

Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration

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Received 8 June 2004; accepted 8 September 2004

Available online 27 October 2004

Abstract

Basic fibroblast growth factor (bFGF) was immobilized to hydrogel scaffolds with retention of mitogenic and chemotactic activity. The bFGF was functionalized in order to incorporate it covalently within polyethylene glycol (PEG) hydrogel scaffolds by reaction with acryloyl-PEG-NHS. Hydrogels were formed by exposing aqueous solutions of PEG diacrylate, acryloyl-PEG-RGDS, and acryloyl-PEG-bFGF to long-wavelength ultraviolet light in the presence of a photoinitiator. These bFGF-modified hydrogels with RGD adhesion sites were evaluated for their effect on vascular smooth muscle cell (SMC) behavior, increasing SMC proliferation by ~41% and migration by ~15%. A covalently immobilized bFGF gradient was formed using a gradient maker to pour the hydrogel precursor solutions and then photopolymerizing to lock in the concentration gradient. Silver staining was used to detect the bFGF gradient, which increased linearly along the hydrogel's length. Cells were observed to align on hydrogels modified with a bFGF gradient in the direction of increasing tethered bFGF concentration as early as 24 h after seeding. SMCs also migrated differentially, up the concentration gradient, on bFGF-gradient hydrogels compared to control hydrogels with and without a constant bFGF concentration. These hydrogel scaffolds may be useful for studying protein gradient effects on cell behavior and for directing cell migration in tissue-engineering applications.

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Keywords: Tissue engineering; Hydrogel; Basic fibroblast growth factor; Gradient; Migration; Smooth muscle cell

1. Introduction

Polyethylene glycol (PEG) diacrylate hydrogel scaffolds may be useful for optimizing engineered tissue formation. These hydrogel scaffolds are highly resistant to protein adsorption due to PEG's hydrophilicity, yet provide the opportunity to incorporate bioactive factors [1–4]. This combination of protein-resistant scaffold and incorporated biofunctionality makes it possible to control the identity and concentration of bioactive factors presented to cells [1,2]. For example, fibroblasts, osteoblasts, and vascular smooth muscle cells (SMCs) have all been shown to adhere to and grow on PEG-

based hydrogel scaffolds when these scaffolds were modified with adhesion peptides [5–8]. In fact, the incorporation of adhesion peptides was required for cells to interact at any significant level with hydrogel scaffolds, while cell attachment and spreading was shown to vary depending on the bulk peptide concentration [5–8]. Thus, these scaffolds provide a blank slate to which cell adhesion peptides can be added in a controlled fashion to enable cell interaction. However, many of these studies have simply focused on studying cell interactions with hydrogels modified with the RGD adhesion peptide. Additional signals that may include a combination of cell adhesion peptides and growth factors will be necessary to elicit cell behavior that is conducive to the formation of engineered tissues [9,10].

Previous studies have demonstrated that it is possible to covalently immobilize growth factors with retained

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effects on proliferation and extracellular matrix production. Kuhl and Griffith-Cima tethered epidermal growth factor (EGF) to glass slides via a PEG-based polymer chain and showed that immobilized EGF stimulated DNA synthesis and morphological changes in rat hepatocytes [11]. These alterations were comparable to those observed in the presence of soluble EGF. Similarly, transforming growth factor- β 2 (TGF- β 2) can retain its effect on collagen tissue deposition when conjugated to collagen via a PEG-based chain [12]. Thus, conjugation to a polymer chain does not impede the interaction between cell receptor binding sites and the growth factors, EGF and TGF- β 2, making it possible to control the growth factor's localized concentration.

This technology for covalently immobilizing growth factors can be readily applied to PEG hydrogel scaffolds to influence cell behavior. For example, EGF and TGF- β have been tethered to PEG chains by reaction with an *N*-hydroxysuccinimidyl ester of PEG monoacrylate [13,14]. These PEG-modified growth factors can then be immobilized to PEG hydrogel scaffolds during photopolymerization. The immobilized EGF retained its mitogenic activity and also promoted cell migration on hydrogel surfaces containing both EGF and the RGD adhesion peptide [13]. The TGF- β -modified scaffolds promoted collagen production by vascular SMCs seeded within the hydrogel scaffolds, and the resultant engineered tissues had improved mechanical properties compared to those formed with scaffolds modified with only RGD [14]. From these two examples, it is clear that growth factor immobilization in the PEG diacrylate hydrogel system is feasible and can help to influence cell behavior within the scaffold to optimize tissue formation.

In this study, hydrogel scaffolds were modified with the growth factor, basic fibroblast growth factor (bFGF), to stimulate two important aspects of tissue formation—proliferation and migration. bFGF was selected because it is a potent mitogen and chemotactic agent for vascular SMCs [15,16]. bFGF is also normally found in the vascular environment as it is associated with the extracellular matrix [15,16]. Thus, this growth factor may play an important role in developing engineered tissues that more closely mimic the structural and functional properties of native blood vessels. Furthermore, we have developed methods that allow immobilization of the growth factor in a concentration gradient. This should mimic the presentation of bioactive factors found *in vivo* to elicit enhanced and directional cell migration, which may be useful for optimizing tissue formation. The ability to create a stable, immobilized gradient with a known concentration profile of a growth factor should improve our understanding of cellular responses to gradients. Such materials should also allow

guidance of cell migration in many tissue-engineering applications.

2. Materials and methods

2.1. Cell maintenance

Human aortic smooth muscle cells (HASMCs) were obtained from Cell Applications (San Diego, CA). They were maintained at 37 °C/5% CO₂ on Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 2mM L-glutamine (Sigma, St. Louis, MO), 1000 U/L penicillin (Sigma, St. Louis, MO), and 100 mg/L streptomycin (Sigma, St. Louis, MO). For the gradient bFGF studies, SMCs were maintained on SMC Growth Medium (Cell Applications) without the bFGF supplement. All experiments were conducted using cells at passage 6–8.

2.2. Synthesis and characterization of the PEG-bFGF conjugate

Recombinant human bFGF (Promega, Madison, WI) was conjugated to PEG by reacting it with acryloyl-PEG-*N*-hydroxysuccinimide (acryloyl-PEG-NHS, 3400 Da; Shearwater Polymers, Huntsville, AL) in a 1:15 (peptide:PEG) molar ratio in 50mM sodium bicarbonate (pH 8.5) for 2h. The polymer solution was then lyophilized and stored at –80 °C.

A Western blot was used to analyze the resulting acryloyl-PEG-bFGF. 480 ng of unmodified and PEG-conjugated bFGF were separated on a 4–15% SDS-PAGE gel (BioRad, Hercules, CA) and transferred to a Trans-blot Transfer Medium nitrocellulose membrane (BioRad). The membrane was incubated overnight at 4 °C with 5% milk in buffer containing 0.1% (vol/vol) Tween 20 in TBS (TBST). The membrane was incubated with rabbit anti-bFGF (Sigma) at a 1:2000 dilution for 1 h at room temperature. After two washes (5 min each) with TBST, peroxidase-labeled goat anti-rabbit IgG (Sigma) was added at 1:3000 dilution and incubated for 1 h at room temperature. The membrane was washed again in TBST and then treated with Opti-4CN chemiluminescence reagent (BioRad) for detection.

2.3. Bioactivity of the PEG-bFGF conjugate

The bioactivity of the PEG-bFGF conjugate was assessed by monitoring cell proliferation in response to medium supplemented with no bFGF, unmodified bFGF, or PEG-conjugated bFGF. HASMCs were seeded in 24-well plates at a density of 7900 cells/cm² and incubated overnight in a 37 °C/5% CO₂ environment. The media were then exchanged for media

supplemented with 0, 2.86 nmol/L unmodified bFGF, or 2.86 nmol/L PEG-conjugated bFGF. After 2 days, cells were rinsed with phosphate-buffered saline, trypsinized, and then counted using a Coulter counter (Multisizer #0646; Coulter Electronics, Hialeah, FL) to determine cell numbers.

2.4. Hydrogel preparation

PEG diacrylate was synthesized by dissolving 12 g dry PEG (6000 Da; Fluka, Milwaukee, WI) in 36 mL anhydrous dichloromethane. 0.25 g triethylamine and then 0.43 g acryloyl chloride (Lancaster Synthesis, Windham, NH) were added dropwise, and the mixture was stirred under argon for 48 h. The resulting solution was then washed with 2 M K₂CO₃ and separated into aqueous and dichloromethane phases to remove HCl. The dichloromethane phase was subsequently dried with anhydrous MgSO₄ (Fisher Scientific, Pittsburg, PA), and the PEG diacrylate was then precipitated in diethyl ether, filtered, and dried under vacuum at room temperature overnight. The resultant polymer was dissolved in *N,N*-dimethylformamide-*d*₇ (Sigma, St. Louis, MO) and characterized via proton NMR (Avance 400 MHz; Bruker, Billerica, MA) to determine the degree of acrylation.

A cell adhesive component was prepared by reacting acryloyl-PEG-NHS with the adhesion peptide, RGDS (American Peptide, Sunnyvale, CA), in 50 mM sodium bicarbonate (pH 8.5) at a 1:1 molar ratio for 2 h. The solution was then lyophilized and stored at –20 °C.

Hydrogels were formed by combining 0.1 g/mL PEG diacrylate and 2.8 μmol/mL acryloyl-PEG-RGDS in 10 mM HEPES buffered saline (pH 7.4). This solution was then sterilized by filtration (0.8 μm prefilter and 0.2 μm filter; Gelman Sciences, Ann Arbor, MI). For hydrogels containing bFGF, 2.86 nmol/L unmodified bFGF or 2.86 nmol/L PEG-conjugated bFGF was added to the polymer solution. 10 μL/mL of 2,2-dimethyl-2-phenyl-acetophenone in *N*-vinylpyrrolidone (300 mg/mL) was added as the photoinitiator. This solution was then placed in a rectangular glass mold (0.2 mm thickness) and exposed to long-wavelength ultraviolet (UV) light (365 nm, 10 mW/cm²) for 60 s.

2.5. bFGF incorporation in hydrogels

Hydrogels (~50 μL volume) were formed in a round mold (32 mm²) as described above, except for the addition of a set of hydrogels containing 0, 2.86, 14.3, 28.6, or 57.2 nmol/L immobilized bFGF. None of these hydrogels contained immobilized RGDS. Hydrogels were allowed to swell for 24 h at 4 °C in 0.250 mL phosphate-buffered saline (Sigma). The supernatant was then collected and stored at –80 °C. The supernatant was subsequently tested using an ELISA kit for bFGF

(R&D Systems Inc., Minneapolis, MN) and compared to standard bFGF concentrations.

2.6. Cell proliferation on hydrogels

A cork borer (10 mm diameter) was used to cut disk-shaped gels, which were individually placed in the wells of a 48-well tissue culture plate. Cells were then seeded on the surfaces of these RGDS-modified PEG hydrogels at a density of 7840 cells/cm². DNA content was determined after 5 days in culture. The cell-seeded hydrogels were hydrolyzed in 0.1 N NaOH overnight at 37 °C, neutralized with 0.1 N HCl, and stored at –20 °C prior to being assayed. To determine DNA content, a fluorescent DNA binding dye, PicoGreen (Molecular Probes, Eugene, OR), was used, and its fluorescence was measured using a fluorometer (excitation filter at 480 nm and emission filter at 590 nm).

2.7. Cell migration on bFGF-modified surfaces

Hydrogels were formed as described above except for the addition of a set of hydrogels containing 14.2 nmol/L immobilized bFGF. The hydrogel scaffolds were used in a fence-style migration assay. Cells were seeded on these gels within the confines of a Teflon mold (17 mm outer diameter, 6 mm inner diameter) at a density of 45,000 cells/cm². After 22 h, the molds were removed and cell culture medium was exchanged for medium containing 0.5 μg/mL mitomycin C (Calbiochem, San Diego, CA) to block proliferation. Photographs were taken of the area covered by the cells 3 days after removing the ring, which formed the initial boundary for determining the extent of migration.

2.8. Preparation and detection of covalently immobilized gradients of bFGF

A hydrogel with a gradient of tethered bFGF was formed using a gradient maker (CBS Scientific Co., Del Mar, CA) to pour the hydrogel precursor solutions prior to photopolymerization. Gradient flow was controlled by a Teflon valve centered between two chambers containing polymer solution with or without the PEG-bFGF conjugate. Both polymer solutions contained 0.1 g/mL PEG diacrylate, 3.5 μmol/mL acryloyl-PEG-RGDS, and 10 μL/mL of 2,2-dimethyl-2-phenyl-acetophenone in *N*-vinylpyrrolidone (300 mg/mL). The polymer solutions were slowly combined in a rectangular glass mold (2 mm thickness) and exposed to long-wavelength UV light to form a hydrogel with tethered bFGF ranging from 0 to 51 nmol/L. A silver stain kit (BioRad, Hercules, CA) was used to detect the tethered bFGF gradient. Briefly, a hydrogel containing a gradient of 0–51 nmol/L tethered bFGF was rinsed in deionized water for 20 min, placed in a staining and

development solution (5 mL silver complex solution, 5 mL reduction moderator solution, 5 mL image development reagent, 35 mL deionized water, and 50 mL development accelerator solution as provided in the silver stain kit) for 20 min, and then placed in a stopping solution (5% acetic acid) for 15 min followed by rinsing in deionized water. The mean staining density at each position was analyzed under light microscopy using Scion Image.

2.9. Cell alignment and migration on hydrogel surfaces with a gradient of tethered bFGF

Hydrogels were also formed from polymer solutions containing 0 or 26 nmol/L of the PEG-bFGF conjugate. These hydrogels contained the same concentrations of PEG diacrylate, acryloyl-PEG-RGDS, and 2,2-dimethyl-2-phenyl-acetophenone in *N*-vinylpyrrolidinone as the hydrogel with a gradient of tethered bFGF. Cells were then seeded on the gels at a density of 12,500 cells/cm² and allowed to attach overnight. After 24 h, hydrogel surfaces were photographed in several areas and assessed for cell alignment under phase contrast microscopy using Scion Image. Cell alignment was determined for each cell by finding the cell's angle relative to the direction of the bFGF gradient. Cell angles were determined for 150 cells on each hydrogel surface, and then these angles were categorized into nine different groups based on their relative angle to the bFGF gradient. These groups included cells positioned relative to the bFGF gradient within ± 0 –10°, 10–30°, 30–50°, 50–70°, or 70–90°.

Cell migration was assessed on hydrogel surfaces with a gradient of tethered bFGF. Cells were seeded within a square stainless-steel mold (7.7 mm × 7.7 mm × 10.1 mm), which was positioned at the gradient hydrogel's midsection and compared to cells seeded on hydrogels without bFGF and a constant concentration (26 nmol/L) of tethered bFGF. In addition to the various concentrations of tethered bFGF, the hydrogels contained 0.1 g 6k PEG diacrylate, 5.0 μmol/mL acryloyl-PEG-RGDS, and 10 μL/mL 2,2-dimethoxy-2-acetophenone in *N*-vinylpyrrolidinone. After 16 h, the stainless-steel ring creating the initial boundary was removed, and the cells were photographed under phase contrast microscopy. Two days later, the cells were photographed again.

2.10. Statistical analysis

Data sets were compared using a Student's *t*-test assuming unequal variances. *P* values less than 0.05 were considered statistically significant. All values are reported as the mean and standard deviation of the mean.

3. Results

3.1. Characterization of PEG-conjugated bFGF

Western analysis demonstrated that bFGF was successfully conjugated to PEG. Visual inspection of the gel showed that only a small percentage of the growth factor remained unmodified, while the majority of the growth factor showed an increase in molecular weight corresponding to the attachment of PEG chains. Multiple PEG chains attached to bFGF, resulting in a product with a range of molecular weights (MW of unmodified bFGF = 17.5 kDa). Thus, bFGF was conjugated to PEG via the reaction between acryloyl-PEG-NHS and primary amines within the growth factor. In order to incorporate bFGF into PEG hydrogels, the growth factor was conjugated to a PEG-based chain by reacting bFGF with acryloyl-PEG-NHS. The success of this conjugation was confirmed by Western analysis as depicted in Fig. 1. Staining intensity differed dramatically for the unmodified bFGF compared to PEG-conjugated bFGF. Staining was present at higher molecular weights as indicated by the protein ladder for PEG-conjugated bFGF samples. This staining was

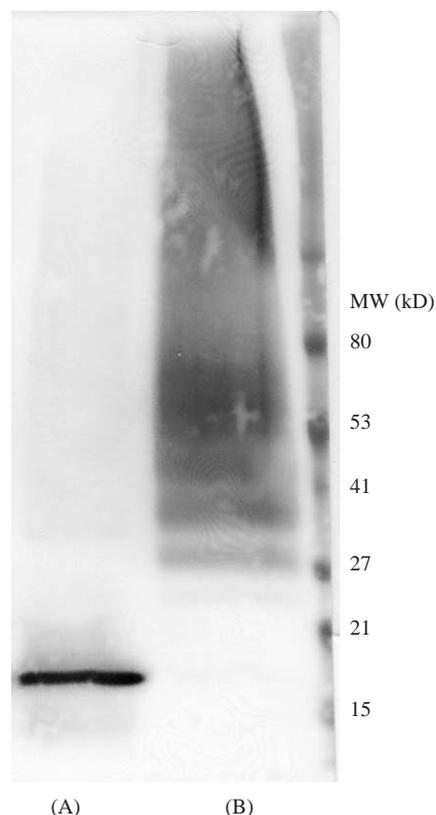


Fig. 1. Western analysis of unmodified bFGF (A, MW = 17.5 kDa) and PEG-conjugated bFGF (B) demonstrated that bFGF was successfully conjugated to PEG (MW 3400). This conjugation was indicated by the increase in MW corresponding to the attachment of PEG chains.

not evident for the unmodified form of the growth factor.

PEG-conjugated bFGF retained the ability to stimulate SMC proliferation. After 48 h, SMCs grown on standard tissue culture polystyrene and treated with unmodified bFGF or PEG-conjugated bFGF (soluble, not photopolymerized into a hydrogel) at a concentration of 2.86 nmol/L showed significant increases in cell number compared to the untreated control cells. SMC number increased by ~24% when treated with unmodified bFGF and by ~21% when treated with PEG-conjugated bFGF ($p < 0.00006$ versus control for both cases). The difference in cell number was not statistically significant between the samples treated with unmodified bFGF and those treated with PEG-conjugated bFGF. Thus, bFGF was successfully conjugated to a polymer tether with retention of its stimulatory effect on SMC proliferation.

PEG-conjugated bFGF was also successfully incorporated within hydrogel scaffolds. After 24 h, less than 0.5% of the bFGF was released into the supernatant. This level of incorporation was also evident at several different immobilized bFGF concentrations ranging from 2.86 to 57.2 nmol/L.

3.2. Cell proliferation and migration in response to covalently immobilized PEG-bFGF

PEG-conjugated bFGF was covalently incorporated into PEG diacrylate hydrogel scaffolds to assess its effects on both SMC proliferation and migration. Cell proliferation on the surface of hydrogels containing unmodified bFGF (2.86 nmol/L) and immobilized bFGF (2.86 nmol/L) were compared. After 5 days, the increase in cell number was ~41% over the amount seen on gels without bFGF ($p < 0.02$). After 5 days, the relative proportion of SMCs was 21% greater on hydrogel surfaces with unmodified bFGF compared to hydrogels without bFGF ($p < 0.30$). However, this increase was less than that seen with covalently immobilized bFGF and not significantly different from SMC number on control hydrogel surfaces.

Cell migration was also improved on hydrogel surfaces modified with tethered bFGF. This was determined using a fence-style migration assay. Migration depended on the bulk concentration of tethered bFGF. On hydrogel surfaces containing 2.86 nmol/L immobilized bFGF, migration was increased ~8% after 72 h in the presence of mitomycin C, which blocks cell proliferation ($p < 0.01$). Migration was greater when the hydrogel surfaces contained 14.2 nmol/L immobilized bFGF such that migration increased ~15% after 72 h ($p < 0.01$).

3.3. bFGF-gradient scaffolds affect vascular SMC behavior

A gradient maker was used to fabricate hydrogel scaffolds with a covalently immobilized bFGF gradient. The gradient maker was used with polymer solutions with and without PEG-conjugated bFGF to generate a continuous linear gradient of the PEG-conjugated bFGF. As the hydrogel precursor was poured into the mold, it was photopolymerized to lock the bFGF gradient in place. The resulting hydrogel was then silver stained to detect the presence of the bFGF gradient, which ranged in concentration from 0 to 51 nmol/L. Silver staining made the bFGF gradient visible under light microscopy, making it possible to analyze the gradient using digital image processing. As depicted in Fig. 2, the staining density increased linearly in the direction of increasing bFGF concentration ($R^2 = 0.85$).

The effects of the immobilized bFGF gradient on cell alignment were assessed. SMCs were seeded on hydrogel scaffolds with a gradient of immobilized bFGF (ranging from 0 to 51 nmol/L) or a constant concentration of bFGF (26 nmol/L) and also without bFGF. As shown in Figs. 3 and 4, SMC alignment was observed on gradient-bFGF scaffolds in the direction of increasing tethered bFGF concentration. This alignment was not evident on the other hydrogel surfaces, which either lacked tethered bFGF or contained a constant concentration of tethered bFGF (26 nmol/L). In addition, SMC alignment on bFGF-gradient scaffolds was more pronounced after 48 h than at 24 h as shown in Fig. 5. SMC alignment also depended on the concentration of tethered bFGF as it was not as evident in regions of the hydrogel scaffold with lower bFGF concentrations (below ~17 nmol/L).

SMC migration on bFGF-gradient hydrogel scaffolds was also examined using a fence-style migration assay. As shown in Fig. 6, cells migrated further when seeded on hydrogel surfaces with a gradient of tethered bFGF (where the cells were initially exposed to immobilized bFGF concentrations ranging from ~23 to ~28 nmol/L) compared to control surfaces lacking tethered bFGF.

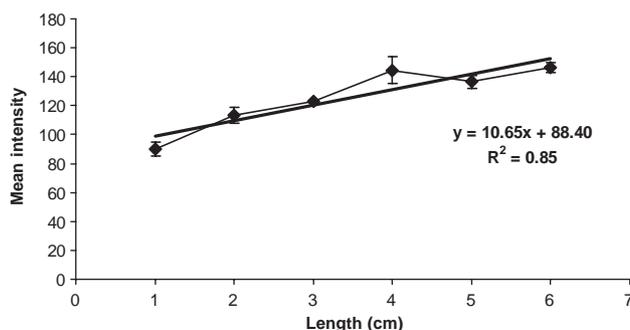


Fig. 2. The bFGF gradient was silver stained, analyzed under light microscopy, and found to be linear.

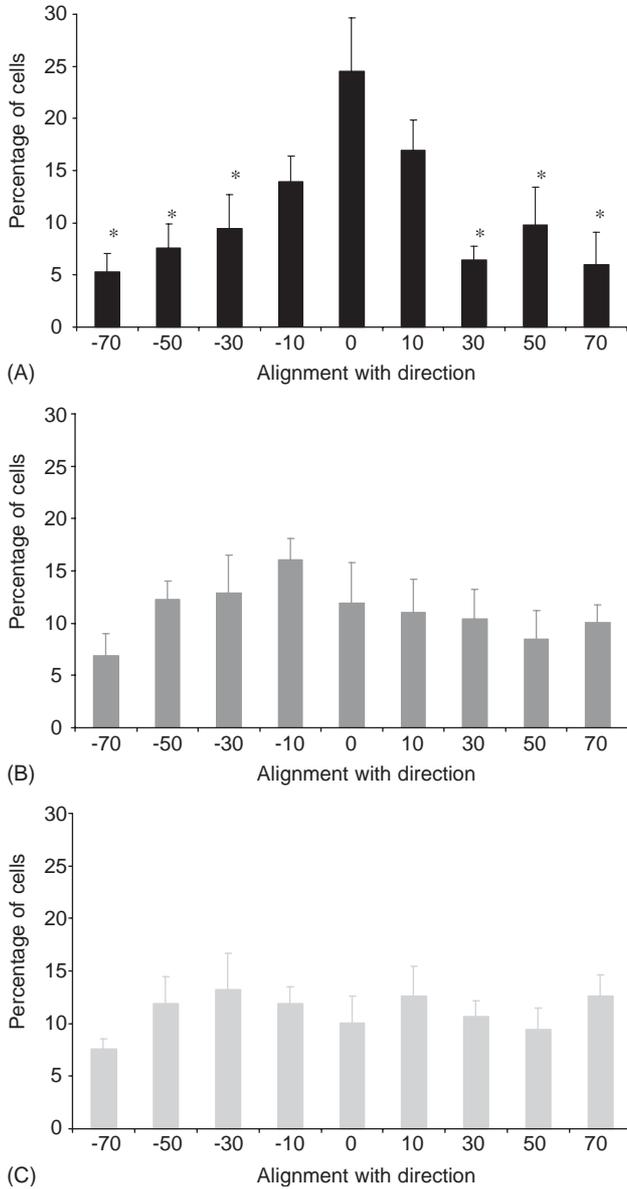


Fig. 3. A gradient of tethered bFGF influenced cell alignment (A). More cells were aligned in the direction of increasing bFGF concentration than on the other hydrogel surfaces (constant bFGF (B); no bFGF (C)), which lacked a gradient of tethered bFGF. * $p < 0.03$ compared to cells aligned with the gradient axis (within $\pm 10^\circ$).

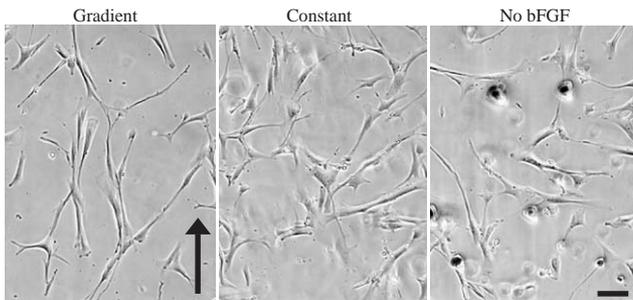


Fig. 4. More cells were aligned on hydrogel surfaces with a bFGF gradient (indicated by arrow) than on the other hydrogel surfaces, which lacked a gradient of tethered bFGF (bar = 10 μ m).

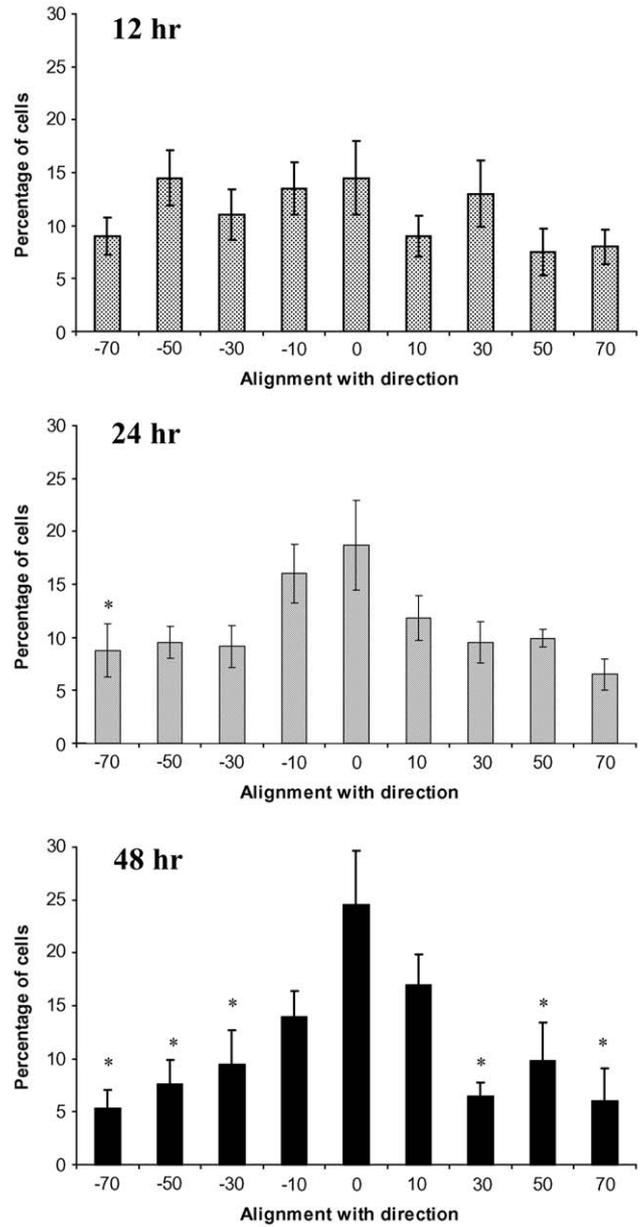


Fig. 5. Cell alignment became more apparent with time on the bFGF-gradient hydrogel surface. * $p < 0.03$ compared to cells aligned with the gradient axis (within $\pm 10^\circ$).

Migration on gradient hydrogels also varied, depending on cell movement up and down the concentration gradient. Cells migrated $\sim 47\%$ further when they moved in the direction of increasing bFGF concentration compared to control surfaces without tethered bFGF. Migration was lower (just 25% greater than cells on control surfaces) when the cells migrated down the concentration gradient. The migration distance was also improved ($\sim 13\%$ greater) in the direction of increasing bFGF concentration compared to surfaces containing a constant concentration of tethered bFGF. Since this study was not performed in the presence of mitomycin

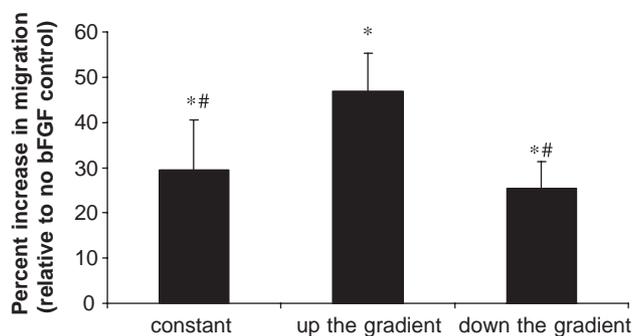


Fig. 6. Cells migrated further on gradient-modified surfaces compared to surfaces with a constant concentration of bFGF when the cells were positioned to move away from areas of high cell density while moving in the direction of increasing bFGF concentration. * $p < 0.005$ compared to hydrogel surfaces without bFGF. # $p < 0.02$ compared to hydrogel surfaces with cells migrating up the concentration gradient.

C, this enhanced effect on migration down the concentration gradient compared to control surfaces without bFGF may have been influenced by cell proliferation.

4. Discussion

PEG-conjugated bFGF was investigated for its effect on vascular SMC behavior when covalently incorporated within PEG-based hydrogel scaffolds. bFGF is a potent mitogenic and chemotactic agent present throughout the vascular environment and as a result may be useful for developing an engineered blood vessel. In PEG-based hydrogels, covalently immobilized bFGF has the potential to elicit cell behavior that is conducive to the formation of engineered tissues, while providing the opportunity to control bioactive signal presentation. Previous studies have investigated the protein-resistant character of these PEG-based scaffolds and the ability to control cell interaction with these hydrogel scaffolds by varying the bulk concentration of incorporated RGD [3–8]. In addition, TGF- β 1 and EGF have been covalently immobilized within PEG-based hydrogels and were shown to retain their respective effects on extracellular matrix production and migration [13,14]. This study has focused on the effects of covalently immobilizing bFGF into PEG-based hydrogels in combination with RGD on vascular SMC proliferation and migration and the potential to control bioactive signal presentation by using a bFGF gradient to influence vascular SMC alignment and migration.

RGD and bFGF were modified with an acrylated PEG chain in order to provide a mechanism for their covalent incorporation within PEG-based hydrogels. Hern and Hubbell showed previously that 85% of an RGD-containing peptide was successfully conjugated to an acrylated PEG chain when reacted in a 1:2

(peptide:acryloyl-PEG-NHS) molar ratio for 2 h, and at least 97% of this product was covalently immobilized to the hydrogel material following photopolymerization [5]. Similarly, Belcheva et al. conjugated NGF to PEG-fluorescein using a 1:10 (NGF: PEG-fluorescein) molar ratio for 24 h and found that PEG chains were successfully attached to NGF [17]. In this study, bFGF was shown to react successfully with acryloyl-PEG-NHS such that the majority of the growth factor was modified with at least one PEG chain, and at least 99.5% of the bFGF was covalently bound to the hydrogel material following photopolymerization.

While PEG conjugation has the potential to reduce bFGF bioactivity by interfering with the binding of bFGF to cell surface receptors, this was not observed in the current study [18–20]. PEG-conjugated bFGF (soluble, not photopolymerized into a hydrogel) increased vascular SMC proliferation by ~21%, and unmodified bFGF at the same concentration increased vascular SMC proliferation by ~24%. Thus, the effect of unmodified bFGF on vascular SMC proliferation was greater than that observed with PEG-conjugated bFGF, but the difference was small and not significant ($p < 0.32$). The minimal impact of PEG conjugation may depend on the lack of a primary amine in bFGF's higher affinity receptor binding surface, which is comprised of tyrosine-103, leucine-140, and tyrosine-24 [18]. Since acryloyl-PEG-NHS targets primary amines, the PEG chain would not attach to this region where it might interfere with bFGF-receptor binding.

Once hydrogels were formed via photopolymerization, covalently incorporated bFGF retained its stimulatory effect on vascular SMC proliferation and migration. Vascular SMC number increased by ~41% after 5 days when exposed to covalently incorporated bFGF compared to an ~21% increase when exposed to unmodified bFGF. The higher SMC density in response to covalently incorporated bFGF may be due to the diffusion of unmodified bFGF from the hydrogels. Covalently incorporated bFGF had a similar enhancement on SMC migration. SMC migration increased by ~15% when seeded on hydrogel surfaces containing 14.2 nmol/L immobilized bFGF compared to SMC migration on hydrogel surfaces lacking immobilized bFGF.

Since bFGF can be covalently bound to photopolymerized hydrogels with good retention of activity, this offers the opportunity to prepare biomaterials with covalently immobilized gradients of the growth factor. These were prepared using a gradient maker to pour the hydrogel precursors into a mold and then immediately photopolymerizing the hydrogel. The resultant bFGF gradient was visualized after silver staining and found to be linear. The bFGF gradient stimulated vascular SMC alignment and migration. This alignment appeared to increase over the first 48 h, indicating that the bFGF

gradient had a sustained effect on SMC behavior. SMCs also migrated differentially on gradient-bFGF hydrogel surfaces compared to control surfaces without bFGF or surfaces containing a constant concentration of bFGF. SMCs migrated furthest up the concentration gradient. Thus, covalent incorporation of bFGF provided the opportunity to present a bFGF-gradient to vascular SMCs that was long-lasting, making it possible to observe changes in SMC alignment and SMC migration over at least 48 h. In comparison, other assay methods provide a transient soluble gradient, which makes it difficult to study behavior by cells that migrate much slower than immune cells. In addition, it may be possible to observe gradient effects on cell migration in three dimensions using this hydrogel system. This application would involve modifying the hydrogel so that proteolytically degradable peptide sequences were incorporated into the backbone of the polymer chain, allowing cells to migrate through the hydrogel [6,21]. This system should allow guidance of engineered tissue formation and provide the opportunity to study detailed mechanisms of chemotaxis in vitro.

Acknowledgments

The authors would like to thank the NIH for financial support. Technical assistance provided by Dr. Wafa Elbjairami, Andre Gobin, and James Moon is greatly appreciated.

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