

Self-assembling short oligopeptides and the promotion of angiogenesis

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Abstract

Because an adequate blood supply to and within tissues is an essential factor for successful tissue regeneration, promoting a functional microvasculature is a crucial factor for biomaterials. In this study, we demonstrate that short self-assembling peptides form scaffolds that provide an angiogenic environment promoting long-term cell survival and capillary-like network formation in three-dimensional cultures of human microvascular endothelial cells. Our data show that, in contrast to collagen type I, the peptide scaffold inhibits endothelial cell apoptosis in the absence of added angiogenic factors, accompanied by enhanced gene expression of the angiogenic factor VEGF. In addition, our results suggest that the process of capillary-like network formation and the size and spatial organization of cell networks may be controlled through manipulation of the scaffold properties, with a more rigid scaffold promoting extended structures with a larger inter-structure distance, as compared with more dense structures of smaller size observed in a more compliant scaffold. These findings indicate that self-assembling peptide scaffolds have potential for engineering vascularized tissues with control over angiogenic processes. Since these peptides can be modified in many ways, they may be uniquely valuable in regeneration of vascularized tissues.

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1. Introduction

Tissue engineering has promise for treatment of a broad variety of diseases by providing alternatives to donor tissues and organs [1]. Because cells isolated from host organs may not have the ability to assemble into functional tissue, success depends on the capability of the scaffold material to support three-dimensional cell organization and provide necessary signals for cell

survival and growth. An adequate blood supply to and within the newly grown tissue is an essential factor. While considerable success has been achieved in growing artificial arteries [2–4], generating a functional microvasculature remains one of the major challenges in tissue engineering [5–7]. Despite recent advances in material science and bioengineering [7,8], most scaffold materials used to support cell growth are unable to promote functional capillary formation and integrate into surrounding tissue [7]. Therefore, development and testing of new scaffold materials able to support vascularization and angiogenesis remains a high priority.

In order to understand the mechanisms of capillary formation, several *in vitro* models of angiogenesis have

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been used, including collagen I, fibrin, alginate gels and Matrigel [6,9]. Although these models contributed to understanding the signaling and molecular mechanisms of capillary network formation and remodeling, in most systems, cultured endothelial cells undergo apoptosis by 28–72 h after embedding, and high concentrations of angiogenic factors are required for cell survival [10–16]. Alternatively, substrates like Matrigel (animal-derived preparation of a basement membrane) already contain a combination of growth factors and have been shown to promote angiogenesis in endothelial cell cultures [9]. However, the presence of these angiogenic factors may result in activation of a variety of signaling pathways in cells [17–19], which can lead to difficulties in isolating mechanisms for specific cell–cell and cell–matrix interactions involved in angiogenesis. In addition to biochemical signals, mechanical interactions between cells and a surrounding biomaterial have been shown to play an important role in promoting or inhibiting vascularization [7,20]. Therefore, new scaffold materials supporting attachment and growth of endothelial cells without addition of specific factors may prove to be useful for studying mechanisms of angiogenesis.

Recently, a new class of biomaterials made through spontaneous self-assembly of short (8–24 amino acids) ionic complementary oligopeptides has been described [21]. These scaffolds support cell attachment and differentiation of a variety of mammalian primary and transformed cells [22–25]. These materials are especially suitable for tissue engineering applications, because the mechanical strength as well as chemical composition of the scaffold can be controlled through manipulation of peptide parameters [21,26,27].

In this study, we demonstrate that a self-assembling peptide scaffold provides an angiogenic environment promoting long-term cell survival and capillary-like network formation in three-dimensional cultures of human microvascular endothelial cells. Our data show that in contrast to collagen type I, the peptide scaffold inhibits endothelial cell apoptosis in the absence of added angiogenic factors, possibly due to enhanced expression of VEGF. In addition, our results indicate that the process of short-term structure formation *in vitro* depends on the initial distance between seeded cells, as well as on the mechanical properties of the scaffold. Importantly, the peptide scaffold described in this study represents a synthetic material with a short sequence and low immunogenicity, which may prove an important advantage over materials like Matrigel for future *in vivo* applications. These findings suggest that self-assembling peptide scaffolds have potential for engineered vascularized tissues with additional control over angiogenic process, as compared with other commonly used scaffolds.

2. Materials and methods

2.1. Cell isolation and culture

Microvascular endothelial cells were isolated from human fat tissue using collagenase I digestion (Worthington), as described previously [28]. Cells were cultured in gelatin-coated tissue culture flasks in Medium 199 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 5% heparin (Sigma), 1% glutamine, 1% penicillin-streptomycin (Gibco) and 1% endothelial cell growth supplement (Biomedical Technologies), and used at passages 4–8. Cell phenotype was assessed using immunohistochemical staining with anti-human von Willebrand factor antibodies (Sigma), low-density lipoprotein incorporation (Biomedical Technologies) and isolectin B4 (Vector Laboratories), which demonstrated that more than 95% of cells were positively stained for those markers (data not shown). In addition, gene expression analysis (see below) showed expression of mRNA for endothelium-specific receptors VEGFR-2 and Tie2.

2.2. Self-assembling peptide scaffold preparation and cell seeding

Lyophilized peptide (peptide sequence AcN-RARA-DADARARADADA-CNH₂, SynPep Corporation) was dissolved in distilled water at a concentration of 10 mg/ml (1%). Fifty microliters of peptide solution was added to 12 mm diameter culture inserts (Millipore), which were immediately immersed in the external well of a 24-well plate containing the culture medium. Exposure of the peptide solution to the culture medium at pH 7.1 resulted in scaffold formation within 5 min. After the scaffold was formed, the inserts were filled with the culture medium and incubated at 37 °C until cell seeding. For each experiment, cells were trypsinized from the culture flasks and seeded on the scaffold at concentrations described below, or embedded in the scaffold. For three-dimensional embedding, cells were re-suspended in 1 volume of distilled water containing 300 mM sucrose (to maintain osmolarity) and then mixed with 1 volume of 2% peptide solution also containing 300 mM sucrose, so that the final peptide concentration was 1%, and the final cell concentration was 5×10^6 cells/ml. Depending on the experiment, cells were cultured for 1–14 days, with medium changed every 2–3 days.

2.3. Collagen gel preparation

Collagen type I solution was prepared as follows. 3.5 ml of 2 mg/ml collagen type I (Invitrogen) was mixed with $10 \times$ Hanks buffered salt solution (Gibco) and 0.2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES) (500 μ l each) with subsequent addition of 5.5 ml phosphate-buffered saline (Gibco). The ready solution was then placed on the bottom of 24-well culture plates (Corning) and allowed to gel for 2 h at 100% humidity and 37 °C. After gel formation, culture medium was added and cells were seeded on the surface of the gels at an appropriate concentration. For three-dimensional embedding, cells were trypsinized, centrifuged, re-suspended in liquid collagen solution described above, placed on the surface of Millipore inserts inside the 24-well plate and allowed to gel for 2 h, with subsequent addition of culture medium.

2.4. Immunohistochemistry

At pre-determined time points (1, 3, 6, 9, 16, 24 h or 1, 3, 7 and 14 days), samples were fixed with 2% paraformaldehyde for 1 h at room temperature. Then, samples were washed with phosphate-buffered saline (PBS), incubated in 0.1% TritonX-100 for 1 h at room temperature, followed by 2 h incubation in blocking buffer (20% FCS, 0.1% TritonX-100 in PBS). Cultures were incubated with primary antibodies (rabbit anti-human anti-Von Willebrand factor (Sigma), mouse anti-actin antibody (Chemicon)) for 45 min at room temperature, with subsequent washing in the blocking buffer overnight followed by incubation with the secondary antibodies (goat anti-rabbit IgG-FITC (Sigma) or Alexa Fluor 488 goat anti-mouse IgG1 (Molecular Probes)) for 30 min. For paraffin embedding, gel samples were removed from the inserts following formaldehyde fixation, dehydrated in series of ethanol and xylene and embedded in paraffin following the standard protocols. Five micrometer thick slices were used to describe a three-dimensional cell organization (tri-chrome staining, or 30 min incubation in 20 μ g/ml solution of isolectin B4 (Vector Laboratories)). Finally, cultures were incubated with 4',6-Diamidino-2-phenylindole (DAPI) (Molecular Probes) to visualize cell nuclei. Samples incubated with secondary antibodies only served as negative controls.

2.5. Time-course study of cell network formation

For this study, cells were seeded on the surface of the peptide scaffold and collagen gel as described above at concentrations of 4, 8, 12 and 16 $\times 10^4$ cells/cm². At pre-determined time points (1, 3, 6, 9, 16, 24 h), 1 well with each density and gel type was fixed and stained with DAPI as described above. Of each well, images of cell nuclei distribution at three different locations were taken using a fluorescent microscope and analyzed to determine the correlation length corresponding to each image, as described below. These experiments were repeated 4 times.

2.6. Network formation on peptide scaffolds with different properties

Peptide scaffolds were prepared as described above using two different peptide concentrations: 1% and 2%. The elastic and loss moduli (G' and G'') of the peptide scaffolds were measured using a rheometer (model AR1000, TA Instruments, Inc., New Castle, DE). Peptide scaffold samples (10 mm diameter, 450 μ m height) were transferred to a parallel-plate setup of the rheometer, the upper plate was then lowered to 400 μ m to ensure full-surface contact, and a constant strain amplitude ($\mu = 0.001$) frequency sweep was performed ($f = 0.1 - 10$ Hz). The values of elastic (G') and loss (G'') moduli were averaged over the entire frequency range for 4 samples for each peptide concentration. Variations in modulus over this range were typically less than 15%. To measure the properties of a collagen I gel, samples (10 mm diameter, 900 μ m height) were subjected to a constant torque (0.1 μ Nm) over the range of 0.01–0.15 Hz, which resulted in average strain values of 0.004 ± 0.001 for 7 different gels, with 6 measurements each. Cells were seeded on the surface of similarly prepared peptide scaffolds or collagen gels at a density of 10^5 cells/cm², and one sample of each concentration was removed and fixed at 1, 3, 6, 9, 16, and 24 h after seeding. Samples were stained with DAPI and images of the cell distribution were taken and analyzed to determine the correlation length (defined below) corresponding to each sample. Each of these experiments was repeated 3 times.

2.7. Cell number assay

To assess cell viability, the CellTiter 96[®] non-radioactive MTS-based cell proliferation assay (Promega Corp., Madison, WI) was used. According to manufacturer instructions, a calibration curve was first obtained by seeding the pre-determined numbers of microvascular endothelial cells (MVECs) and culturing them for 1 day, followed by incubation in the soluble tetrazolium salt (MTS)-containing medium for 3 h. At that time, 50- μ l aliquots were taken from cell culture wells and placed in 96-well plate. The absorbance of the aliquots was measured at 490 nm using an ELISA plate reader. The resulting curve was linear over the entire range of cell numbers used in this experiment (data not shown). For cell viability and proliferation assessment, cells were seeded at the density of 10^5 cells/cm² (3 samples for each time point) and cultured up to 14 days. At days 1, 3, 7 and 14 cell numbers were determined using absorbance measurements.

2.8. Apoptosis assay

Apoptosis was determined using a TUNEL kit (Roche) according to the manufacturer's instructions.

Briefly, samples were fixed, permeabilized and exposed to a reaction mix containing terminal deoxynucleotidyl transferase and nucleotide mixture in equilibration buffer for 60 min at 37 °C. Samples of at least three different experiments were used to calculate the ratio of TUNEL-positive cells to the total cell number (determined using DAPI or propidium iodide nuclear staining).

2.9. Ribonuclease protection assay

Cells were seeded on the surface of a 1% peptide scaffold or a collagen gel at a concentration of 10^5 cells/cm² and cultured for 3 days. The peptide scaffold with endothelial cell networks was removed from the inserts, washed with PBS and centrifuged. For collagen gel cultures, cells were removed from the gel by trypsin digestion and centrifugation. RNA was extracted using Tri-reagent (Sigma). ³²P-UTP-labeled riboprobes containing a custom template set for human vascular endothelial growth factor A (VEGF-A), angiopoietins Ang1 and Ang2, VEGF receptor VEGFR-2, and angiopoietin receptor Tie2 (Pharmingen) were generated by *in vitro* transcription. 3 μg of sample RNA were hybridized with riboprobes using Human Angiogenesis Multi-Probe Template set (Pharmingen) and digested with RNase A. Samples were separated by electrophoresis and the gel was exposed by radiography.

2.10. Correlation analysis of cell networks

In order to characterize capillary structure formation, we developed a method based on correlation analysis of the images of cell distribution on the scaffold surface. In this analysis, a correlation function $F(a,b)$ is calculated for a gray scale DAPI image of the cell network at a given time (e.g., Fig. 2), using the equation, where (x,y) represent pixel coordinates on the original image, (a,b) represent correlation distances in pixels along the x and y directions, $I(x,y)$ is image intensity at (x,y) , “ $\langle \rangle$ ” denotes

$$F(a,b) = \frac{\langle (I(x,y) - I_{ave})(I(x+a,y+b) - I_{ave}) \rangle}{\sigma^2}$$

averaging over (x,y) , I_{ave} is average image intensity, and σ^2 is standard deviation for $I(x,y)$. The physical meaning of the correlation function in this analysis can be illustrated as follows: if a cell is located at position (x,y) on the original image, then the correlation function $F(a,b)$ represents the probability of finding another cell at $(x+a,y+b)$. After the function $F(a,b)$ was calculated for a given image, it was then averaged over all angles to obtain the correlation function $F_r(r)$, which represents distance-dependent correlations only. $F_r(r)$ was then fit using a single exponential $\exp(-r/L_c)$, where L_c (correlation length) is a parameter used to

characterize the cell distribution for each image. As illustrated in Fig. 2, L_c for a random cell distribution roughly reflects the size of the cell nuclei, while for the cell networks and clusters it reflects the characteristic size of the structure.

3. Results

3.1. Human microvascular endothelial cells (MVEC) form networks on self-assembling peptide scaffold

When seeded on the surface of the peptide scaffold, MVEC underwent rapid spatial organization resulting in formation of interconnected networks by 16 h after seeding (Fig. 1A–C), with further transformation of some parts of networks into clusters by 24 h. In contrast, MVEC seeded on the surface of collagen I gels did not exhibit any noticeable spatial re-arrangement and remained attached and randomly distributed (Fig. 1D–F). Interestingly, several other studies reported formation of cell aggregates or clusters on Matrigel after 24 h [29,31] similar to our findings, although we observed that the morphology of final structures depended on initial seeding density, with higher density (12×10^5 cells/cm²) resulting in formation of both clusters and stable networks. Correlation analysis of cell structures demonstrated that for cells cultured on the peptide scaffold, correlation length increased with time (Fig. 2), reflecting an increase in network characteristic size from a single-cell nucleus (around 7 μm, 1 h after seeding when all cells are separate) to multi-cell networks of >40 μm. In contrast, no change in correlation length for collagen gel cultures was observed, reflecting the absence of spatial redistribution of endothelial cells cultured on the collagen gel. Therefore, the correlation length can be used as a single parameter representing the size of the structures formed by endothelial cells on the peptide scaffold.

3.2. Effect of the seeding density and scaffold properties on network formation

Because the peptide scaffold is made from a short peptide sequence without any known integrin attachment sites, cells likely attach via non-specific interactions. In our experiments, the cell structures appeared to form around the “nucleation” centers, with evidence of smaller structures merging into larger ones. Therefore, we hypothesized that the peptide scaffold acts as a mechanical mediator of cell–cell interactions, with cells at a nucleation center interacting with cells at a distance to create a tension or matrix strain gradient toward this center. If this hypothesis is valid, then the size of the final structures will be determined by the ability of the cells to produce scaffold deformation. One might expect

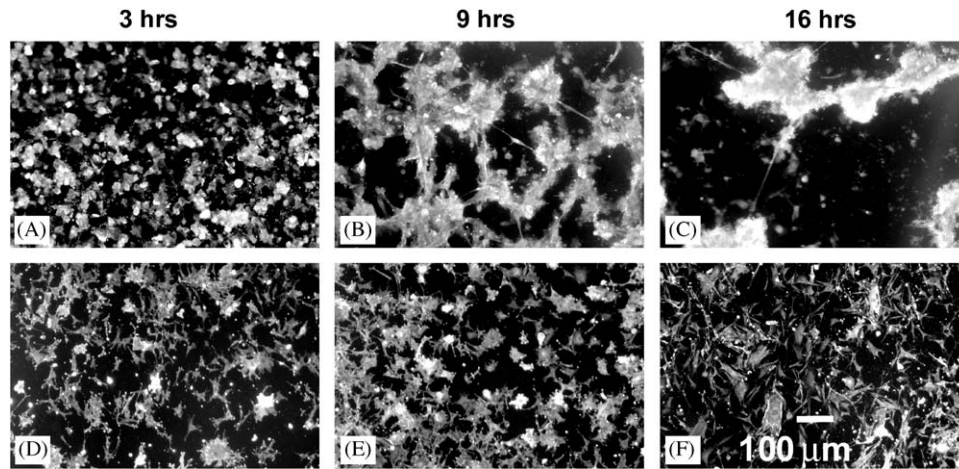


Fig. 1. Capillary-like network formation by human microvascular endothelial cells (MVEC, actin staining) seeded on the surface of the peptide gel (A–C). In contrast, MVEC seeded on the collagen gel maintained their initial random distribution (D–F).

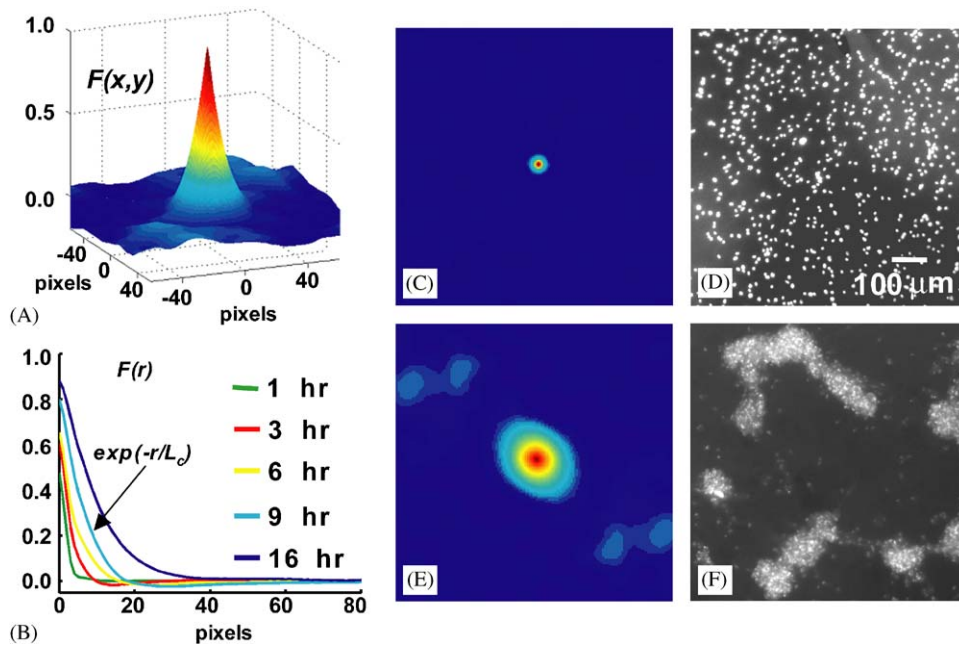


Fig. 2. Analysis of network formation following cell seeding on 1% peptide scaffold. (A) An example of a 3D plot of correlation function at 16 h. (B) Correlation length L_c increases with time, as cells form networks. (C–F) Correlation functions at 1 h (C) and 16 h (E) and (D,F)—corresponding images with DAPI staining.

scaffolds with different properties but seeded with the same cell density to promote formation of structures of different size, provided that the initial distance between cells is smaller than the range of interactions permitted by the scaffold. Alternatively, in the case of a chemotactic mechanism for network formation, the final dimension of cell structures will not depend on the scaffold properties, but will rather depend exclusively on the initial distance between the cells, with higher cell density resulting in larger structures. To explore this, we performed two sets of experiments. First, we studied the effect of initial distance between the

seeded cells on the size of the final network structures. Second, we performed experiments with cells seeded at the same density on 1% and 2% peptide scaffolds. Measurements of the peptide scaffold material properties demonstrated that the 1% and 2% peptide scaffolds have significantly different material properties, with the values for 1% peptide scaffold, $G' = 12.1 \pm 3.5$ kPa and $G'' = 1.5 \pm 0.5$ kPa, reflecting a more compliant and in general more fluid-like material behavior, as compared with 2% peptide scaffold, $G' = 68.3 \pm 5.2$ kPa and $G'' = 10.5 \pm 1.5$ kPa (mean \pm SD, $n = 4$). In contrast to the peptide scaffold, the collagen I gel at the concentrations

used in these experiments was found to be a much softer material, with moduli of $G' = 292 \pm 67$ Pa and $G'' = 66 \pm 14$ Pa.

We observed (Fig. 3) that if the distance between the cells is less than $35 \mu\text{m}$ (densities at or above 8×10^4 cells/cm²), the cells migrate actively and form networks and multi-cellular dense structures, with no difference in structure dimensions for 8, 10 or 12×10^4 cells/cm² cultures ($p > 0.7$, ANOVA). Interestingly, in the cultures with relatively low seeding density (4×10^4 cells/cm²) corresponding to the average distance between cells of $50 \mu\text{m}$, cells remain relatively sparse even at 24 h after seeding and do not form dense structures, as reflected in the significantly smaller values for the correlation length relative to higher densities ($p < 0.001$, ANOVA). When cells were seeded at 10×10^4 cells/cm² on the 1% or 2% scaffold, the structures formed on more rigid scaffolds (2% peptide) were significantly larger and more extended than those formed on more compliant scaffold (1% peptide) (Fig. 4, $p < 0.001$, ANOVA). In addition, on the 2% scaffold we observed merging of smaller clusters into larger ones, while on the 1% scaffold most of smaller clusters remained stable. These data are consistent with the hypothesis of a mechanical basis of network formation and suggest that the 2% scaffold may be a better mediator of long-range cell–cell interactions than the 1% scaffold.

3.3. Cell networks formed on the peptide scaffold remain viable and stable

To investigate whether the peptide scaffold supports long-term cell survival, cell cultures on the surface of the peptide scaffold or collagen I gel (10^5 cell/cm²) were maintained for 2 weeks. MTS-based cell viability assay demonstrated that percentages of cells survived in the

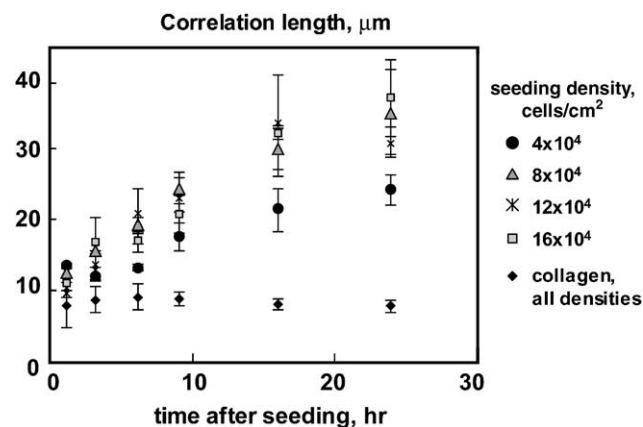


Fig. 3. Effect of seeding cell density on network formation (1% peptide scaffold). Low cell density resulted in significantly smaller structures than higher densities ($p < 0.001$, ANOVA), while there was no significant difference in the size of structures between cell densities of 8, 12 or 16×10^4 cells/cm².

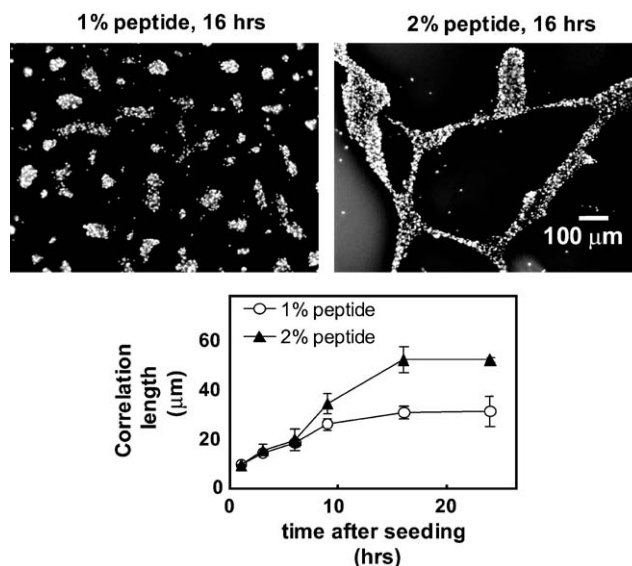


Fig. 4. Cells (DAPI staining) seeded on a more compliant scaffold (1% peptide) form smaller, less-connected structures, as compared with cells cultured on more rigid scaffold (2% peptide) (mean \pm SD, $p < 0.001$, ANOVA).

surface-seeded peptide and collagen gel cultures were not significantly different ($65 \pm 10\%$, $53 \pm 7\%$ and $56 \pm 7\%$ of seeded cells on the peptide scaffold vs. $49 \pm 12\%$, $53 \pm 11\%$ and $63 \pm 17\%$ of seeded cells on the collagen gel at day 3, 1 week and 2 weeks, respectively). As expected, the collagen gel cultures displayed typical behavior for a confluent cell monolayer with a relatively small increase in cell number and no changes in cell morphology or distribution (Fig. 5D) [30]. In contrast, network structures formed on the peptide scaffold displayed signs of active remodeling (Fig. 5A–C), with some clusters undergoing regression and some clusters showing growth and appearance of sprouts (Fig. 5B). Analysis of histological cross-sections of cell clusters revealed a circular cell orientation resembling that of a blood vessel, with evidence of cells sprouting from the cluster and migrating through the scaffold (Fig. 5C). TUNEL staining demonstrated similar levels of cell apoptosis for both substrates, with $2.9 \pm 1.6\%$ and $3.6 \pm 0.5\%$ of apoptotic cells on the peptide scaffold vs. $3.1 \pm 1.1\%$ and $3.3 \pm 1.8\%$ of apoptotic cells on the collagen gel at 1 and 2 weeks in culture, respectively. Overall, these results demonstrate that endothelial cells cultured on the peptide scaffold undergo activation and change phenotype in a manner similar to the process of sprouting in vivo [39].

3.4. MVEC embedded in the peptide scaffold form three-dimensional capillary-like structures

To study whether the peptide scaffold is able to support endothelial cell survival in a true three-dimen-

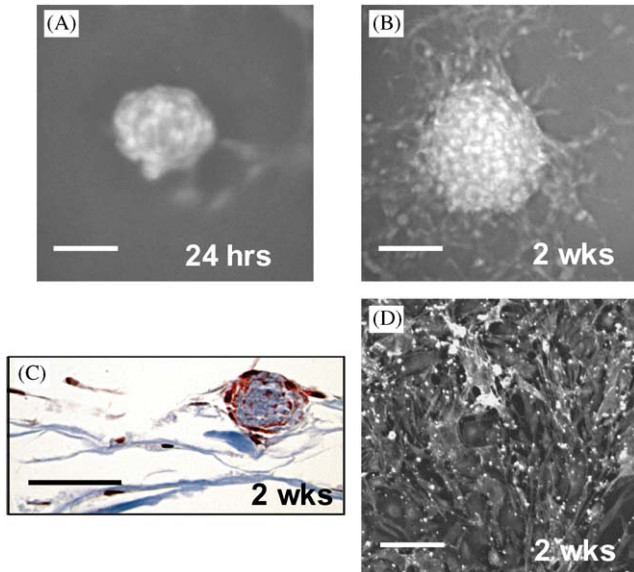


Fig. 5. Typical cluster appearance at 24 h (A) and 2 weeks in culture (B,C) on the peptide scaffold and at 2 weeks on the collagen gel (D) (A, B, D—actin staining, C—trichrome staining of a cluster cross-section). Some clusters on the peptide scaffold showed cell growth and formation of sprouts (B,C), while other clusters underwent regression and disappeared (not shown). In contrast, cells cultured on the collagen gel remained in monolayer with little growth (D). Bars—100 μm.

sional configuration, MVECs were embedded in the 1% peptide scaffold (5×10^6 cells/ml) or collagen I gel as a control and cultured for up to 2 weeks. The analysis of histological sections of samples of the peptide scaffold cultures obtained at 3, 7 and 14 days demonstrated that cells remained viable and formed capillary-like structures (Fig. 6), with both single- and multi-cell lumens present. In contrast, cells embedded in collagen I gels and cultured under the same conditions showed high rates of apoptosis as early as day 1 in culture, and were dead at 1 week, consistent with previous studies [11,12,14,15]. TUNEL staining of histological sections demonstrated significantly higher ratios of apoptotic to normal cells in the collagen gel (Fig. 7), as compared with the peptide scaffold, at both day 1 and day 3 ($p < 0.01$, ANOVA). Thus, on day 1, 28% of all cells in the collagen gel were apoptotic vs. 17% in the peptide scaffold, whereas on day 3, the percentage of apoptotic cells in the collagen gel increased to 52%, while in the peptide scaffold it decreased to 14%. Importantly, the improved survival of endothelial cells in the peptide scaffold as compared with collagen I gel was obtained at 10% FBS conditions in the absence of external VEGF or bFGF, while previous studies of angiogenesis in vitro demonstrated that supplementation of large amounts of exogenous angiogenic factors in addition to serum was necessary for cell survival [11,14,15].

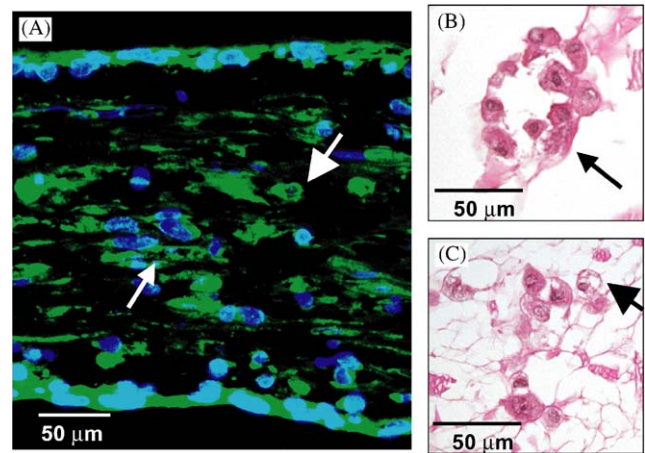


Fig. 6. Endothelial cells embedded into the peptide gel survive for at least 2 weeks and form capillary-like networks. (A) Histological section of a 2-week culture showing viable cells (isolectin, green and DAPI, blue). (B,C) Same culture stained with H&E. Note capillary-like structures with multi-cell (thin arrows) and single-cell (thick arrows) lumens.

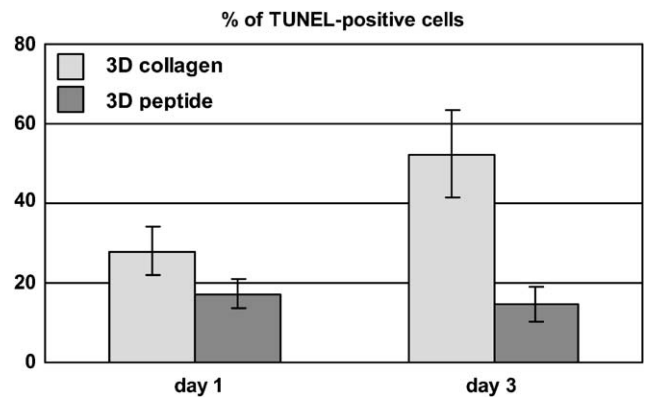


Fig. 7. TUNEL staining of histological sections demonstrated significantly higher ratios of apoptotic to normal cells embedded into the collagen gel, as compared with the peptide scaffold, at both day 1 and day 3 ($p < 0.01$, ANOVA).

3.5. The peptide scaffold induces VEGF gene expression by microvascular endothelial cells

To investigate whether the activation and improved survival of endothelial cells cultured in the peptide scaffold was related to expression of angiogenic genes, we performed RNase-protection assays to measure the levels of gene expression of two key angiogenic growth factors (VEGF-A and Angiopoietin 1) and their functional receptors (Flk-1 and Tie2) by microvascular endothelial cells in the peptide scaffold and collagen I cultures. Cells cultured on the peptide scaffold expressed significantly higher levels of VEGF-A mRNA 3 days after seeding, as compared with cells cultured on the surface of collagen I gel (Fig. 8). In contrast, we did not observe changes in gene expression for VEGF receptor 2 (Flk-1), Angiopoietin 1 or Angiopoietin 1 receptor

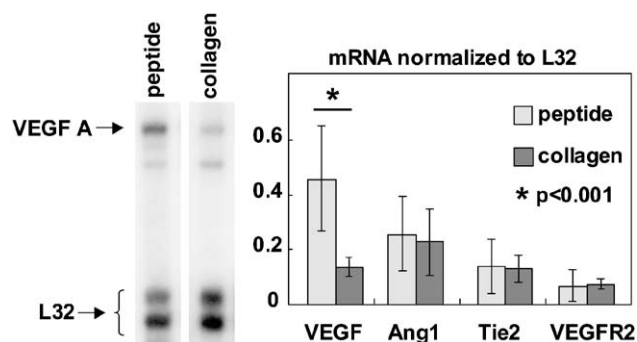


Fig. 8. Cells seeded on 1% peptide scaffold express significantly higher levels of VEGF mRNA, as compared with cells seeded on the collagen gel (day 3 in culture, $n = 5$, $p < 0.001$, ANOVA). In contrast, no difference in mRNA expression for Ang1, Tie2 and VEGFR2 between peptide and collagen gel cultures was detected.

Tie-2. These results indicate that the peptide scaffold may promote activation of endothelial cells similar to the process of angiogenesis observed *in vivo*, when some cells in the capillary remain quiescent, while others become activated and migrate outside the capillary wall to form sprouts. In addition, increased expression of VEGF mRNA may be related to the improved cell survival in long-term cultures described above, since VEGF is an anti-apoptotic and pro-survival factor [10,11,14–16].

4. Discussion

In this study, we demonstrate that a self-assembling peptide scaffold provides a unique three-dimensional environment for *in vitro* angiogenesis. Our results demonstrate that human microvascular endothelial cells are able to attach to the peptide scaffold and undergo activation with rapid formation of cell networks. These results are similar to previously reported capillary-like networks and clusters formed by endothelial cells seeded on other substrates, including Matrigel, fibrin and collagen I gels, but in the presence of VEGF and bFGF [10,14,15,29,30]. The cell networks remain viable over a period of at least two weeks and show evidence of morphologic remodeling similar to the angiogenic process *in vivo*. While the exact nature of cell adhesion to the materials of this class is not yet clear, the absence of a known integrin-attachment motif and growth support of a variety of cell types [21] suggests that non-specific interactions with the scaffold are probably responsible. This is supported by our findings that cell migration and network formation on the peptide scaffold are unlikely to occur through integrin or cell–cell binding-induced release of chemotactic signals, as was reported for fibrin matrices [33,34]. In preliminary studies, we have found that inclusion of RGD adhesion sequences in the peptides did not change cell

adhesion. However, it needs to be determined whether other integrins that do not bind RGD sequences may be involved in cell–scaffold interactions, as well as what scaffold modifications can influence cell attachment and behavior. Previous studies suggested that matrix-transduced tractional forces may be responsible for directional capillary sprouting in two-dimensional Matrigel cultures [35,36] and three-dimensional collagen I cultures of endothelial cells [37]. In addition, a recent theoretical model showed that for a two-dimensional culture, isotropic strain-stimulated cell traction is sufficient to form capillary-like structures on viscoelastic substrates similar to those observed experimentally [38].

Our data for endothelial cells embedded into the peptide scaffold give evidence that this scaffold is providing cells with a soft three-dimensional support, allowing them to form capillary-like structures and survive without active remodeling of the surrounding matrix. Importantly, the pore size of the peptide scaffold, similar to native extracellular matrix, is on the order of 50–100 nm. Previous studies (also confirmed by our results) have shown that in other substrates, such as collagen I or fibrin gels, cell embedding is followed by rapid activation of proteases and gel degradation, resulting in the induction of apoptosis [13,14,40,41]. In contrast, we did not observe any noticeable peptide scaffold degradation or an increase in cell apoptosis over a period of two weeks. Moreover, the peptide scaffold promotes expression of a survival factor—VEGF—by endothelial cells, as suggested by our results for increased VEGF mRNA expression in the peptide cultures relative to the collagen I substrate. One possible explanation for the observed cell spatial reorganization in the peptide scaffold is that cells migrate by breaking peptide filaments and “parting” the matrix, rather than by enzymatic degradation. Overall, understanding the mechanism of cell attachment to the peptide scaffold and cell–scaffold interactions is very important for any future application of this material for tissue engineering, and future studies are needed to elucidate this mechanism.

One of the implications of our results is that the process of capillary formation and the size and spatial organization of cell networks may be controlled through manipulation of the scaffold properties. Our data demonstrate that a more rigid scaffold with a higher G'/G'' ratio (more solid-like behavior) may promote longer range cell–cell interactions, resulting in more extended structures with a larger inter-structure distances (see Fig. 4), as compared with more dense structures of smaller size observed on a more compliant scaffold with a lower G'/G'' ratio. Our preliminary studies indicate that variation of the peptide concentration between 0.5% and 2% does not affect cell viability when endothelial cells are embedded in the scaffold, suggesting that a wide range of adjustment of the scaffold properties may be used to optimize it for

different applications. It is important to note, however, that in addition to altering scaffold stiffness, variations in peptide concentration result in changes in the pore size of the matrix. The difference in the matrix pore size may affect cell behavior and attachment and thus provide an alternative mechanism for regulating the process of network formation. In general, the ability to manipulate angiogenesis through alteration of scaffold properties may potentially be an important advantage for the use of the self-assembling peptide scaffolds in tissue engineering applications.

In this study, we present a new method to characterize network formation by endothelial cells. Previously, different methods of network quantitation have been used, including using outlines of cell clusters with counting sprouts [20], percentage vacuolization [36,42], sprouting index [34], number, length and width of the tubes [14,16,32]. Our goal was to develop an automated and simple method to describe changes in network formation. As demonstrated in Fig. 2, the parameter proposed in this study—the correlation length—reflects the characteristic size of the structures in a wide range of conditions, starting from the size of a single cell nucleus (at 1 h after cell seeding) to the dimensions of the cell networks at equilibrium (16 and 24 h after seeding). This analysis can also be applied to confocal slices or histological sections, allowing for examination of these structures in three dimensions.

One limitation of this study is that we performed all our experiments with a single peptide sequence, therefore, we cannot exclude the possibility that some of the effects we observed are specific to this protein sequence. However, for this class of materials, the peptide sequence is one of the parameters that can be varied while still preserving the material's ability to self-assemble at physiological pH. Therefore, our results may serve as a basis for further optimization of the peptide scaffold for particular applications.

5. Conclusions

In this study we used the self-assembling peptide scaffold to establish a three-dimensional angiogenic environment promoting capillary-like network assembly and long-time survival of human microvascular endothelial cells. This scaffold consists of a 16-amino acid sequence, which undergoes self-assembly within minutes after subjecting it to physiological pH, and can therefore be used as an injectable scaffold for a variety of applications [43,44]. Our results suggest that the process of angiogenesis can be controlled through manipulation of scaffold material properties, with the scaffold serving as a mediator of cell–cell signaling, thus regulating the size and density of cell networks. In addition to varying

material properties, this scaffold can potentially be engineered to include signaling sequences, as well as to adjust its density and assembly process, in order to provide a better control over cell adhesion characteristics, ease of degradation, filament diameter and pore size.

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