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Binding and release of basic fibroblast growth factor from heparinized collagen matrices

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Abstract

Endothelial cell seeding is a promising method to improve the performance of small-diameter vascular grafts. Growth of endothelial cells seeded on the luminal surface of synthetic vascular grafts, coated with a matrix suitable for cell seeding (e.g. collagen), can be accelerated by local, sustained release of basic fibroblast growth factor (bFGF). In this study two potential matrices for in vivo endothelial cell seeding were studied with respect to bFGF binding and release: collagen crosslinked using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), as well as heparinized EDC/NHS-crosslinked collagen. bFGF binding was determined after incubation of circular samples (10 mm diameter) with 0.25 ml bFGF solution for 90 min. Immobilization of increasing amounts of heparin, also using EDC and NHS, to crosslinked collagen containing 14 free primary amino groups per 1000 amino acid residues (E/N14C) resulted in binding of increasing amounts of bFGF. A plateau in bFGF binding was observed for heparinized E/N14C containing approximately 2.0–3.0 wt% of immobilized heparin which was obtained using a molar ratio of EDC to heparin–carboxylic acid groups of 0.4 during heparin immobilization (E/N14C–H(0.4)). At concentrations up to 840 ng bFGF/ml, 10% of the added bFGF bound to E/N14C, while binding of bFGF to E/N14C–H(0.4) amounted to 22%. Both E/N14C and E/N14C–H(0.4) pre-loaded with bFGF showed sustained bFGF release. A burst release of 30% in endothelial cell culture medium (CM) was observed for E/N14C during the first 6 h, compared to 2% release from E/N14C–H(0.4). After 28 days, the bFGF release from E/N14C and E/N14C–H(0.4) in CM amounted to 100 and 65%, respectively. Combined results of binding and release of bFGF indicate that compared to E/N14C, E/N14C–H(0.4) is the substrate of choice for bFGF pre-loading and subsequent endothelial cell seeding.

Keywords: Vascular grafts; Collagen coating; Heparin immobilization; Endothelial cell seeding; Basic fibroblast growth factor; Sustained release

1. Introduction

Vascular grafts made of Dacron or expanded Teflon are successful in large-diameter applications [1]. In small-diameter positions (inner diameter less than 5 mm), however, graft performance is disappointing. Due to thrombus formation and stenosis especially long-term patency rates are low [2–5].

Endothelial cell seeding is a recognized strategy to improve the performance of small-diameter vascular grafts [6,7]. However, for successful endothelialization of synthetic vascular grafts a number of problems have to be dealt with. Dacron and expanded Teflon are poor substrates for endothelial cell seeding [8–10]. Regarding commercially available albumin-, gelatin- and collagen-coated synthetic vascular graft materials, glutaraldehyde or formaldehyde used for crosslinking of the protein coating evokes cytotoxic reactions, thus hampering endothelialization of the luminal graft surface [11,12]. Secondly, the supply of autologous endothelial cells is limited, leaving (large) parts of the thrombogenic graft surface exposed to blood directly after cell seeding. Vascular grafts with a confluent lining of endothelial cells can be obtained after expansion of cell numbers by cell culture in vitro. Drawbacks of this approach are the time interval between the need of an endothelialized vascular

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graft and its availability, as well as the increased risk of bacterial infection, limiting its use to non-emergency situations [13]. Per-operative seeding therefore is the preferred method for endothelialization of vascular grafts. As a result, low seeding densities have to be coped with.

We have previously developed a new collagen coating for the existing vascular graft materials, crosslinked using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). EDC/NHS-crosslinked collagen can be heparinized using EDC and NHS as well. During EDC/NHS-crosslinking of collagen, carboxylic acid groups of aspartic and glutamic acid residues in collagen react with EDC and NHS. This results in formation of NHS-activated carboxylic acid groups, which upon reaction with ε-amino groups from lysine and hydroxy lysine residues form peptide-like crosslinks and release NHS. Although EDC/NHS-crosslinked collagen is reported to be non-cytotoxic, both in vitro [14] and in vivo [15,16], collagen is a thrombogenic material. Immobilization of the anticoagulant heparin is a widely applied approach to reduce thrombus formation at blood–biomaterial interfaces [17,18]. Immobilization of heparin to collagen, therefore, may prevent platelet adhesion and blood coagulation [19,20], notably when the collagen coating is not yet completely covered by seeded endothelial cells.

In addition to providing a suitable substrate for endothelial cell seeding, proliferation of seeded endothelial cells might be further improved by local, sustained release of basic fibroblast growth factor (bFGF, a heparin binding protein). Controlled release of growth factors like bFGF is widely explored in tissue engineering. Vascularization of implanted matrices [21,22] or proliferation of cells (seeded) on various matrices [23–26] could be improved by local release of bFGF.

Local, sustained bFGF release has been shown to improve the proliferation of (seeded) endothelial cells in synthetic vascular grafts implanted in experimental animals. A fibrin glue sealant containing bFGF was developed to induce spontaneous endothelialization when applied as coating on synthetic vascular graft materials [27]. Greisler et al. studied endothelialization of ePTFE grafts coated with a mixture of fibrin glue, heparin and either aFGF or bFGF. By adjusting the ratio of heparin and growth factor in the fibrin glue, proliferation of endothelial cells was accelerated while proliferation of smooth muscle cells could be inhibited in vitro [28]. Spontaneous endothelialization of these vascular grafts was observed when implanted in rats, dogs and rabbits [29–31]. Polyurethane grafts coated with a mixture of photoreactive gelatin, heparin and bFGF, also demonstrated spontaneous endothelialization as a result of transmural in-growth of endothelial cells, when implanted in rat aortas [32].

In the present study, the binding of bFGF to and the release of bFGF from (heparinized) EDC/NHS-crosslinked collagen was determined.

2. Materials and methods

2.1. Materials

Unless otherwise stated, chemicals were obtained from Merck (Darmstadt, Germany), and were of the highest purity available.

2.2. Collagen films

All experiments were carried out using flat collagen films as model substrates. Type I insoluble collagen (1 g) derived from Bovine Achilles Tendon (Sigma, St. Louis, MO) was swollen overnight in 0.52 M acetic acid solution (50 ml) at 4°C. The mixture was dispersed with 50 g of crushed ice for 4 min in a Philips Blender and thereafter homogenized for 30 min at 4°C using an Ultra-Turrax T25 (IKA labortechnik, Staufen, BRD). The resulting slurry was filtered through a series of filters (Cellector screen, Bellco, Feltham, UK), with a pore size decreasing from 140 to 10 μm, mounted in 47 mm diameter Swinnex disc filter holders (Millipore, Etten-Leur, The Netherlands). After de-aeration at a pressure of 0.06 mbar, the resulting suspension was casted as a film with a thickness of 3 mm on a flat poly(ethylene terephthalate) (PET) surface, using a casting knife. After drying at room temperature, a collagen film with a thickness of approximately 50 μm was obtained.

2.3. Collagen crosslinking

Collagen films were crosslinked using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in a 0.05 M buffer of 2-morpholinoethane sulfonic acid (MES buffer, pH 5.40) [33]. Before crosslinking, dried collagen films were incubated with MES buffer for at least 30 min. Subsequently, the films were immersed in a solution of EDC and NHS in MES buffer under gentle shaking. For the crosslinking reaction, 1.731 g EDC and 0.415 g NHS in 215 ml MES buffer were used per gram of collagen, corresponding to a molar ratio of EDC : NHS : collagen–carboxylic acid groups of 7.0 : 2.9 : 1.0. Using these conditions, crosslinking was completed in 4 h. Thereafter, the collagen was washed with 0.1 M Na_2HPO_4 solution (2 h) and demineralized water (four times for 30 min) [33,34]. The residual number of free primary amino groups in collagen after crosslinking was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, from Fluka, Buchs, Switzerland), according to a slightly modified procedure as described by Wang et al. [35–37]. The shrinkage temperature of (crosslinked) collagen, indicating the resistance against thermal denaturation, was determined using differential scanning calorimetry (DSC) [38].

2.4. ^{3}H-labeling of heparin

Heparin sodium salt (Bufa Chemie, Castricum, the Netherlands) was used. This heparin preparation from porcine mucosa has the following characteristics [39]: M_n = 12,500 g/mol (molecular weight distribution 3000–30,000 g/mol), activity = 195 IU/mg, 18.75 mol of carboxylic acid groups (Hep–COOH) per mol of heparin.

Heparin was tritiated using the method described by Hatton et al. [40], with slight modifications. Briefly, heparin (3.00 g) was dissolved in distilled water (400 ml), and the pH was adjusted to 8.0 with NaOH solution (4 M). NaB^3H_4 (100 mCi, 7.5 Ci/mmol, Amersham, UK) was added, and the reaction was allowed to proceed for 3 h at room temperature. The resulting ^{3}H-labeled heparin was purified by dialysis at 4°C against subsequently 2 M NaCl in phosphate-buffered saline (PBS, from NPBI, Emmer Compascuum, the Netherlands, pH 7.40), PBS (2 times) and distilled water (3 times). ^{3}H-heparin was isolated by lyophilization and stored in a desiccator at room temperature. The yield was 2.80 g, with a specific activity of 76.9 kBq/mg. Free label content was less than 0.5%, as determined by gel filtration using a PD10-Sepharose column (Pharmacia, Upsala, Sweden).

2.5. Heparin immobilization

Crosslinked collagen films were incubated with 0.05 M MES-buffer (pH 5.60) for at least 30 min. Carboxylic acid groups of heparin (Hep–COOH) were activated using EDC and NHS at a fixed molar ratio of 0.6, and a molar ratio of EDC : Hep–COOH of 0–8.0. To a 2% (w/v) solution of (^{3}H-labeled) heparin in 0.05 M MES-buffer (pH 5.60), EDC and NHS were added. After 10 min, 1 g of crosslinked collagen (containing 14 free primary amino groups per 1000 amino acid residues, E/N14C) was reacted with 188.3 ml of EDC/NHS-activated heparin solution, giving a molar ratio of heparin to free primary amino groups of 2. After 2 h of incubation, the heparinized E/N14C (E/N14C–H) was washed with 0.1 M Na_2HPO_4 (2 h), 4 M NaCl (4 times for 24 h) and distilled water (3 times for 24 h).

The amount of immobilized heparin was determined either by measurement of the radioactivity of the collagen samples after immobilization of ^{3}H-labeled heparin, or using toluidine blue as described elsewhere [41].

2.6. ^{125}I-labeling of bFGF

Human recombinant basic fibroblast growth factor (bFGF, Gibco, Paisley, UK) was labeled with ^{125}I using Iodobeads [42,43]. Briefly, to 3 Iodobeads (Iodobeads iodination reagents, Pierce, Rockford, IL) 500 μCi ^{125}I-Na (Amersham) in 100 μl phosphate buffer (100 mM, pH 7.0) was added. After 5 min, bFGF solution in phosphate buffer (1 ml, 100 μg bFGF/ml) was added and iodination was carried out under gentle shaking at room temperature. After 15 min, the beads were removed and rinsed in 150 μl phosphate buffer. To the total aliquot of phosphate buffer, 600 μl bovine serum albumin (BSA, Sigma A7030) solution in phosphate buffer was added, giving a final albumin concentration of 1 mg/ml. Residual ^{125}I was removed from the ^{125}I-labeled bFGF solution by purification over a series of 3 PD10-columns. Thereafter, the bFGF solution (with a specific activity of 36.4 ± 1.7 kBq/μg bFGF) was aliquotted and stored at −20°C until use.

2.7. ^{125}I-bFGF binding studies

Circular films of E/N14C and heparinized E/N14C with a diameter of 10 mm were incubated overnight in 5 ml PBS. After blotting dry, the films were incubated with 0.25 ml bFGF solution (0–320 ng bFGF/ml) in PBS (NPBI, Emmer Compascuum, The Netherlands) containing 1 mg/ml BSA, for 90 min at room temperature. Thereafter, the samples were washed in 5 ml PBS (2 times for 5 min, removing all non-bound bFGF as determined previously), and the radioactivity of the samples was measured using a Compugamma 1282 γ-counter (LKB, Stockholm, Sweden). Alternatively, bFGF binding was investigated using bFGF solutions in PBS (1 mg/ml BSA) containing 0–2.5 M NaCl. In order to mimic endothelial cell culture conditions, (heparinized) E/N14C samples were incubated overnight in 5 ml PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (both from Gibco, Paisley, UK) before bFGF binding.

2.8. bFGF release experiments

Circular collagen films with a diameter of 10 mm were loaded with bFGF as described above, either using unlabeled bFGF (840 ng/ml) or ^{125}I-labeled bFGF (280 ng/ml). After washing with PBS, the samples were transferred to release medium (5 ml), which consisted of endothelial cell culture medium (CM) containing 5% human serum as used for endothelial cell culture. Endothelial cell culture medium (CM) consisted of a mixture of equal volumes of RPMI 1640 and M199, containing 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone, 2 mM L-glutamax (all from Gibco). Release of bFGF was measured at 37°C. Medium was replaced every 24 h, and stored at −20°C until assayed. The release of unlabeled bFGF was studied for 10 days. The bFGF concentration in the supernatant was determined using a sandwich ELISA (Quantikine bFGF ELISA, R & D Systems, Abingdon, UK). The release of ^{125}I labeled bFGF was studied for 28 days.

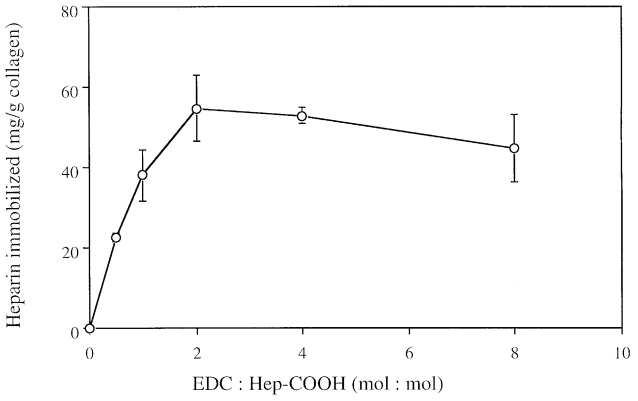

Fig. 1. Immobilization of ^{3}H-heparin to E/N14C, as a function of the molar ratio of EDC to Hep–COOH used for immobilization ($n = 4$, mean $\pm$ SD).

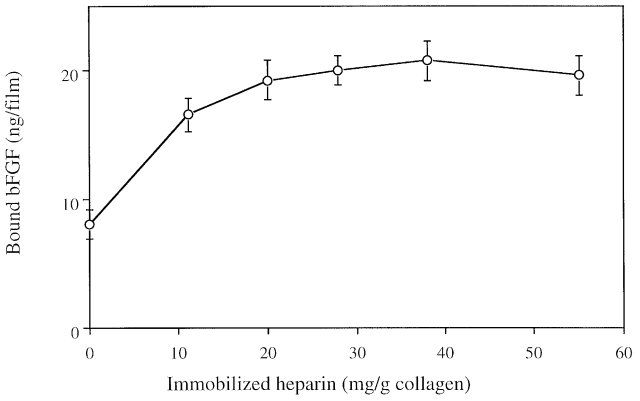

Fig. 2. Binding of ^{125}I-labeled bFGF to heparinized E/N14 collagen, as a function of the amount of heparin immobilized ($n = 4$, mean $\pm$ SD). Circular films with a diameter of 10 mm were incubated for 90 min with 0.25 ml ^{125}I-bFGF solution (360 ng/ml).

3. Results

3.1. Collagen crosslinking

Upon crosslinking with EDC and NHS, the number of free primary amino groups per 1000 amino acid residues decreased while the shrinkage temperature increased. At the conditions used, collagen crosslinking resulted in a material containing approximately 14 free primary amino groups per 1000 amino acid residues (E/N14C), compared to 27 in native collagen [44]. The shrinkage temperature increased from 55.4°C for native collagen to 75.9°C for E/N14C.

3.2. Heparin immobilization

Heparin was covalently immobilized to E/N14C, as shown in detail elsewhere [45]. In Fig. 1, the amount of immobilized heparin is given as a function of the molar ratio of EDC to carboxylic acid groups of heparin (Hep–COOH) used for immobilization. The amount of immobilized heparin increased with increasing ratios of EDC : Hep–COOH to a maximum of approximately 5–5.5% heparin (w/w) per gram of collagen at a molar ratio of EDC : Hep–COOH of 2. Heparin was immobilized homogeneously through the entire thickness of the crosslinked collagen film, as shown using Alcian Blue staining of paraffin coupes of the material [45].

3.3. bFGF binding

Binding of ^{125}I-labeled bFGF from PBS (1 mg/ml BSA) to E/N14C and heparinized E/N14C reached a plateau value within 90 min of incubation (data not shown). Immobilization of increasing amounts of heparin to E/N14C led to increased binding of bFGF (Fig. 2). A plateau value in bFGF binding was observed for materials obtained after heparin immobilization using a molar ratio EDC : Hep–COOH of 0.4–0.6. This corresponds to approximately 20–30 mg heparin immobilized per gram of collagen, which is lower than the maximally achievable amount of immobilized heparin (55 mg heparin/g collagen).

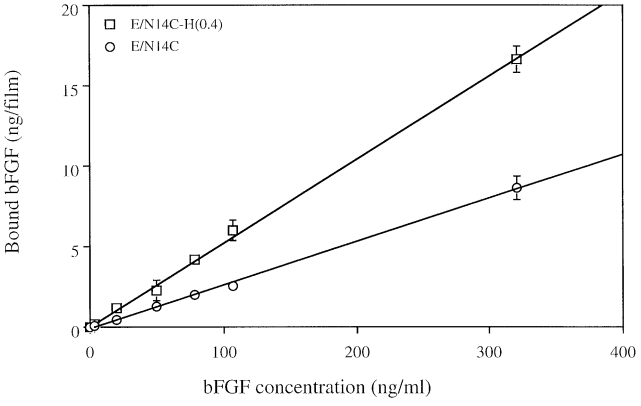

Fig. 3. Binding of ^{125}I-labeled bFGF to E/N14 collagen and E/N14 collagen–heparin(0.4), as a function of the bFGF concentration before incubation ($n = 3$, mean $\pm$ SD). Circular films with a diameter of 10 mm were incubated for 90 min with 0.25 ml ^{125}I-bFGF solution (0–320 ng/ml).

For further experiments, E/N14C and E/N14C heparinized using a molar ratio EDC : Hep–COOH of 0.4 (E/N14C–H(0.4)) were used. Binding of bFGF to E/N14C and E/N14C–H(0.4) showed a linear correlation with the concentration of bFGF used for incubation (Fig. 3). Up to concentrations of 320 ng/ml bFGF, bFGF binding to E/N14C and E/N14C–H(0.4) amounted to 10 and 22%, respectively. In separate experiments, similar observations were made for bFGF concentrations up to 840 ng/ml (data not shown).

In a control experiment, bFGF binding from PBS containing 20 mg/ml BSA was studied. Higher albumin concentrations are usually applied to prevent loss of bFGF e.g. due to adsorption to the walls of test-tubes

during experiments. When using a solution of bFGF in PBS containing 20 mg/ml BSA, bFGF binding to both E/N14C and E/N14C–H(0.4) showed a linear correlation with bFGF concentration. Binding of bFGF to both matrices, however, was approximately 25% lower when compared to bFGF binding from PBS containing 1 mg/ml BSA (data not shown).

In a second control experiment, bFGF solutions (100 ng bFGF/ml, 1 mg BSA/ml in PBS) used for incubation with E/N14C or E/N14C–H(0.4) were used for a second incubation with fresh substrates. In this experiment bFGF-binding to E/N14C as first substrate was $11.1 \pm 0.7\%$. Subsequent incubation of the partially depleted bFGF solution with E/N14C or E/N14C–H(0.4) resulted in additional bFGF binding of 3.6 and 9.2%, respectively, when bFGF binding was expressed as percentage of the bFGF present in the original 100 ng/ml bFGF solution (Table 1). When using E/N14C–H(0.4) as first substrate, bFGF binding amounted to $22.6 \pm 0.5\%$. The second incubation of this bFGF solution with E/N14C and E/N14C–H(0.4) resulted in bFGF binding of 1.0 and 2.8%, respectively. Substantial bFGF binding to a second substrate was only observed after subsequent incubation of a bFGF solution with E/N14C and E/N14C–H(0.4) (9.2%). Combined bFGF binding to both substrates was $20.3 \pm 2.6\%$, which was not significantly different from bFGF binding to E/N14C–H(0.4) alone ($22.6 \pm 0.5\%$ in this experiment). These results indicate that only a fraction of the bFGF was capable of binding to (heparinized-) EDC/NHS-crosslinked collagen.

Overnight incubation of E/N14C and E/N14C–H(0.4) with a solution of penicillin and streptomycin in PBS, a procedure used prior to seeding of HUVECs on these substrates, did not influence binding of bFGF from PBS (1 mg/ml BSA) (data not shown). Binding of bFGF to E/N14C and E/N14C–H(0.4) was dependent on the NaCl concentration (Fig. 4). Solutions of bFGF dissolved in phosphate buffer (1 mg/ml BSA) containing increasing NaCl concentrations were used for loading of E/N14C and E/N14C–H(0.4). For E/N14C, maximal bFGF binding was observed at a physiological NaCl concentration. bFGF binding from PBS (0.14 M NaCl) to E/N14C was approximately 2-fold higher when compared to solutions without NaCl. Compared to bFGF binding from PBS, bFGF binding gradually decreased with increasing NaCl concentration. For E/N14C–H(0.4) maximal bFGF binding was observed at 0 to 0.6 M NaCl; at higher NaCl concentrations bFGF binding also gradually decreased.

Table 1
Binding of ^{125}I-labeled bFGF to E/N14 collagen and E/N14 collagen–heparin (0.4) during second incubation

First substrate	Binding to second substrate	
	E/N14C	E/N14C–H(0.4)
E/N14C	$3.6 \pm 0.4\%$	$9.2 \pm 0.6\%$
E/N14C–H(0.4)	$1.0 \pm 0.1\%$	$2.8 \pm 0.9\%$

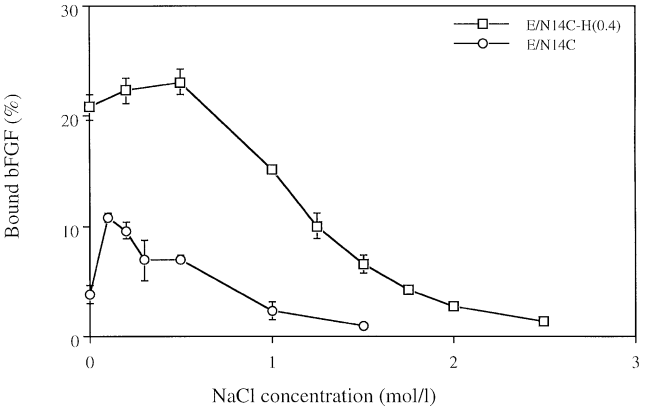

Fig. 4. Binding of ^{125}I-labeled bFGF to E/N14 collagen and heparinized E/N14 collagen as function of the NaCl concentration ($n = 3$, mean $\pm$ SD). Circular films with a diameter of 10 mm were incubated for 90 min with 0.25 ml ^{125}I-bFGF solution (100 ng/ml).

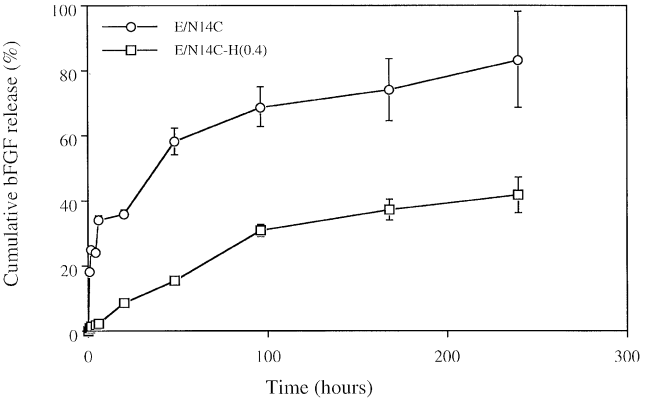

Fig. 5. Release of bFGF from E/N14 collagen and E/N14 collagen–heparin(0.4) in culture medium supplemented with 5% serum ($n = 3$, mean $\pm$ SD). Circular films with a diameter of 10 mm were loaded for 90 min with 0.25 ml unlabeled bFGF solution (840 ng/ml). bFGF concentrations in supernatant were determined using a bFGF-ELISA.

3.4. Release of bFGF

In Fig. 5, the release of bFGF from E/N14C and E/N14C–H(0.4) at 37°C in endothelial cell culture medium (CM) supplemented with 5% human serum is plotted. E/N14C and E/N14C–H(0.4) films were incubated with bFGF solutions, resulting in 27.2 ± 1.3 and 49.0 ± 1.9 ng bFGF bound per film, respectively. The initial release of bFGF from E/N14C–H(0.4) was slower, compared to the release of bFGF from E/N14C. During the first 6 h, 9.3 ± 0.3 ng bFGF (34%) was released from E/N14C, while in the same period only 1.0 ± 0.1 ng bFGF (2%) was released from E/N14C–H(0.4). After

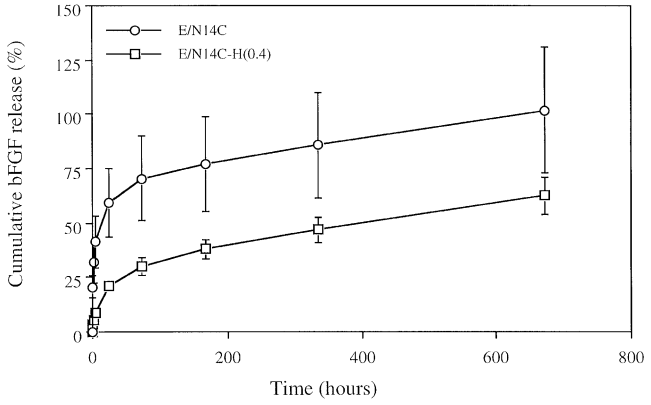

Fig. 6. Release of ^{125}I-labeled bFGF from E/N14 collagen and heparinized E/N14 collagen ($n = 3$, mean $\pm$ SD). Circular films with a diameter of 10 mm were loaded for 90 min with 0.25 ml ^{125}I-labeled bFGF solution (280 ng/ml).

10 days, however, the cumulative absolute bFGF release from E/N14C equaled 22.4 $\pm$ 4.0 ng (83 $\pm$ 14%), which was comparable to the absolute bFGF release from E/N14C–H(0.4) (20.5 $\pm$ 2.6 ng, 42 $\pm$ 5%).

In a separate experiment the release of ^{125}I-labeled bFGF from E/N14C and E/N14C–H(0.4) at 37°C in CM supplemented with 5% human serum was measured for 28 days (Fig. 6). E/N14C and E/N14C–H(0.4) films were pre-loaded with 8.1 $\pm$ 1.2 and 19.4 $\pm$ 1.6 ng bFGF, respectively. Regarding ^{125}I-labeled bFGF release, results were similar to results obtained using unlabeled bFGF in combination with a bFGF ELISA. Initial release of ^{125}I-labeled bFGF from E/N14C was faster compared to the bFGF release from E/N14C–H(0.4). During the first 4 h 3.6 $\pm$ 0.2 ng bFGF was released from E/N14C, while 1.6 $\pm$ 1.1 ng bFGF was released from E/N14C–H(0.4). After 168 h, cumulative absolute bFGF release from E/N14C (6.2 $\pm$ 1.1 ng, 77 $\pm$ 22%) was comparable to absolute bFGF release from E/N14C–H(0.4) (7.3 $\pm$ 0.7 ng, 38 $\pm$ 5%).

Using E/N14C matrices with varying amounts of heparin immobilized (obtained using a ratio of EDC: Hep–COOH of 0.2 to 2.0, Fig. 1), release profiles similar to the release profile of bFGF from E/N14C–H(0.4) were obtained (data not shown).

4. Discussion

Growth factors can generally be defined as proteins which promote proliferation and migration of cells, by interaction with specific cell membrane receptors. Basic fibroblast growth factor (bFGF) is an 18 kDa protein, which induces, inter alia, the proliferation of endothelial cells, fibroblasts, smooth muscle cells and chondrocytes [46,47]. Basic FGF is also known as heparin-binding growth factor, because of its high affinity for heparin and heparan sulfate. At physiological pH and temperature, the in vitro half-lifetime of bFGF activity is approximately 12 h [48]. Binding of bFGF to heparin induces a conformational change in the bFGF molecule [49], resulting in an increased resistance against thermal denaturation and enzymatic degradation, and a reduced inactivation at acidic pH [48–51]. Furthermore, binding of bFGF to heparin or heparan sulfate facilitates the binding of bFGF to high-affinity cell membrane receptors [49,52]. Although bFGF is localized in almost any tissue examined [53], suggesting that it is needed continuously, the turnover time of target cells like for example endothelium is measured in years. Heparan sulfate is abundantly present in the extracellular matrix (ECM) of endothelial cells. The high affinity of bFGF for glycosaminoglycans suggests that the ECM may function as a storage pool for bFGF [54,55]. When the vascular wall is damaged, bFGF can be released through several mechanisms [56] and proliferation of endothelial cells will be induced. By binding of bFGF to a heparinized collagen-coated vascular graft, this process of induction of cell growth can be mimicked.

Increased binding of radio-labeled bFGF was observed with increasing amounts of immobilized heparin, giving maximal bFGF binding for materials obtained after heparin immobilization using a molar ratio of EDC: Hep–COOH of 0.4–0.6. In a previous study the anticoagulant activity of heparin immobilized to E/N14C was determined [45]. E/N14C matrices heparinized using a molar ratio of EDC: Hep–COOH of 0.4 showed maximal thrombin inhibitory activity and a maximal reduction in contact activation. Combining these results, for further experiments E/N14C matrices heparinized using a molar ratio of EDC to Hep–COOH of 0.4 (E/N14C–H(0.4)) were used.

Binding of bFGF to E/N14C and E/N14C–H(0.4) showed a linear correlation with bFGF concentrations up to 840 ng/ml. Depending on molecular weight, heparin can bind up to 13 molecules of bFGF per molecule of heparin in solution [51,57]. In the case of E/N14C–H(0.4), maximal one molecule of bFGF was bound per 1000 molecules of immobilized heparin, indicating that saturation of binding will occur only at much higher bFGF concentrations. The low Kd reported for binding of bFGF to cell surface heparan sulfate or heparin in solution (10^{-8} to 10^{-9} M) [52] led us to expect high-efficiency binding of bFGF from solution to heparinized crosslinked collagen matrices. Low binding of bFGF was observed, however, which was most likely not caused by material characteristics, but probably due to depletion of the fraction of bFGF capable of binding to E/N14C and E/N14C–H(0.4) (Table 1). When both substrates were incubated with fresh bFGF solution after a first incubation with bFGF, during both first and second incubation, an illustrative bFGF binding of 10 and 22% was observed for E/N14C and E/N14C–H(0.4),

respectively (data not shown). One might speculate that low binding is caused by e.g. deficient folding of the recombinant protein during manufacturing, resulting in a fraction of bFGF not capable of binding [58].

Binding of bFGF and heparin is mediated by ionic interaction between both 2-0-sulfate groups and *N*-sulfate groups of heparin molecules [59,60] and certain lysine and arginine residues in bFGF [61,62]. Studies concerning binding of bFGF to collagen are not reported in literature, but binding of bFGF to collagen is likely to involve non-specific ionic interactions. Additionally, hydrophobic interactions might account for the increased bFGF binding from PBS to E/N14C observed when increasing the NaCl concentration from 0 to 0.15 M. This, however, is speculative, and a conclusive explanation cannot be given. In this study, binding of bFGF to E/N14C and E/N14C–H(0.4) decreased with increasing NaCl concentrations, indicating the presence of ionic interactions between bFGF and (heparinized) E/N14C. The gradual decrease in bFGF binding with increasing NaCl concentrations suggests the involvement of multiple binding sites with a range of binding strengths located on both E/N14C and E/N14C–H(0.4).

When used for cell culture in our laboratory, (heparinized) crosslinked collagen is incubated overnight in a solution containing penicillin and streptomycin, a procedure used to decrease the risk of bacterial infection during cell culture. Following incubation with penicillin and streptomycin, matrices are pre-loaded with bFGF and subsequently endothelial cells are seeded. Incubation of these matrices with antibiotics did not influence bFGF binding despite the fact that these antibiotics are positively charged and capable of binding to heparin.

Release of bFGF was measured in CM supplemented with 5% serum, resembling the medium used for culture of HUVECs seeded on bFGF-loaded (heparinized) collagen matrices [63]. Release of bFGF from E/N14C was initially faster than bFGF release from E/N14C–H(0.4), probably due to the higher affinity of bFGF for heparin. However, because loading of E/N14C–H(0.4) with bFGF was approximately two-fold higher compared to E/N14C, after 10 days in CM 5% comparable amounts of bFGF were released from both materials. bFGF released from both E/N14C and E/N14C–H(0.4) was biologically active, and able to stimulate endothelial cell proliferation in vitro, as described elsewhere [63].

As there was some doubt regarding the stability of radio-labeled bFGF, its release was also measured using unlabeled bFGF in combination with an ELISA. Release profiles of unlabeled bFGF and ^{125}I-labeled bFGF were in good agreement. Although initial release from E/N14C–H(0.4) as measured using ^{125}I-bFGF was somewhat higher compared to release measured using unlabeled bFGF (deviation maximal 10%) results expressed as percentage release show no difference after longer periods of time (e.g. 4 days). These observations are in agreement with results obtained by others when measuring bFGF release using ^{125}I-bFGF, in combination with additional techniques [64,65].

When injected intravenously, bFGF is cleared rapidly from blood. In rats, half-lifetimes of 1.5–3 min are reported [66,67]. When E/N14C or E/N14C–H(0.4) are used in vivo as matrix for endothelial cell seeding of vascular grafts, a rapid clearance of bFGF *released* from these matrices is to be expected too. For bFGF bound to E/N14C, a burst-release of 30–35% was observed during the first 6–20 h, in contrast to E/N14C–H(0.4) from which bFGF release was more gradual, resulting in a substantial loss of bFGF pre-loaded to E/N14C. In addition to a slower release rate of bound bFGF, heparin immobilization to E/N14C resulted in approximately two-fold higher bFGF loading. Therefore, compared to E/N14C, improved proliferation of seeded HUVECs on E/N14C–H(0.4) over a prolonged period of time is to be expected. Our results indicate that E/N14C–H(0.4) is a candidate matrix for bFGF pre-loading and subsequent endothelial cell seeding.

5. Conclusions

Immobilization of 2–3 wt% heparin to E/N14C results in approximately two-fold increase of bFGF binding. Both E/N14C and E/N14C–H(0.4) pre-loaded with bFGF show sustained bFGF release in endothelial cell culture medium over a prolonged period of time. Because of the increased bFGF binding and the slower, more gradual release of bFGF from E/N14C–H(0.4), E/N14C–H(0.4) pre-loaded with bFGF is expected to be a better substrate for endothelial cell seeding than E/N14C without immobilized heparin.

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