

Synthetic Biomimetic Hydrogels Incorporated with Ephrin-A1 for Therapeutic Angiogenesis

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Eph receptors and ephrin ligands are essential for vascular development and angiogenic remodeling. In this work, we developed biomimetic poly(ethylene glycol)-diacrylate hydrogels incorporated with ephrin-A1 and examined their angiogenic properties. Ephrin-A1 was covalently immobilized on the surface of hydrogels by chemical modification and photopolymerization. Ephrin-A1 immobilized on hydrogels was found to retain its capacity to stimulate endothelial cell adhesion in a dose-dependent manner as similar findings were observed on polystyrene culture wells pre-adsorbed with ephrin-A1. Cell adhesion stimulated by ephrin-A1 was abolished by treatment with soluble RGDS and anti- $\alpha_v\beta_3$ integrin but not anti- $\alpha_v\beta_5$ integrin antibodies, suggesting that ephrin-A1 activates cell adhesion through $\alpha_v\beta_3$ integrins. Also, surface immobilized ephrin-A1 was found to induce endothelial tubule formation with luminal diameters ranging 5–30 μm on hydrogels. The results of these studies demonstrate that pro-angiogenic properties of ephrin-A1 are preserved in hydrogels and suggest potential applications of this hydrogel system in regenerative medicine and tissue engineering.

Introduction

Assembly and maintenance of organized tissue patterns are tightly regulated by adhesive interactions among neighboring cells and migration of the constituent cells. Within these processes, Eph receptors and their ephrin ligands mediate cell adhesion and migration by regulating cell position, organization, repulsion, and attraction.¹ Hence, the Eph and ephrin families, which are predominately expressed in endothelial cells (ECs) and nerves, have been recognized as the key players in a wide spectrum of developmental processes such as tissue border formation and neuronal guidance, and of particular importance to us are their prominent roles in vascular assembly and remodeling.²

The objective of this research was to exploit the regulatory roles of the Eph and ephrin families in vascular assembly to stimulate blood vessel formation for regenerative medicine. Specifically, we sought to incorporate a potent angiogenic subclass of the Eph and ephrin proteins into a synthetic biomaterial and examine its capacity to present the angiogenic moieties to cells and ultimately induce blood vessel growth. Covalent incorporation of angiogenic agents into the biomaterial would allow their continuous presentation to attached cells, and this, in turn, can facilitate therapeutic angiogenesis in synthetic materials for tissue engineering applications.

Although there have been tremendous efforts to replace and restore various tissues and organs since the first FDA approval of engineered epidermis for burn patients, most successes in tissue engineering have been limited to thin or avascular tissues such as skin and cartilage.³ As the tissue becomes thicker, diffusion limits are approached, and cells deep inside the tissue do not receive sufficient oxygen and nutrients. To develop more complex and clinically relevant tissues, recent focus has been placed on vascularization of engineered tissue constructs prior to transplantation.

Numerous studies have tested a range of natural as well as synthetic materials to induce vascularization of scaffolds and reported varying successes.^{4,5} Growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have been delivered to local environments using controlled release drug delivery systems.^{6–8} As an alternative to these traditional approaches, Zisch et al. have developed fibrin matrices coupled with ephrin-B2 and shown that fibrin-bound ephrin-B2 stimulated EC angiogenic responses.⁹ This work has demonstrated that matrix-bound ephrins can evoke prolonged and local signaling events in adjacent cells and tissues. However, there are other members of the Eph and ephrin families that possess more potent angiogenic properties, and we propose in this work to take advantage of these subclasses to lay groundwork for solving the problem of vascularizing engineered tissues.

Eph receptors are transmembrane receptor tyrosine kinases with extracellular ephrin binding domains. Ephrins ligands are classified into two subgroups according to the mechanism of attachment on cell membranes. Ephrin-A proteins are attached to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor, whereas ephrin-B proteins have transmembrane and highly conserved cytoplasmic domains.¹ Upon engagement, the Eph receptor–ephrin ligand complex propagates bidirectional signaling events. In this unique binding event, forward and reverse signals are initiated by the Eph receptor and ephrin ligands to two opposing cells to regulate various aspects of cellular functions (for a review, see ref 10).

Previous studies of Eph receptors and ephrin ligands indicate that among the 16 subclasses of Eph receptors and nine ephrin ligands identified to date,¹⁰ the EphA2 receptor expressed on ECs is one of the key components that govern angiogenesis. The EphA2 receptor is activated by the ephrin-A1 ligand, and subsequent to this EphA2 activation, ECs initiate migration and capillary invasion.¹¹ On the other hand, blocking EphA2 activation inhibits migration, vascular assembly, and tumor- and VEGF-induced angiogenesis.^{11–13} Activation of the EphA2 receptor via ephrin-A1 treatment is considered to be intimately

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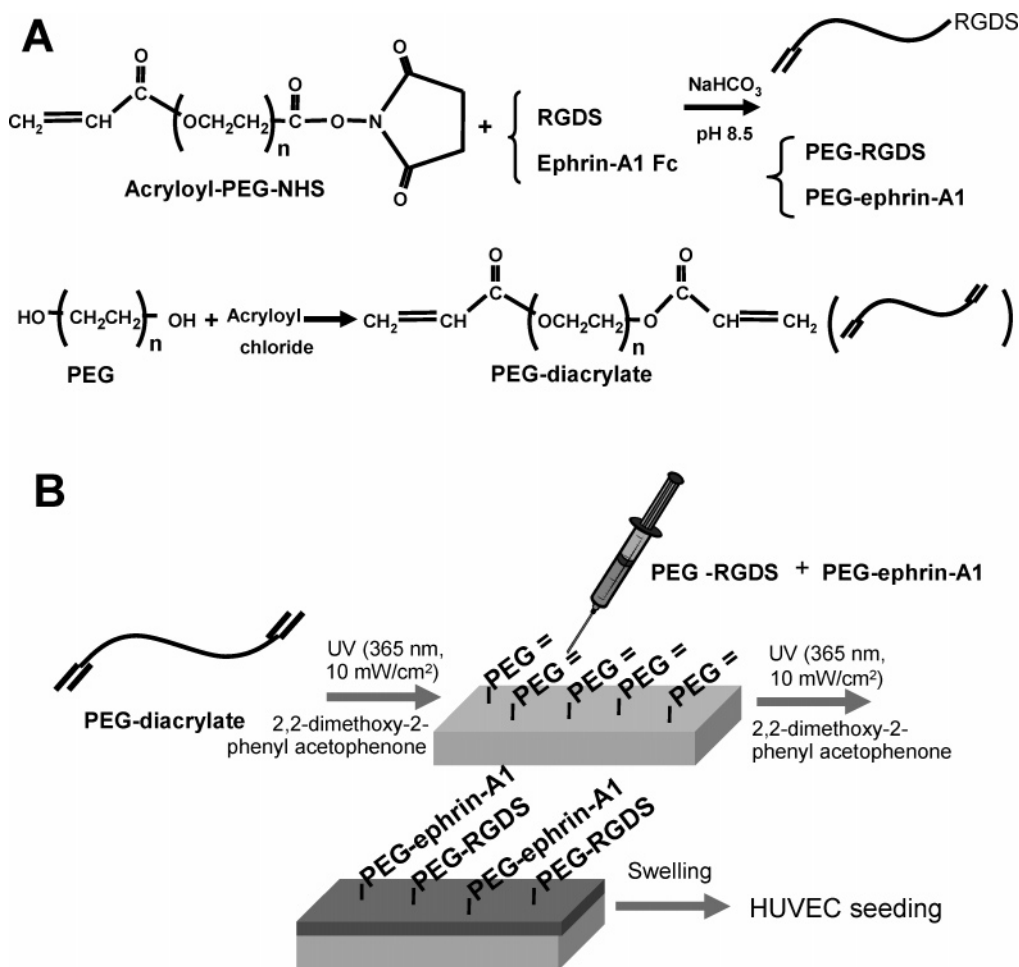


Figure 1. Scheme of surface immobilization technique. (A) RGDS and ephrin-A1/Fc were modified to monoacrylate-PEG via *N*-hydroxysuccinimide chemistry. PEGDA was synthesized from PEG in a reaction with acryloyl chloride. (B) Using two photopolymerization steps, PEG-RGDS and PEG-ephrin-A1 were immobilized on the surfaces of PEGDA hydrogels.

associated with angiogenesis, and we have thus chosen to utilize the angiogenic properties of ephrin-A1 for potential therapeutics.

We have previously developed a biomimetic hydrogel scaffold based on poly(ethylene glycol)-diacrylate (PEGDA). PEGDA hydrogels are hydrophilic and highly resistant to protein adsorption and subsequent nonspecific cell adhesion.¹⁴ However, bioactive factors can be incorporated within these materials to allow specific cellular interactions with the scaffold. In previous studies, non-adhesive PEGDA hydrogels have been derivatized with various cell adhesive ligands to support cell adhesion, proliferation, and migration of many different cell types including fibroblasts, smooth muscle cells, and pre-adipocytes.^{14–18}

Growth factors can be covalently immobilized on biomaterials while retaining their specific bioactivity. For instance, an epidermal growth factor (EGF) covalently coupled to glass slides via a PEG linker stimulated DNA synthesis and cell rounding responses in rat hepatocytes.¹⁹ Those responses were comparable to those observed in the presence of soluble EGF treatment. In our laboratory, EGF as well as transforming growth factor- β (TGF- β) and basic fibroblast growth factor (bFGF) were grafted into a PEGDA hydrogel network and shown to retain bioactivity.^{14,20,21}

Since proteins and peptides can be immobilized on PEGDA hydrogels while minimizing nonspecific protein adsorption, we hypothesized that the cell surface protein, ephrin-A1, immobilized onto PEGDA hydrogels would maintain its angiogenic properties and provide a useful system to stimulate therapeutic angiogenesis. Our findings demonstrate that ephrin-A1 im-

mobilized on PEGDA hydrogels indeed retains its capacity to stimulate EC adhesion and spreading in a dose-dependent manner and that the $\alpha_v\beta_3$ integrin is the primary mediator of these processes. Also, surface immobilized ephrin-A1 was found to induce EC capillary formation, highlighting the therapeutic potential of the current hydrogel system.

Experimental Procedures

Cell Maintenance. Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex (East Rutherford, NJ) and were grown on endothelial cell medium EGM-2 (Cambrex) supplemented with 2 mM L-glutamine, 1000 U/mL penicillin, and 100 mg/L streptomycin (Sigma, St. Louis, MO). Cells were incubated at 37 °C in a 5% CO₂ environment. All experiments were conducted using cells from passage 3–8.

Synthesis of Poly(ethylene-glycol)-diacrylate (PEGDA). PEGDA was synthesized by reacting 12 g of dry poly(ethylene-glycol) (PEG) (6000 Da; Fluka, Milwaukee, WI) in 36 mL of anhydrous dichloromethane with 0.25 g of triethylamine and 0.43 g of acryloyl chloride (Lancaster Synthesis, Windham, NH) under argon overnight (Figure 1A). The resulting solution was washed with 2 M K₂CO₃ and separated into aqueous and organic phases. The organic phase was dried with anhydrous MgSO₄, and PEGDA was precipitated in diethyl ether, filtered, and dried under vacuum. The polymer was analyzed by proton NMR (Avance 400 Hz; Bruker, Billerica, MA).

Synthesis of PEG-ephrin-A1 and PEG-RGDS. Recombinant fusion proteins of ephrin-A1 were used in this study, comprised of the Fc

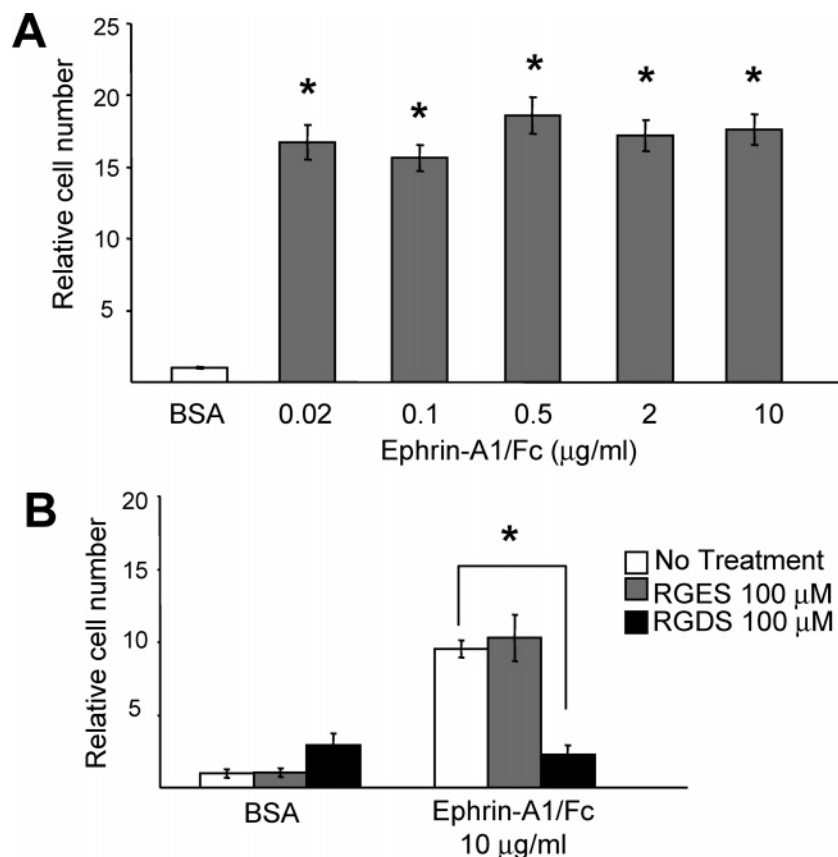


Figure 2. Effect of ephrin-A1 on HUVEC adhesion. (A) HUVECs were seeded on tissue culture wells pre-adsorbed with ephrin-A1/Fc and blocked with 3% BSA. After 30 min, cell adhesion was quantified by counting adherent cell numbers and normalized to that of the control group. (B) Cell adhesion in the presence of soluble competitive inhibitors. Data represent mean \pm SEM ($n = 4$). * $P < 0.01$, analyzed by two-way ANOVA followed by Tukey's HSD test, as compared to the corresponding control.

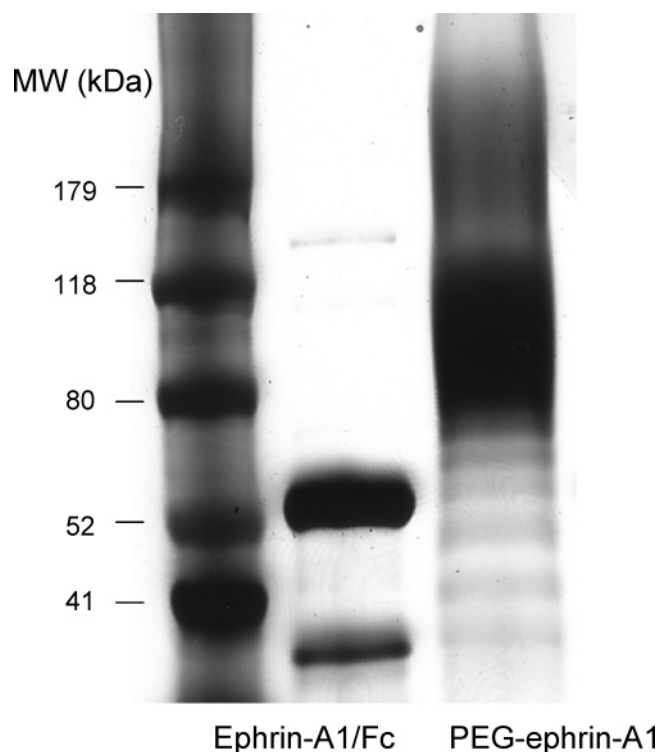


Figure 3. Analysis of unmodified ephrin-A1/Fc and PEG-ephrin-A1 with gel electrophoresis. SDS-PAGE followed by silver staining shows changes in molecular weight of ephrin-A1/Fc after PEG conjugation.

portion of human IgG and the extracellular cell binding domain of ephrin-A1 (R & D Systems, Minneapolis, MN). This protein is

henceforth referred to as ephrin-A1/Fc. Ephrin-A1/Fc was conjugated to PEG monoacrylate by reaction with a 50-fold molar excess of acryloyl-PEG-*N*-hydroxysuccinimide (3400 Da; Fluka, Milwaukee, WI) in 50 mM sodium bicarbonate buffer (pH 8.5) for 2 h. The resultant products are referred to as PEG-ephrin-A1 (Figure 1A). SDS-PAGE followed by silver staining was used to confirm conjugation to PEG. The cell adhesive ligand, Arg-Gly-Asp-Ser (RGDS, American Peptide, Sunnyvale, CA), was conjugated to PEG monoacrylate in a 1:1 molar ratio under similar conditions. The resultant product, PEG-RGDS, was dialyzed, lyophilized, and stored at -80°C . A gel permeation chromatography system equipped with UV-vis and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA) was used to analyze the products.

Surface Immobilization on PEGDA Hydrogels. We have shown previously that peptides or proteins can be immobilized on the surface of PEGDA hydrogels with great fidelity and that the amount bound can be controlled by varying their initial concentration in the prepolymer solution or the duration of exposure to the ultraviolet (UV) lamp during photopolymerization.²² Surface immobilization on PEGDA hydrogels involved two successive photopolymerization steps (Figure 1B). First, base PEGDA hydrogels were prepared by pouring 0.1 g/mL PEGDA in 10 mM HEPES buffered saline (pH 7.4) into rectangular glass molds (0.5 mm thickness) and exposing the polymer solution to long-wavelength UV light (365 nm, 10 mW/cm²) for 30 s. After rinsing the hydrogels with phosphate buffered saline (pH 7.4), the surfaces of the hydrogels were covered evenly with 50 μL of a second polymer solution containing various concentrations of PEG-ephrin-A1 along with PEG-RGDS. A second exposure to UV light for 90 s formed a covalently-bound layer on the surface of the base PEGDA hydrogels. All polymer solutions contained 10 μL/mL 2,2-dimethyl-2-phenyl-1-aceptophenone in *N*-vinylpyrrolidone (300 mg/mL) as the photoinitiator. Unbound peptides or proteins were rinsed from the hydrogels during 2 day

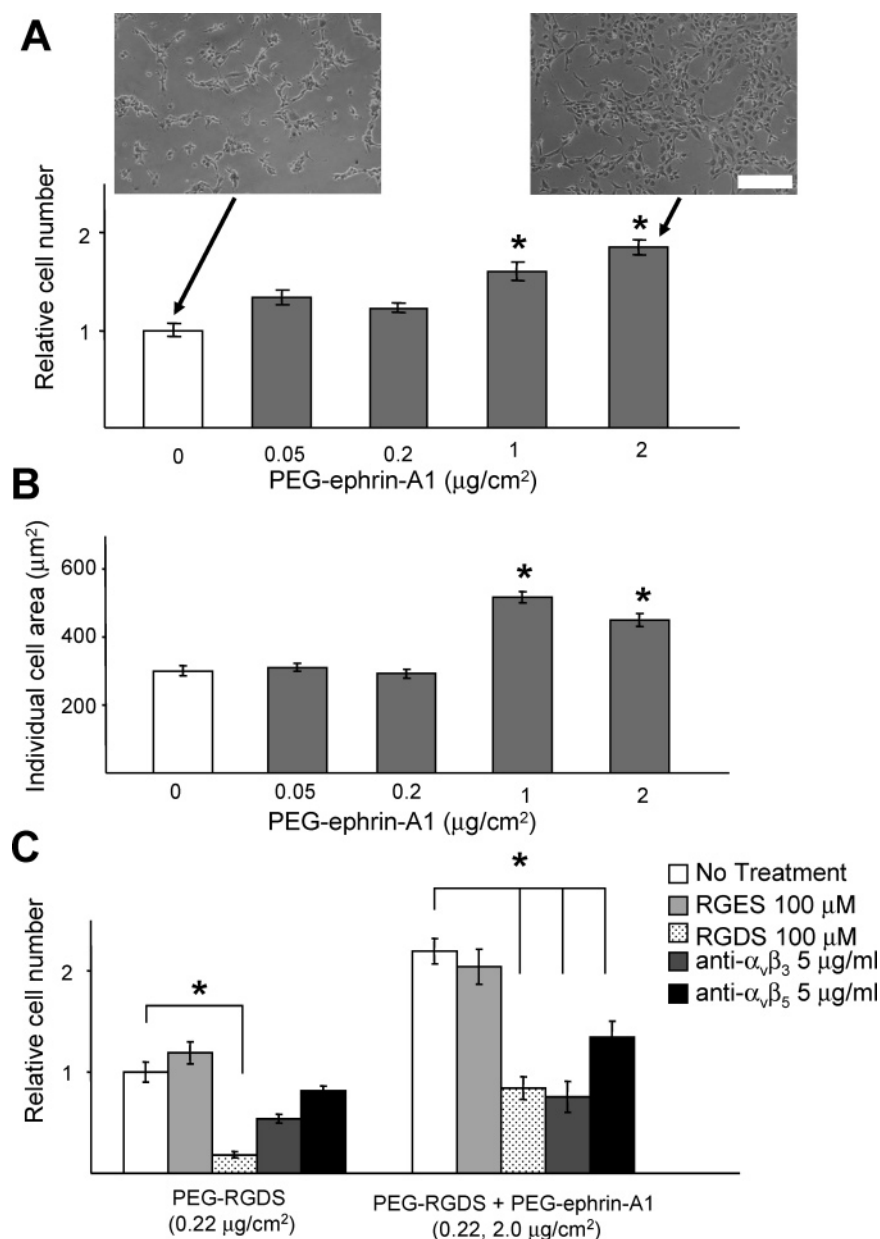


Figure 4. HUVEC adhesion and spreading on PEGDA hydrogels with immobilized PEG-ephrin-A1 and PEG-RGDS. (A) HUVEC adhesion was quantified by counting adherent cell numbers after 5 h of seeding and normalized to that on PEG-RGDS-only hydrogels. Phase-contrast photographs show HUVECs attached on hydrogels at specified PEG-ephrin-A1 concentrations. (B) Cell spreading was obtained by measuring the individual cell area. (C) Cell adhesion in the presence of soluble competitive inhibitors. Data represent mean \pm SEM ($n = 4$). * $P < 0.01$, analyzed by two-way ANOVA followed by Tukey's HSD test, as compared to the corresponding control. Scale bar = 250 μm .

incubation in EGM-2 media. Finally, the hydrogels were cut into discs with a 2.0 cm diameter and placed in 24-well plates.

Cell Adhesion and Spreading. Cell adhesion and spreading were evaluated on both hydrogels and polystyrene culture wells. Hydrogel samples were prepared with a fixed minimal concentration of PEG-RGDS (0.22 $\mu\text{g}/\text{cm}^2$) and various concentrations of PEG-ephrin-A1 (0–2.0 $\mu\text{g}/\text{cm}^2$). Throughout this paper, the PEG conjugated products are reported in weights of the original peptide or proteins prior to modification with PEG chains for convenient comparison with the unmodified counterparts. In parallel studies, various concentrations of unmodified ephrin-A1/Fc were adsorbed on 96-well plates at 37 $^{\circ}\text{C}$ for 2 h, and then the plates were washed twice with PBS. The wells were incubated with 3% bovine serum albumin (BSA) at 37 $^{\circ}\text{C}$ for 2 h to block nonspecific binding sites and washed twice with PBS. HUVECs (45 000 cells/ cm^2) were plated on the hydrogels containing immobilized PEG-ephrin-A1 and also in the wells adsorbed with ephrin-A1/Fc. In competitive inhibition studies, cells were preincubated with soluble competitive inhibitors for 15 min and then plated. These soluble

inhibitors included RGDS and its negative control, Arg-Gly-Glu-Ser (RGES, American Peptide), each at 100 μM as well as anti- $\alpha_v\beta_3$ (LM609) and anti- $\alpha_v\beta_5$ integrin (P1F6) antibodies (Chemicon, Temecula, CA) each at 5 $\mu\text{g}/\text{mL}$. After 5 h of incubation, non-adherent cells were removed with a PBS wash. To aid in visualization, cells were fixed in 3.7% formaldehyde for 15 min and stained in a 0.5% crystal violet solution for 3 min. Five random areas from four wells for each sample were photographed with a digital camera (Nikon) mounted on a phase-contrast microscope (Axiovert 135; Carl Zeiss, Thornwood, NY). Cell attachment and spreading were evaluated by measuring the number of adherent cells and the area of individual cells using Scion Image.

Tube Formation and Visualization. HUVECs (45 000 cells/ cm^2) were cultured for 16 days on hydrogels with immobilized PEG-RGDS (0.22 or 2.2 $\mu\text{g}/\text{cm}^2$) in the presence or absence of PEG-ephrin-A1 (2.0 $\mu\text{g}/\text{cm}^2$). After 1 day in the culture, five random areas from three wells for each sample were photographed, and cell adhesion was measured by counting the number of attached cells. After 16 days in the culture,

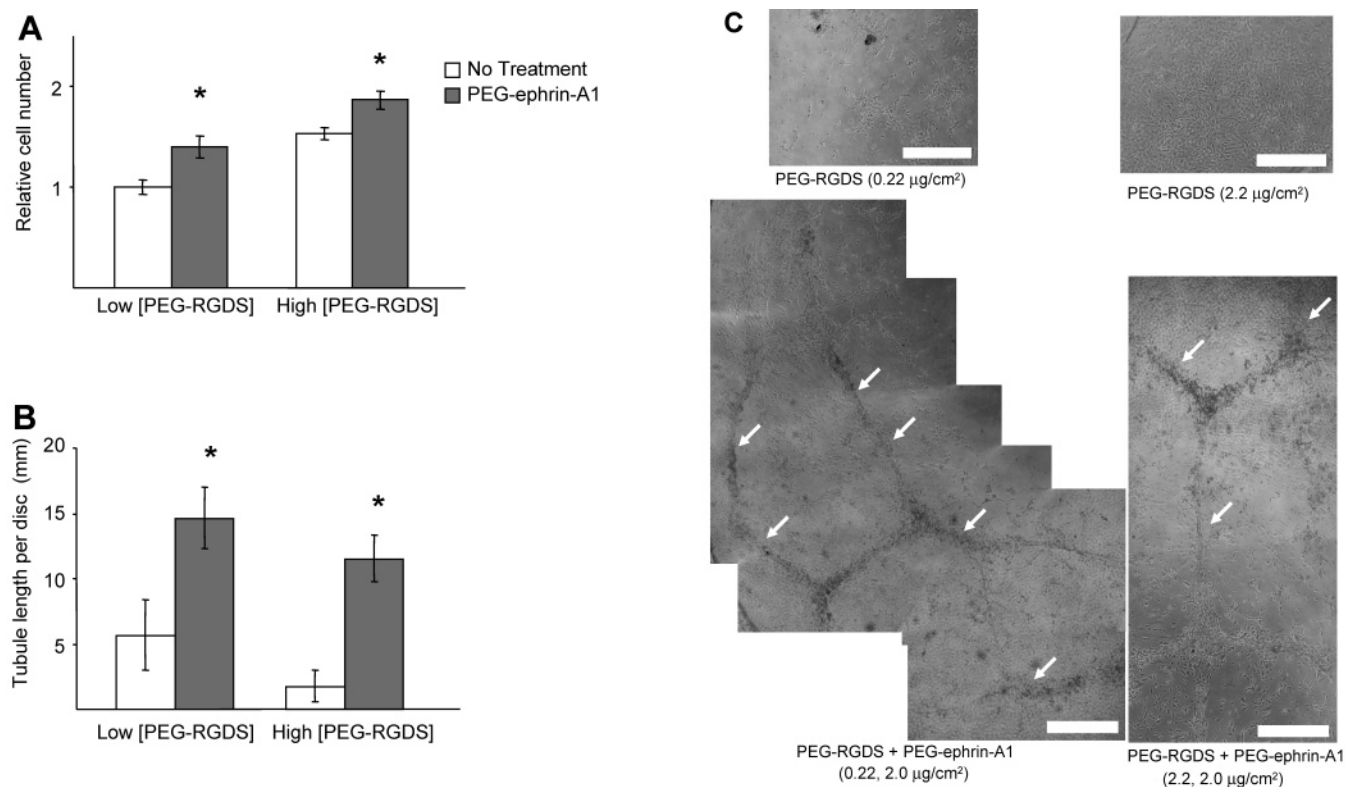


Figure 5. Pro-angiogenic properties of PEG-ephrin-A1. HUVEC adhesion (A) and total tubule length on each hydrogel disc (B) were measured with low and high concentrations of PEG-RGDS (0.22 and 2.2 $\mu\text{g}/\text{cm}^2$) in the presence or absence of PEG-ephrin-A1 (2.0 $\mu\text{g}/\text{cm}^2$). Ephrin-A1 stimulated HUVEC adhesion and tubule formation independently from RGDS concentration. (C) Merged phase-contrast images show tubule networks on hydrogels as indicated by arrows. Data represent mean \pm SEM ($n = 3$). * $P < 0.05$, analyzed by two-way ANOVA followed by Tukey's HSD test, as compared to the corresponding control. Scale bar = 800 μm .

a series of photographs was taken in each well, and the images were merged using Adobe Photoshop Elements. Tubule length in each well was measured with Scion Image. HUVECs with tubule structures on hydrogels were fixed in 3.7% formaldehyde in PBS for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min. For actin and nuclei visualization, the specimens were incubated with TRITC-conjugated phalloidin (5 U/mL, Sigma) for 1 h and DAPI (300 nM, Invitrogen, Carlsbad, CA) for 5 min to stain actin and cell nuclei, respectively. Fluorescence was visualized via confocal microscopy (Zeiss LSM 510 META).

Statistical Analysis. Statistical analysis was performed with JMP 5.1 (SAS Institute Inc, Cary, NC). Data sets were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's HSD test for multiple comparisons. P values less than 0.05 were considered statistically significant. All values are reported as mean \pm standard error of mean.

Results and Discussion

Surface-Adsorbed Ephrin-A1 Promotes HUVEC Adhesion via Integrins. To understand the effects of ephrin-A1 on EC adhesion, we evaluated HUVEC adhesion on polystyrene culture wells pre-adsorbed with various concentrations of ephrin-A1 ectodomain fusion protein, ephrin-A1/Fc. Thirty minutes after seeding, HUVECs attached poorly on non-treated wells blocked with BSA, while the number of adherent cells increased by more than 15-fold in the wells pre-adsorbed with 0.02–10 $\mu\text{g}/\text{mL}$ ephrin-A1/Fc (Figure 2A). Similar treatments with human IgG, a negative control corresponding to the Fc portion of the fusion proteins, had no effect on HUVEC adhesion in concentrations between 0.5 and 60 $\mu\text{g}/\text{mL}$ (data not shown).

There are reports suggesting that the Eph and ephrin families mediate cellular adhesion, not through a mechanical tethering mechanism but by activation of integrins.^{23,24} To test whether

HUVEC adhesion was supported by integrins, RGDS, an integrin ligand, was added in media during cell adhesion. Soluble RGDS at 100 μM effectively inhibited HUVEC adhesion promoted by ephrin-A1/Fc, while RGES, a control peptide sequence, did not have any effects (Figure 2B). These results suggest that integrins are the major mediator of HUVEC adhesion and that ephrin-A1 promotes HUVEC adhesion via activation of integrins.

Characterization of PEG Modified Materials. Proton NMR analysis has demonstrated that two terminal groups of PEG were successfully converted to acrylates as PEGDA showed the methylene protons of PEG as a triplet at 3.6–3.7 ppm and the acrylate protons at 6.0–6.5 ppm. PEG-RGDS analyzed by gel permeation chromatography displayed a distinctive shift in the molecular weight distribution peak after acrylate-PEG conjugation to RGDS.

SDS-PAGE followed by silver staining confirmed that PEG was successfully conjugated to ephrin-A1/Fc (Figure 3). Molecular weight of the recombinant protein, ephrin-A1/Fc, is 47 kDa, but due to glycosylation, the majority of the products appear in the 50–55 kDa range, while the minor band at 35 kDa is likely from degradation of ephrin-A1/Fc. The band corresponding to PEG-ephrin-A1 showed an increase in molecular weight as compared to the unmodified counterpart. The smeared bands in the lane for PEG-ephrin-A1 indicate that multiple PEG chains were conjugated to ephrin-A1/Fc. Additionally, the staining intensity of the band corresponding to the unmodified ephrin-A1/Fc was negligible, indicating that the majority of ephrin-A1/Fc was conjugated to PEG. To verify that PEG conjugation did not negatively affect the bioactivity of ephrin-A1, HUVEC adhesion was observed on polystyrene culture wells pre-adsorbed with PEG modified and unmodified

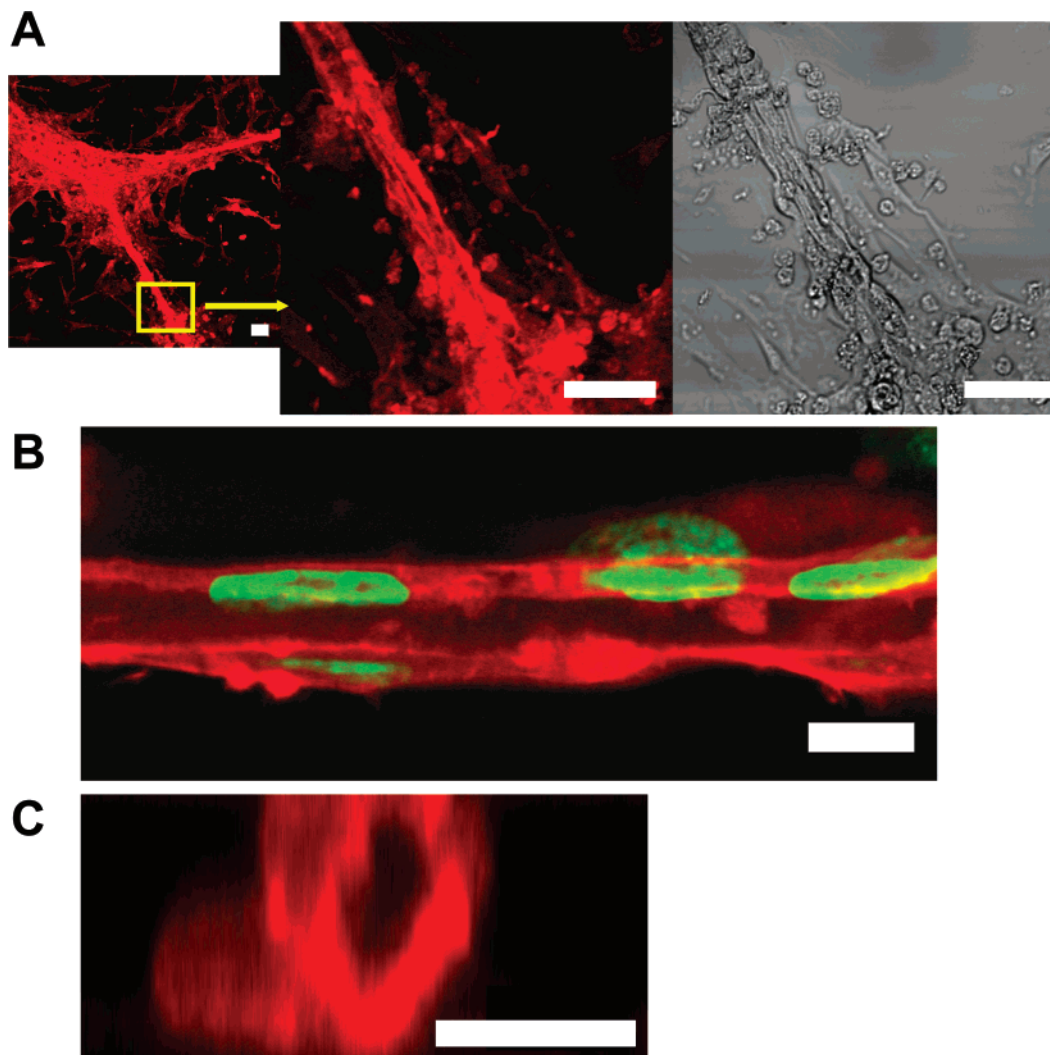


Figure 6. Visualization of capillary-like structures formed by HUVECs on PEGDA hydrogels. (A) Confocal and phase-contrast images show tubular interconnections formed by HUVECs on PEGDA hydrogels with immobilized PEG-RGDS and PEG-ephrin-A1 at 0.22 and 2.0 $\mu\text{g}/\text{cm}^2$, respectively. Ephrin-A1 immobilized on the hydrogels stimulated formation of capillary-like structures with lumens. The capillary-like structures were visualized in longitudinal (B) and vertical (C) cross-sections. Cell staining with phalloidin-TRITC and DAPI are pseudo-colored in red and green, respectively. Scale bars = 50 μm in panel A and 10 μm in panels B and C.

ephrin-A1/Fc. PEG-ephrin-A1 indeed retained the bioactivity of the original protein as comparable degrees of cell adhesion were observed with both forms of ephrin-A1/Fc (data not shown).

Ephrin-A1/Fc Immobilized on PEGDA Hydrogels Promotes HUVEC Adhesion via $\alpha_v\beta_3$ Integrins. To present ephrin-A1 in a highly controlled system, PEG-ephrin-A1 was immobilized on the surface of PEGDA hydrogels via photopolymerization. Since PEGDA hydrogels with PEG-ephrin-A1 alone did not support HUVEC adhesion (data not shown), a minimal concentration of PEG-RGDS (0.22 g/cm^2) was added to all hydrogels. PEG-ephrin-A1 immobilized on PEGDA hydrogels promoted a dose-dependent increase in HUVEC adhesion and spreading after 5 h of cell seeding (Figure 4A). Specifically, at concentrations of 1.0 and 2.0 $\mu\text{g}/\text{cm}^2$, the number of adherent cells was increased by approximately 60 and 85%, respectively ($P < 0.01$). In a similar manner, cell spreading, measured by the individual cell area, was significantly enhanced by 1.0 and 2.0 $\mu\text{g}/\text{cm}^2$ PEG-ephrin-A1 ($P < 0.01$). Representative phase-contrast images are shown in Figure 4A.

To determine the subclass of integrins responsible for HUVEC adhesion, we performed competitive inhibition assays during cell adhesion on PEGDA hydrogels with immobilized

RGDS (0.22 $\mu\text{g}/\text{cm}^2$) with or without PEG-ephrin-A1 at 2.0 $\mu\text{g}/\text{cm}^2$ (Figure 4C). Consistent with the results seen on polystyrene culture wells, solution-phase RGDS, but not RGES, abrogated HUVEC adhesion induced by PEG-ephrin-A1 immobilized on PEGDA hydrogels. The addition of an anti- $\alpha_v\beta_3$ integrin antibody significantly inhibited cell adhesion on hydrogels immobilized with PEG-ephrin-A1, whereas a decrease in cell adhesion was only modest with the addition of the anti- $\alpha_v\beta_5$ integrin antibody. This suggests an important role for $\alpha_v\beta_3$ integrin as a major mediator of ephrin-A1 stimulated HUVEC adhesion.

Regulation of cell adhesion by the Eph and ephrin families has been linked in part to integrins. Interaction between the EphB1 receptor and the ephrin-B1 ligand induce integrin-mediated attachment and migration in human renal microvascular ECs.^{23,24} Similarly, ephrin-A5 modulates fibroblast adhesion via activation of integrins.²⁵ Our data corroborate with these evidence in that ephrin-A1 stimulated HUVEC adhesion is found to be mediated by integrins, specifically by $\alpha_v\beta_3$ integrin rather than $\alpha_v\beta_5$ integrin. Cell repulsion is also known to be regulated by the interaction between integrins and Eph and ephrin families. Activation of EphA2 with ephrin-A1/Fc induces inactive conformation of integrins and inhibits spreading and migration

of PC-3 prostate epithelial cells.²⁶ It is noteworthy that the inhibitory effect of ephrin-A1/Fc treatment on the spreading of epithelial cells is reversed in ECs as seen in the present study, indicating cell type specificities of the Eph and ephrin families.

Ephrin-A1 Induces HUVEC Adhesion with Low and High Concentrations of RGDS. In the cell adhesion experiments on PEGDA hydrogels (Figure 4), a small concentration of PEG-RGDS (0.22 $\mu\text{g}/\text{cm}^2$) was immobilized in all hydrogels to support basal cell adhesion. To confirm that the adhesive effect of ephrin-A1 was not restricted to this particular concentration of RGDS, we chose to compare HUVEC adhesion on hydrogels with PEG-RGDS either at 0.22 or 2.2 $\mu\text{g}/\text{cm}^2$ either with or without PEG-ephrin-A1 at 2.0 $\mu\text{g}/\text{cm}^2$.

When measured after 1 day of culture, HUVEC adhesion was found to be promoted by PEG-ephrin-A1 at a low PEG-RGDS concentration of 0.22 $\mu\text{g}/\text{cm}^2$ as discussed earlier (Figure 5A). As expected, in the absence of PEG-ephrin-A1, a higher PEG-RGDS concentration of 2.2 $\mu\text{g}/\text{cm}^2$ increased the number of adherent cells as compared to a lower PEG-RGDS concentration at 0.22 $\mu\text{g}/\text{cm}^2$ (53% increase). PEG-ephrin-A1 was able to promote HUVEC adhesion even at a high concentration of PEG-RGDS at 2.2 $\mu\text{g}/\text{cm}^2$. Although the percent increase in cell adhesion in the presence of PEG-ephrin-A1 was reduced from 40% at 0.22 $\mu\text{g}/\text{cm}^2$ PEG-RGDS to 22% at 2.2 $\mu\text{g}/\text{cm}^2$ PEG-RGDS, there was a general trend of increased cell adhesion by HUVECs stimulated with PEG-ephrin-A1.

Ephrin-A1 Promotes HUVEC Tubule Formation with Low and High Concentrations of RGDS. The current system was found to be highly conducive for angiogenesis as HUVECs spontaneously formed tube-like networks on the hydrogels as early as 9 days after seeding. After 16 days of cell seeding, tube-like structures were photographed, and the tubule length in each well was measured (Figure 5B,C). On hydrogels with immobilized PEG-ephrin-A1 at 2.0 $\mu\text{g}/\text{cm}^2$, the tubule formation was significantly enhanced independently from PEG-RGDS concentrations, as indicated by the increased tubule length. In contrast, HUVECs did not form a tubule network but maintained a normal EC monolayer on hydrogels with PEG-RGDS alone at both concentrations of 0.22 and 2.2 $\mu\text{g}/\text{cm}^2$.

HUVECs Form Capillary-Like Structures on Ephrin-A1 Immobilized PEGDA Hydrogels. The tube-like structures formed on hydrogels immobilized with PEG-ephrin-A1 were fixed on day 9 and stained for actin and nuclei with phalloidin-TRITC and DAPI and examined under confocal microscopy (Figure 6). Cells stained with phalloidin-TRITC show interconnections of cell bodies highly organized into tube-like structures (Figure 6A). When examined more closely in longitudinal cross-sections, HUVECs appeared to be aligned with a central lumen (Figure 6B). As identified by nuclear staining, the central lumens were enveloped by one or two cells along the central axis, which is reminiscent of capillaries. The vertical cross-section showed that HUVECs extended upward from the surface to form a capillary-like structure with 5–30 μm diameter lumens (Figure 6C).

Conclusion

In this work, we have examined pro-angiogenic properties of ephrin-A1 and successfully incorporated it into biomimetic hydrogels for studies of angiogenesis and future applications in therapeutic angiogenesis. Covalent immobilization of ephrin-A1/Fc onto PEGDA hydrogels permitted continuous presentation of its cell binding domains to HUVECs and stimulation of cell adhesion and capillary formation with lumens. Ephrin-A1

promoted HUVEC adhesion and spreading in a dose-dependent manner, and data from the competitive inhibition study suggest that ephrin-A1 stimulates HUVEC adhesion via $\alpha_v\beta_3$ integrin rather than $\alpha_v\beta_5$ integrin. The angiogenic properties of ephrin-A1 were demonstrated further as it enhanced cell adhesion, increased the total length of tubes formed, and acted independently from RGDS concentration.

We have demonstrated here that ephrin-A1 treatment at all concentrations tested enhanced EC angiogenic responses (cell adhesion and tubule formation) and that these findings are consistent with other studies.^{11,12} Ephrin-A1/Fc induced EC migration and capillary invasion,¹¹ while ECs lacking EphA2, the receptor for ephrin-A1, failed to undergo vascular assembly in response to ephrin-A1 treatment.¹² EphA2 expression seems to be specifically localized in tumor-associated endothelium in the pancreatic islet and mammary carcinoma models, and EphA2 inactivation with soluble EphA2/Fc or EphA3/Fc inhibits EC migration and tumor angiogenesis.¹¹ In addition, VEGF, a potent mediator of angiogenesis, is thought to increase ephrin-A1 expression in ECs, leading to juxtacrine activation of the EphA2 receptor and inducing subsequent angiogenic responses. These lines of evidence indicate that activation of the EphA2 receptor with the ephrin-A1 ligand is a critical step in angiogenesis.¹³

There have been enormous efforts in the cardiovascular as well as cancer research community to understand the mechanism behind angiogenesis. The work presented here aimed to investigate the EC responses in angiogenesis mediated by ephrin-A1 and to translate this pro-angiogenic agent identified previously in basic research into synthetic biomaterials, which in the future may provide suitable tools for therapeutic angiogenesis as well as vascularization of tissue engineered constructs.

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