Proliferation of endothelial cells on surface-immobilized albumin–heparin conjugate loaded with basic fibroblast growth factor

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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 on bFGF-loaded albumin–heparin conjugate bound to CO_2 gas-plasma-treated polystyrene. 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 shortly after seeding, resulting within 3 days in confluent cell monolayers with high density. 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 and the biological activity of bFGF towards ECs were not influenced by the (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. This limits the use of labeled bFGF to short-term (hours) experiments. In conclusion, bFGF loading of vascular graft surfaces through complexation of bFGF with a heparin-containing matrix probably will lead to more rapid formation of a confluent monolayer of ECs on graft surfaces upon seeding of the cells. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 44, 330–340, 1999.

Key words: small-diameter vascular grafts; endothelial cell seeding; albumin–heparin conjugate; basic fibroblast growth factor (bFGF); stability

INTRODUCTION

Synthetic vascular grafts of Dacron or expanded Teflon (ePTFE) with diameters 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 hemostasis.[8,9] Unlike in animal species, the spontaneous outgrowth of endothelium on implanted vascular prostheses generally does not occur in humans.[3,10] The seeding of ECs on the luminal surface of vascular grafts therefore is a promising method for improving the patency of small-diameter prostheses.[11–14]

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Since the availability of autologous ECs needed for seeding is limited, the graft surface generally will not be completely endothelialized immediately after seeding. When an 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 that 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] The precoating of small amounts of fibronectin onto albumin–heparin-coated PS-CO_2 promotes the binding of HUVECs to the substrate. Thus the coating of vascular grafts with albumin–heparin conjugate in combination with the 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 may be found in the local release of growth factors, which may accelerate the formation of a confluent cell layer. ECs then could be seeded subconfluently 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 glycosamino glycans 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 2 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 the aortas of rats for 4 weeks significantly enhanced the extent of endothelialization proceeding from the anastomotic sites as well as the 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 bound to a surface of gas-plasma-modified polystyrene provided with a monolayer of albumin–heparin conjugate (Fig. 1).

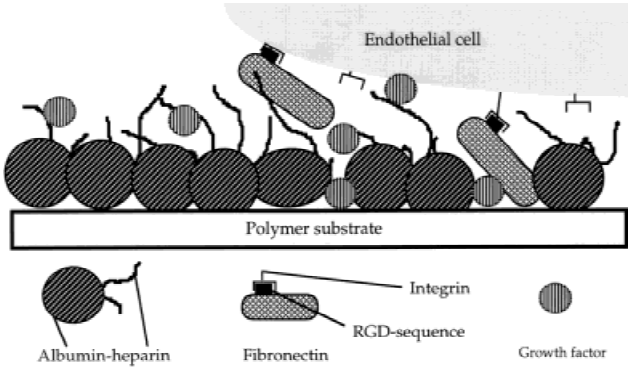

Figure 1. Schematic representation of endothelial cells using heparin-binding adhesive proteins to adhere to albumin–heparin conjugate immobilized on a polymeric substrate. bFGF is bound to the matrix to stimulate endothelial cell proliferation. In the case of endothelial cell binding to fibronectin, the integrins can be either VLA-5 (α_5, β_1) or the vitronectin receptor (α_v, β_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). 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) petri dishes (35 mm in diameter) were from Greiner GmbH (Frickenhausen, Germany). 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 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, Minnesota). Tri-(hydroxymethyl)-aminomethane hydrochloride (Tris), lysine, bovine serum albumin (BSA, fraction V), and agarose–heparin were from Sigma Chemical Company (St. Louis, Missouri). 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).

^{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.[34] In this way, ^{125}I is incorporated into tyrosine residues. $Na^{125}I$ (500 μCi, 15.5 mCi/μg) was added to three 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 the eluent. Alternatively, in one experi-

ment (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 of 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 petri dishes.

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

Human recombinant bFGF was labeled with ^{35}S employing t-butoxycarbonyl-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, 0.1*M*, 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, 0.2*M*, pH 8.5). A NaCl solution in PBS (900 μL, 3.33*M*) was added to the mixture, and subsequently the labeled bFGF was isolated using a series of PD10 columns with PBS as the 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 of free label was determined by PD10 column separation. For comparison, the amount of free label of ^{35}S-albumin (labeled with SLR in a procedure similar to that of 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 nonreducing polyacrylamide SDS gel electrophoresis. Equivolume samples of either bFGF solution or molecular weight markers and SDS in Tris buffer (10 mg/mL SDS, 0.5*M* 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 Excel-Gel® 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 of 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 of 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 and 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 of packed gel, final volume 2 mL) in order to determine if bFGF that did not bind during the first incubation would bind during the second incubation. The 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 tempera-

ture. Phenylmethylsulfonylfluoride (0.1 m*M*) was added as a protease inhibitor to all buffers. FNc (200 mL, 2.5 mg/mL in 50 m*M* of 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 m*M*, pH 7.5), 2 volumes of NaCl solution (1*M* of NaCl in 50 m*M* of Tris-HCl, pH 7.5), and again with Tris-HCl buffer (50 m*M*, pH 7.5). Elution of fibronectin was carried out with 4*M* of urea in Tris-HCl-buffer (50 m*M*, 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 min at 37°C with trypsin–EDTA solution in PBS [0.05 and 0.02% (w/v)]. After the HUVECs were harvested, cell culture medium supplemented with 20% (v/v) human serum (CMS 20, see below) was added to inactivate the trypsin. The medium was exchanged for fresh medium, and the cells were plated in fibronectin-coated TCPS flasks and grown at 37°C in 95% air/5% CO_2 saturated with water vapor 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 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 vapor.

The culture media used were complete cell culture media (CM) supplemented with 5 or 20% (v/v) pooled human serum (CMS 5 and CMS 20, respectively) 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 m*M*), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (2.5 μg/mL). The 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.

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

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

Gas plasma treatment and coating of polystyrene petri dishes

Polystyrene petri dishes 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 petri dishes (10.9 cm^2) by incubating the dishes with the protein or alb–hep conjugate (1 mL, 15 mg/mL) in borate buffer (5 m*M*, pH 8.2) for 45 min. Subsequently, the petri dishes were rinsed three times with PBS, three times with PBS containing 1*M* NaCl that had been brought to pH 3.5, and three times with PBS containing 1*M* NaCl that had been brought to pH 10.5. 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 min, 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 I. Initial surface concentrations were determined using radiolabeled compounds (albumin, alb–hep conjugate, and fibronectin).[16] Small quantities of the immobilized compounds were released after one day's incubation in a serum-containing cell culture medium whereas almost all the FN was released from the surface of PS-CO_2/alb–hep + FN.[16]

^{125}I-bFGF-loading of surfaces

PS-CO_2 surfaces, uncoated or coated with albumin, alb-hep conjugate, or a combination of alb–hep conjugate and

TABLE I
Surface Concentrations of Albumin, Alb–Hep Conjugate (AH) and Fibronectin (FN) After Immobilization on PS-CO_2.[16]

Coating	^{14}C-alb (μg/cm^2)	^{14}C-alb–hep (μg/cm^2)	^{125}I-FN (μg/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

fibronectin, were incubated with ^{125}I-bFGF (1 mL, 137 ng/mL) in PBS containing 20 mg of 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 the radioactivity bound to the surface was determined.

Effect of surface-immobilized bFGF on cell proliferation

Proliferation of HUVECs was investigated using petri dishes 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 (Fig. 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).

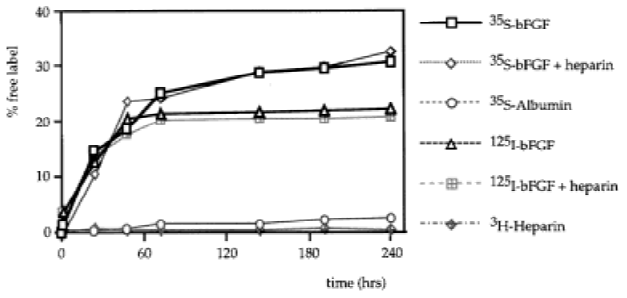

Figure 2. Percentage of free label of ^{125}I- and ^{35}S-labeled bFGF with or without heparin as a function of storage time in PBS/BSA (20 mg BSA/mL) at 37°C, determined by PD10 column separation. For comparison, free label of ^{35}S-albumin and ^{3}H-heparin are presented. Free label present prior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

During the first 48 h, the release of free label from ^{35}S-bFGF was similar to that from ^{125}I-bFGF (Fig. 2). Subsequently, release of ^{125}I leveled 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.

Dimerization or even multimerization of bFGF as a result of labeling with ^{125}I was not observed directly after labeling (Fig. 3, lane 2). However, during storage of ^{125}I-bFGF at 37°C, tetra- and trimers were formed (Fig. 3, lanes 4 and 8). Still, a band at 17 kD remained present. The addition of albumin stabilized the growth factor to some extent (Fig. 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).

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 (Fig. 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).

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 (Fig. 5). To elucidate whether 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, Fig. 5). In the second control experiment, bFGF capable of binding to heparin was obtained by means of Sepharose–HiTrap heparin. Using this bFGF solution, about 75 percent of the bFGF bound to the agarose–heparin (data not shown).

In a separate experiment, in which a concentration

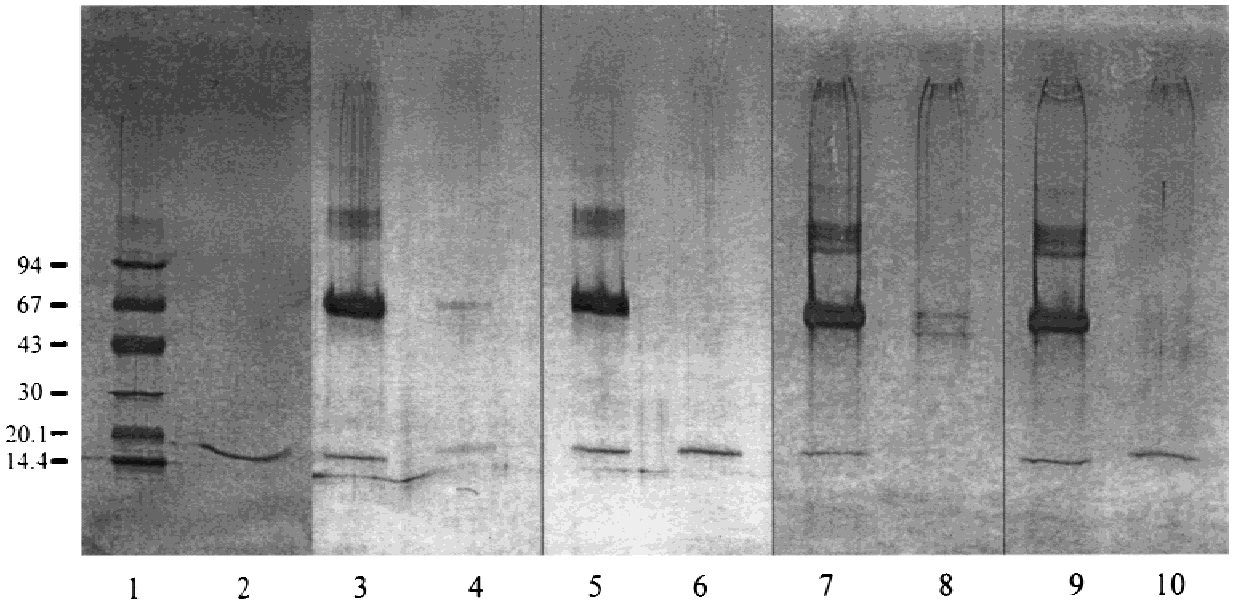

Figure 3. Electrophoresis gels of ^{125}I-bFGF and ^{125}I-bFGF/albumin as a function of storage time at 37°C Lane 1: LMW markers (14.4, 20.1, 30, 43, 67, and 94 kD); lane 2: ^{125}I-bFGF prior to storage; lanes 3 and 4: ^{125}I-bFGF in the presence (lane 3) or absence (lane 4) of albumin, nonreduced after 2 days of storage at 37°C; 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.

range of 10 to 100 ng/mL of 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).

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

To study the effect of the iodine labeling of bFGF on its biological activity with respect to the stimulation of endothelial cell growth, bFGF was labeled with nonradioactive 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 (Fig. 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

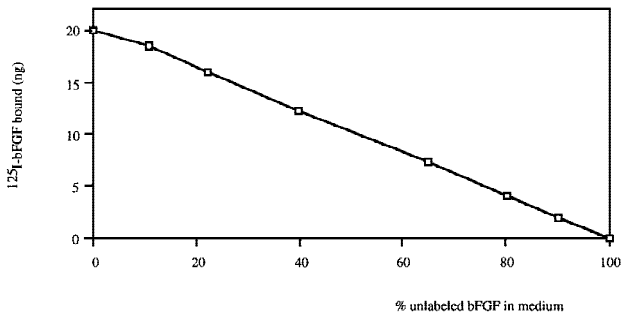

Figure 4. Binding of ^{125}I-bFGF to agarose–heparin beads upon serial dilution with unlabeled bFGF. bFGF concentrations as determined by ELISA were identical for all mixtures (115 ± 5 ng/mL). n = 5, ±SD.

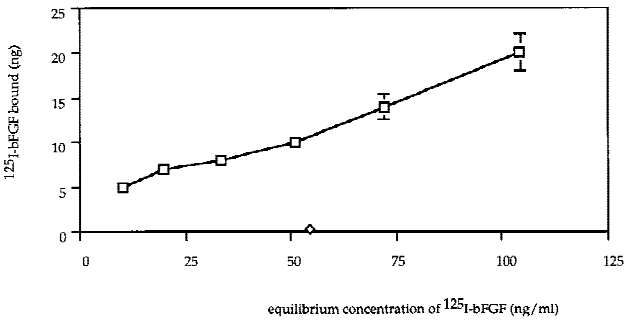

Figure 5. Concentration-dependence of bFGF binding to agarose–heparin beads (50 μL packed gel, 2 mL total volume) after 30 min at room temperature. The supernatant of the experiment in which the highest bFGF concentration was used was diluted after incubation to a concentration of 54 ng/mL and incubated again with fresh agarose–heparin beads (□). n = 5, ±SD.

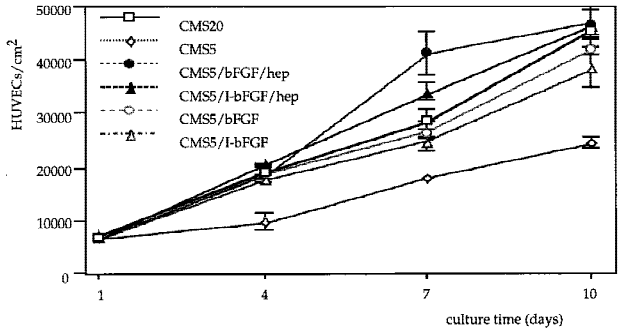

Figure 6. Comparison of the effects of nonradioactive I-labeled bFGF (I-bFGF) and unlabeled bFGF on the proliferation of HUVECs plated on fibronectin-coated TCPS in the presence of CMS 5 with or without 5 U/mL of heparin or in the presence of CMS 20. n = 3, ±SD.

when CMS 20 was used as the culture medium. When besides the growth factor 5 U/mL of heparin were 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 the 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).

^{125}I-bFGF-loading of surfaces

^{125}I-bFGF was bound from PBS/BSA (20 mg of 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 (Fig. 7). More bFGF was immobilized on surfaces containing heparin than on surfaces without heparin. When besides alb–hep conjugate fibronectin also 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.

Effect of surface-immobilized bFGF on cell proliferation

For the first proliferation experiment (Fig. 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 (CMS 5/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 subconfluent monolayers. All other surfaces showed confluent monolayers after 8 days of proliferation.

In the second experiment (Fig. 9), besides alb–hep conjugate-coated PS-CO_2, albumin-coated PS-CO_2 was used, applying a solution with a high bFGF concen-

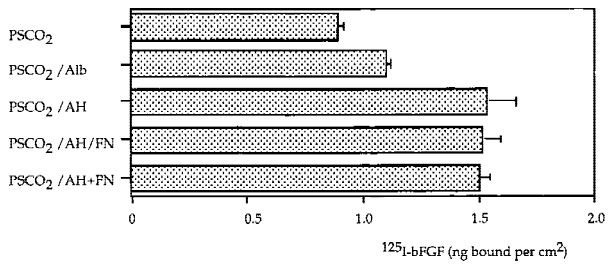

Figure 7. Quantity of ^{125}I-bFGF immobilized after incubation for 90 min at ambient temperature using 1 mL of 137 ng/mL of bFGF per well on uncoated PS-CO_2 or on PS-CO_2 coated with albumin, alb–hep conjugate (AH), or alb–hep conjugate in combination with fibronectin either added in a separate second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

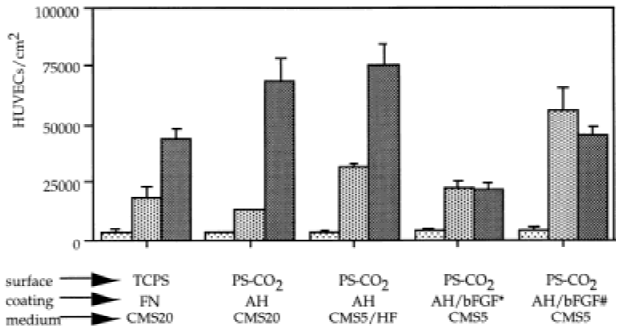

Figure 8. Number of endothelial cells on alb–hep conjugate (AH)-coated PS-CO_2 with or without immobilized bFGF and on fibronectin-coated TCPS at different proliferation times: ⚄ day 1, ▨ day 4, ■ day8. Two bFGF concentrations were used during immobilization: 28 ng/mL (bFGF*) and 280 ng/mL (bFGF#). Media used are presented in the graph. CMS 5 and CMS 20 are cell culture media with 5% and 20% human serum, respectively; CMS 5/HF is CMS 5 with 5 U/mL of heparin and 0.3 ng/mL of bFGF. n = 3, ±SD.

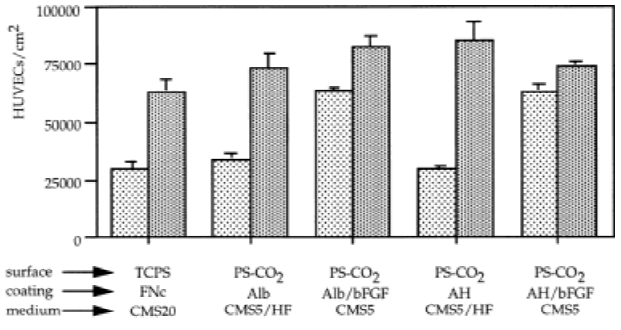

Figure 9. Number of endothelial cells on fibronectin-coated TCPS and on albumin- or alb–hep conjugate-coated PS-CO_2 with or without immobilized bFGF and at different proliferation times: ⚄ day 3, ▨ day 10. bFGF was immobilized using a concentration of 205 ng/mL. The media used are presented in the graph. CMS 5 and CMS 20 are cell culture media with 5%, 20% human serum, respectively; CMS 5/HF is CMS 5 with 5 U/mL of heparin and 0.3 ng/mL of bFGF. n = 3, ±SD.

tration (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 surfaces loaded with bFGF or the 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.

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 (Fig. 10). Moreover, no difference was found in the morphology of HUVECs grown on albumin-coated PS-CO_2, alb–hep conjugate-coated PS-CO_2, or fibronectin-coated TCPS (not shown).

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 tumor 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 ws 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 that was present in the solutions prior to storage at elevated temperature, the total release of ^{125}I and ^{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 be inhibited only to a certain extent by the addition of heparin. Since heparin is known to stabilize bFGF,[23–25] it is postulated that the tendency of labeled bFGF towards

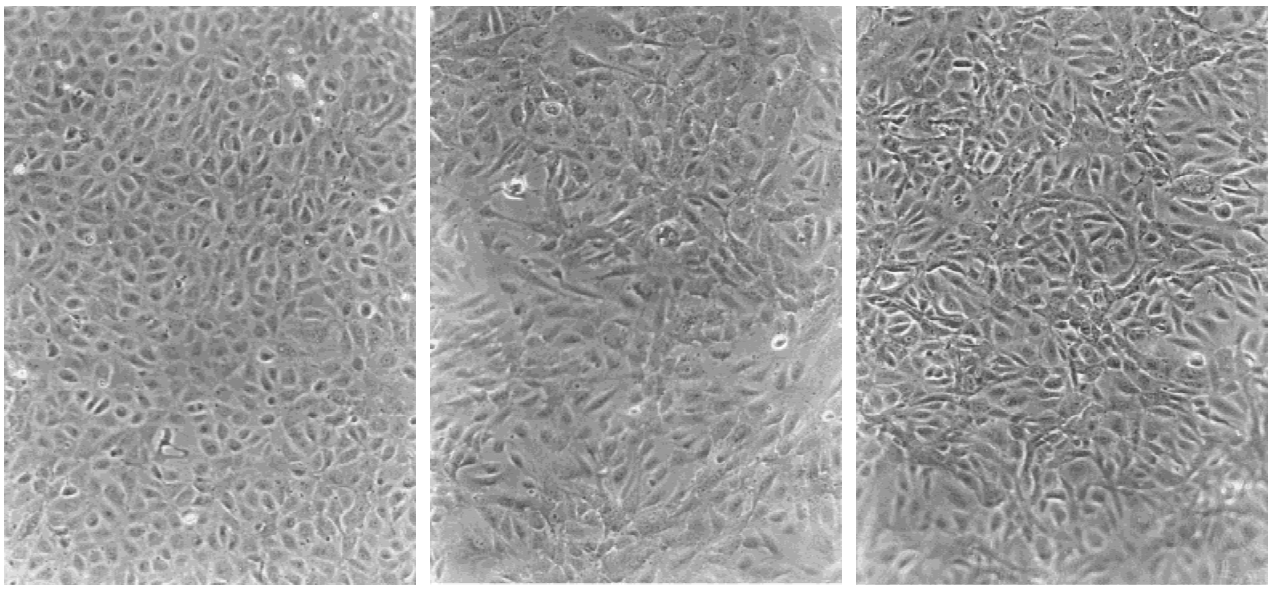

Figure 10. Light-microscopic images of HUVECs grown for 3 days on fibronectin-coated TCPS (A, CMS20) and on alb–hep conjugate immobilized on PS-CO_2 with bFGF either in the medium (B, CMS5/HF) or immobilized to the surface (C, CMS5). Original magnification: ×128.

degradation is a result of the labeling itself and is not caused merely by elevated temperatures. The increased stability of bFGF in the presence of heparin reported in the 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 days 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, such as, 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 when 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 of 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 super natant 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 n*M*)[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 either was 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 posttranslational modifications of the protein, as suggested by Linemayer et al.[44]

I-labeled bFGF (nonradioactive) 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 same 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 hydrophilicity upon albumin coating of the PS-CO_2 surface. In general, higher amounts of proteins adsorb to materials that are less hydrophilic.[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. The 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 and 3 ng/cm^2 for albumin and alb–hep conjugate, respectively. In the case of alb–hep conjugate, this corresponds to about one 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 four 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 of bFGF/mL were 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 3 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 has 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, for example, for 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 necessary 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.

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