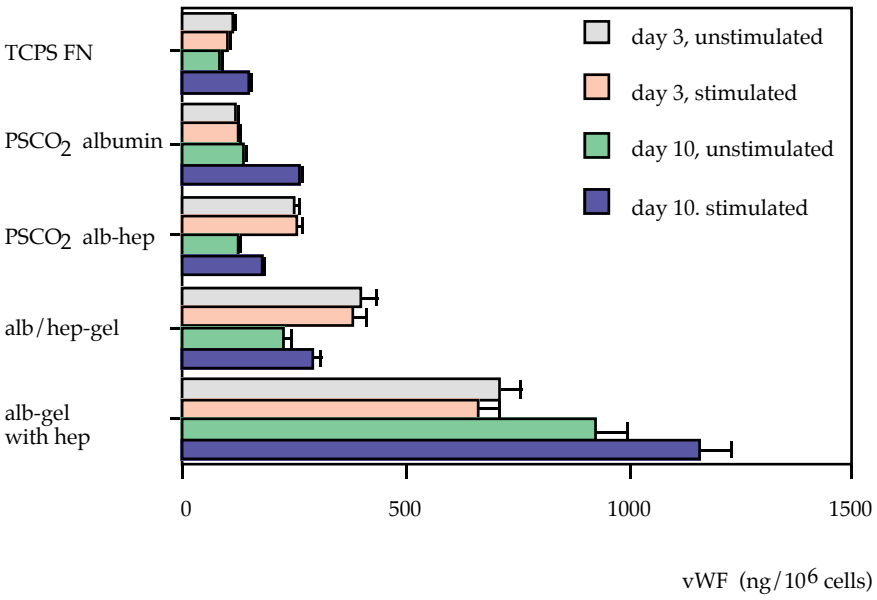

Figure 4 *Basal- and Ca^{2+}-ionophore A23187-stimulated secretion of vWF by HAMVECs on fibronectin-coated TCPS, on albumin- and on alb-hep conjugate coated $PSCO_2$, and on crosslinked gels of albumin and heparin after 3 and 10 days of culture. (n=3 ± st.dev.)*

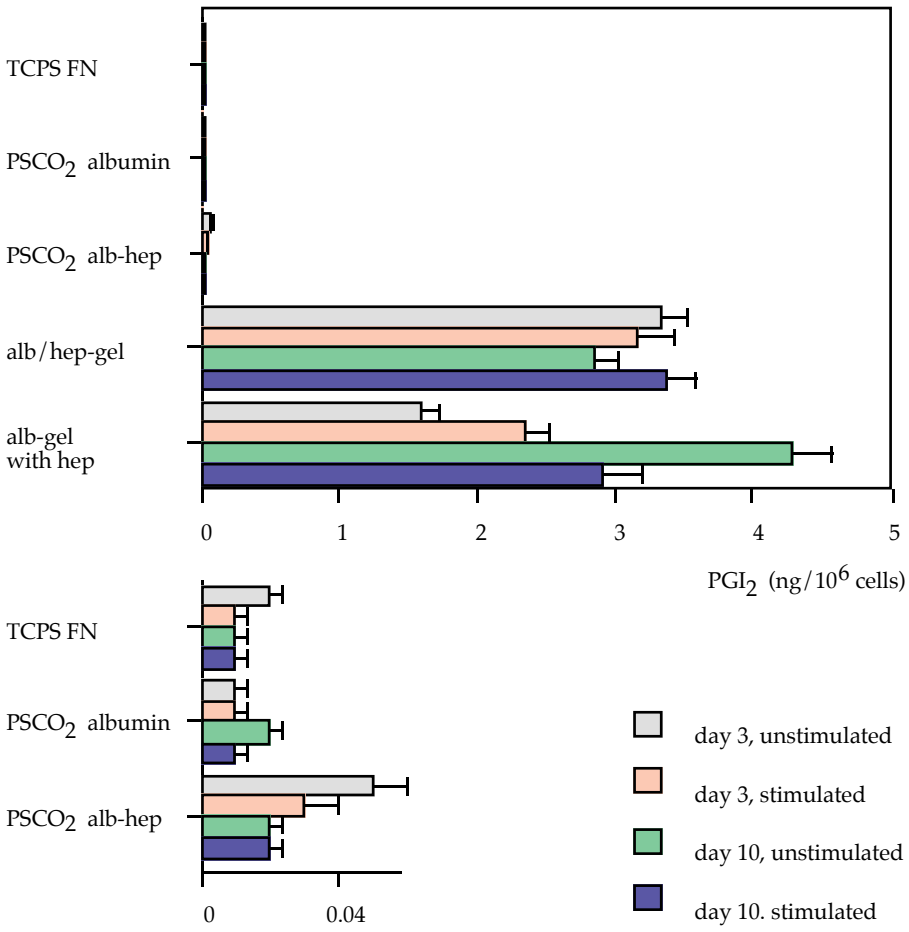

Figure 5 *Basal- and Ca^{2+}-ionophore A23187-stimulated secretion of PGI_2 by HAMVECs on fibronectin-coated TCPS, on albumin- and on alb-hep conjugate coated $PSCO_2$, and on crosslinked gels of albumin and heparin after 3 and 10 days of culture. (n=3 ± st.dev.)*

When the released quantities are plotted versus cell density (figures 6 and 7), an inverse correlation is found between the amount of vWF and PGI_2 secreted per cell and the cell density.

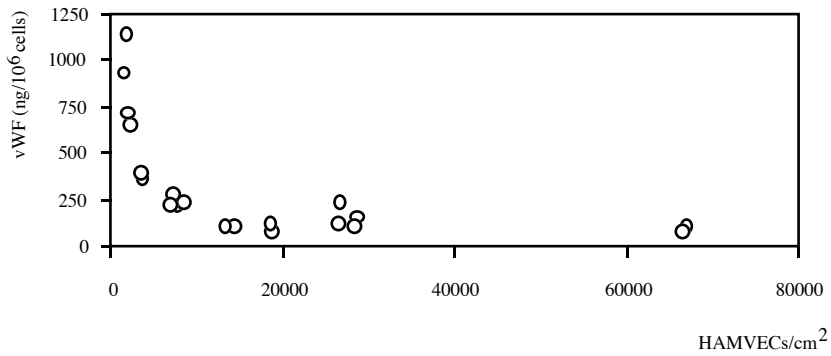

Figure 6. *Quantity of vWF released per 10^6 cells as a function of cell density.*

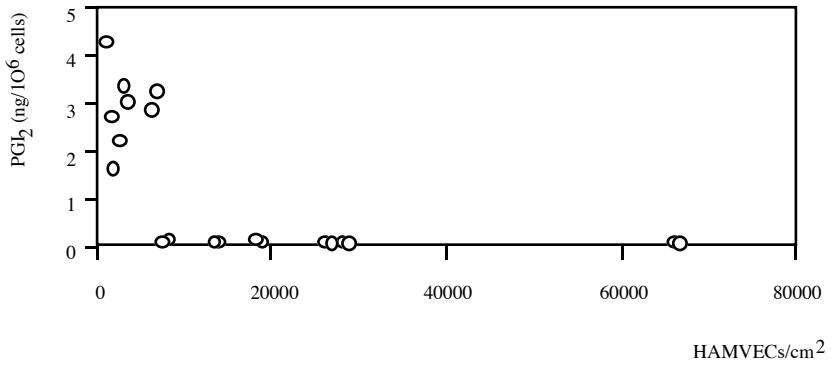

Figure 7. *Quantity of PGI_2 released per 10^6 cells as a function of cell density.*

Discussion

Since in humans, in contrast to animal species, the luminal surface of vascular prostheses is not spontaneously overgrown by endothelium, seeding of ECs is used to obtain endothelialization of vascular grafts in order to prevent thrombotic complications.[10-13] A prerequisite for successful cell seeding is the optimal adhesion of autologous ECs to the graft surface. The present study was undertaken to investigate the isolation and culture of ECs from microvessels present in adipose tissue.

Using an enzyme digestion method, cells were isolated from minced adipose tissue of different origin and from liposuction material. In the limited number of samples studied, there was no significant difference regarding the age and gender of the donor. The resulting culture was usually composed of a mixture of ECs, fibroblasts, and other cells, as found by others.[19,20,33] As was reported by Williams *et al.*,[34] the number of cells isolated per gram of tissue differs between individuals.

Using a series of cell selection procedures with *UEA-I*-coated para-magnetic beads, it was possible to obtain a homogeneous culture of ECs. The necessity of repetitive selection was reported previously.[33] *UEA-I* binds selectively to human ECs,[35] but not to animal endothelium[36] and human fibroblasts.[37] Binding occurs via an endothelial cell surface glycoprotein that contains an α-L-fucosylgroup.[35] After selection of the ECs, the beads can be separated from the outer cell membrane by competitive binding with L-fucose. In order to obtain homogeneous HAMVEC cultures, it was necessary to purify the cell culture when ECs were more abundant than fibroblasts.[20,33,38] Non-specific binding of fibroblasts to the beads may be the reason that contamination with fibroblasts is not completely prevented. Non-specific binding to Dynabeads has been reported to depend on the kind of cells used.[39] For some cells (e.g. normal bone marrow cells) non-specific binding to uncoated beads was less than 1 percent, whereas for other cells (e.g. neuroblastoma cells) it amounted to more than 30 percent.[39] An important fac-

tor, therefore, is to optimize the number of beads per cell. If too many beads are present, too much non-specific binding will occur, and positive cell selection will be hindered. On the other hand, if the number of beads is insufficient, not all ECs will bind to the beads, resulting in decreased cell numbers. From our results it can be estimated that four beads per cell is optimal to select enough ECs and to minimize non-specific binding of other cells. The manufacturer of the beads recommends to use at least 5 beads per cell.

Shortening the trypsin time during detachment of the cells appeared to remove at least part of the fibroblasts from cell cultures, which is in agreement with the observation that the binding of fibroblasts to solid substrates is stronger than that of ECs.[40] Overgrowth of ECs by fibroblasts can thus be prevented temporarily. This method looks promising (in terms of time and effort) when combined with endothelial cell selection using *UEA-I*-coated beads.

Homogeneous HAMVEC cultures were cultured to passage 4 or beyond, as reported for other microvessel ECs.[20,33,38,41] Most homogeneous cultures showed a cobblestone appearance, but occasionally less regular cell shapes were observed. This has also been observed by Williams *et al.*.[42] Senescence, present in some HAMVEC cultures after about eight to twelve passages, was also reported for microvascular ECs from human dermis.[41]

To determine if the isolated cells were of endothelial origin, four of the homogeneous HAMVEC cultures were characterized by means of immunofluorescence microscopy. Previously, several authors reported to have isolated ECs from omentum; such cells later turned out to be mesothelial cells (MCs).[19,29] We compared the fluorescent staining of HAMVECs, MCs and HUVECs. Differentiating between these cell types is essential, as MCs may overgrow ECs in cultures,[29] and MCs are thrombogenic in contrast to ECs.[43] In our study, von Willebrand factor was detected in both ECs and MCs. However, vWF-derived immunofluorescence of MCs was faint and diffuse throughout the cells.[19,44,45] In

endothelial cells (HUVECs and HAMVECs), however, staining was granular and more intense, emanating from Weibel-Palade bodies, which are known to contain and secrete vWF.[46] Granular immunofluorescence of vWF was also reported for ECs present in capillaries of native human omentum;[19,47] but not in omental MCs. The different vWF-staining patterns in MCs reported here and in literature, may be due to the specific antibody used. Visser *et al.*[29] reported either faint and diffuse staining or no staining at all, depending on the antibody used.

Endoglin and PECAM-1 were present in HUVECs and HAMVECs, and absent in MCs. Fluorescent staining of PECAM-1, characteristically expressed along the cell borders, was reported also by Kräling *et al.* for microvascular ECs derived from human dermis.[41] Cytokeratin 8 (CK8), which is typical for MCs, was present in Mcs and not in HUVECs and HAMVECs. Staining of cytokeratin 18 (CK18) showed less clear results. Although all cell types stained positively, the most intense fluorescent signal was observed in MCs. Chung-Welch *et al.*,[48] reported positive staining of CK8 and negative staining of CK18 in pulmonary microvascular ECs (PMVECs), whereas staining of CK8 in MCs was more intense than in PMVECs. Thus, although there are differences between staining of cytokeratins in microvascular ECs, the expression of cytokeratins is significantly less than in MCs.

Previously we showed that HAMVECs also express leukocyte adhesion molecules like ICAM-1, VCAM-1, and E-selectin.[49] Expression of ICAM-1, VCAM-1, and E-selectin can be stimulated by IL-1β. Stimulated expression of ICAM-1 and VCAM-1 was also reported by Kräling *et al.* for microvascular ECs derived from human dermis.[41]

Based on the above mentioned results, we conclude that the isolated HAMVECs in this study were of endothelial origin. This is in agreement with the results of a recently reported study regarding the characterization of cells derived from subcutaneous fat. Subcutaneous adipose tissue consists mainly of adipocytes and microvessels, without MCs.[50]

Furthermore, our studies demonstrate that HAMVECs adhere to and proliferate on albumin and alb-hep conjugate, covalently immobilized onto PS-CO_2. No difference between HAMVEC cell densities for albumin and alb-hep conjugate was observed. However, the numbers of HAMVECs were lower than when HUVECs were plated on these substrates.[16] Especially in the case of the crosslinked gels, proliferation of HAMVECs was worse compared with HUVECs, which reached cell densities between 20,000 and 40,000 cells/cm^2 on heparin-containing crosslinked albumin-gels.[51] Furthermore, it was observed that HAMVECs, in contrast to HUVECs, quickly detached from the surface when rinsed with PBS. Apparently, the binding of HAMVECs to a specific substrate is weaker than the binding of HUVECs to the same substrate. Improvement of adherence of the cells to these substrates, for instance by adding fibronectin to the coating, may be necessary.

HAMVECs cultured on the above mentioned substrates released both vWF, which provides a bridge between adhering platelets and (sub-)endothelium, and prostacyclin, a vasodilator and inhibitor of platelet aggregation. However, in contrast to the results obtained with HUVECs,[16,51] stimulation of the HAMVEC cultures with Ca^{2+}-ionophore A23187 had no or hardly any effect on the release of either vWF or PGI_2. This behaviour may indicate that the HAMVECs were activated even in the absence of the Ca^{2+}-ionophore. However, since cells from only one isolation were used in this experiment, it can not be concluded that this behaviour is typical for HAMVECs.

The quantities of released vWF per 10^6 cells from HAMVECs grown on TCPS-FN, PS-CO_2-albumin, or PS-CO_2-alb-hep, were comparable to data obtained for HUVECs.[16] When seeded on crosslinked alb/hep-gel or alb-gel with hep, HAMVECs produced significantly more vWF per cell than HUVECs.[51] PGI_2 secretion of HAMVECs was significantly lower compared to HUVECs, the difference being a factor 50 to 100 for TCPS-FN, PS-CO_2-albumin, and PS-CO_2-alb-hep, and a factor 5 to 10 for

crosslinked alb/hep-gel and alb-gel with hep.[16,51] From the release and proliferation data, it appeared that secretion of both vWF and PGI_2 is not primarily influenced by the cell culture substrate, but merely by the cell density. An inverse correlation was observed between the secreted amounts of both vWF and PGI_2 and cell density. Similar results were reported by others for unstimulated secretion of vWF and PGI_2 by human ECs, isolated from the saphenous vein of adults.[52] Secretion of vWF by HAMVECs described in this study, again indicates that the HAMVEC cultures represent ECs, since vWF was not found in the medium of MCs.[47]

PGI_2 production by HAMVECs has been reported by others,[38,42] but no absolute values were presented. This absence of values, together with other procedures and different presentations of the results, makes it difficult to compare data. Koyama *et al.*,[38] for instance, reported PGI_2 production of 15.9 ± 3.3 ng/mg protein/h by HAMVECs versus 30.5 ± 12.1 ng/mg protein/h by HUVECs. Although in their study release of PGI_2 from HAMVECs is lower than from HUVECs, the difference is much smaller than reported here.

Several studies have demonstrated that thrombogenicity was decreased and patency was improved by seeding microvascular ECs on vascular grafts implanted *in vivo* . The thrombogenicity of ePTFE grafts in a rat aortic graft model, for instance, was decreased after sodding with microvascular ECs derived from intraperitoneal fat pads.[53] Similarly, the patency of ePTFE grafts in a canine model was improved by seeding with ECs derived from microvessels of falciform ligament fat, which is histologically similar to human liposuction fat.[54]

Because HAMVEC isolations need to be purified several times, immediate seeding onto vascular grafts after isolation is not feasible. However, *in vitro* culture of endothelial cell seeded vascular grafts has recently been shown to improve the patency of vascular grafts.[12,13] Therefore, after purification the HAMVECs can be seeded on the inner surface of the grafts, and subsequently be cultured to confluency prior to implantation.

Conclusions

ECs obtained by enzymatic digestion of subcutanous fat tissue from knee cavity or abdomen, and purified by repeated positive selection with the aid of *Ulex Europaeus Agglutinin-I* lectin-coated Dynabeads, are of endothelial origin. Immunofluorescent staining of these HAMVECs is positive for vWF, endoglin and PECAM-1. HAMVECs are able to adhere to and subsequently proliferate on albumin and alb-hep conjugate, immobilized to PS-CO_2. Although cell numbers are low compared to HUVECs grown on these substrates, confluent HAMVEC monolayers are formed. Adhesion and slow proliferation of HAMVECs occurs on crosslinked alb/hep-gels and alb-gels with hep. When compared with HUVECs, HAMVECs secrete normal amounts of vWF, and relatively low amounts of PGI_2. This secretion is inversely related to the cell density.

HAMVECs, isolated from subcutaneous fat tissue, are candidate cells for seeding of vascular prostheses.

Acknowledgement

We wish to thank Dr. de Smit, Dr.de Bruijn and the staff of the operating rooms (Medisch Spectrum Twente, Enschede, The Netherlands), for providing the fat tissue, Dr. A. Pronk (Diaconessen huis, Utrecht, The Netherlands) for his gift of MCs, and Ir. H.R. Eppinga for crosslinking of the albumin and albumin/heparin gels.

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Summary

Many individuals, notably in western society, suffer from the complications of vessel wall degeneration known as atherosclerosis, when blood vessels narrow progressively and eventually become occluded. Besides, thrombi narrowing the vascular lumen may be released from the vessel wall, and subsequently block small or medium-sized blood vessels. Occlusion of blood vessels may cause life threatening situations like myocardial or cerebral infarction, and eventually cause death.

Up till now, saphenous veins are used for reconstructive vascular surgery of medium and small-diameter arteries. However, increased flow and pressure in the arterial circulation may cause deterioration of grafted veins. Moreover, the number of veins for vascular reconstruction is limited and structural changes may render them unsuitable for use.

Alternatively, synthetic grafts are used to replace atherosclerotic blood vessels. Vascular grafts of Dacron (polyethylene terephthalate) or Teflon (polytetrafluoroethylene), in combination with treatments using anticoagulants and platelet inhibitors, have been used successfully to replace blood vessels with a large inner diameter (i.e. more than 5 mm). However, despite the use of medication, small-diameter synthetic grafts often occlude rapidly when placed in low-flow high-resistance locations.

Endothelial cells (ECs), which form the inner lining of the natural vessel wall, perform a key regulatory role in haemostasis. Unlike in a number of animal species, spontaneous outgrowth of endothelium from the anastomoses on vascular prostheses implanted in humans does generally not occur. Seeding of ECs on the luminal surface of the vascular graft, therefore, may be a promising method to avoid occlusion of small-diameter prostheses in humans. ECs used for seeding need to be autologous and, depending on the size of the vascular graft, numerous ECs are required to allow the formation of a confluent monolayer. In many cases the number of ECs avail-

able for seeding is limited. Because in most cases the layer of seeded ECs will be incomplete immediately after seeding, the prosthetic material itself should display antithrombogenic properties and be able to promote both adherence and proliferation of ECs.

The research described in this thesis is focused on the synthesis and evaluation of a vascular graft coating consisting of albumin and the anticoagulant heparin (alb-hep conjugate), which may fulfill the above mentioned requirements. Previous research demonstrated that coating of surfaces with alb-hep conjugates improves blood compatibility of graft surfaces. Moreover, a number of adhesive proteins such as cellular fibronectin have binding sites for heparin, and may therefore mediate the binding of ECs to the alb-hep conjugate coating. In the present model study, polystyrene is modified by gas plasma treatment to generate chemical groups at the surface which are used to covalently immobilize the alb-hep conjugate. The coupling chemistry and the stability of the coatings have been investigated. Subsequently, this substrate (either with or without added fibronectin) was tested for its capability to allow adherence and proliferation of ECs. Moreover, the effect of basic fibroblast growth factor (bFGF), loaded onto surface-immobilized alb-hep conjugate, on the proliferation of seeded ECs has been studied. Next, blood compatibility of surfaces coated with alb-hep conjugate is reported, as well as the effect of seeded ECs on platelet deposition to these substrates. In addition, blood compatibility of crosslinked gels of albumin and heparin, adhesion and proliferation of ECs on these substrates, as well as the effect of seeded ECs on platelet deposition have been investigated. Finally, the isolation and characterization of microvascular ECs from adipose tissue are reported, as well as their culture on surface-immobilized alb-hep conjugate.

An overview of the efforts made to improve vascular grafts used for replacement of small-diameter blood vessels is presented in **chapter 2**. The strategies used to improve the patency of these small-diameter grafts, the current status of clinical trials, and further perspectives in the field of artificial vascular graft development are reviewed. It is concluded that, in view of recent developments in tissue engineering approaches, small-diameter vascular prosthesis with an adequate coating or sealant combined with seeded endothelial cells may have good perspectives for future application.

In **chapter 3**, polystyrene (PS) discs and petridishes were treated with argon or carbon dioxide gas plasma, which resulted in the introduction of hydroxyl, epoxide, carbonyl, and carboxylic acid groups at the surface. Using ^{14}C-labeled alb-hep conjugate, it was shown that formation of a monolayer, which is relatively stable in contact with aqueous solutions at physiological pH, required preactivation of carboxylic acid groups at the surface of argon plasma-treated PS discs. However, for carbon dioxide plasma-treated PS discs (PS-CO_2) preactivation of carboxylic acid groups was not necessary, indicating that binding of conjugate can occur through other groups than surface carboxylic acid groups. Subsequent investigations were carried out using PS-CO_2-petridishes, without preactivation. Coupling of ^{14}C-labeled methylamine to PS-CO_2 petridishes suggests that epoxide and/or aldehyde groups at the surface play a role in the covalent attachment of albumin and alb-hep conjugate. The surface concentration of alb-hep conjugate and albumin, immobilized onto PS-CO_2 and tissue culture PS petridishes (TCPS), as well as the stability of these coatings, were strikingly similar. It was concluded that a relatively stable monolayer of albumin or alb-hep conjugate on polystyrene can be obtained by first pretreating the PS-surface with CO_2 gas plasma, followed by incubation with an aqueous protein (conjugate) solution at pH 8.2.

In **chapter 4**, the adherence and proliferation of ECs derived from human umbilical veins (HUVECs) on alb-hep conjugate immobilized to PS-CO_2 and on fibronectin-coated TCPS is described. The number of HUVECs adhering and proliferating on these surfaces were comparable. However, the structure and size of the HUVECs proliferating on surface-immobilized alb-hep conjugate were more irregular than on a fibronectin coating. Long-term adherence of HUVECs was improved by adding a small amount of fibronectin to the alb-hep conjugate-coated surface. HUVECs cultured on either surface showed secretion of both von Willebrand factor and prostacyclin. The secretion of both endothelial cell products was stimulated by Ca^{2+}-ionophore A23187. It was concluded that alb-hep conjugate coating allows seeding and proliferation of ECs, especially when small amounts of fibronectin are present.

In **chapter 5**, the culture of HUVECs on bFGF-loaded alb-hep conjugate coating on PS-CO_2 is described. To allow the formation of a confluent monolayer of HUVECs, 2 to 3 ng/cm^2 bFGF had to be immobilized. The most prominent effect of surface-immobilized bFGF was stimulation of the proliferation of HUVECs shortly after seeding, resulting in confluent cell monolayers with high density within 3 days. In contrast, when 0.3 ng/ml bFGF was present in the culture medium instead of bound to the surface, it took almost a week before the cell layers reached confluency. Only a minor part of the bFGF used in this study displayed heparin affinity. Furthermore, in time degradation and multimerization of labeled bFGF occurred when the growth factor was stored at 20-37°C in PBS containing 20 mg/ml albumin, either with or without heparin. Therefore, the use of labeled bFGF is limited to short term (hours) experiments, like for instance determination of the amount of bFGF bound to a matrix. It was concluded that bFGF-loading of vascular graft surfaces through complexation of bFGF with a heparin-containing matrix will probably lead to more rapid formation of a confluent monolayer of ECs on the graft surfaces upon seeding of the cells.

In **chapter 6**, it was shown that alb-hep conjugate immobilized on PS-CO_2 significantly increased the recalcification time of blood plasma exposed to this surface. Furthermore, surface-immobilized alb-hep conjugate was able to inhibit thrombin. Heparin activity was reduced by adding fibronectin on top of a monolayer of alb-hep conjugate, but not by simultaneous coating of fibronectin and alb-hep conjugate. Coating of PS-CO_2 with alb-hep conjugate significantly decreased contact activation (FXII activation). The number of platelets deposited from blood plasma on PS-CO_2 coated with albumin respectively alb-hep conjugate with relatively low anticoagulant activity was similar, whereas on alb-hep conjugate with higher anticoagulant activity the number of deposited platelets increased two-fold. Addition of fibronectin to alb-hep conjugate-coated PS-CO_2 had no significant effect on the number of adhered platelets. Seeding of the substrates with ECs significantly reduced platelet adhesion when tested at stationary conditions. However, on surfaces seeded with ECs the number of deposited platelets slightly increased with endothelial cell density. Platelets deposited onto endothelialized surfaces were primarily found on endothelial cell edges. As alb-hep conjugate-coated surfaces display anticoagulant activity and ECs adhering to and proliferating on these coatings significantly decrease the number of adhering platelets, it was concluded that alb-hep conjugate-coated surfaces form a suitable substrate for seeding of ECs at low density. Application of fibronectin on top of the coating, although decreasing anticoagulant activity to some extent, may be useful in view of the improved adherence of ECs to the coating.

Besides alb-hep conjugate coatings, crosslinked gels of albumin and heparin (Alb/hep-gel) and crosslinked gels of albumin to which heparin is immobilized (Alb-gel with hep), which are potential sealants for prosthetic vascular grafts as well as substrates for endothelial cell seeding, were studied with regard to *in vitro* stability, binding of bFGF and cellular interactions

(**chapter 7**). During storage for 14 days in serum-containing cell culture medium at 37°C, heparin release from these gels was 21 - 25 percent, whereas release of albumin did not occur. HUVECs rapidly adhered and subsequently spread on these crosslinked gels as well as to crosslinked albumin-gels without heparin (Alb-gel), but proliferation was only observed if heparin was present in the gel. HUVECs cultured on these gels secreted both von Willebrand factor (vWF) and prostacyclin (PGI_2), and the expression of both endothelial cell products could be stimulated by Ca^{2+}-ionophore A23187. An inverse correlation between the quantity of secreted vWF and PGI_2 per cell and the cell density on various gels and on fibronectin-coated TCPS was observed. Growth of HUVECs occurred on Alb-gel with hep loaded with bFGF, but not on Alb-gel loaded with bFGF. The number of platelets deposited under stationary conditions onto heparinized gels was more than twice the number found on Alb-gels without heparin. Seeding of ECs on Alb-gel with heparin, significantly reduced the number of adhering platelets. Moreover, spreading of platelets was not observed on substrates seeded with ECs. Most platelets were deposited near the edges of HUVECs, and only rarely on areas between ECs. It was concluded that crosslinked gels of albumin to which heparin is immobilized, are candidate sealants for prosthetic vascular grafts and suitable substrates for endothelial cell seeding.

One way to collect sufficient ECs for complete coverage of the vascular graft surface, is the use of adipose tissue as a source of ECs (**chapter 8**). Following isolation of human ECs from adipose microvessels (HAMVECs), contaminating cells such as fibroblasts were eliminated from the primary and subsequent cultures by repeated positive selection of ECs, using *Ulex Europaeus Agglutinin-I* lectin-coated para-magnetic beads. The endothelial origin of the cells was demonstrated by positive immunofluorescent staining of the isolated cells for vWF, endoglin and platelet endothelial cell adhesion molecule-1. HAMVECs grew to confluency on PS-CO_2 coated with alb-hep

conjugate. Although release of vWF by HAMVECs under stationary conditions was comparable to release by HUVECs, stationary release of PGI_2 by HAMVECs was significantly lower. Since isolated HAMVECs need to be purified several times, immediate seeding onto vascular grafts after isolation is not feasible, which restricts the use of these cells to non-emergency vascular replacements. It was concluded that adipose tissue is a candidate source to obtain ECs for seeding of vascular prostheses, provided that these cells release sufficient PGI_2 under flow conditions.

In conclusion, covalently bound albumin and heparin, either in the form of a conjugate or in the form of crosslinked gels, are candidate coatings respectively sealants for prosthetic vascular grafts and suitable substrates for endothelial cell seeding. bFGF-loading of vascular graft surfaces through complexation of bFGF with these heparin-containing matrices will probably lead to more rapid formation of a confluent monolayer of ECs on the graft surfaces upon seeding of the cells. Alb-hep conjugate-coated surfaces display anticoagulant activity, and ECs adhering to and proliferating on these coatings significantly decrease the number of platelets which adhere to the surface. Application of fibronectin on top of the coating, although decreasing the anticoagulant activity to some extent, may be useful in view of the improved adherence of ECs to the coating. Therefore, surfaces coated with alb-hep conjugate or sealed with crosslinked albumin and heparin, which are subsequently loaded with bFGF, form a suitable substrate for seeding of ECs at low density.

An alternative for growth factor accelerated *in vivo* proliferation of ECs seeded at relatively low density may be the *in vitro* expansion of endothelial cell cultures prior to seeding followed by implantation of cultured endothelial cell seeded vascular grafts. Culture of seeded grafts before implantation, to allow maturation of the endothelial cell cytoskeleton, has recently

been shown to significantly improve the patency of the prostheses compared to unseeded grafts during 7 years of implantation.

The future of small-diameter vascular prosthesis looks promising. Completely tissue engineered vascular grafts are being developed which, compared to traditional polymer based implants, have the advantage of being able to grow upon implantation in young patients. Limited willingness of surgeons to use tissue engineered grafts without polymeric reinforcement, and time and effort consuming regulatory issues may delay the wide-spread use of this promising approach. Meanwhile, seeding of ECs on vascular graft materials, impregnated or coated with biologically relevant proteins (e.g. collagen, albumin, fibronectin, and laminin) and additives such as anticoagulants, growth factors and antibiotics, remains the method of choice to improve the patency of small-diameter vascular grafts.

Samenvatting

Veel mensen, met name in de westerse maatschappij, leiden aan de gevolgen van vaatwand degeneratie. Deze ziekte, die atherosclerose ofwel aderverkalking heet, kenmerkt zich door het geleidelijk vernauwen van de bloedvaten, die uiteindelijk dichtslibben. Bovendien kunnen stolsels die een bloedvat vernauwen loslaten en vervolgens kleine of middel-grote bloedvaten afsluiten. Verstopping van bloedvaten kan leiden tot levensbedreigende situaties, zoals hart- of herseninfarcten, die uiteindelijk de dood zullen veroorzaken.

Momenteel wordt de vene saphena vaak gebruikt voor reconstructieve operaties van middel-grote en kleine bloedvaten. Echter, de verhoogde stroming en druk in de arteriele bloedcirculatie kan afbraak van deze geimplanteerde bloedvaten veroorzaken. Ook is er slechts een beperkt aantal bloedvaten geschikt voor gebruik in reconstructieve operaties, en ze kunnen bovendien zelf aangetast en daardoor onbruikbaar zijn.

Als alternatief worden kunststof bloedvaten gebruikt ter vervanging van bloedvaten die aangetast zijn door atherosclerose. Bloedvat prothesen van Dacron (polyethyleen tereftalaat) of Teflon (polytetrafluorethyleen), in combinatie met het toedienen van anticoagulantia en plaatjesremmers, worden met succes toegepast om bloedvaten met een grote diameter (meer dan 5 mm) te vervangen. Kleine-diameter kunststof bloedvaten verstoppen echter snel als ze bij lage stroomsnelheid en hoge weerstand worden toegepast, zelfs wanneer medicamenten worden toegediend.

Endotheelcellen, die de bekleding van natuurlijke bloedvaten vormen, spelen een belangrijke regulerende rol in de bloedstolling. In tegenstelling tot een aantal diersoorten, treedt in mensen geen spontane uitgroei van endotheel op vanaf de uiteinden van de prothese. Het zaaien van endotheelcellen aan de binnenzijde van het bloedvat lijkt daarom een veelbelovende techniek om het dichtslibben van kleine-diameter prothesen in mensen te voorkomen. Endotheelcellen die gebruikt worden voor

het zaaien moeten lichaams-eigen zijn. Bovendien zijn er, afhankelijk van de afmetingen van het bloedvat, veel endotheel-cellen nodig om de vorming van een aaneensluitende monolaag cellen mogelijk te maken. Omdat in de meeste gevallen de laag gezaaide cellen niet confluent is direct na zaaien, moet de prothese zelf antistollende eigenschappen vertonen en daarnaast in staat zijn de hechting en groei van endotheelcellen te bevorderen.

Het onderzoek dat is beschreven in dit proefschrift is gericht op de synthese en evaluatie van een coating voor kunststof bloedvaten die bestaat uit albumine en de anticoagulant heparine (alb-hep conjugaat). Deze coating voldoet wellicht aan de bovengenoemde eisen. In voorafgaand onderzoek werd vastgesteld dat door het coaten van oppervlakken met alb-hep conjugaat, de bloedcompatibiliteit van prothese-oppervlakken verbetert. Verder hebben een aantal hechtingseiwitten, zoals cellulair fibronectine, bindingsplaatsen voor heparine. Daardoor kunnen ze een brug vormen tussen endotheelcellen en de alb-hep conjugaat coating. In de hier gepresenteerde model-studie wordt polystyreen behandeld met een gas-plasma om chemische groepen aan te brengen aan het oppervlak, die vervolgens gebruikt worden om alb-hep conjugaat covalent te binden. De koppelingschemie en de stabiliteit van de coating zijn onderzocht. Vervolgens werd getest of op dit substraat, met of zonder additioneel fibronectine, hechting en groei van endotheelcellen mogelijk was. Verder is onderzocht wat het effect is van het beladen met basische fibroblast groeifactor (bFGF) van met alb-hep conjugaat gecoate oppervlakken op de groei van endotheelcellen. Vervolgens wordt de bloed-compatibiliteit van met alb-hep conjugaat gecoate oppervlakken beschreven, en het effect dat gezaaide endotheelcellen hebben op de afzetting van bloedplaatjes. Tenslotte wordt de isolatie en karakterisering van microvasculaire endotheelcellen uit vetweefsel gerapporteerd, en het kweken hiervan op alb-hep conjugaat coatings.

In **hoofdstuk 2** wordt een overzicht gepresenteerd van de pogingen die gedaan zijn om kunststof bloedvat prothesen, die gebruikt worden voor het vervangen van kleine-diameter bloedvaten, te verbeteren. Beschreven worden de strategien die gebruikt worden om de doorgankelijkheid van deze prothesen te verbeten, de huidige status van het klinisch onderzoek, alsmede de toekomstverwachting voor de ontwikkelingen op het gebied van kunstmatige bloedvat prothesen. Er wordt geconcludeerd dat, gezien de recente ontwikkelingen op het gebied van weefsel technologieen (tissue engineering), kleine diameter bloedvat prothesen met een afdoende coating of impregnering, gecombineerd met gezaaide endotheelcellen, goede perspectieven bieden voor toekomstige toepassing.

In **hoofdstuk 3** worden polystyreen (PS) schijfjes en petrischaaltjes behandeld met een argon of carbondioxyde gasplasma, waardoor hydroxyl, epoxide, carbonyl, en zuurgroepen zijn geïntroduceerd aan het oppervlak. Met ^{14}C-gelabeld alb-hep conjugaat werd aangetoond dat voor de vorming van een monolaag, die relatief stabiel is bij blootstelling aan waterige oplossingen bij fysiologische pH, pre-activering van zuurgroepen op het oppervlak van argon-behandelde PS schijfjes noodzakelijk is. Voor carbondioxyde-behandelde PS schijfjes (PS-CO_2) is pre-activering echter niet noodzakelijk. Dat geeft aan dat de binding van het conjugaat plaatsvindt door andere groepen dan zuurgroepen aan het oppervlak. Verder onderzoek werd uitgevoerd met PS-CO_2 petrischaaltjes, zonder pre-activering. Het koppelen van ^{14}C-gelabeld methylamine aan PS-CO_2 petrischaaltjes duidt erop dat epoxide en/of aldehyde groepen aan het oppervlak een rol spelen in de covalente binding van albumine en alb-hep conjugaat. De oppervlakte concentratie van alb-hep conjugaat en albumine, geïmmobiliseerd op PS-CO_2 en weefselkweek polystyreen (TCPS), en de stabiliteit van deze coatings vertoonde opvallende gelijkenis. Er werd geconcludeerd dat een relatief stabiele monolaag albumine of alb-hep conjugaat op polystyreen kan worden gerealiseerd door het

PS-oppervlak eerst te behandelen met een CO_2 gas-plasma, en vervolgens dit oppervlak te incuberen met een waterige eiwit/conjugaat oplossing met pH 8,2.

In **hoofdstuk 4** wordt de hechting en de groei beschreven van uit menselijke navelstreng venen geisoleerde endotheelcellen (HUVECs) op alb-hep conjugaat geimmobiliseerd op PS-CO_2 en op met fibronectine gecoat TCPS. Het aantal HUVECs dat hecht en groeit op deze oppervlakken is vergelijkbaar. De structuur en de afmeting van de HUVECs die groeien op een alb-hep conjugaat coating zijn echter minder regelmatig dan op een fibronectine coating. De lange-termijn hechting van HUVECs kan worden verbeterd door een kleine hoeveelheid fibronectine toe te voegen aan het met alb-hep conjugaat gecoate oppervlak. HUVECs, gekweekt op een van beide oppervlakken, scheiden zowel van Willebrand factor als prostacycline uit. De uitscheiding van beide endotheel cell producten wordt gestimuleerd door Ca^{2+}-ionofoor A23187. Er werd geconcludeerd dat coating met alb-hep conjugaat coldoende mogelijkheid biedt voor hechting en groei van gezaaide endotheelcellen, met name wanneer een kleine hoeveelheid fibronectine in de coating aanwezig is.

In **hoofdstuk 5** wordt het kweken van HUVECs op een met bFGF-beladen coating van alb-hep conjugaat op PS-CO_2 beschreven. Voor de vorming van een aaneensluitende monolaag HUVECs moest 2 tot 3 ng/cm^2 bFGF worden geïmmobiliseed. Het meest opvallende effect van oppervlak-geïmmobiliseerd bFGF was de groeistimulatie van HUVECs direct na het zaaien. Binnen 3 dagen was een confluente cellaag met hoge dichtheid gevormd. In tegenstelling, wanneer 0,3 ng/ml bFGF werd toegevoegd aan het kweekmedium in plaats van aan het oppervlak, duurde het bijna een week om dit te bereiken. Slechts een klein gedeelte van het bFGF dat in deze studie is gebruikt heeft affiniteit voor heparine. Bij opslag van de groeifactor bij 20 - 37 °C in PBS met 20 mg/ml albumine, met of zonder heparine, treedt in de loop der tijd degradatie en multimerisatie op van radioactief gelabeld bFGF. Het gebruik van gelabeld bFGF dient daarom te worden beperkt tot korte termijn (uren) experi-

menten, zoals het bepalen van de hoeveelheid bFGF die gebonden is aan een matrix. Er werd geconcludeerd dat het beladen van vaatprothese-oppervlakken met bFGF door complexatie van bFGF aan een heparine-bevattende matrix waarschijnlijk zal leiden tot snelle vorming van een aaneensluitende monolaag endotheelcellen op het oppervlak van de prothese na zaaien van de cellen.

In **hoofdstuk 6** werd aangetoond dat coating van PS-CO_2 met alb-hep conjugaat de recalcificeringstijd van bloedplasma dat wordt bloodgesteld aan dit oppervlak significant vermindert. Verder remt het geïmmobiliseerde alb-hep conjugaat thrombine. De activiteit van heparine werd gereduceerd door het aanbrengen van fibronectine bovenop de alb-hep conjugaat coating, maar niet door het gelijktijdig aanbrengen van fibronectine en conjugaat. Het coaten van PS-CO_2 met alb-hep conjugaat verminderde ook de contact activering (FXII activering) significant. De afzetting van bloedplaatjes vanuit bloedplasma op PSCO_2 dat is gecoat met albumin of met alb-hep conjugaat met lage antistollende werking was vergelijkbaar, terwijl op alb-hep conjugaat met een hogere antistollende werking de plaatjes depositie twee maal zo hoog was. Het toevoegen van fibronectine aan de alb-hep coating had geen significant effect op het aantal afgezette plaatjes. Zaaien van endotheelcellen op de verschillende substraten verminderde de depositie van bloedplaatjes onder stationaire condities significant. Echter op oppervlakken met gezaaide endotheelcellen nam het aantal plaatjes licht toe met toenemende celdichtheid. De plaatjes die afgezet werden op oppervlakken met gezaaide endotheelcellen bevonden zich met name aan de rand van deze cellen. Omdat met alb-hep conjugaat gecoate oppervlakken antistollende eigenschappen vertonen en endotheelcellen die hechten en groeien op dit oppervlak het aantal afgezette plaatjes significant vermindert, werd geconcludeerd dat oppervlakken die zijn gecoat met alb-hep conjugaat een geschikt substraat zijn voor het zaaien van endotheelcellen in een lage dichtheid. Hoewel toevoegen van fibronectine aan de alb-hep

conjugaat coating de anti-stollende werking tot op zekere hoogte vermindert, kan het nuttig zijn vanwege de verbeterde hechting van endotheelcellen aan de coating.

Naast alb-hep conjugaat coatings zijn gecrosslinkte gelen van albumine en heparine (Alb/hep-gel) en gecrosslinkte gelen van albumine waarop later heparine is geïmmobiliseerd (Alb-gel met hep) bestudeerd (**hoofdstuk 7**). Deze materialen kunnen gebruikt worden als impregne-ringsmiddel voor poreuze bloedvatprothesen. Tevens kunnen ze dienen als substraat voor gezaaide endotheelcellen. De gecrosslinkte gelen werden bestudeerd met betrekking tot *in vitro* stabiliteit, binding van bFGF en cellulaire interacties. Bij opslag gedurende 14 dagen in serumbevattend celkweek medium bij 37 °C lekte 21 – 25 procent van het heparine uit de matrix, terwijl er geen albumine vrijkwam. HUVECs hechten en spreiden goed op deze gecrosslikte gelen. Dat gebeurde ook op gecrosslinkte gelen van albumine zonder heparine (Alb-gel), maar groei van de cellen trad alleen op wanneer er heparine aanwezig was in de gel. HUVECs die werden gekweekt op deze gecrosslinkte gelen scheidden van Willebrand gactor en prostacycline uit, en de expressie van deze endotheelcel producten kon worden gestimuleerd door Ca^{2+}-ionofoor A23187. Een negatieve correlatie tussen de hoeveelheid uitgescheiden van Willebrand factor en prostacycline per cel en de celdichtheid op verschillende gelen en op met fibronectine gecoat TCPS werd waargenomen. Groei van HUVECs vond plaats op met bFGF beladen Alb-gel met hep, maar niet op met bFGF beladen Alb-gel. Het zaaien van endotheelcellen op Alb-gel met hep verminderde het aantal afgezette plaatjes, waarbij er geen spreiding van de plaatjes optrad. De meeste plaatjes werdebn afgezet bij de randen van de endo-theelcellen en slechts af en toe op plekken tussen de cellen in. Er werd geconcludeerd dat gecrosslinkte gelen van albumine waarop heparine is geimmobiliseerd, kandidaten zijn voor gebruik als impregneermiddel voor kunststof bloedvat prothesen en dat deze gelen geschikt zijn voor het zaaien van endotheelcellen.

Een manier om voldoende endotheelcellen te isoleren voor een volledige bedekking van het bloedvat-prothese oppervlak is het gebruik van vetweefsel als bron voor endotheelcellen (**hoofdstuk 8**). Na isolatie van menselijke endotheelcellen uit microvaatjes in vetweefsel (HAMVECs) werden verontreinigende cellen zoals fibroblasten verwijderd uit de primaire en volgende kweken door herhaalde positieve selectie van endotheelcellen met paramagnetische bollen die zijn gecoat meet Ulex Europaeus Agglutinine-I lectine. Dat de geisoleerde cellen endotheelcellen waren, bleek uit positieve immunofluorescente aankleuring van de cellen voor van Willebrand factor, endogline en plaatjes endotheelcel adhesie molecuul-1 (PECAM-1). HAMVECs groeiden uit tot een confluente laag op PS-CO2 dat was gecoat met alb-hep conjugaat. Hoewel afgifte van van Willebrand factor onder stationaire condities vergelijkbaar was met die van HUVECs, was de afgifte van prostacycline onder stationaire condities lager. Omdat geisoleerde HAMVECs verschillende malen gezuiverd moeten worden, is het niet mogelijk deze cellen direct na isolatie te zaaien in een bloedvat prothese. Het gebruik van deze cellen is dus beperkt tot niet-urgente bloedvat vervangingen. Er werd geconcludeerd dat vetweefsel een kandidaat bron is voor het verkrijgen van endotheelcellen om te zaaien in een kunststof bloedvat prothese, mits deze cellen voldoende prostacycline uitscheiden onder stromings condities.

Er wordt geconcludeerd dat covalent gebonden albumine en heparine, ofwel in de vorm van een conjugaat of in de vorm van een gecrosslinkte gel, bruikbaar zijn als coating respectievelijk impregneringsmiddel van kunststof bloedvatprothesen, en geschikt zijn als substraat voor gezaaide endotheelcellen. Belading van bloedvat prothesen met bFGF door complexatie van bFGF met deze heparine bevattende matrices zal waarschijnlijk leiden tot de snellere vorming van een aaneengesloten monolaag endotheelcellen op het oppervlak van de prothese na het zaaien van de cellen. Oppervlakken die zijn gecoat met alb-hep conjugaat vertonen anti-stollende eigenschappen, en endo-

theelcellen die groeien op deze oppervlakken verminderen de depositie van bloedplaatjes significant. Hoewel toevoegen van fibronectine aan de alb-hep conjugaat coating de antistollende werking tot op zekere hoogte vermindert, kan het nuttig zijn vanwege de verbeterde hechting van endotheelcellen aan de coating. Daarom zijn oppervlakken die zijn gecoat met alb-hep conjugaat of geimpregneerd zijn met gecrosslinkt albumine en heparine, en die vervolgens zijn beladen met bFGF, geschikte substraten voor het zaaien van endotheelcellen in een lage celdichtheid.

Een alternatief voor de door bFGF gemedieerde in vivo versnelling van de groei van in lage celdichtheid gezaaide endotheel cellen, is het *in vitro* kweken van de endotheelcellen voorafgaand aan het zaaien, gevolgd door implantatie van met gekweekte endotheelcellen gezaaide bloedvat prothesen. Onlangs is aangetoond dat het kweken van gezaaide prothesen voorafgaand aan implantatie, om het cytoskelet van de endotheelcellen te stabiliseren, de doorgankelijkheid van de prothesen significant verbetert ten opzichte van niet-gezaaide prothesen, gedurende 7 jaar implantatie.

De toekomst van bloedvat prothesen met een kleine diameter ziet er veelbelovend uit. Momenteel worden vaatprothesen ontwikkeld die volledig uit weefsel worden geconstrueerd. Deze prothesen hebben ten opzichte van de traditionele op polymeren gebaseerde bloedvat prothesen het voordeel, dat ze mee kunnen groeien, bijvoorbeeld bij implantatie in jonge patienten. Algemene toepassing van deze veelbelovende techniek wordt echter vertraagd door beperkte bereidheid van chirurgen om deze volledig uit weefsel geconstrueerde prothesen zonder polymeer versterking te gebruiken, en door tijd en inspanning vergende zaken in verband met regelgeving. Ondertussen blijft het zaaien van endotheelcellen op bloedvat-prothese materiaal, geimpregneerd of gecoat met biologisch relevante eiwitten, zoals collageen, albumine, fibronectine en laminine, en voorzien van additieven zoals anticoagulantia, groeifactoren en antibiotica, de aangewezen

methode om de doorgankelijkheid van bloedvat prothesen met een kleine diameter te verbeteren.

Curriculum Vitae

Gert Bos werd geboren te Lienden op 4 oktober 1968. Hij groeide hier ook op en behaalde het VWO-diploma in 1987 aan het Ichthus College te Veenendaal.

In dat zelfde jaar vertrok hij naar Enschede en begon de studie Chemische Technologie aan de Universiteit Twente. In 1993 studeerde hij af in de vakgroep van Prof. Dr. Jan Feijen onder begeleiding van Dr. Gerard Engbers. Een deel van het afstudeerwerk werd op de Strathclyde University onder supervisie van Prof. Jim Courtney PhD uitgevoerd.

De daarop volgende jaren verrichtte hij het in dit proefschrift beschreven onderzoek aan de Universiteit Twente te Enschede, onder leiding van Prof. Dr Jan Feijen, Prof. Dr. W.G. van Aken en Dr A.A. Poot, binnen de vakgroep Polymeerchemie en Biomaterialen. De resultaten van dit onderzoek worden beschreven in het voorliggende proefschrift.