Effects of Epidermal Growth Factor on Fibroblast Migration through Biomimetic Hydrogels

Andrea S. Gobin and Jennifer L. West*

Department of Bioengineering, Rice University, Houston, Texas 77005-1892

We have previously reported on the development and use of synthetic hydrogel extracellular matrix (ECM) analogues that can be used to study the mechanisms of migration. These biomimetic hydrogels consist of bioinert poly(ethylene glycol) diacrylate derivatives with proteolytically degradable peptide sequences included in the backbone of the polymer and adhesion peptide sequences grafted into the network. Cells adhere to the hydrogel via interaction between the grafted adhesion ligands and receptors on the cell surface. The cells migrate through the three-dimensional system by secreting the appropriate proteolytic enzymes, which are involved in cell migration and are targeted to the peptide sequences incorporated in the backbone of the polymer. It was observed that cell migration has a biphasic dependence on adhesion ligand concentration, with optimal migration at intermediate ligand levels. In this study, we demonstrate that we can covalently attach epidermal growth factor (EGF) to PEG and graft them into the hydrogels. It was observed that EGF when tethered maintained mitogenic activity. It was also observed that fibroblast migration significantly increased in the presence of the grafted EGF through the collagenase-sensitive hydrogels. In addition, the increase in migration was found to be independent from the proliferative response of the cells. These synthetic ECM analogues allow one to systematically control identities and concentrations of biomolecules and are useful tools to study mechanisms of cell migration.

Introduction

Cell migration is an important and predominant function during inflammation and wound repair. There are three general phases of wound healing. The first phase is inflammation, which involves emigration of neutrophils and monocytes. Chemical signals, such as growth factors and cytokines, are released from these cells, as well as from the plasma and the injured tissue. Thus, the second phase is initiated, which involves migration and mitosis of fibroblasts and vascular endothelial cells to form granulation tissue. Last, the recruited cells remodel the granulation tissue and synthesize new extracellular matrix (ECM) proteins, such as collagen and proteoglycans (*1*). One of the important chemical mediators in this process is epidermal growth factor (EGF). EGF is thought to play an important role in the mechanisms in wound healing by stimulating neovascularization and chemotaxis of wound cells (*2*).

Epidermal growth factor is a 6 kDa mitogenic polypeptide that is present in many cell types, including fibroblasts, vascular endothelial cells, and epithelial cells, and various body fluids, such as blood, urine, and saliva. Depending on the cell type and its state of differentiation, EGF can either stimulate or inhibit proliferation and differentiation as well as other metabolic activities (*3*). For example, EGF can stimulate ion transport, enhance endogenous protein phosphorylation, alter cell morphology, and stimulate DNA synthesis (*3*). The EGF receptor (EGFR) is a 17 kDa transmembrane glycoprotein receptor, which when bound activates the tyrosine kinase activity in the receptor's cytoplasmic domain (*3*, *4*).

It has been reported that EGFR activation, via binding of EGF, increases cell migration (*5*, *6*) independent of EGF's mitogenic effects (*5*, *7*). This increased migration is believed to be the result of an integration of several effects of EGF. First, EGF increases lamellipodial extension and membrane ruffling (*6*, *7*). Second, EGF reduces the strength of the cell-substratum adhesion (*7*), thus allowing easier cell detachment. Finally, the combination of the above effects leads to increased random movement of the cell and thus increases migration (*5*, *6*).

This report will discuss the effects of EGF on fibroblast migration through a three-dimensional biomimetic hydrogel system. This system has been previously used to assess the effects of adhesivity on cell migration (*8*). In three-dimensional biomimetic hydrogels containing both proteolytically degradable peptides sequences and RGDS adhesion sequences, cell migration had a biphasic dependence on substrate adhesiveness. To study the effects of EGF on fibroblast migration in the current study, hydrogels with incorporated degradation sequence GGGLGPAGGK in the polymer backbone targeted by collagenase, with Arg-Gly-Asp-Ser (RGDS) adhesion ligands, were used. EGF was also covalently immobilized to the hydrogel via a flexible linker. We found that EGF can be tethered to a PEG spacer chain and still remain active. In addition, we also observed that EGF had a stimulatory effect with adhesion on cell migration.

Methods and Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

* To whom correspondence should be addressed. Ph: (713) 348-5955. Fax: (713) 348-5877. Email: jwest@rice.edu.

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Synthesis of Poly(ethylene glycol) Diacrylate. Poly(ethylene glycol) (PEG) diacrylate was prepared by combining 0.1 mmol/mL dry PEG (6000 Da; Fluka, Milwaukee, WI), 0.4 mmol/mL acryloyl chloride, and 0.2 mmol/mL triethylamine in anhydrous dichloromethane (DCM) under argon overnight. The resulting PEG diacrylate was then precipitated with ether, filtered, and dried in vacuo. The polymer was analyzed by proton NMR (Avance 400 MHz; Bruker, Billerica, MA; solvent, *N*,*N*-dimethylformamide-d_7) to determine the degree of acrylation.

Synthesis of Poly(ethylene glycol) Derivatives Containing Degradable Peptide Sequences. Acrylated ABA block copolymers of PEG (A) and peptides (B) that are substrates for proteolytic enzymes were synthesized. The degradable peptide sequence, NH_2-GGLG-PAGGK-COOH (collagenase sensitive), was synthesized using standard dicyclohexylcarbodiimide (DCC) activation and 9-fluorenylmethoxycarbonyl (Fmoc) protection chemistry on an Applied BioSystems peptide synthesizer (model 431A; Foster City, CA). The peptide sequence was then reacted with acryloyl-PEG-*N*-hydroxysuccinimide (acryloyl-PEG-NHS, 3400 Da; Shearwater Polymers, Huntsville, AL) in a 1:2 (peptide:PEG) molar ratio in 50 mM sodium bicarbonate (pH 8.5) for 2 h. During the reaction, the NHS groups of the acryloyl-PEG-NHS are replaced by the amine groups on the peptide, one at the N-terminus of the peptide and another on the amine group of the lysine at the C-terminus of the peptide. The product (acryloyl-PEG-peptide-PEG-acryloyl) was then dialyzed (MWCO 5000 Da; Fisher Scientific, Pittsburgh, PA), lyophilized, and stored frozen under argon. Gel permeation chromatography with UV (260 nm) and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA) was used to analyze the peptide sequence, the resulting PEG-peptide copolymer and PEG standards (Polymer Laboratories).

Synthesis of Poly(ethylene glycol) Derivatives Containing Adhesive Peptide Sequences. The adhesive peptide sequence used for all studies was Arg-Gly-Asp-Ser (RGDS). The peptide was conjugated to PEG monoacrylate by reacting the peptide with acryloyl-PEG-NHS (3400 Da) in 50 mM sodium bicarbonate (pH 8.5) at a 1:1 molar ratio for 2 h. The mixture was then dialyzed (MWCO 3500 Da), lyophilized, and stored frozen under argon. Gel permeation chromatography was used to determine the coupling efficiency.

Synthesis of Poly(ethylene glycol) Derivatives Containing Adhesive Epidermal Growth Factor. Human EGF was conjugated to PEG monoacrylate in a similar fashion as to the adhesive ligands. EGF was first dissolved in 10 mM sterile acetic acid (2 μg/μL). Acryloyl-PEG-NHS (3400 Da) was dissolved in 50 mM sterile sodium bicarbonate (pH 9.0, 5 mg/mL) and added to the EGF solution at a 1:1 molar ratio. The mixture was then adjusted to pH 8.5 and allowed to react for 2 h. The product was then frozen, lyophilized, reconstituted in sterile 10 mM HEPES buffered saline (pH 7.4, HBS), and stored frozen. Bicinchoninic acid (BCA; Pierce Endogen, Rockford, IL) protein assays were used to determine coupling efficiency. EGF is not soluble in water unless coupled to PEG, and thus the BCA assay can be used with aqueous samples to assess the degree of PEG conjugation.

Hydrogel Photopolymerization. Hydrogels were prepared by combining 0.1 g/mL PEG diacrylate derivative containing the degradable sequence (GGGLGPAG-GK) and 0.01 g/mL (2.8 μmol/mL) acryloyl-PEG-RGDS in 10 mM HEPES buffered saline (pH 7.4, HBS). The solution was then sterilized via filtration (25 mm Acrodisc, 0.8/0.2 μm Supor membrane; Pall Gelman Laboratories, Ann Arbor, MI). Next, 100 ng/mL of the sterile acryloyl-PEG-EGF was added to the solution, and 10 μL/mL of 2,2-dimethyl-2-phenyl-acetophenone in *N*-vinylpyrrolidone (600 mg/mL) was then added as the photoinitiator. The resulting solution was then exposed to UV light (365 nm, 10 mW/cm^2) for 30 s to convert the liquid polymer to a hydrogel. It has been previously shown that >97% of the PEG-coupled species are incorporated into the hydrogel material following photopolymerization (*11*). PEG diacrylate hydrogels and PEG diacrylate hydrogels with 100 ng/mL acryloyl-PEG-EGF were prepared as controls. Degradable hydrogels with acryloyl-PEG-EGF alone were also prepared as controls.

Cell Maintenance. Human dermal fibroblasts (HDFs; Clonetics, San Diego, CA) were maintained on Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD), 2 mM L-glutamine, 500 U penicillin, and 100 mg/L streptomycin. Cells were incubated at 37 °C in a 5% CO_2 environment.

Effects of Tethered EGF on Fibroblast Proliferation. To assess if coupling the growth factor to a PEG spacer chain inhibits its activity, proliferation studies were used to assess the activity of the tethered EGF. Proliferation was assessed two ways: by Coulter counting and by immunostaining for proliferating cell nuclear antigen (PCNA). PCNA is an intranuclear polypeptide whose rate of synthesis is maximum during the S-phase of the cell cycle; thus proliferating cells stain positive for PCNA. HDFs were plated in 48-well plates (20,000 cells/well; passage 7) in serum-free DMEM and incubated overnight. The media was then changed to either contain tethered EGF (100 ng/mL), unmodified EGF (100 ng/mL), or no EGF (n = 6 for each condition). As a control, a parallel study was conducted with the addition of an antibiotic and carcinostatic agent, mitomycin C (0.5 μg/mL; Calbiochem-Novabiochem, San Diego, CA), which inhibits DNA synthesis by blocking the cell cycle at the G2 phase. After a 48 h incubation period, the cells were either trypsinized and counted using a Coulter counter (Multisizer 0646; Coulter Electronics, Hialeah, FL) or stained with a PCNA staining kit (Dako, Carpinteria, CA) to determine the number of proliferating cells. Cells were fixed in 10% buffered formalin, permeabilized with methanol, incubated with mouse anti-PCNA IgG (Dako, Carpinteria, CA), incubated with HRP-conjugated anti-mouse IgG (Dako, Carpinteria, CA), incubated with 3-amino-9-ethylcarbazole (AEC) substrate chromogen (Dako, Carpinteria, CA), and finally counter-stained with Mayer's hematoxylin. The numbers of proliferating and nonproliferating cells in each well were counted under light microscopy (Axiovert 135, Carl Ziess, Thornwood, CA).

Effects of Grafted EGF in Hydrogels on Fibroblast Proliferation. Activity of EGF when covalently grafted into hydrogels was also assessed. PEG diacrylate hydrogels with 2.8 μmol/mL acryloyl-PEG-RGDS were prepared with 100 ng/mL acryloyl-PEG-EGF. The hydrogels were then allowed to swell in serum-free DMEM overnight to reach equilibrium swelling. For controls, PEG diacrylate hydrogels with 2.8 μmol/mL acryloyl-PEG-RGDS were prepared with 100 ng/mL EGF and allowed to swell in serum-free DMEM with 100 ng/mL EGF overnight. HDFs (passage 14; 20,000 cells/gel) were seeded upon the hydrogel surface and incubated for an additional 48 h. After incubation the cells were trypsinized and counted.

Cell Migration through Bioactive Polyethylene Glycol Hydrogels. A modified Boyden chamber assay was used to evaluate cell population migration through the hydrogel networks. Collagenase sensitive hydrogels containing 0.1 g/mL PEG-diacrylate derivative containing GGLGPAGGK (for collagenase degradation) with 2.8 μmol/mL acryloyl-PEG-RGDS and 100 ng/mL acryloyl-PEG-EGF were prepared as described above. 50 μL of the polymer solution was placed into a transwell cell culture insert (6.4 mm diameter, 8 μm pore PET membrane; Becton Dickinson, Franklin Lakes, NJ) and photopolymerized. The resulting hydrogel was 1 mm thick. As controls, PEG diacrylate hydrogels and degradable hydrogels with either grafted adhesion ligand or grafted EGF were also prepared as described above. The inserts were then placed into 24-well companion plates (Becton Dickinson) and DMEM containing 2% FBS was added to the insert and the well and the plates were incubated at 37 °C with 5% CO_2. After a 24 h, human dermal fibroblasts (HDFs) were seeded on top of the hydrogel layer (50,000 cells/insert). After 7 days of culture, cells were removed from the top of the hydrogel layer and from below the insert membrane by trypsinization and counted using a Coulter counter. The migration index was calculated as the number of cells that had migrated through the hydrogel and insert membrane divided by the sum of the cells that had migrated beneath the insert membrane and those that remained on top of the hydrogel layer.

Statistical Analysis. Data sets were compared using two-tailed, unpaired t-tests. *P* values less than 0.05 were considered significant. Error bars in figures represent standard deviations

Results

Effects of Tethered EGF on Fibroblast Proliferation. The goal of the current studies was to assess the effects of covalently grafted EGF in a hydrogel on fibroblast migration. To allow the EGF to be accessible to the cells a PEG space linker was attached to the amine terminus of the polypeptide. To assess if the EGF retained its activity after a soluble polymer chain had been covalently attached, proliferation studies were conducted. Fibroblasts were seeded into tissue cultured 48-well plates in serum-free media. After quiescence was achieved, fresh media with 2% serum was added with or without EGF. Figure 1a shows that in the presence of either unmodified EGF or acryloyl-PEG-EGF there is a significant increase in the number of cells as compared to cells grown in the absence of EGF. In addition, the percentage of PCNA-positive cells was significantly higher than those in cells not exposed to EGF (Figure 1b). In a set of controls, mitomycin C was added under the same conditions, and the number cells were counted. In all conditions, little or no proliferation was seen in the presence of the EGF.

Effects of Grafted EGF in Hydrogels on Fibroblast Proliferation. In addition to the above proliferation studies, we wanted to be certain that once the EGF was grafted into a three-dimensional hydrogel network, the EGF was not prohibited from interacting with the cell's receptors as a result of steric hindrance. Proliferation studies were conducted again; however, the EGF was covalently grafted into the hydrogel via photopolymerization. As a control, hydrogels were formed with entrapped unmodified EGF and swelled in the presence of EGF. Cells were seeded upon the surface and incubated for 48 h, after which cells were Coulter counted. Figure 2 represents the total number of cells found on each

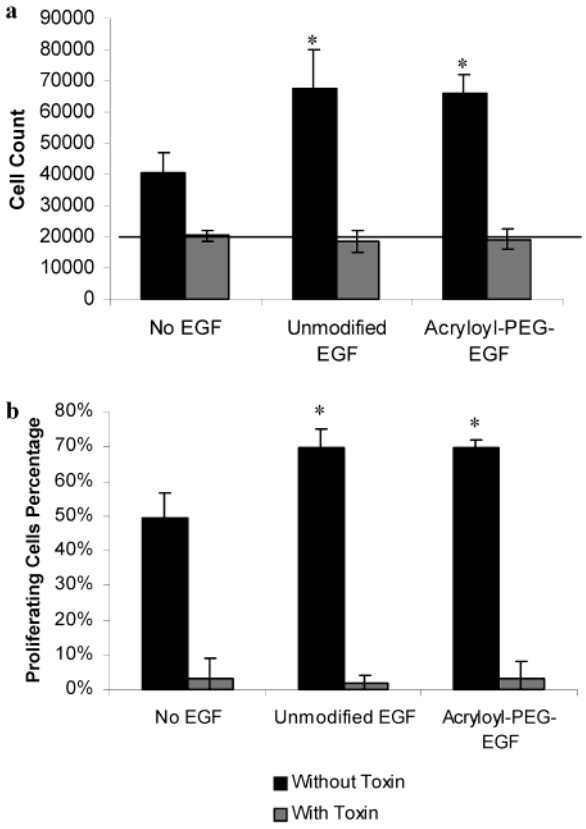

Figure 1. Effects on fibroblast proliferation due to conditioned media. Media with 2% serum contained no EGF, unmodified EGF (100ng/mL), or acryloyl-PEG-EGF (100 ng/mL). (a) Total number of cells per well with and without toxin. Bar line represents initial seeding density. (b) Percentage of proliferating cells per well with and without toxin. Data represents mean and standard deviation of 6 samples. (∗) $p < 0.01$ compared to samples without EGF in the media.

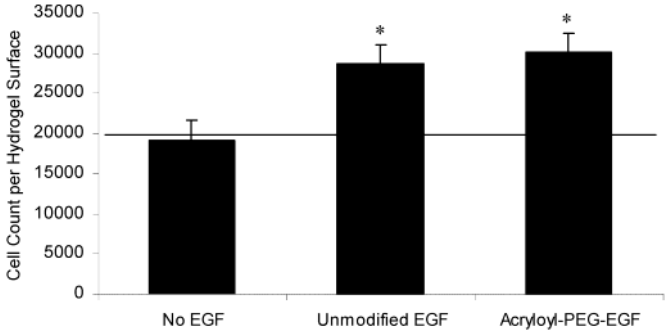

Figure 2. Effects on fibroblast proliferation when EGF is grafted into hydrogel network. Hydrogel samples were prepared either without EGF, soluble EGF in hydrogel precursor solution, or EGF covalently immobilized in hydrogel after photopolymerization. Bar line represents initial seeding density. Data represents mean and standard deviation of 7 samples. (∗) $p < 0.001$ compared to samples without EGF.

surface type. It was observed that a significantly higher number of cells grew upon the surfaces that contained EGF as compared to hydrogels without EGF.

Cell Migration through Bioactive Poly(ethylene glycol) Hydrogels. The effects of EGF on cell migration through collagenase-sensitive hydrogels were observed using a population-based assay in a modified Boyden chamber system. Hydrogels contained either covalently grafted EGF, covalently grafted adhesion ligand (RGDS), or both bioactive signals. After a 7 day incubation period, the number of cells that had migrated through the 1 mm

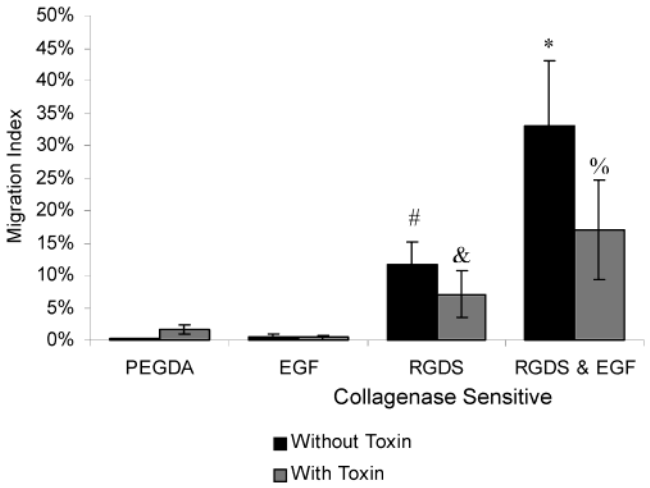

Figure 3. Effects on fibroblast migration when EGF is covalently grafted into hydrogel network. PEGDA, PEG diacrylate; EGF, collagenase-sensitive hydrogels with grafted EGF; RGDS, collagenase-sensitive hydrogels with covalently grafted adhesion ligand RGDS; RGDS & EGF, collagenase-sensitive hydrogels with both EGF and RGDS covalently grafted in network. Data represents mean and standard deviation of 4 samples. (∗) $p < 0.01$ when compared to RGDS. (#) $p < 0.01$ when compared to PEGDA or EGF. (%) $p < 0.05$ when compared to RGDS. (&) $p < 0.05$ when compared to PEGDA or EGF.

thick layer was counted and a migration index was calculated. Figure 3 shows that cells can migrate through hydrogels when both the degradable and adhesive components are present. However, EGF does not provide adhesive properties sufficient to promote cell migration, and thus its migration index is comparable to PEG diacrylate hydrogels alone. The migration index for fibroblasts migrating through hydrogels with both adhesive and degradable components was 11.71 ± 3.37% (mean ± SD). The addition of EGF into the system increased the index to 32.93 ± 10.04% ($p = 0.007$ when compared to no EGF in hydrogel). When the same study was conducted in the presence of the mitomycin C, to abolish proliferative response, similar trends were observed, though the total cell numbers were lower.

Discussion and Conclusions

In previous reports, we have shown that we can develop and use biomimetic materials to investigate some of the mechanisms of cell migration. These materials are photopolymerizable hydrogels based on acrylated derivatives of poly(ethylene glycol). These materials contain proteolytically degradable peptide sequences, targeted for degradation by specific enzymes involved in cell migration, in the polymer backbone (*9*). Cell adhesion peptides, such as RGDS, are also grafted into these hydrogels during photopolymerization to promote interaction with specific cell surface receptors (*10*, *11*). We have shown that we can develop a synthetic collagen analogue containing the proteolytically degradable sequence GGGLGPAGGK and adhesive ligands RGDS and that fibroblasts could migrate through the hydrogel layer at migration indices comparable to those of collagen layers of the same thickness (*10*). Furthermore, we have demonstrated that cell migration has a biphasic dependence on substratum adhesiveness, with optimal migration at intermediate levels of adhesion (*8*).

To further mimic the natural environment of a cell, growth factors can also be incorporated into the system. For this report, epidermal growth factor is covalently grafted into the hydrogel network. Mann et al. (*12*) has already demonstrated that TGF-β can be linked to a PEG polymer chain and remain active to stimulate matrix production from smooth muscle cells. It has also been shown by Kuhl et al. (*13*) that EGF could be tethered to poly(ethylene oxide) (PEO) and then immobilized on solid surfaces. Kuhl observed that when EGF was immobilized in this fashion, biological activity was retained. In contrast, when EGF was physically adsorbed onto the surface, no activity was seen.

For this report, EGF was modified by covalently attaching a PEG chain, which allows flexibility of the growth factor to interact with the cells' receptors. When EGF was tethered to PEG linker chains, it was observed that proliferative activity was retained. Furthermore, when the acryloyl-PEG-EGF was grafted into the hydrogel network, cells were still able to recognize the polypeptide and have mitogenic responses. To assess the effects of EGF on cell migration a modified Boyden chamber was used. We found that EGF increased migration indices 32% over cells migrating through hydrogels that contained only the degradable and adhesive bioactive signals. Moreover, it was seen that this increase was synergistic to adhesion, since migration was not seen in hydrogels that contained degradable sequences and acryloyl-PEG-EGF. To assess that increases in migration was primarily due to an increase in random migration and not mitogenic responses, parallel studies were conducted in the presence of Mitomycin-C (an antibiotic toxin, which inhibits cell proliferation). It was seen that migration was induced by EGF through signaling pathways other than mitogenic pathways. Furthermore, when migration indices are compared between collagenase sensitive hydrogels with RGDS and the same system in the presence of mitomycin C, there is no significant difference, suggesting that cell migration is due to random migration and that proliferative events contribute little.

With biomimetic systems we are able to control the identity and concentrations of bioactive signals presented to cells. We have shown that EGF has integrative effects on cell migration. Our results provide supporting evidence that cell migration is regulated simultaneously by extracellular matrix molecules and growth factors. By placing individual components together in a stepwise fashion, we will be able to investigate more of the mechanisms of cell migration and assess how they interact to influence a cell's behavior.

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