Published in final edited form as:

Acta Biomater. 2012 January ; 8(1): 154–164. doi:10.1016/j.actbio.2011.08.029.

REGULATION OF ENDOTHELIAL CELL ACTIVATION AND ANGIOGENESIS BY INJECTABLE PEPTIDE NANOFIBERS

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Abstract

RAD16-II peptide nanofibers are promising for vascular tissue engineering and were shown to enhance angiogenesis *in vitro* and *in vivo*, although the mechanism remains unknown. We hypothesized that the pro-angiogenic effect of RAD16-II results from low-affinity integrin-dependent interactions of microvascular endothelial cells (MVECs) with RAD motifs. Mouse MVECs were cultured on RAD16-II with or without integrin and MAPK/ERK pathway inhibitors, and angiogenic responses were quantified. Results were validated *in vivo* using mouse diabetic wound healing model with impaired neovascularization. RAD16-II stimulated spontaneous capillary morphogenesis, increased β_3 integrin phosphorylation and VEGF expression in MVECs. These responses were abrogated in the presence of β_3 and MEK inhibitors or on the control peptide without RAD motifs. Wide-spectrum integrin inhibitor echistatin completely abolished RAD16-II-mediated capillary morphogenesis *in vitro* and neovascularization and VEGF expression in the wound *in vivo*. Addition of the RGD motif to RAD16-II did not change nanofiber architecture or mechanical properties, but resulted in significant decrease in capillary morphogenesis. Overall, these results suggest that low-affinity non-specific interactions between cells and RAD motifs can trigger angiogenic responses via phosphorylation of β_3 integrin and MAPK/ERK pathway, indicating that low-affinity sequences can be used to functionalize bio-compatible materials for the regulation of cell migration and angiogenesis, thus expanding the current pool of available motifs that can be used for such functionalization. Incorporation of RAD or similar motifs into protein engineered or hybrid peptide scaffolds may represent a novel strategy for vascular tissue engineering and will further enhance design opportunities for new scaffolds materials.

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1. Introduction

Tissue engineering is a promising field in medicine which aims to develop biological substitutes that restore tissue functions [1]. Capillary formation (angiogenesis) is a key process in the body during natural wound healing. However, supplying proper vascularization to provide oxygen and nutrients to the newly forming tissues is one of the major hurdles for success of tissue engineering [2]. Therefore, providing a suitable pro-angiogenic microenvironment is of central importance when designing a scaffold material for tissue engineering applications.

Hydrogels have been used for vascular tissue engineering and delivery of cells and growth factors [3–7]. Recently, a subset of hydrogels called self-assembling peptides has gained growing attention for tissue engineering scaffold applications [8–12]. Scaffolds made from the self-assembling peptides have been shown to support cell attachment, differentiation and proliferation of a variety of mammalian cells, exhibit excellent biocompatibility, and their mechanical strength can be controlled through manipulation of peptide parameters [8, 13–16]. Because the amino-acid sequences of a large number of these peptides are not naturally found in living system and are thought to be biologically non-functional [17, 18], significant efforts have been focused on functionalization of these materials by incorporating biologically active motifs for specific applications and cell types [19–22]. However, there are several particular sequences, including RAD16-II nanofibers (RARADADA)₂, that have been shown to provide an angiogenic environment and enhance survival of ECs and angiogenesis both *in vitro* and *in vivo* without addition of any functional motifs [9, 10, 23–26]. This material has been used to improve neovascularization and heart repair after myocardial infarction [27] and as a robust system for protein and cell delivery to the injured tissue [27–29]. Despite high interest and promise of this type of materials for vascular tissue engineering applications, the underlying molecular mechanisms of their pro-angiogenic action remain not known. Therefore, the objective of this study was to determine the mechanisms that may regulate microvascular endothelial cell interactions with RAD16-II nanofibers and what molecular pathways may be involved in nanofiber-mediated angiogenic cell responses.

The process of angiogenesis includes endothelial cell activation by angiogenic growth factors or changes in the extracellular environment, followed by cell migration, proliferation, formation of nascent capillaries, vasculature remodeling and maturation [30]. Angiogenesis is mediated via interactions between integrins, which are expressed on the surface of activated ECs, and their ligands in the extracellular matrix [31, 32]. It has been reported that ECs express up to 10 different integrins depending on their location and activation state [33], which include vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$, fibronectin receptor $\alpha_5\beta_1$, and laminin receptor $\alpha_6\beta_4$ [34]. Integrin-mediated angiogenic signaling can involve a direct signal transduction from integrin phosphorylation through cytoplasmic domain of β subunit upon ligand binding to the extracellular matrix (ECM), as well as signaling via a synergism between integrins and pro-angiogenic growth factor pathways (via receptor tyrosine kinases and downstream MAPK/ERK cascade), including such major mediators of angiogenesis as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) [33, 35–38]. In endothelial cells, integrin $\alpha_v\beta_3$ is the most abundant and influential receptor regulating angiogenesis [39]. For $\alpha_v\beta_3$ integrin-dependent function, phosphorylation of β_3 cytoplasmic domain is crucial: it regulates $\alpha_v\beta_3$ affinity, avidity, ligand binding strength [40–42], as well as basic cellular functions such as cell spreading and survival through interactions with intracellular signaling proteins [43]. The integrin $\alpha_v\beta_3$ binds to its ligands via Arg-Gly-Asp (RGD) binding motif [44]. In contrast, an RGD homolog, the RAD motif, exhibits a low affinity interaction with the $\alpha_v\beta_3$ integrin, which follows a non-specific binding curve [45, 46]. It has been previously shown that RGD homologs, such as RLD, can

be directly involved in cell binding to $\alpha_v\beta_3$ integrins [47]. Therefore, we hypothesized that pro-angiogenic effects of the RAD16-II nanofibers may be triggered by the low-affinity interactions between RAD motifs on the nanofibers and $\alpha_v\beta_3$ integrins on the endothelial cells, which result in phosphorylation of β_3 integrin cytoplasmic domain and angiogenic cell responses. We have tested this hypothesis by quantifying angiogenic responses of mouse microvascular endothelial cells both *in vitro*, i.e. seeded on the peptide nanofiber scaffolds, and *in vivo* using a mouse model of diabetic wound healing, where wound treatment with the RAD16-II nanofibers can significantly improve diabetes-impaired neovascularization [23].

2. Materials and methods

2.1. Microvascular endothelial cell isolation and culture

Primary microvascular endothelial cells (MVECs) were isolated from mouse (C57BL/6J, Jackson Laboratory) lung tissues using collagenase digestion and sequential double sorting by anti-CD-31 (BD Pharmingen, San Jose, CA) and anti-CD-102 (BD Pharmingen, San Jose, CA) antibodies with appropriate secondary antibodies conjugated to the magnetic beads (Dynabeads®, Invitrogen Corporation, Carlsbad, CA). Cells were cultured in gelatin-coated dishes in Medium 199 (HyClone, Logan, UT) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% antibiotic/antimycotic (Atlanta Biologicals, Lawrenceville, GA), 10 $\mu\text{g/ml}$ heparin (Sigma Aldrich, St. Louis, MO), and 0.2 ng/ml growth supplement (Sigma Aldrich, St. Louis, MO). To confirm cell phenotype, cells (up to passage 14) were immunostained using antibodies against von Willebrand factor (vWf, Sigma Aldrich, St Louis, MO), with more than 95% of the cells showing positive staining. Cells between the 5th and 12th passage in culture were used for all *in vitro* experiments.

2.2. Capillary morphogenesis assay

In vitro capillary morphogenesis assay was performed as described previously [10, 48]. Briefly, MVECs were seeded on the surface of the 1% (w/v) peptide nanofibers in culture plate inserts (13-mm diameter, 0.4- μm pore size; Millipore, Billerica, MA) at a cell seeding density of 6.25×10^4 cells/ cm^2 . Cells were cultured in no growth factor medium (cell culture medium without additional growth factor supplementation) or in serum-free medium (M199 without FBS or growth factor supplementation) for 24 hours. Cells were either pre-stained with CellTracker Dyes (Invitrogen Corporation, Carlsbad, CA) before seeding or stained with Phalloidin-TRITC (Sigma Aldrich, St Louis, MO) after fixing at 24 hours. All peptides used in this work were obtained from SynBioSci Corporation (Livermore, CA) and included RADA16-II (AcN-RARADADARARADADA-CNH₂), functionalized RAD16-II (RGD-RAD16-II, AcN-RGD-GG-RARADADARARADADA-CNH₂), and negative control peptide KFE-8 (AcN-FKFEFKFE-CNH₂), which has similar hydrogel structure and material properties but does not contain RAD or RGD binding site. Following seeding on the RAD16-II nanofibers, MVECs undergo spontaneous capillary morphogenesis which does not require addition of stimulating growth factors [10, 48], in contrast to collagen, fibrin or Matrigel systems for *in vitro* angiogenesis [49, 50]. At 24 hours, cells were fixed in 2% paraformaldehyde and 5 images of the cell networks were taken from each insert at 4X magnification using an inverted fluorescent microscope (Olympus IX81; Olympus America Inc., Center Valley, PA). The images of the cell networks were used to determine the characteristic size of capillary-like networks using correlation analysis and custom-written Matlab code (Mathworks, Natick, MA) as described previously [10]. In this analysis, the characteristic size of capillary-like network represents a single parameter that for a random cell distribution roughly reflects the size of the single cell, while for the cell networks it reflects the characteristic, “averaged” size of the structure (including contribution from both length and width) formed by endothelial cells on the nanofiber matrix. This analysis

underestimates the length of the network branches, and is more conservative than the measurement of the length alone; however, it is fully automated, fast, and user-independent. The images with phalloidin-TRITC staining were used for qualitative analysis of the cell networks, while the images of CellTracker-stained networks were used in the correlation analyses, with no significant difference in characteristic network size detected between the two staining methods (ANOVA, $n=3$ samples/group).

For three-dimensional network characterization, a built-in integrated 3D imaging system was used (Plus Imaging System, Olympus) which included motorized Z-drive with 10 nanometer step size and imaging/analysis software (ImagePro, Media Cybernetics, Inc.). Lumens were visualized using Z-stack images of cells stained with Phalloidin-TRITC (Sigma Aldrich, St Louis, MO) with a spacing of 0.5 μm between frames [48].

To determine the role of integrins and MAPK/ERK pathway in RAD16-II –mediated angiogenic cell responses, capillary morphogenesis assays were performed in the presence or absence of integrin β_3 blocking antibody [50 $\mu\text{g/ml}$] (BD Pharmingen, San Jose, CA), wide-spectrum integrin inhibitor echistatin [10 $\mu\text{g/ml}$] (Sigma Aldrich, St. Louis, MO), or MEK inhibitor U0126 [10 μM] (Sigma Aldrich, St. Louis, MO) which completely blocks activation of downstream ERK [51].

2.3. Quantification of integrin β_3 subunit phosphorylation

Cells were serum starved for 12 hours prior to seeding. To quantify integrin β_3 subunit phosphorylation, equal numbers of cells were either kept in suspension for 30 minutes to prevent adhesion-induced activation (negative control), or seeded on the RAD16-II nanofibers, or dishes coated with vitronectin (Abcam, Cambridge, MA) which is a known β_3 subunit activator (positive control), and allowed to adhere for 30 minutes in M199 media without serum or growth factor supplements. Cells were lysed using 1% NP-40 lysis buffer containing protease and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO). Whole cell lysates were centrifuged at 4°C to remove insoluble materials. Equal amounts of protein were immunoprecipitated using rabbit anti-mouse integrin β_3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The unbound fraction was saved and used as a loading control. Immunocomplexes were denatured in Laemmli sample buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. The membrane was probed with rabbit anti-mouse anti-integrin β_3 (pTyr747) phosphospecific antibody (Invitrogen Corporation, Carlsbad, CA), then stripped and reprobed with goat anti-mouse integrin β_3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The bands were visualized by reaction with a HRP-conjugated secondary antibody (Invitrogen Corporation, Carlsbad, CA) and developed with an enhanced chemiluminescent substrate (ECL) substrate (Thermo Scientific, Rockford, IL), followed by autoradiography. For the loading control, the unbound fraction was blotted and probed with C4 anti-actin mouse monoclonal antibody (Seven Hills Bioreagents, Cincinnati, OH).

2.4. VEGF protein expression

VEGF protein expression by MVECs was quantified using Mouse VEGF Quantikine® ELISA kit (R&D Systems, Minneapolis, MN). Briefly, MVECs were seeded on 1% (w/v) RAD16-II peptide nanofibers in culture plate inserts ($n=3$), and cultured in medium without growth factor supplement for 12 hours. At 12 hours, medium was collected and VEGF content was determined using VEGF ELISA and the manufacturer's protocol. To determine the role of integrins in RAD16-II–mediated angiogenic cell responses, VEGF protein expression was determined for cells cultured on the RAD16-II nanofibers in the presence or absence of integrin β_3 blocking antibody [50 $\mu\text{g/ml}$] (BD Pharmingen, San Jose, CA).

2.5. In vivo model of diabetic wound healing

In order to validate that the *in vitro* results for integrin-mediated angiogenic responses of endothelial cells can be translated to *in vivo* conditions, a mouse db/db model of diabetic wound healing was used. This model is characterized by a delayed wound healing with markedly reduced neovascularization of the repair tissue [52]. We have recently reported that wound treatment with the RAD16-II nanofibers results in dramatic improvement in wound neovascularization and accelerated healing in this model [23]. In the present study, we aimed to demonstrate that the RAD16-II-mediated enhanced angiogenesis in the wound tissue depends on the integrin-mediated endothelial cell interactions with the RAD16-II nanofibers in the diabetic wound microenvironment.

All procedures were approved by the Institutional Animal Care and Use Committee. Seven- to 8-week-old female, diabetic B6.BKS(D)-*Lepr^{db}/J* (db/db) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Two full thickness circular excisional wounds were created side-by-side on the dorsum of each animal (Figure 5A), leaving the underlying muscle (panniculus carnosus) intact, as described previously [52]. A sterile transparent dressing was applied to the skin to cover the wounds and held in place with benzoin tincture. Peptide nanofiber solution (NFS) (RAD16-II or KFE8, 10 mg/ml) with or without integrin inhibitor echistatin (10 ug/ml), or phosphate-buffered saline (PBS) was topically administered (40 ul each) on the wound under the transparent dressing using a Hamilton syringe. Each mouse received either NFS/PBS, or NFS+echistatin/PBS, or PBS/PBS (left/right, respectively) (Figure 5A). The PBS treatment on the right side wound served as an internal control. The animals were euthanized at day 7; wound tissues were harvested, fixed, and paraffin embedded. To identify blood vessels in the newly formed tissue, immunoperoxidase staining of 5- μ m paraffin sections was performed using platelet endothelial cell adhesion molecule (PECAM) rat anti-mouse CD31 monoclonal antibody (BD Pharmingen, San Jose, CA) as described previously [52]. Biotinylated lectin at 1 mg/ml in PBS (Sigma-Aldrich, St. Louis, MO) was injected into the mice via tail vein prior to euthanization to determine that newly formed capillary lumens were anastomosed with the host vasculature. The wound sections from these animals were processed and stained with ABC-DAB (Vector laboratories, Burlingame, CA) which produces a brown staining at the site of blood vessels where injected lectin has bound to host endothelium.

2.6. Microscopy and image analyses of the repair tissue

Two morphological parameters were used to quantify wound healing, including the gap between encroaching epithelial margins (epithelial gap) and granulation tissue area [52], using hematoxylin and eosin (H&E) stained images of 4X edge-to-edge wound sections by morphometric image analysis (ImagePro, Media Cybernetics, Silver Spring, MD, and Metamorph, Molecular Devices, Downingtown, PA). Epithelial gap was measured as the distance (in mm) along the panniculus carnosus (Figure 5B, depicted by black lines in each wound) between encroaching epithelial margins (Figure 5B, depicted by black arrows), and the granulation tissue area was measured as an entire wound area (in mm²) above the panniculus muscle within the epithelial margins.

To quantify neovascularization in the repair tissue, twelve random high-powered fields (40X images) were obtained per each section using Olympus IX81 inverted microscope. Within the newly-formed granulation tissue, CD31-positive blood vessels were identified and the areas of the lumens were measured by the blinded observer using the freehand drawing tool of NIH ImageJ v1.43u [53]. Wound sections were stained with ABC-DAB to visualize the presence of biotinylated lectin in newly-formed that anastomosed with the host vasculature (Figure 6B). Neovascularization was expressed as the area of CD31-positive blood vessels in each high power field (Figure 6C).

2.7. Characterization of the nanofiber architecture and mechanical properties of the nanofiber hydrogels

For nanofiber architecture characterization, transmission electron microscopy was used. Peptide was dissolved within deionized water at 0.5 mg/ml; the solutions were incubated for 2 hours (RGD-RAD and RAD peptide were mixed 50/50 at 30 min). Same volume of PBS was added to gel the solution and to render the final concentration to be 0.25 mg/ml. Peptide was then incubated for another 2 hours before applied to 400-mesh carbon-filmed copper grid. Samples were analyzed with a Tecnai F30 STEM.

Elastic moduli (G') and loss moduli (G'') of 1% RAD16-II, RGD-RAD16-II, 1:1 RAD16-II:RGD-RAD16-II, and KFE-8 peptide nanofiber scaffolds were measured with a parallel-plate rheometer (Bohlin Instruments Inc., East Brunswick, NJ). Using molds, circular constructs of 8-mm diameter and approximately 500- μ m height were formed on glass slides, covered with Media 199 for gel formation and allow to set for 1 hour at room temperature. For testing, glass slides were transferred and secured to the bottom plate of the rheometer and the top parallel plate was lowered to a gap height which ensured complete contact with the sample. A constant strain amplitude ($\gamma=0.01$) frequency sweep ($f=0.1$ –10 Hz) was applied, with the measured elastic and loss moduli (G' and G'' , kPa) serving as indicators of overall construct stiffness and viscosity, respectively. Moduli values measured at 0.1 Hz are reported as average $\pm$ SD.

2.8. Statistical analyses

All *in vitro* experiments were repeated at least 3 times. Statistical comparisons between groups were performed by one-way ANOVA using SPSS 16 (SPSS Inc., Chicago, IL). For multiple comparisons, one-way ANOVA with Bonferroni post hoc comparison was carried out. p values <0.05 were considered statistically significant. All bar graphs represent mean $\pm$ standard deviation.

3. Results

3.1. Nanofiber-mediated capillary morphogenesis depends on the peptide amino acid sequence and does not require surface coating with serum proteins

MVEC seeding on the RAD16-II nanofibers resulted in three-dimensional cell migration and spontaneous formation of robust capillary-like network structures at 24 hours (Figure 1), consistent with the previous results by our group [10, 48]. The appearance of the endothelial networks was similar to the capillary morphogenesis in this material reported previously by our group [48] and Sieminski et al [26], as well as capillary-like networks observed in fibrin [54, 55] or collagen I gels [49]. Consistent with previous results [48], Z-stack images of endothelial structures clearly show the three-dimensional nature of the networks and formation of hollow lumens (Figure 1B). This network formation does not require addition of external angiogenic factors, in contrast to capillary morphogenesis reported for Matrigel, collagen or fibrin-based hydrogels [49, 50]. It has been previously suggested that RAD16-II surface coating with serum proteins may play a role in endothelial cell interactions with these nanofibers [25]. To test this, MVECs were seeded on the RAD16-II nanofibers in M199 medium without serum or growth factor supplements. At 24 hours, capillary-like network structures were still observed (Figure 1), suggesting that nanofiber coating with serum proteins is not required for capillary morphogenesis by endothelial cells on the RAD16-II nanofibers. We did notice a slightly different cell appearance under these conditions, which could be related to the long-term (24 hrs) starvation in the absence of serum and was consistent with results reported by Sieminski et al [25]. Next, experiments were conducted to determine whether nanofiber-mediated capillary morphogenesis depends on the particular amino acid sequence. MVECs were seeded on KFE-8 nanofibers, which are

similar in structure to RAD16-II (Figure 7A) but do not contain RAD or RGD motifs. Previously, non-RAD-based sequences (KEF-8 and KLD-12) have been shown to support only minimal cell spreading at 3 hours after seeding, in sharp contrast with extensive cell spreading on two RAD-containing sequences (RAD16-I and RAD16-II) [25]. Consistent with those observations, our experiments showed that after 24 hours, there was limited cell attachment (less than 10%) and no structure formation on the KFE-8 nanofibers (Figure 1). Overall, these results together with the findings by Sieminski et al suggest that nanofiber-mediated capillary morphogenesis may depend on the presence of the RAD, but not KFE or KLD motifs. Because of the negative effects of the KFE-8 control peptide nanofibers on endothelial cell attachment and spreading, this group was not included in further experiments regarding the role of integrins and MAPK/ERK pathway in the nanofiber-mediated angiogenic cell responses.

To determine the role of de novo protein synthesis in the RAD16-II-mediated capillary morphogenesis, the experiments were performed in the presence of 10 $\mu\text{g/ml}$ of general protein synthesis inhibitor cycloheximide (CH) (Sigma) in the presence or absence of 10% FBS. Consistent with the results by Sieminski et al [25], addition of cycloheximide resulted in decrease in cell spreading in the absence of serum but no effects on cell spreading in the presence of serum (data not shown). Capillary morphogenesis at 24 hours was significantly decreased in the absence of serum when cycloheximide was added ($16 \pm 1 \mu\text{m}$ vs $21 \pm 1 \mu\text{m}$, CH w/o FBS vs RAD16-II w/o FBS, data not shown). These results indicate that protein synthesis is important in nanofiber-mediated capillary morphogenesis. For example, long-term (24 hrs or longer) [10, 48] pro-angiogenic effects of RAD16-II may rely on de novo expression of cytokines or other signaling proteins by the cells (such as VEGF, Figure 4), or it may involve deposition of ECM proteins as has been suggested by Sieminski et al [25]. However, the exact molecular mechanisms are still unknown and will be the subject of the future studies.

3.2. Capillary morphogenesis on RAD16-II nanofibers is integrin-mediated

To establish whether cell interactions with RAD motifs on the nanofibers and capillary morphogenesis involve integrins, similar to cell interactions with RGD motifs on native substrates, MVECs were cultured for 24 hours on RAD16-II in the presence of integrin β_3 -blocking antibody, or echistatin, which is a wide-spectrum integrin inhibitor blocking β_1 , β_3 (and possibly more) integrin subunits. The presence of β_3 -blocking antibody resulted in the significant reduction of the size of capillary-like networks as compared with RAD16-II control (Figure 2). Capillary morphogenesis was completely abolished in the presence of echistatin (Figure 2). These results suggested that the capillary morphogenesis by MVECs on RAD16-II is mediated by integrins, and specifically by β_3 and β_1 integrin subunit, despite the absence of RGD or other known integrin binding sites on the nanofibers.

3.3. Microvascular endothelial cell interactions with RAD16-II nanofibers result in tyrosine 747 phosphorylation of β_3 integrin subunit

Phosphorylation of the integrin β subunit is required for the downstream pathway activation and angiogenic cell responses [42]. To determine whether the endothelial cell interactions with the RAD16-II nanofibers result in the activation of β_3 integrin subunit, phosphorylation of β_3 at tyrosine 747 (Tyr747) was quantified at 30 min after MVEC seeding on the RAD16-II nanofibers, cell seeding on the $\beta_v\beta_3$ ligand vitronectin (positive control) and cells maintained in suspension (negative control). MVECs seeded on the RAD16-II nanofibers displayed high levels of β_3 phosphorylation (quantified as a ratio of phosphorylated β_3 to the total β_3 levels) that was significantly greater than the minimal β_3 phosphorylation in cells kept in suspension (negative control group) (Figure 3A–B). These results suggest that the low-affinity interactions of cells with RAD motifs can elicit activation of integrin β_3 .

subunits, similar to the specific interactions between the cells and RGD motifs in the vitronectin, and also may upregulate overall β_3 integrin expression.

3.4. RAD16-II-mediated angiogenic responses by microvascular endothelial cells involve MAPK/ERK signaling pathway

We next tested whether the RAD16-II-mediated capillary morphogenesis involves angiogenic intracellular MAPK/ERK pathway which is known to be triggered by phosphorylation of β_3 integrin [56]. Capillary morphogenesis by MVECs on RAD16-II nanofibers at 24 hours was significantly inhibited in the presence of specific MEK inhibitor U0126 (Figure 4A). To further confirm that β_3 -MAPK/ERK axis is indeed involved in RAD16-II-mediated angiogenic response of MVECs, we quantified VEGF protein expression by MVECs cultured on the RAD16-II nanofibers for 24 hours in the presence or absence of β_3 - or MEK- inhibitors. VEGF protein expression is another angiogenic endothelial response (in addition to capillary morphogenesis) regulated via MAPK/ERK pathway [57, 58]; moreover, we have previously shown that VEGF gene expression is upregulated when MVECs are seeded on the RAD16-II nanofibers, as compared to collagen I hydrogel [10]. Similar to the results for capillary morphogenesis, we observed that MVECs seeded on the RAD16-II nanofibers in the presence of both β_3 - or MEK-inhibitors had significantly reduced levels of VEGF protein expression, as compared with RAD16-II controls (Figure 4B). Overall, these results suggest β_3 -MAPK/ERK-VEGF axis plays a significant role in RAD16-II-mediated angiogenesis *in vitro*.

3.5. Enhanced healing and neovascularization in the diabetic wounds treated with RAD16-II nanofibers is mediated by integrins

For *in vivo* validation of the experimental results presented above, mouse db/db model of diabetic wound healing was used. This model recapitulates important features of diabetes-associated impaired healing in human patients, including delayed formation of granulation tissue and impaired neovascularization. We have recently shown that wound treatment with RAD16-II nanofibers resulted in significantly enhanced angiogenesis and improved healing at day 7 post wounding, as compared to treatment with PBS and hyaluronic acid hydrogel [59]. To demonstrate that pro-angiogenic effect of RAD16-II nanofibers *in vivo* depended on the peptide sequence, wounds were treated with RAD16-II nanofibers or KFE-8 nanofibers, which is a control peptide with similar nanofiber structure (Figure 7) but without RAD or RGD binding motifs. Importantly, previous studies by Sieminski et al [25] showed that both RAD16-II and KFE-8 can retain significant amount of protein from external solution. Therefore, the KFE-8 group served as an additional control to determine the possible contribution of proteins from the plasma in the nanofiber-mediated wound healing. To explore the role of integrins, an additional treatment group was included, where wounds were treated with RAD16-II nanofibers with added integrin inhibitor echistatin.

As expected, RAD16-II nanofibers resulted in noticeable wound closure and granulation tissue formation at day 7, which were significantly greater than similar measures in the PBS-treated group (Figure 5B–D). In contrast, no granulation tissue formation or healing was observed in KFE-8-treated wounds at day 7 (Figure 5B), suggesting that nanofiber-mediated healing response depends on the nanofiber sequence and that nanofiber coating or other contribution of extracellular matrix proteins from the plasma are not likely to play the major role in this response. Because of the absence of healing in KFE-8, further analyses were not performed on this group. The stimulating effect of RAD16-II on wound healing was abolished in the presence of echistatin, resulting in no significant differences in epithelial gap closure and granulation tissue formation between RAD16-II with echistatin and PBS groups (Figure 5C–D). Wound treatment with RAD16-II nanofibers resulted in significantly increased VEGF protein levels in the wound tissue (Figure 5), while addition of echistatin

abolished this effect. Furthermore, neovascularization of the granulation tissue of the wounds treated with RAD16-II nanofibers was significantly enhanced (Figure 6C), as compared with wounds treated with PBS. Tail vein injection of biotinylated lectin demonstrated that most of the neo-vessels were anastomosed with the host vasculature by day 7 in the RAD16-II –treated wounds (Figure 6B). Addition of integrin inhibitor echistatin, however, completely abrogated this effect and resulted in significantly impaired neovascularization, as compared with RAD16-II nanofibers or PBS treatments (Figure 6C). Taken together, these results are consistent with the *in vitro* findings and suggest that the pro-angiogenic effect of the RAD16-II nanofibers *in vivo* is likely mediated by interactions between the integrins and RAD motifs on the nanofibers.

3.6. Nanofiber-mediated capillary morphogenesis decreases with increasing RGD concentration

Capillary morphogenesis depends on the overall cell adhesion strength to the substrate, which is a function of both of individual bond strength and surface ligand concentration. In the peptide nanofiber system, the strength of individual interactions between integrins on the cell surface and the nanofibers depends on the amino-acid sequence (e.g., high-affinity binding for RGD-containing motifs vs. low-affinity interactions with RAD motifs), while surface ligand concentration is determined by the peptide concentration (1% w/v peptide in our experiments). In order to characterize the role of RAD motif and integrin-mediated interactions in RAD16-II-induced capillary morphogenesis, the sequence of the RAD16-II nanofibers was modified by inserting a RGD binding motif (known native ligand of $\beta_v\beta_3$, $\alpha_5\beta_1$, and other integrins) to the carboxyl terminus separated by two glycine linkers (RGD-RAD16-II), and formation of capillary-like structures was quantified for three RGD concentrations: 0% (RAD16-II), 50% and 100% of RGD-containing nanofibers, while maintaining the total peptide concentration of 1%. The analyses of the nanofiber architecture (TEM, Figure 7A) and scaffold mechanical properties (elastic and loss moduli, G' and G'' , Figure 7B) demonstrated that these properties were consistent with previously reported values [25, 48] and were not affected by the addition of the RGD motif. Previously, Genove et al [21] showed that addition of longer (5 amino acids or more) functional sequences to another RAD-based nanofiber backbone (RAD16-I) resulted in significant decrease in the hydrogel G' and G'' moduli, suggesting that mechanical properties of the functionalized nanofiber hydrogels may depend on the length of the added motif and nanofiber sequence. KFE-8 had a nanofiber architecture that was similar to RAD16-II (Figure 7A), and as expected [25] was stiffer than RAD-containing samples (Figure 7B), with the G' value still within the range of stiffness values permissible for capillary morphogenesis in RAD-based hydrogels [25].

Surprisingly, we observed that MVECs cultured for 24 hours on the mixture of RAD16-II and RGD-RAD16-II nanofibers containing varying amount of RGD-RAD16-II showed significantly decreased capillary morphogenesis with increasing RGD-RAD16-II concentration, as compared with RAD16-II control (Figure 7C). Cells seeded on RGD-containing substrates showed more spreading, and there were more single cells not associated with any network in 100% RGD-RAD16-II, as compared with RAD-only nanofibers, where the majority of cells were involved in coordinated cell migration and formation of multi-cellular networks.

4. Discussion

The results of this study demonstrate that endothelial cell interactions with self-assembling peptide RAD16-II nanofibers result in angiogenic cell activation, stimulation of VEGF expression and capillary morphogenesis via the mechanism that involves integrins, specifically, β_3 and β_1 integrin subunits, and MAPK/ERK pathway. Importantly, β_3 integrin

activation was observed shortly (30 min) after cell seeding on the nanofibers in the serum- and growth-factor free medium and before any extracellular matrix proteins could be produced by the cells themselves. Also, these effects were not observed on the control KFE-8 nanofibers. Our data suggest, therefore, that pro-angiogenic effects of the RAD16-II nanofibers are likely mediated by interactions between cells and RAD motifs on the nanofibers and do not occur due to nanofiber coating by proteins in the serum or growth factors in the media or cell-produced ECM, as has been suggested previously [25]. The RAD16-II nanofiber sequence contains 3 RAD motifs, which have a close homology to RGD, the major motif responsible for integrin-mediated cell interactions with the variety of extracellular matrix proteins [60]. Interactions between β_3 integrin subunit and RAD motif are low-affinity in nature (i.e. follow a low-affinity binding kinetics) and thus differ from interactions between β_3 and RGD, which exhibit the characteristic saturation binding curve [45]. Our findings demonstrate that nanofiber-mediated angiogenic cell responses *in vitro* were decreased in the presence of β_3 integrin inhibitor and abolished on the control KFE-8 peptide that did not contain RAD sequence. In addition, blocking of both β_1 and β_3 integrins by echistatin further reduced the extent of *in vitro* endothelial capillary morphogenesis on RAD16-II nanofibers, as compared with blocking of β_3 integrin alone by functional blocking antibody. Our findings of the possible role of RAD motif in integrin-mediated cell interactions with the substrate are consistent with the results of the previous study where another RGD homolog, RLD, was shown to be directly involved in neuron binding to $\alpha_v\beta_3$ integrin on astrocytes [47]. Therefore, the important implication of the results of this study is that low-affinity interactions between RAD motifs on the substrate and integrins on the cell surface may be sufficiently strong to stimulate cell attachment, integrin activation, intracellular signaling response and capillary morphogenesis, while not requiring the full strength of RGD – integrin binding. In the right microenvironment, there can be an additional benefit in combination of materials with RGD and RAD motifs. A recent study showed that cytoprotective effects of RAD-containing nanofiber scaffolds for cardiac stem cells is further improved by adding an RGD sequence to the nanofibers [61]. Given the high current interest in different approaches to functionalize bio-compatible materials for the regulation of cell migration and angiogenesis [6, 7, 18–22, 62, 63] the concept of utilizing low-affinity sequences for regulation of cell behavior potentially expands the current pool of available motifs that can be used for such functionalization. To achieve this, however, further studies would be required to better understand the exact molecular processes involved in initiating angiogenic cell responses by low-affinity interactions with the substrate and specific integrins that may participate in these processes.

The results for the role of integrins and RAD motif in nanofiber-mediated angiogenesis *in vitro* are consistent with our *in vivo* findings using a mouse model of diabetic wound healing, which is characterized by the significantly impaired angiogenesis and neovascularization in repair tissue of excisional skin wounds of diabetic mice, as compared with background-matched wild type controls [64]. We have recently reported that diabetic wound treatment with the RAD16-II nanofibers results in significantly enhanced wound neovascularization and improved wound healing [23]. In the present study, we also show that diabetic wound treatment with RAD16-II nanofibers results in significant increase in VEGF protein levels in the wound tissue, which is similar to the RAD16-II-mediated increase in VEGF expression observed *in vitro*. Our *in vivo* data is consistent with the concept that the pro-angiogenic effect of RAD16-II nanofibers depends on the peptide sequence (it was absent in the control treatment with non-RAD peptide) and is mediated by integrins (it was abolished in the presence of wide-spectrum integrin inhibitor echistatin, which is known to block β_1 - and β_3 - containing integrins [65]). Overall, the results indicate that similar mechanisms may regulate pro-angiogenic effects of RAD16-II nanofibers *in vitro* and *in vivo*, which may have important implications for using this or other materials of this class to develop novel therapeutic pro-angiogenic strategies, such as therapies for

healing of diabetic chronic wounds, cardiac regeneration or cardiovascular stent coating. Indeed, one of the main advantage of self-assembling peptides is that multiple specific bio-functionalities can be realized by extending the self-assembling domain with a short ligand of interest, such as integrin binding, surface binding, growth factor binding, or specific proteolytic susceptible domain [21, 66, 67]. For example, a recent study presented a self-assembling peptide matrix that contains nitric oxide (NO) donating residues, endothelial cell adhesive ligands composed of YIGSR peptide sequence, and enzyme-mediated degradable sites, which has a potential for coating of cardiovascular implants to overcome restenosis and thrombosis that hamper the long-term clinical success [68]. Better understanding the cellular and molecular mechanisms that are involved in endothelial cell interactions with these materials will help facilitate translation of these findings into the clinical setting.

The findings of the β_3 - and MEK- dependent RAD16-II –mediated expression of VEGF in both *in vitro* and *in vivo* experiments are consistent with the existing model for angiogenic endothelial signaling via β_3 /VEGFR2-MAPK/ERK-VEGF axis in other systems. Indeed, experimental evidence suggests that there is a cross-talk between the integrins and growth factor receptors, in particular, that binding of $\beta_v\beta_3$ to its ligands promotes phosphorylation and activation of vascular endothelial growth factor receptor 2 (VEGFR-2) and enhances mitogenic activity [49] of VEGFs, and that VEGF stimulation triggers β_3 tyrosine phosphorylation [37, 69], with downstream signaling via MAPK/ERK pathway [70]. We have previously observed significantly increased level of VEGF mRNA expression by ECs seeded on RAD16-II nanofibers as compared with cells cultured on collagen I [10, 71]. Together with the previous findings, the results of this study suggest, therefore, that phosphorylation of β_3 integrins, which is triggered by endothelial cell interactions with the nanofibers, may also stimulate VEGFR-2 activation, capillary morphogenesis and VEGF expression via MAPK/ERK pathway. Thus, increased phosphorylation of β_3 may further enhance the mitogenic activities of VEGF, which when taken together, increases angiogenic cell responses in an autocrine positive feedback manner. Importantly, VEGF mediation of cell interactions with the substrate does not occur exclusively via β_3 -containing integrins: numerous studies have shown that VEGF stimulates angiogenesis in other *in vitro* systems, such as collagen I [49, 72, 73] and fibrin [73] gels, where cell adhesion to the substrate mostly involves $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ integrins [74]. In addition, $\alpha_5\beta_1$ integrin can interact with Tie2 (a receptor for the angiogenic growth factor angiopoietin-1) [38], and is significantly upregulated on angiogenic ECs [75]. The role of cross-talk between integrins and growth factors in nanofiber-mediated angiogenesis is the focus of the ongoing research in our laboratory.

Interestingly, we observed increased number of individual cells not associated with any network and reduced capillary morphogenesis with increasing RGD concentration. These results were surprising, because RGD motif is a native cell binding motif that is recognized by many different integrins and has been shown to be the key player in several *in vitro* models of angiogenesis [33, 76]. However, these findings are consistent with recent work by Moon and co-workers [77], who observed that endothelial cord formation on the 50 μ m wide stripes with PEG-RGDS was stimulated for intermediate concentration of RGDS, and it was inhibited at higher RGDS concentrations, where cells remained in monolayer. Overall, the observed variations in endothelial cell responses to pure RAD- and RAD-RGD substrates are consistent with a known model of a biphasic relationship between cell migration speed and cell adhesion strength [78] and the model for aggregate cell morphogenesis by Powers and Griffith [79]. Indeed, coordinated migration of activated endothelial cells is the major component of capillary morphogenesis *in vitro*. As the biphasic model suggests, cell migration is slow if substrate is not adhesive enough or if it is too adhesive, and the migration is the fastest at the intermediate adhesion strength [80]. Ultimately, the ability of

cells to aggregate and form networks depends on the balance between actin-mediated traction forces generated by the cell and cell-substratum (and cell-cell) adhesive forces [79]. It is well established that modulation of adhesion strength between the cell and the substrate can be achieved through variation of the affinity and surface density of peptide ligands [63, 81]. In the studies of the *in vitro* capillary morphogenesis, adhesion strength is often modulated by the protein (or ECM) concentration of the gel [26, 49, 54], which also alters mechanical properties of the matrix surrounding the cells, or by pharmacological agents [55]. In this study, we adopted an alternative approach, where cell adhesion to the nanofibers was modulated by a) the variation in the affinity of interactions between integrins and peptide ligands (low - RAD vs. high - RGD [45]); and b) the variation in the concentration of RGD-containing nanofibers, while the protein concentration and mechanical properties of the peptide hydrogels were retained between all RAD- and RGD-containing nanofibers where capillary morphogenesis was observed. Therefore, in our system, the strength of cell attachment is the lowest for KFE-8 nanofibers [25], and it is likely to increase with increasing RGD content of the substrate due to higher strength of cell interactions with RGD vs. RAD motifs. As a result, the most robust cell migration and capillary morphogenesis on the peptide nanofibers could be expected at the “optimal” cell attachment strength (which appears to be the case for pure RAD nanofibers), and these would be expected to decrease with increasing substrate adhesiveness (i.e., with increasing RGD concentration in RAD-RGD nanofiber mixtures), which is consistent with our findings. Further validation of this model will require the detailed characterization of cell adhesion to the peptide motifs, as has been done in other systems [81].

5. Conclusions

Peptide-based biomaterials [82] have emerged as a new class of scaffold materials due to their potential to produce precisely defined, hierarchical 3D structures [83] with highly tunable nature, and have shown great promise for biomedical tissue engineering applications. The possibility of selectively placing functional motifs into a protein structure in these materials allows the manipulation of response to physiological stimuli. The results of this study suggest a novel mechanism for activation of angiogenic signaling via relatively weak low-affinity interactions between endothelial cells and RAD motifs in the RAD16-II nanofibers, which can trigger phosphorylation of β_3 integrin and stimulate angiogenesis and VEGF expression via MAPK/ERK pathway. Our results suggest that non-specific cell-matrix interactions may be preferable for promoting angiogenesis-related endothelial cell migration, as compared with RGD-rich scaffolds, indicating that the nature of cellular interactions with certain non-natural binding sequences, such as RAD motifs, may be an important parameter to consider when designing or functionalizing tissue engineering scaffold materials. Overall, incorporation of RAD or similar motifs peptide-based biomaterials such as protein engineered or hybrid peptide scaffolds [83] may represent a novel strategy for vascular tissue engineering and will further enhance design opportunities for new scaffold materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH/NDDK (1R21DK078814-01A1), American Heart Association Beginning Grant-In-Aid (BGIA-533 0765425B), and startup funds from University of Cincinnati Department of Biomedical Engineering to DAN.

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Figure 1. Capillary morphogenesis by MVECs on self-assembling peptide nanofibers

(A) At 2 hours after seeding on the RAD16-II nanofibers, cells have attached and are distributed randomly (top, left). At 24 hrs following seeding on the RAD16-II nanofibers in 10% FBS without additional growth factors, capillary-like structures are spontaneously formed by MVECs (top, right). Capillary morphogenesis occurred on RAD16-II nanofibers in the absence of serum or angiogenic growth factors (bottom, left). No capillary morphogenesis was observed on the control peptide (KFE-8) nanofibers (10% FBS) which do not contain RAD motif (bottom, right). The scale bars are 200 μm . Cells were stained with phalloidin-TRITC. (B) 3-D reconstruction of Z-stack images of single endothelial

structure at 24 hrs was used to visualize lumen formation. Hollow endothelial lumens were observed in (xx) and (yy) planes (through the structure). The scale bar represents 50 μm .

Characteristic Network Size (μm)

Figure 2. Capillary morphogenesis on RAD16-II nanofibers is integrin-mediated

Capillary morphogenesis on the RAD16-II nanofibers at 24 hours was significantly reduced in the presence of β_3 blocking antibody, and completely abolished in the presence of wide-spectrum integrin inhibitor echistatin, as compared with RAD16-II nanofibers alone. The scale bars are 200 μm . Cells were stained with phalloidin-TRITC.

Figure 3. Microvascular endothelial cell interactions with RAD16-II nanofibers result in tyrosine 747 phosphorylation of β_3 integrin subunit

(A–B) MVECs were either kept in suspension or plated on RAD16-II nanofibers or vitronectin for 30 minutes. Cells plated on the RAD16-II nanofibers showed high levels of β_3 phosphorylation at tyrosine 747 (Western blot analyses, relative units, pTyr747 intensity / total β_3 intensity), which was similar to that of the positive control, and significantly greater than the phosphorylation levels in the negative control group ($p<0.05$).

Figure 4. Pro-angiogenic effects of RAD16-II nanofibers involve MAPK/ERK signaling pathway

(A) Presence of MEK inhibitor U0126 resulted in significant reduction in capillary morphogenesis ($p < 0.001$) by MVECs at 24 hours, as compared with cells seeded on the RAD16-II alone. (B) Similar to capillary morphogenesis results, VEGF protein expression by MVECs was significantly reduced by both β_3 integrin inhibitor ($p < 0.001$) and U0126 ($p < 0.001$) at 12 hours (ELISA), as compared with RAD16-II control group.

Figure 5. Enhanced healing in the diabetic wounds treated with RAD16-II nanofibers is integrin-mediated

(A) A schematic of wound sites and treatments on the dorsum of a diabetic mouse. (B–D) Quantitative morphological analysis of H&E stained wound sections and, (E) VEGF ELISA of the wounds at day 7. RAD16-II nanofibers resulted in noticeable wound closure and granulation tissue formation at day 7 (B), which were significantly greater than similar measures in the PBS-treated group (C–D, $p < 0.005$). In contrast, no granulation tissue formation or healing was observed in KFE-8-treated wounds at day 7; therefore, further analyses were not performed on this group. The stimulating effect of RAD16-II on wound healing was abolished in the presence of echistatin, resulting in no significant differences in epithelial gap closure and granulation tissue formation between RAD16-II with echistatin and PBS groups (C, D). Wound treatment with RAD16-II nanofibers resulted in significantly increased VEGF protein levels in the RAD16-II- wound tissue (E, $P < 0.001$), while addition of echistatin abolished this effect.

Figure 6. Enhanced neovascularization in the diabetic wounds treated with RAD16-II nanofibers is integrin-mediated

(A) At day 7 post-wounding, diabetic wound treatment with RAD16-II nanofibers resulted in marked increase in neovascularization of the repair tissue (CD31-positive brown staining), as compared with PBS. This effect was abolished by addition of echistatin to the RAD16-II nanofibers. (B) Mice were injected with biotinylated lectin prior to euthanization and staining showed lumens with red blood cells within the CD31-stained vessels, indicating the anastomoses of the newly-formed vessels in the granulation tissue to host vasculature. (C) Images (n=12) of each section were obtained and image analysis software (NIH ImageJ c1.4.3u) was used to measure the area of CD31-positive blood vessels (shown in red) in tissue sections from each treatment group (n=3 per each group). The analysis showed that integrin inhibition by echistatin significantly reduced RAD16-II-mediated neovascularization of diabetic wounds *in vivo*. Scale bars represent 25 μm .

Figure 7. Nanofiber-mediated capillary morphogenesis decreases with increasing RGD concentration

In order to characterize the role of RAD motif and integrin-mediated interactions in RAD16-II-induced capillary morphogenesis, the sequence of the RAD16-II nanofibers was modified by inserting a RGD binding motif to the carboxyl terminus separated by two glycine linkers (RGD-RAD16-II), and formation of capillary-like structures was quantified for three RGD concentrations: 0% (RAD16-II), 50% and 100% of RGD-containing nanofibers, while maintaining the total peptide concentration of 1%. (A) Transmission Electron Microscopy analysis demonstrated that addition of RGD motif did not alter fiber architecture. The scale bars are 100 nm. (B) Rheometry testing showed that there was no significant difference in

elastic (G') and loss moduli (G'') between the hydrogel formed using RAD16-II with or without RGD-RAD16-II nanofibers. KFE-8 had a nanofiber architecture that was similar to RAD16-II and it was stiffer than RAD-containing samples, as expected based on previous results by Sieminski et al (2008). (C) MVECs cultured for 24 hours on the mixture of RAD16-II and RGD-RAD16-II nanofibers containing varying amount of RGD-RAD16-II showed significantly decreased capillary morphogenesis with increasing RGD-RAD16-II concentration, as compared with RAD16-II control. Cells seeded on RGD-containing substrates showed more spreading, and there were more single cells not associated with any network in 100% RGD-RAD16-II, as compared with RAD-only nanofibers, where the majority of cells were involved in coordinated cell migration and formation of multi-cellular networks. The scale bars are 200 μm .