The promotion of microvasculature formation in poly(ethylene glycol) diacrylate hydrogels by an immobilized VEGF-mimetic peptide

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Microvascularization of tissue engineered constructs was achieved by utilizing a VEGF-mimicking peptide, QK, covalently bound to a poly(ethylene glycol) hydrogel matrix. The 15-amino acid peptide, developed by D’Andrea et al., was modified with a PEG-succinimidyl ester linker on the N-terminus of the peptide, then photocrosslinked onto the surface or throughout PEG hydrogels. PEGylation of the peptide increased its solubility and bioactivity, as evidenced by endothelial cell proliferation. PEG-QK showed equal or superior ability to promote angiogenesis in vitro, on the surface of hydrogels and within three-dimensional collagenase-degradable hydrogels, compared to RGDS only or PEG-VEGF hydrogels. Endothelial cells were shown to form tubule structures, migrate, and make cell–cell contacts in response to covalently-bound PEG-QK. In vivo a mouse cornea micropocket angiogenesis assay, PEG-QK hydrogels promoted more complete coverage of host microvasculature within the hydrogel. PEG-QK was shown to enhance vessel branch points and vessel density as well as space filling properties of fractal dimension and lacunarity. This report shows the ability to promote angiogenesis in tissue engineered constructs using a covalently-bound small peptide rather than a large protein and may point to an advance in designing biomimetic cellular environments.

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1. Introduction

Many approaches of promoting microvascularization utilize signaling moieties, most often growth factor proteins, either released from or attached to a tissue engineering matrix. The use of growth factor proteins has inherent disadvantages, such as immunogenicity and loss of bioactivity. Although fibronectin was originally used for cell attachment to matrices, many researchers found the simplicity of the Arg-Gly-Asp (RGD) peptide sequence, the bioactive integrin ligand derived from fibronectin [1], to be advantageous in matrix design. Similarly, it could be beneficial to use a shorter bioactive peptide sequence derived from a growth factor protein if it could confer the same bioactivity and cellular response.

A synthetic 15-amino acid peptide, based on a region of the vascular endothelial growth factor (VEGF) binding interface, has been shown to possess similar biological activity to that of the VEGF protein. This peptide, called QK, was based on the region of VEGF that binds to VEGFR-1 domain 2. The VEGF binding region, Phe-17-Tyr-25, forms an α-helix conformation in its native bound state. D’Andrea et al. synthesized the exact peptide sequence in the binding domain, but found that the peptide did not form an α-helix conformation in water, most likely from the lack of surrounding sequences that would stabilize the bioactive conformation. The unstructured VEGF15 peptide did not display bioactivity. To stabilize the α-helix conformation, alterations in amino acid sequence were included; this designed peptide was called QK. The α-helix of the sequence was considered a necessity for bioactivity, as the tertiary structure of the sequence determined ligand–receptor binding [2]. The sequences of the investigated physiological VEGF binding region and the QK peptide are compared below (Table 1).

In D’Andrea’s studies, QK was able to bind and activate both KDR (VEGFR-2) and Flt-1 (VEGFR-1) receptors in vitro. In a proliferation assay, similar bioactivity levels were found between 1 mol VEGF and 385 mol QK. QK was shown to activate ERK1/2, which is a required cell signaling pathway step in VEGF-modulated angiogenesis. ERK1/2 was activated to different levels depending on dosage of QK, and QK and VEGF activation was additive. Additionally, D’Andrea et al. showed that soluble QK induced tubulogenesis within 11 d in a Matrigel model. D’Andrea et al. also showed that QK...
could cause VEGF receptor dimerization and subsequent activation despite its small molecular weight [2]. This novel, bioactive VEGF-mimicking peptide is another promising option to promote and control angiogenesis in tissue engineering matrices because it shows similar bioactivity to VEGF yet provides the advantages of a peptide, including smaller size, ease of use in chemical reactions, less likelihood to trigger an immune response, and ability to be more easily synthesized with variations allowing tuning of a biomimetic matrix system. The current work aims to determine whether incorporating a small bioactive peptide, instead of a bulky protein, into a tissue engineering matrix will produce angiogenic behavior in vitro and in vivo. If so, then a giant step can be taken in the field to move toward incorporating bioactive peptides, with the above-mentioned advantages, instead of proteins, in designing bioactive matrices which can direct cell behavior.

Poly(ethylene glycol) diacylate (PEGDA) is a well-known synthetic polymer that can be crosslinked into hydrogels used as biomimetic tissue engineering matrices [3,4]. PEG hydrogels offer several advantages over natural polymers including ease in varying mechanical strength and consistent characterization. Because PEG hydrogels can be formed using non-cytotoxic photoinitiators, they can support cell attachment, encapsulation, and activity [3,5,6]. The incorporation of collagenase-degradable PEG derivatives supports cell encapsulation into 3D hydrogels as well as cell migration into the matrix, through cell-secreted protease cleavage of the proteolytically-degradable peptide backbone within the PEG chain [7]. PEG hydrogels can also be modified with covalently-linked cell-adhesive ligands and bioactive proteins and peptides to promote specific cell activity on or within the hydrogel [4,8].

The use of PEGDA hydrogels as tissue engineering matrices allows the fine tuning of matrix–cell interactions. Previous work [9,10] shows that incorporation of PEG–modified peptide RGDS and protein VEGF confers bioactivity upon the PEG hydrogel, promoting angiogenic activity. Release of biological signals must be limited in tissue engineering applications to reduce unwanted systemic effects. As D’Andrea already showed that soluble QK can induce angiogenic behavior, the current work aims to determine whether covalently bound QK can do the same, thus tightly controlling the signaling location. The biological signal should be sequestered to act as a base catalyst. Acryloyl-PEG-succinimidy ester (PEG-SMC, Laysan, MW = 3400 Da) was similarly dissolved at a concentration of 1.5 molar equivalent Ag2O (Sigma), 1.1 molar equivalent acryloyl chloride (Sigma) and 30 mM NaI (Sigma) in anhydrous DCM at 0–4 °C overnight. The product was filtered in solution using Celite 521 (Spectrum Chemical Manufacturing Corp, Gardena, CA) to remove silver. The filtered product was then directly dissolved in phosphate buffer (pH 7.4) followed by altering the pH to pH 3–5 using HCl. The solution was then heated to 35 °C for 1 h, and activated charcoal (Fisher) was added to remove iodine. The solution was then again filtered using Celite 521. NaCl was added with DCM, followed by extraction of DCM. Phase separation using 2 molar NaCl removed chloride ions and acid. Resulting monoacrylated PEG was dried using pentane and concentrated via vacuum. PEG monoacrylate was then reacted with 4 molar excess diisuccinimidyl carbonate (Sigma) in anhydrous dichloromethane and pyridine (Sigma) overnight under argon. The resulting solution was then diacetylated in acetate buffer (0.1 M, pH 4.5, 15% NaCl), dried using anhydrous MgSO4, filtered in ethyl ether, and vacuum filtered. PEG monoacrylate was then reacted with 4 molar excess diisuccinimidyl carbonate (Sigma) in anhydrous dichloromethane and pyridine (Sigma) overnight under argon. The resulting solution was then diacetylated in acetate buffer (0.1 M, pH 4.5, 15% NaCl), dried using anhydrous MgSO4, filtered in ethyl ether, and filtered overnight under vacuum. PEG-SMC was characterized by 1H NMR and MALDI-TOF and stored at −80 °C under argon until use.

2.2.2. Preparation and purification of PEG-QK

The angiogenic peptide Ac–KLTVQELYQL[K(Ac)][Y(Ac)][G(Ac)]–amide was designed to react with PEG-SMC at the first lysine (K) residue only, by protecting all other free amines with acryloyl groups (represented by Ac). QK (MW = 2036 Da, Aaptec, Louisville, KY) was dissolved in DMSO at a concentration of 7.4 mM. N,N-diisopropylcarbodiimide (DCM; Sigma) was added at a molar ratio of 200 molar equivalents to PEG-SMC with a concentration of 0.1 M in acetate buffer (0.1 M, pH 4.5, 15% NaCl), dried using anhydrous MgSO4, filtered in ethyl ether, and filtered overnight under vacuum. PEG-QK was then lyophilized and stored in HEPES buffered saline (HBS) with 0.1% bovine serum albumin (BSA) at 4 °C until use. Conjugation was characterized by MALDI-ToF (Bruker Daltonics, Billerica, MA).

2.2.4. Preparation and purification of PEG-RGDS

The cell-adhesive peptide RGD (American Peptide, Sunnyvale, CA) was dissolved in DMSO at a concentration of 30 mM. Dipea was added to the solution to act as a base catalyst. Acryloyl-PEG-N-hydroxysuccinimide (PEG-SMC, Laysan, MW = 3400 Da) was similarly dissolved at a concentration of 1.5 molar equivalents of PEG-SMC was added dropwise to RGD in a 3:1 molar ratio with slow mixing and allowed to react 4 d at 25 °C. The product was precipitated in cold isopropanol and dried overnight. PEG-RGD was then lyophilized and stored at −80 °C under argon until use. Conjugation was characterized by gel permeation chromatography (GPC) using a PLgel column (5 μm, 500 A, Polymer Laboratories, Ambergate, MA) with 0.1% triethylamine in DMF solvent, and evaporative light scattering (ELS) detector (Polymer Laboratories), run against unreacted PEG-SMC for comparison.

2.2.5. Synthesis of collagenase-degradable PEG-PQ-PEG

A collagenase-sensitive peptide (PQ), CCGGDPWQGQCK, was prepared on a peptide synthesizer (Aaptec, Louisville, KY) using standard Fmoc chemistry. The peptide was cleaved from the resin using 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS) in water and precipitated in ether. The peptide was reacted with PEG-SMC (Laysan, MW = 3400 Da) in a 2:1 ratio (PEG-SMC/ PEG) in DMSO and Dipea (2 mol per mol PEG) for 4 d at 25 °C to generate a PEG-diacylate derivative with PQ in the polymer backbone. PEG-PQ-PEG was dialyzed against DI H2O for 8 h using a membrane with a 3500 Da molecular weight cutoff (Spectrum Laboratories, Rancho Dominguez, CA). PEG-RGDS was then lyophilized and stored at −80 °C under argon until use. Conjugation was characterized by gel permeation chromatography (GPC) using a PLgel column (5 μm, 500 A, Polymer Laboratories, Ambergate, MA) with 0.1% triethylamine in DMF solvent, and evaporative light scattering (ELS) detector (Polymer Laboratories), run against unreacted PEG-SMC for comparison.

2.2.6. Synthesis of PEG-VEGF

PEG-VEGF was synthesized as previously described in detail [10]. In brief, VEGF15 (Sigma) was reacted with acryloyl-PEG-SMC in a 1:2000 molar ratio in sterile 50 mM sodium bicarbonate buffer (pH 8.0, 5 °C) for 4 d. PEG-VEGF was then lyophilized under sterile conditions and stored in HEPES buffered saline (HBS) with 0.1% bovine serum albumin (BSA) at 4 °C until use. Conjugation was confirmed via Western blot by visualizing an increase in molecular weight as previously described in detail [10].
2.3. Bioactivity assay

Bioactivity of PEG-QK was determined by measuring its pro-mitotic effect on endothelial cells. HUVECs were seeded at 105 × 103 cells/cm2 in EGM-2 medium without VEGF on Day 1. On Day 4, medium was replaced with EGM-2 medium without VEGF and FGF, with an addition of either 0.13 pmol/ml VEGF (positive control), 50 pmol/ml QK, 50 pmol/ml PEG-QK, or no additives (negative control). The levels of QK were chosen to correspond with the bioactivity levels reported in D’Andrea’s original paper on the peptide, which showed that the bioactivity of 1 mol VEGF was equal to that of 385 mol QK [2]. This medium was replaced after 4 h with EGM-2 without VEGF and FGF. On Day 6, cells were treated with a Hoechst 33342 dye (Bis-Benzimide, Sigma), which labels cell nuclei. Hoechst dye was added at a concentration of 5 μg/ml in EGM-2 without VEGF and FGF, and cells were incubated with this media for 1 h. Fluorescent images were taken immediately after washing and incubation with 4–6 images per well, using a fluorescent microscope with excitation − 350 nm, emission − 460 nm. Cell nuclei were quantified using ImageJ. Statistical differences between groups were analyzed using ANOVA, followed by Tukey’s Least Significant Difference (LSD) post-hoc analysis, with p < 0.05 considered statistically significant.

2.4. Formation of modified PEGDA hydrogels

2.4.1. Formation of PEGDA hydrogels

Hydrogels were formed as previously described in detail [10]. In brief, 6 kDa PEG was dissolved in HEPES buffered saline (HBS) in a 10% w/v solution and sterile filtered. Photoinitiator, 10 μl of 300 mg/ml 2,2-dimethoxy-2-phenylacetophenone in N-vinylpyrrolidone (NVP), was added to the solution. The polymer solution was pipetted into molds and crosslinked through exposure to long wave-length ultraviolet light (B-200SP UV lamp, UVP, 365 nm, 10 mW/cm2) for 30 s. After crosslinking, the mold was removed, and the PEGDA hydrogel slab was placed in sterile PBS with 0.1% sodium azide until further use.

2.4.2. Surface modification of PEGDA hydrogels

Hydrogel slabs were soaked for 1 h in sterile PBS to remove sodium azide. 5 mm diameter circles were punched from PEGDA hydrogel slabs. A polymer solution consisting of 173 nmol/ml PEG-QK, 30 pmol/ml PEG-RGDS, 1 pmol/ml eosin Y, and 3.95 μl/ml NVP was prepared. From this solution, 10 μl was pipetted onto the top surface of the gel, completely covering the surface. The gel and polymer solution were exposed to a 532 nm laser at 30 mW/cm2 for 30 s. Positive control hydrogels were washed with 4% acryloyl-PEG-QK instead of PEG-QK, and negative control hydrogels contained PEG-RGDS only. Each surface-modified gel was then soaked in sterile PBS for 1 h to allow non-reacted polymer, excess photoinitiator, and residual sodium azide to diffuse from the gel.

2.4.3. Formation of Three-dimensional protein-covalently degradable PEG hydrogels

Collagenase-degradable hydrogels with encapsulated HUVECs (3 × 103 cells/ml, labeled with CellTracker Red) were prepared. Briefly, PEG-PQ-PQ (0.1 g/ml), acryloyl-PEG-RGDS (3.5 μmol/ml), and acryloyl-PEG-QK (152 nmol/ml or 760 nmol/ml) were mixed with a cell suspension and photocrosslinked by exposing to long wavelength ultraviolet light (385 nm, 10 mW/cm2) for 7 min, using Irgacure 2959 as the photoinitiator (0.3% w/v). Two concentrations of PEG-QK were investigated; the lower corresponds to the bioactivity levels reported in D’Andrea et al. [2]. Solutions were crosslinked via exposure to UV light for 2 min. Hydrogels for implantation were prepared by making a 10% polymer weight percentage (0.1 g/ml) prepolymer solution of PEG-PQ-PQ, 3.5 μmol/ml PEG-RGDS, 10 μl/mıl acetonaphone stock solution, and 640 ng soluble VEGF per gel with or without 1.65 mg PEG-VEGF or 32 ng PEG-QK per gel. Hydrogels did not contain encapsulated cells. Prepolymer solution (0.12 μl) was injected between glass slides separated by a 0.005 in thick polytetrafluoroethylene (PTFE) spacer secured with binder clips and crosslinked via exposure to UV light for 2 min.

Hydrogels were implanted into the mouse cornea micropocket angiogenesis assay following the protocol outlined in Poche et al. [11]. All animals were used under an approved protocol of the American Association of Laboratory Animal Science and the Institutional Animal Care and Use Committee at Baylor College of Medicine. FkI-myr:mCherry transgenic mice were utilized, which enable visualization of vessel coverage via endothelial cell specific fluorescence [12]. Briefly, a partial thickness incision of approximately 30 μm in depth was made in the cornea of anesthetized mice. A Von Graefe knife was used to separate the layers of the cornea, creating a micropocket approximately 700 μm in length where the implants could be placed approximately 400–400 μm away from the limbus vessels with great reproducibility. Mice were euthanized 14 d after implantation, and corneas were collected and fixed in 4% parafomaldehyde. Tissue flatmounts were prepared and imaged on a Zeiss LSM 510 META confocal microscope using a 40× oil immersion objective (working distance 0.28 mm with a 543 nm laser for excitation of the mCherry fluorophore. Images of vessels on the hydrogel were compiled from projections of z-stacks 22 μm in thickness, spaced 1 μm apart. These images were then compiled into z-stacks, and the three-dimensional data set was used to count branching and vessel diameters, using the image browser in the LSM software. Next, the stacks were processed into projections using a maximum intensity projection setting in the LSM Image examiner software. Projections were processed into grayscale images and by threshold to preserve the vessel elements. The images were then opened in MATLAB and analyzed for vessel density and several scale-dependent space filling parameters including fractal dimension and lacunarity using a program available free online through the MATLAB central exchange [13–16]. Briefly, the fractal dimension quantifies the amount of space filled by an object, and lacunarity quantifies how it fills space [13,17,18]. Finally, all groups were compared using ANOVA with Tukey’s LSD to identify statistically significant differences.
3. Results

3.1. Polymer characterization

Conjugation of QK to acryloyl-PEG-SMC was confirmed by MALDI-ToF, which showed a distribution of PEG-QK species around 5000–7000 Da, corresponding to one acryloyl-PEG molecule per QK, with the distribution due to the polydispersity of the acryloyl-PEG-SMC used in the reaction. Each species is about 44 Da different from the next species, which corresponds to the molecular weight of one ethylene glycol repeat in the PEG chain. Additionally, the peptide QK was not detected by MALDI-ToF at its molecular weight of 2036 Da, suggesting complete reaction of all peptide in the synthesis reaction. Conjugation of RGDS to acryloyl-PEG-SMC was confirmed via GPC, which showed the product PEG-RGDS to have a higher molecular weight and hydrodynamic size than PEG-SMC. Conjugation of PEG-PQ-PEG was similarly confirmed via GPC. Conjugation of VEGF to acryloyl-PEG-SMC was confirmed via Western blot.

3.2. Pro-mitotic effects of PEG-QK

Bioactivity of PEG-QK was measured by its pro-mitotic effects on endothelial cells. Proliferation was measured by counting cell number after treatment with bioactive media containing VEGF, QK, PEG-QK, or no additional additives (Control). PEG-QK increased endothelial cell number 1.27 ± 0.02 fold and VEGF increased endothelial cell number 1.25 ± 0.05 fold, compared to a 1.09 ± 0.10 fold increase in response to un-PEGylated QK. The apparent low bioactivity of un-PEGylated QK may be due to its low solubility in cell medium. PEG-QK appears to have sufficient solubility in water for ease of use. The PEG-QK and VEGF results were not statistically different from each other and are in range of VEGF proliferation expected results [9]. Both VEGF, as the positive control, and PEG-QK promoted a proliferation response that was statistically higher than the control, with ***p < 0.01 between PEG-QK and Control as well as VEGF and Control, and *p < 0.05 between PEG-QK and QK as well as VEGF and QK (Fig. 1).

3.3. Quantification of PEG-QK, PEG-VEGF, and PEG-RGDS on the surface of hydrogels

For surface-modified hydrogels, PEG-QK hydrogels had an average of 2.59 ± 1.17 nmol/cm² PEG-QK on the surface. PEG-VEGF hydrogels had an average of 4 ± 0.84 pmol/cm² PEG-VEGF on the surface. Although added amounts were calculated to equalize bioactivity levels between gels, it appears that more PEG-QK was successfully attached to the surface, leading to a 1.7 fold increase in bioactivity level on the PEG-QK gels as compared to the PEG-VEGF gels. All hydrogels had an average of 8.0 ± 2.11 nmol/cm² PEG-RGDS on the surface. Levels of PEG-RGDS were appropriate for long-term cell attachment and equal between all experimental groups.

3.4. Tubulogenic activity of surface-immobilized VEGF and QK

In previous work, PEG-VEGF promoted endothelial tubulogenesis on the surface of hydrogels by day 30, with tubules appearing as early as day 19 on some hydrogels [10]. In this experiment, angiogenic behavior was observed by day 15 on PEG-VEGF and PEG-RGDS hydrogels, and by day 5 on PEG-QK hydrogels. PEG-QK promoted endothelial tubulogenesis after only 5 days in culture, with extensive branching networks of endothelial tubes on hydrogels modified with PEG-QK and PEG-RGDS. Control hydrogel surfaces modified with PEG-VEGF and PEG-RGDS or only PEG-RGDS allowed HUVEC attachment and growth, but did not promote tubulogenesis by this early time point. Hydrogels modified with PEG-RGDS and PEG-QK promoted significantly more tubulogenesis at day 5 than those modified with PEG-RGDS and PEG-VEGF or PEG-RGDS alone (Fig. 2; p < 0.05). Endothelial cells grown on hydrogels modified with PEG-QK and PEG-RGDS, cultured without VEGF in the medium, formed tubules totaling 395 ± 302 μm/mm², while cells on hydrogels modified with PEG-RGDS and PEG-VEGF, cultured without VEGF in the medium, formed tubules totaling 72 ± 62 μm/mm², and cells on hydrogels modified with PEG-RGDS only, cultured without VEGF in the media, formed tubules totaling 117 ± 200 μm/mm². Levels of tubulogenesis on hydrogels with PEG-VEGF and PEG-RGDS were not significantly different than PEG-RGDS only. A common behavior in angiogenesis is remodeling and regression of tubules after initial formation [19]. By day 15, some endothelial tubules on PEG-QK hydrogels had regressed (total tubule length/area = 286 ± 145 μm/mm²). Additionally, by this time point, there were more tubules on PEG-VEGF (535 ± 82 μm/mm²) and PEG-RGDS (519 ± 704 μm/mm²) hydrogels, although there were no significant differences between groups at day 15. These experiments suggest that the wave of tubule formation, followed by regression, occurred earlier on PEG-QK hydrogels than PEG-VEGF hydrogels.

3.5. Angiogenic activity of immobilized QK in 3D hydrogels

To further study the dynamic events occurring on surface-modified hydrogels, time lapse experiments were performed using three-dimensional hydrogel cultures with encapsulated cells. In 3D PEG hydrogels with immobilized QK, endothelial cells exhibited extensive angiogenic behavior, as observed by time lapse confocal microscopy. Between 17 and 35 h after encapsulation, HUVECs formed elongated multiple-cell structures in hydrogels with RGDS and QK or RGDS and VEGF homogeneously and covalently bound to the matrix, but less so in hydrogels with RGDS only (Fig. 3A).

Total tubule length per area was significantly higher in QK and VEGF hydrogels compared to RGDS-only controls (Fig.3B, p < 0.01). Quantification of cell migration showed an increase in hydrogels with the high dose of incorporated QK, compared to VEGF hydrogels (**p < 0.01) and RGDS-only hydrogels (*p < 0.05) (C). Cell–cell contact number was normalized to the number of cells in the initial frame of the time lapse data. Cells in high-dose QK-modified hydrogels formed significantly more cell–cell
contacts than cells in hydrogels with RGDS only, and cells in low-dose QK-modified hydrogels showed similar cell–cell contact formation activity to hydrogels with VEGF (D). In cell migration and cell–cell contact quantification, low-dose QK and VEGF hydrogels showed trends for increased angiogenic behavior, and high-dose QK hydrogels confirmed these trends with significantly more angiogenic behavior. Both migration and cell–cell contact formation through surface projections are fundamental characteristics of angiogenesis [20].

Time lapse confocal microscopy showed endothelial tubes regressing after 44 h for QK hydrogels and 54 h for those with VEGF. HUVEC migratory behavior in QK hydrogels continued until the study ended, suggesting that the covalently-bound QK peptide retained bioactivity throughout the study. The time lapse studies confirmed that covalently bound PEG–QK is able to induce angiogenic behaviors: endothelial cell–cell contact formation, migration, and endothelial tubule network formation.

3.6. Vessel coverage by immobilized VEGF and QK in vivo

To investigate the role of the immobilized VEGF-mimicking peptide in vivo, bioactive hydrogels were implanted in a mouse cornea micropocket – an established, reproducible, and quantifiable assay [21–23]. These matrix metalloproteinase-sensitive hydrogels contained non-bound, diffusible VEGF to stimulate an angiogenic response from the surrounding limbus into the normally avascular cornea. Bound forms of VEGF and QK were then added to the implant to prolong the bioactive signaling to newly formed vessels on the hydrogel. Vessel coverage of the hydrogels is illustrated with three representative images of each treatment group (Fig. 4A). Multiple images from each sample were used to obtain quantitative measurements of traditional vessel parameters, including the number of branch points and vessel diameter.

Additional characterization was carried out by evaluating the space filling properties of vessel density, fractal dimension, and lacunarity, which provide scale-invariant methods to parse out differences in vessel morphology that may not otherwise be quantified. Vessel density identifies how much of each image is filled with neovessels. Fractal dimension quantifies the complexity of the vessel network via a measurement of the space in each image that is filled with vessels [24]. Lacunarity quantifies the uniformity of vessels to describe the gaps between vessels and is reported in terms of the inversely related lacunarity parameter, b [25]. PEG–QK was shown to enhance vessel branch points (B) and vessel density (C), as well as fractal dimension (D) and lacunarity (E). Additionally, the functional vessel diameter of capillaries (near 8 μm), which can permit perfusion of single red blood cells, was maintained in all samples (F). Although the morphological parameters do not show a significant difference between VEGF alone and VEGF, PEG-VEGF did increase the percent of hydrogels with vessel coverage (G).

4. Discussion

D’Andrea et al. made a useful discovery when they found that a modified version of a peptide mimicking the receptor binding region of VEGF would activate receptors responsible for the induction of angiogenesis. Their experiments showed that the 15-amino-acid peptide, QK, could promote endothelial cell proliferation and tubulogenesis in a Matrigel assay when added to culture medium [2].

D’Andrea did not examine the effects of sequestering QK in a matrix. Because PEG hydrogels have been shown to support angiogenic activity when modified with bioactive peptides and proteins, they were used as a matrix material to examine the angiogenic effects of covalently linked QK. This work showed that QK retains bioactivity when linked to a 3.4 kDa PEG chain, as
demonstrated by its effects on endothelial cell proliferation. Additionally, PEG-QK induced a significant tubulogenic response on the surface of PEG hydrogels. When covalently linked into a three-dimensional proteolytically-degradable hydrogel network in vitro, PEG-QK also promoted a significant increase in tubule network formation compared to hydrogels without an angiogenic signaling factor. The activity of PEG-QK was compared to that of PEG-VEGF in 2D and 3D. Studies show that PEG-QK induced an accelerated angiogenic response on the surface of hydrogels compared to PEG-VEGF, with tubule networks appearing as early as day 5 on QK-modified hydrogels. High levels of PEG-QK promoted significant cell migration and cell-cell contact formation, while lower levels, corresponding to bioactivity of PEG-VEGF hydrogels, showed similar results to PEG-VEGF hydrogels, trending toward increases in cell migration. These results suggest that the covalent incorporation of the angiogenic peptide QK could be used to promote angiogenesis in tissue engineered matrices. Most strikingly, in vivo work using hydrogels without encapsulated cells in the mouse cornea micro-pocket angiogenesis assay shows that PEG-QK supports a high level of vessel coverage, outshining that of unbound VEGF and PEG-VEGF hydrogels. These results may point to the greater ease of incorporating the smaller angiogenic peptide, compared to the whole protein, into hydrogels.

The current work shows the success of incorporating a covalently-linked 15-amino acid biomimetic peptide into a tissue engineering matrix to promote angiogenesis. This novel work may lay the foundation for other similar studies in promoting desired cellular behaviors. Published work in the field of matrix modification has concentrated on incorporating full proteins into scaffold materials to promote cellular behaviors, with the one exception of ECM-mimetic peptides to promote cell adhesion. There is no known current published research in which small bioactive peptides are incorporated into tissue engineering matrices to promote angiogenesis. Indeed, it appears that all studies incorporating peptides use ECM-mimetic peptides to promote cell adhesion or protein attachment to ECM proteins (e.g. collagen binding peptides). Due to the popularity of incorporating small cell adhesion peptides, it is possible to imagine the possibilities of using biomimetic angiogenic peptides for therapy. There may be clear advantages to incorporating a small peptide instead of a large protein. First, the use of a synthesized peptide allows more flexibility of engineered design for desired bioactivity without the requirement for modifying protein expression in bacterial production. QK was synthesized to allow only one PEG chain per peptide. The native VEGF protein dimer can be modified with up to
approximately 50 PEG chains, leading to a distribution of reaction products. Excess PEG chains on a VEGF molecule may cause steric hindrance during ligand-receptor binding. Additionally, smaller peptides may be easier to incorporate into bioactive scaffolds, as diffusion into a scaffold may be faster and more complete. This finding could specifically be used in three-dimensional photopatterning of collagenase-degradable hydrogels, in which the hydrogel is first formed, followed by diffusion of the bioactive moiety into the hydrogel, subsequent photocrosslinking, and removal of uncrosslinked factors. Furthermore, this work shows that the bioactive QK peptide retains its bioactivity in reaction conditions containing organic solvents, in this case DMSO. Native proteins characteristically lose bioactivity in such solvents. This characteristic allows more workability using QK than VEGF in chemical formulations. Additionally, it is unlikely that the body would mount an immune response to a 15-amino-acid peptide. Therefore, PEG-QK bioactivity may be prolonged compared to PEG-VEGF in vivo and should be studied in the future.

5. Conclusion

PEG-QK has been shown to promote angiogenesis in PEG hydrogels, without the use of sequestered growth factor proteins. Just as researchers have come to agree that it is more advantageous to include cell-adhesive peptides rather than full-sized ECM proteins in engineered matrices, this novel work introduces the use of a covalently linked angiogenic peptide, PEG-QK, as a comparable and possibly more favorable angiogenic factor than PEG-VEGF for incorporation in scaffolds to promote angiogenesis.

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References


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