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Bioactive peptides derived from vascular endothelial cell extracellular matrices promote microvascular morphogenesis and wound healing in vitro

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Abstract

Studies in our laboratory indicate that collagenase from *Clostridium histolyticum* promotes endothelial cell and keratinocyte responses to injury *in vitro* and wound healing *in vivo*. We postulate that matrix degradation by Clostridial collagenase creates bioactive fragments that can stimulate cellular responses to injury and angiogenesis. To test this hypothesis we performed limited digestion of defined capillary endothelial derived extracellular matrices using purified human or bacterial collagenases. Immunoprecipitation with antibodies recognizing collagen I, II, III, IV and V, followed by mass spectrometry reveals the presence of unique fragments in bacterial, but not human enzyme digested matrix. Results show that there are several bioactive peptides liberated from Clostridial collagenase treated matrices, which facilitate endothelial responses to injury, and accelerate microvascular remodeling in vitro. Fragments of collagen IV, fibrillin-1, tenascin X and a novel peptide created by combining specific amino acids contained within fibrillin 1 and tenascin X each have profound pro-angiogenic properties. The peptides used at 10–100 nM increase rates of microvascular endothelial cell proliferation by up to 47% and *in vitro* angiogenesis by 200% when compared to serum-stimulated controls. Current studies are aimed at revealing the molecular mechanisms regulating peptide-induced wound healing while extending these *in vitro* observations using animal modeling.

Keywords

Clostridial collagenase; matrix metalloproteinases; matrix fragments

INTRODUCTION

Full thickness cutaneous wound healing is a well-organized process that leads to reestablishment of the skin's physical and mechanical integrity. The normal repair process can be divided into several temporally and spatially overlapping phases that include coagulation, inflammation, formation of granulation tissue (proliferative phase), remodeling

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and scarring (1). Platelets and inflammatory cells initiate the wound-healing cascade and produce growth factors and cytokines that induce migration and proliferation of epidermal and dermal cells – keratinocytes and fibroblasts. These cells are the key players in the re-epithelization of the wound, formation of granulation tissue and scarring (1). The function of all cells contributing to wound healing process relies on the presence of an adequate blood supply as a source of nutrients, oxygen and cytokines. Therefore, cellular responses to injury are critically angiogenesis-dependent. In turn, angiogenesis of normal wound healing depends on two major processes: recruitment of the endothelial progenitor cells from the circulation and sprouting of resident endothelial cells from existing adjoining microvascular circuits bordering the wound bed (2). In the latter case, capillary endothelial cells (CEC) become activated in response to injury-induced growth factors such as platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) mainly released by activated platelets, macrophages and keratinocytes within the wound bed (3,4). Growth factor mediated stimulation of the endothelium leads to an increase in CEC proliferation and migration. Both of these processes require and depend on the presence of a permissive microenvironment shaped by the extracellular matrix (ECM). In addition to providing a substrate for CEC migration, native and cleaved ECM molecules bind to and activate endothelial integrin receptors (particularly $\alpha v \beta 3$) that trigger dynamic endothelial responses and sustain angiogenesis (5).

An important prerequisite for wound healing angiogenesis is basement membrane (BM) degradation. Indeed, BM turnover is regulated by a number of matrix remodeling enzymes - serine proteases and matrix metalloproteinases (MMPs), which include collagenases and gelatinases (6) produced by resident endothelial cells. Altogether, BM remodeling helps to coordinate cellular injury responses, including wound healing angiogenesis. Further, ECM degradation allows for efficient cell migration since MMPs liberate matrix-bound growth factors such as basic fibroblast growth factor (bFGF) and VEGF (6,7) that enhance the angiogenic cascade and wound healing. Finally, proteolytic matrix degradation gives rise to pro- and anti-angiogenic ECM fragments that regulate endothelial morphogenesis *in vitro* and angiogenesis *in vivo* (8–11).

Because of the importance of matrix remodeling for wound healing, it has been suggested that exogenous ECM-remodeling proteases, such as collagenase derived from gram-positive bacteria *Clostridium histolyticum* may be beneficial for wound healing (12). Indeed, previous work in our laboratory has demonstrated that this enzyme stimulates epithelial and endothelial response to injury both *in vitro* and *in vivo* (13,14). The question of how bacterial collagenase stimulates wound-healing responses remains. We hypothesize that in addition to the debriding effects, digestion of components of the ECM by the bacterial enzyme leads to the release of bioactive peptides that stimulate wound closure. To test our hypothesis and determine whether matrix remodeling by Clostridial collagenase leads to production of biologically active fragments, we use the well-characterized ECM synthesized by vascular endothelial cells (15). Results reveal that there are several unique small peptide fragments of tenascin X, fibrillin-1 and collagen Type IV released during matrix degradation by bacterial, but not human collagenase. These Clostridial collagenase-liberated and capillary endothelial matrix-derived peptides stimulate proliferation of CEC, enhance microvascular remodeling in two-dimensional (2D) model on Matrigel and induce endothelial sprouting in a novel three-dimensional (3D) model of injury repair.

METHODS

Cells

CEC were isolated as described previously (15–18). Animal tissues for cell isolation were obtained from an abattoir and therefore, no institutional animal use committee approval was

required or obtained. Briefly, bovine retinal capillary fragments were prepared and plated in DMEM supplemented with bovine calf serum (BCS, Atlanta Biologicals, Inc., Lawrenceville, GA) and antibiotics (Invitrogen, Carlsbad, CA). Selection of endothelial cells was performed using selective attachment, media containing 5% human platelet-poor plasma and ultimately, cloning. After isolation, cells were stored in liquid nitrogen and then cultured in DMEM supplemented with 1–5% BCS and antibiotics and used at passages 7–13. These cells were characterized by: (i) labeling with antibodies against Factor VIII (16), (ii) ability to bind and internalize di-I-acyl LDL, (iii) failure to be stained with anti-3G5, a pericyte-specific marker (Nayak and Herman, 2001; (19)) and (iv) their ability to form capillary-like structures in vitro that bear a resemblance to capillaries formed during angiogenesis in vivo, i.e. endothelial-lined ‘sprouts’ that possess ‘lumenal’ compartments (see Supplemental Figure 1).

Matrix preparation

The extracellular matrix was prepared as described (15,20). Briefly, CEC at 7–10 days post-confluence were washed 3 times with phosphate buffered saline (PBS) (pH=7.15). Cells were removed with 0.5% sodium deoxycholate buffered with 20 mM Tris-Cl (pH 8.0) containing 15 mM NaCl, 1 mM EGTA (pH 7.0), 1 mM phenylmethyl sulfonyl fluoride. The matrix then was washed with PBS, immediately collected and used for enzymatic degradation and peptide identification.

Enzymatic degradation of the matrices

Enzymatic matrix degradation was performed as described (13). Purified Clostridial collagenase was obtained from Advance Biofactures (Lynbrook, NY), dissolved in 1M TRIS-HCl (pH=7) at 1mg/mL and stored at –20C before use for no longer than one week. Immediately before the experiments the enzymes were thawed, diluted in calcium buffered saline at 16U/mL and added to the plates for 1h at 37C. Human MMP-1 (Calbiochem, La Jolla, CA; Cat. # 444208) was activated according to manufacturer instructions, and used for 1h at 37C to perform matrix degradation. Both human and bacterial enzymes were used at the same protein concentration.

Peptide identification and synthesis

For peptide identification matrices treated with bacterial collagenase or human MMP-1 were prepared as described above and scraped into immunoprecipitation (IP) buffer containing 0.125% bovine serum albumin, 30mM Tris-Cl (pH=8), 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 and supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, MO; Cat. #P8340). Immunoprecipitation was performed using antibody directed either against collagen I (Abcam, Cambridge, MA; Cat. #292) or mixed antibodies against collagens I-V (Abcam, Cambridge, MA; Cat. #ab24117). Protein A Sepharose beads were washed three times in distilled water and resuspended in IP buffer in the presence of protease inhibitors (Sigma-Aldrich, St. Louis, MO; Cat. #P8340). Antibodies were added to resuspended beads at a final concentration of 225 µg/ml and incubated with agitation for 1h at room temperature to allow for bead-antibody interactions.

ECM prepared as described above was digested with either bacterial or human collagenases, scraped in 0.1x IP buffer, lyophilized overnight and reconstituted in 1/10 volume of distilled water. Before addition to antibody-bound beads matrix preparations were pre-cleared by incubation with protein A sepharose (GE Healthcare, Uppsala, Sweden) in the absence of the antibody. Pre-cleared ECM samples were applied onto antibody-bound beads and rotated overnight at 4C in the presence of protease inhibitors to allow for immunoprecipitation. The precipitated complex was washed several times with IP buffer at 4C. Proteins were eluted using boiling SDS sample buffer containing 2% 2-mercaptoethanol (Sigma Aldrich, St.

Louis, MO), subjected to gel electrophoresis and stained with Coomassie blue dye for protein detection. Protein bands present bacterial, but not human digests, were excised, washed 2 times in 50% acetonitrile and submitted to Tufts University Core Facility (TUCF) for protein identification. At TUCF the gel bands were degraded further using proteomics-grade trypsin and subjected to liquid chromatography mass spectrometry (21). In total over 100 protein fragments were identified, of those 12 containing 10–22 amino acids were selected and submitted to TUCF for synthesis by FastMoc Chemistry (22).

Cell proliferation assay

The unique ECM-derived peptides released by bacterial collagenases were tested for growth-promoting potential toward CEC. CEC were plated at 2×10^3 cells/well in three 48 well plates in DMEM containing 5% BCS. On the next day, cells in one of the plates were washed with PBS, detached from the substrate using trypsin and counted using a Coulter Counter model Z-II (Beckman Coulter, Inc, Fullerton, CA) according to the manufacturer's instructions. Cells in the remaining two plates were fed with DMEM/1% BCS with or without the peptides mixed in the media at concentrations ranging from 1–100nM at day 1 and 3 after plating. Cell counts were performed as described above at days 3 and 5 post-plating. Three wells were used per condition and each experiment was repeated at least three times. In this and following assays low serum-containing media were used in order to minimize possible masking effects of serum components on cellular responses to peptide treatment. Under these experimental conditions, no cell detachment or death is observed (15,23).

2D in vitro endothelial morphogenesis assay

In this assay we used growth factor-reduced (GFR) Matrigel (BD Biosciences, Bedford, MA; Cat. #354230). The GFR Matrigel was thawed on ice overnight in the cold room and used immediately. To prevent premature matrix polymerization, all procedures were performed at 4C. Stock (25 mM) solutions of the peptides were prepared in sterile water or 1M TRIS (pH 8.0), added to the GFR Matrigel at a final concentration of 100 nM and mixed thoroughly. Thus prepared peptide-containing GFR Matrigel was placed into the wells of 8-well chamber slides (BD Biosciences, Bedford, MA). Control wells contained GFR Matrigel alone. For positive control we used GFR Matrigel blended with 10 ng/ml (0.6 nM) of pro-angiogenic growth factors bFGF or VEGF (24). The chamber slides containing GFR Matrigel preparations containing the peptides, bFGF or 1% BCS then were placed into the tissue culture incubators at 37C for 45–60 minutes to allow for matrix polymerization. After matrix polymerization, CEC at 5×10^4 cells per well were plated on the surface of polymerized Matrigel in DMEM containing 1% BCS in the presence or absence of the corresponding peptides or bFGF. Control wells contained DMEM supplemented with 1% BCS. Formation of endothelial sprouts was monitored at 7 and 24 hours post-plating. Visualization of the cells was performed with Axiovert 200M microscope (Carl Zeiss MicroImaging, Thornwood, NY) using 5× or 10× objective lenses. Image analysis and measurement of the length of the structures were performed using ImageJ (available from NIH). Both the length of individual sprouts and total sprout length per field were measured.

3D model of *in vitro* angiogenesis

Unlike the 2D model described above, and in an effort aimed at better reflecting the *in vivo* microenvironment, the 3D construct contains cells that are embedded within a well defined extracellular matrix rather than having the cells being plated upon the matrix surface (25). The matrix is created by combining GFR Matrigel with rat tail collagen Type I (BD Biosciences, Bedford, MA; Cat # 354236) to the final concentration of collagen 0.7 mg/mL, in the presence of 5 mM NaOH and DMEM supplemented with 1% BCS and placed into the wells of 8 well chamber slides (250 uL/well). After matrix polymerization, 1.5×10^5 CEC

were plated in 250 μ L of DMEM supplemented with 1% BCS and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA). After cell attachment, approximately 1h post-plating, the non-adherent cells and excess media were carefully removed, then the second layer of Matrigel-collagen mixture was applied and allowed to polymerize for 45–60 minutes at 37°C creating 3D structures with CEC “sandwiched” in between two layers of Matrigel-collagen mixture. Small (1221.7 $\pm$ 83 μ m diameter) or large (3724.2 $\pm$ 115 μ m diameter) circular wounds were made in the middle of each well using a blunt 22 G needle (Tyco Healthcare Group LP, Mansfield, MA) attached to a vacuum line inside the tissue culture hood. Immediately after injury, both large and small wounds were filled with the Matrigel-collagen mixture blended with the peptides, bFGF, VEGF or DMEM supplemented with 1% BCS. Wounds were imaged immediately after filling and at days 1, 3, 7 post-wounding using a 5 $\times$ objective lens. Fifteen to 20 images were taken per wound at each day of observation and merged using Adobe Photoshop CS2 (Adobe, San Jose, CA). The merged images then analyzed manually to estimate the number of angiogenic sprouts formed by cells invading the Matrigel-collagen plugs. Data were expressed as mean values, $\pm$ SD with statistical analyses performed as described below.

Data analysis and statistics

All experiments were performed at least three times. Counts obtained in cell proliferation assay, in 2D in vitro morphogenesis assay and in a 3D model of wound healing were recorded manually and analyzed using Microsoft Excel (Microsoft, Redmond, WA). Results are presented as mean $\pm$ SEM. Statistical significance of the findings was analyzed by two-tailed t-test. The value of $p < 0.05$ was considered significant.

RESULTS

Identification and synthesis of matrix fragments released by bacterial collagenase

We subjected CEC-derived matrices (26) to limited digestion using purified bacterial collagenase or human MMP-1 as described in Materials and Methods.

Matrix digestion was followed by immunoprecipitation with polyclonal antibodies directed against collagen I or collagens I–V. As can be seen in figure 1, several distinct polypeptides of high (120 kDa), medium (45–50 and 80 kDa) and low (35 kDa) molecular weight were found in matrices degraded by bacterial, but not human collagenase. To reveal the identity of these unique matrix fragments we used liquid chromatography mass spectrometry (21). As shown in Table 1, immunoprecipitation succeeds in enriching for both collagenous (collagens I and IV) and non-collagenous (tenascin X and fibrillin 1) matrix fragments. Several fragments were synthesized using FastMoc Chemistry at TUCF (Table 1). Fragments derived from collagens I and IV include: col1-hyp and col1-leu, derived from the α 1 chain of collagen I and col4-1, col4-2 and col4-c originated from non-collagenous domain of α 3 of collagen IV (Table 1). Fragments of tenascin X and fibrillin 1 present non-collagenous polypeptides. Ten1, ten3 and ten4 are fragments of EGF-like domain rich regions of tenascin X, ten2 is a fragment of fibronectin III-like domain of tenascin X (Table 1). Fibr1, fibr2 and fibr3 are derived from EGF-like domains of fibrillin 1. In addition, we have created combinatory peptide (comb1) by combining fragments of EGF-like domains of tenascin X and fibrillin 1. The length of the polypeptides varies between 10 and 25 amino acids (Table 1).

Peptides derived from endothelial matrix stimulate endothelial cell proliferation in vitro

The effects of matrix-derived peptides on endothelial proliferation were evaluated in 5-day assays using CEC plated at low density as described in Materials and Methods. As shown in figure 2, four peptides induce a statistically significant increase in endothelial proliferation.

Namely, combinatorial peptide comb1 increases cell growth by 28%; fragments of fibrillin 1 (fibr2), and collagen IV (col4-1) enhance endothelial proliferation by 35%. Strikingly, a fragment of tenascin X (ten1) increases endothelial proliferation rate by 47%. For col4-1, ten2 and comb1 stimulatory effects are present at peptide concentration of 10–100 nM. Fibr2 on the other hand exhibits a dose dependent response and is only active at 100 nM inducing a 35% increase in endothelial proliferation. It is important to emphasize that while proliferation is an essential step during angiogenesis, increase in migration, morphogenesis, sprout and lumen formation by activated endothelial cells are comparably critical for successful formation of a nascent and stabile capillary network. Therefore, we directly tested whether the peptides promote capillary endothelial morphogenesis using 2-D and 3-D in vitro models.

Matrix-derived peptides stimulate microvascular remodeling in vitro: 2D modeling

Next, we examined the ability of the peptides to induce endothelial morphogenesis using a well-established Matrigel-based assay as described in Materials and Methods. The assay enables a quantitative analysis of endothelial morphogenesis as a function of time or experimental condition (23). Based on previously published observations indicating that endothelial cells plated on Matrigel form lumen-containing structures within 6–12 hours (27), we chose to study peptide effects on microvascular morphogenesis at 7 and 24 hours post-plating. At both time points studied several peptides stimulate CEC morphogenesis and sprout formation in vitro. Phase-contrast images shown in figure 3 demonstrate that two fragments of fibrillin 1 (fibr2 and fibr3) and collagen IV (col4-c) induce a 25–65% improvement in the rate of endothelial morphogenesis and lead to an increase in the number and total length of endothelial structures formed at an early time point (7h) post cell plating. Combinatorial peptide (comb1), a fragment of collagen IV (col4-1) and two fragments of Tenascin X (ten 2 and ten4) induce almost doubling of the total length of sprouts formed on Matrigel within 7h post-plating (Figure 3). Peptide effects on angiogenic induction were comparable or superior to bFGF and VEGF (Figure 3A–D, Figure 5A).

By 24h post-plating the angiogenic sprouts ‘mature’ as they elongate, acquiring a ‘tube-like appearance. By this time the differences in the length of the structures formed by control and treated cells diminish as control cultures acquire the ‘equilibrium’ features observed in peptide-treated endothelial cultures (Figure 5B). Nonetheless, tubes formed in the presence of VEGF, bFGF (Figure 4B, C, Figure 5B) or comb1, ten4 and col4-1 (Figure 4D, E and F respectively and Figure 5B) are 34–57% longer than those formed in wells containing Matrigel supplemented with 1% serum, alone (Figure 4A, Figure 5B).

Matrix-derived peptides stimulate microvascular remodeling in vitro: 3D modeling

In an effort to learn whether the peptides can be active in a more complex environment and to better approximate their wound healing potential, we tested the most promising peptides in a 3D model of wound healing (25). Three peptides comb1, col4-1 and ten4 that stimulate endothelial proliferation and/or endothelial morphogenesis on Matrigel, were tested as shown schematically in figure 6 and as described in Materials and Methods (25). Imaging of the defects, which are refilled with Matrigel-collagen immediately after injury, demonstrates that a reliable and reproducible ‘wound’ and associated cellular removal can be achieved. And, addition of the Matrigel-collagen plug post-injury does not disrupt the integrity of the wound edge.

In both large and small wounds (Figure 7) visible cellular responses are first seen at 24 hours post-injury. At this time cells begin to establish a plexus and form a microvascular network in the areas proximal to the injury site, which has been re-filled with Matrigel-collagen mixture containing pro-angiogenic peptides, VEGF or bFGF (Figures 7 and 8). In

larger wounds, an ‘angiogenic’ network can be seen in proximity to the wounds filled with Matrigel-collagen mixtures supplemented with 1% serum; but, these wounds contain only a few short sprouts. This response to ‘injury’ is similar to ‘wounds’ filled with Matrigel-collagen mixtures containing serum, or those defects that have been filled/supplemented with the peptides or growth factors (e.g. bFGF). On the other hand, smaller diameter wounds (at 24h post-injury) contain many, many long sprouts and an elaborate, post-injury angiogenic network (Figure 7). Indeed, CEC within these smaller diameter wounds, and which have been treated with either growth factors or the pro-angiogenic peptides, form significantly more sprouts and more robust angiogenic network when compared to control CEC treated with serum/Matrigel-collagen mixtures, alone (Figures 7 and 8). As the CEC network continues to penetrate the wound defect, e.g. 3 days post-injury, differences between the treatment groups are minimized as cells treated under control conditions begin forming more sprouts, resembling those treated with the peptides or growth factors. In larger wounds at day 3 post-wounding, a robust angiogenic response is seen in wells treated with comb1, ten 4 and bFGF (Figure 9), while only small number of structures formed in controls observed at the same timepoint (Figure 9). Thus, data derived using the 3D model of injury-repair confirm that the peptides produced by bacterial collagenase degradation of endothelial extracellular matrices, as well as those combinatorial peptides created ex vivo, stimulate a marked endothelial angiogenic response to injury.

DISCUSSION

Collagenase derived from the gram-positive bacterium *Clostridium histolyticum* has been used for wound debridement since the 1960s (28). More recently, it has been suggested that bacterial collagenase promotes wound healing by stimulating post-injury cellular responses within the epidermal and dermal compartments, which enhances re-epithelialization, wound healing angiogenesis and wound closure (13,14).

We have identified several fragments that are uniquely present in extracellular matrix digested with bacterial collagenase (Figure 1). The differences in matrix degradation patterns reflect differential cleavage sites between the host (mammalian) and bacterial enzymes (29). The mammalian collagenase cleaves specific Gly-Ile or Gly-Leu bonds of $\alpha 1$ and $\alpha 2$ chains releasing two fragments that represent 1/3 and 2/3 the intact parent collagenous substrate. Bacterial collagenase on the other hand, attacks the parent collagenous backbone much more frequently, especially at Y-Gly bonds, therein releasing multiple fragments (29). Our data validate these earlier reports and lend credence to the notion that the resultant products of matrix digestion may be linked to key Clostridial collagenase wound healing properties.

Isolation of the specific peptides released by bacterial collagenase revealed the presence of fragments of several ECM molecules that have been reported to be produced by endothelial cells (26,30–32). Furthermore, matrix macromolecules (tenascin X, collagen IV and fibrillin 1) are glycoproteins that possess multiple domains, including regions that respectively interact with collagen I, III, V, decorin, and fibronectin, elastin and latent transforming growth factor β -binding protein (33,34). That tenascin X is present within the anti-collagen I IPs is of particular interest since this matrix component has been previously implicated in tissue injury and repair in humans and mice (35,36).

Using both 2- and 3D models we found that the peptides released from the ECM by Clostridial collagenase induce key wound healing responses embodied by purified Clostridial collagenase (Table 1). Several peptides significantly increase the rate of sprout formation in Matrigel-based 2D model. Interestingly, the effects of the peptides on endothelial morphogenesis in this model are more pronounced at the earlier time point 7h as

compared to 24h post-plating. This suggests that in addition to enhancement of the rate of cell-cell interactions, which leads to cellular alignment and tube formation, the peptides also enhance endothelial motility - an early step of angiogenic process.

Our results agree with several earlier reports showing biological activity of matrices degraded by bacterial collagenase. It has been demonstrated that fragments of human eschar degraded by bacterial collagenase stimulate fibroblast proliferation both *in vitro* and *in vivo* (37). Moreover, it has been reported that bacterial collagenase degraded collagens, are chemotactic for human fibroblasts (38) and neutrophils (39). The effects of these fragments on endothelial cells have not been reported. To our knowledge no ECM fragments released by Clostridial collagenase have anti-angiogenic effects. This is in striking contrast to mammalian matrix degrading enzymes that liberate both pro- and anti-angiogenic moieties from naturally occurring matrices (10,11).

It has been reported that several elastin-derived peptides containing GXXPG sequence enhance endothelial migration, tube formation on collagen type I *in vitro* and in chick chorio-allantoic membrane model *ex vivo* via induction of MT1-MMP production by endothelial cells (11). Two of our peptides comb1 and ten2 also contain this stretch of amino acids, and both induce morphogenesis in a Matrigel-based assay. However, only a larger combinatorial peptide (comb1), with presumably more complex tertiary structure and two GXXPG domains, also stimulated cellular proliferation. While both ten2 and comb1 can probably stimulate MMP production by cells necessary for endothelial morphogenesis, comb1 is likely to activate other yet undetermined pathways mediating cell proliferation.

Importantly, the chemical structure of other bioactive ECM fragments released by bacterial collagenase and described here have not been reported by others. The receptors and signaling pathways activated by the peptides remain to be discovered. The receptors could include pro-angiogenic integrins $\alpha v \beta 3$, $\alpha v \beta 5$, mediating both migration and proliferation of endothelial cells necessary for angiogenesis. Other potential receptors may include well-established growth factor/tyrosine kinase-dependent receptors (e.g. epidermal factor receptor), that can be activated by proteolytically exposed cryptic sites within matrix molecules (40).

The experiments performed using a novel 3D model of injury repair (Figure 6) we demonstrate that the peptides can also endothelial responses to injury in complex environment (Figures 7–9). To our knowledge, this is the first report where this unique model (25) is used to compare the compounds with different angiogenic potential. Unlike standard Matrigel-based assays (27) here the cells remain viable for a prolonged time (up to 1 week). Thus, in addition to the identification of promising pro-angiogenic or anti-angiogenic therapeutics, such 3D constructs can be employed to study the stability of wound healing compounds and to evaluate drug delivery systems.

In conclusion, we have shown that degradation of endothelial extracellular matrices with bacterial collagenase releases pro-angiogenic peptide fragments. Based on our previous observations ((13,14); Demidova-Rice, Herman unpublished data) we suggest that the peptides described here can also stimulate epithelial responses to injury and wound healing *in vivo*. Therefore we propose (Figure 10) that bacterial collagenase has multiple direct and indirect positive effects on cellular responses to injury and wound healing: it degrades wound matrix easing dermal and epidermal cell motility, releases ECM and cell surface-bound growth factors. Moreover, it liberates biologically active ECM fragments, which in turn can stimulate angiogenesis and epithelialization during wound healing. Future work will determine whether the peptides can stimulate cellular responses to injury *in vivo* and establish the mechanisms of their activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Identification of bioactive matrix fragments released by bacterial collagenase from endothelial matrices

Endothelial matrices synthesized by post-confluent CEC were degraded by either human, or bacterial collagenase and subjected to immunoprecipitation with antibodies directed against collagen I (lanes 1, 2) or collagens type I–V (lanes 3, 4). Arrows indicate unique matrix fragments present in matrices digested by bacterial (lanes 2 and 4), but not human collagenases (lanes 1 and 3).

Figure 2. Bioactive matrix fragments stimulate endothelial proliferation

CEC grown in 48 well plates were treated with ECM-derived peptides at 10 or 100 nM at days 1 and 3 post-plating. Cell counting was performed using Coulter Counter at days 1, 3 and 5. Relative proliferation compared to control is shown. Error bars represent standard error of the mean; *- $p < 0.05$ compared to serum-stimulated control.

Figure 3. Matrix-derived peptides enhance early events during endothelial morphogenesis on Matrigel

(A–F) Photomicrographs of capillary-like structures formed by CEC cultured on Matrigel for 7h in the absence (A) or presence of 100 nM comb1 (D), ten4 (E) or col4-1 (F). Basic FGF and VEGF are used as positive controls (B and C respectively). Image background was corrected using pseudo flat field function (Image J) and high pass filters (Adobe Photoshop CS2).

Figure 4. Effects of bioactive peptides on endothelial morphogenesis in vitro at 24h post-plating (A–F) Photomicrographs of capillary-like structures formed by CEC cultured on Matrigel for 24h in the absence (A) or presence of 100 nM concentration comb1 (D), ten4 (E) or col4-1 (F). Basic FGF and VEGF are used as positive control (B and C respectively). Image background correction was performed as described for figure 3.

Figure 5. Quantitative evaluation of endothelial morphogenesis in a 2D model

The length of endothelial sprouts was measured as described in Materials and Methods at 7 and 24 hours post-plating. Three fields are evaluated in each of three independent experiments and plotted as relative tube length compared to control. (A) relative sprout length at 7h post-plating, (B) relative sprout length at 24h post-plating. Error bars represent standard errors; *- $p < 0.05$ compared to serum-stimulated control. Scale bar 200 μ m.

Figure 6. Schematic representation of the three-dimensional model of angiogenesis

The construct consists of two layers of Matrigel-collagen mixture and a layer of endothelial cells sandwiched in between. Full thickness injury is created by aspiration and defect is filled with matrices containing serum, pro-angiogenic growth factors or peptides.

Figure 7. ECM-derived peptides stimulate angiogenesis and early responses to injury in a novel 3D model of angiogenesis

Small full thickness wounds in 3D constructs are filled with the matrices containing 1% serum (A), bFGF (B), VEGF (C), and peptides comb1 (D), ten 4 (E) or col4-1 (F). Images are taken at 1 day post injury. Image background correction was performed as described for figure 3. Arrows indicate endothelial sprouts; asterisks show the areas of dense angiogenic networks formed by the CEC. Scale bar 200 μm .

Figure 8. Quantitative evaluation of sprout formation by endothelial cells in 3D model of wound healing at day 1 post-wounding

Cellular structures extended into small wounds by day 1 post-injury are counted manually using merged low power images. The mean number of structures found within wounds in three independent experiments is plotted. Error bars represent standard error of the mean; *-p<0.05 compared to serum-stimulated control.

Figure 9. Effects of the peptides on endothelial responses to injury in 3D environment: 3 days post wounding

(A–D) Photomicrographs of large wounds created in 3D matrices containing CEC and treated with 1% BCS (A), 10 ng/mL bFGF (B), 100 nM comb1 (C), and col4-1 (D). Representative images taken at 3 days post-wounding corresponding to each treatment group are shown. The image background correction was performed as described for figure 7. Scale bar 200 μ m. Arrows indicate angiogenic sprouts formed by the cells. (E) Quantitative evaluation of angiogenic sprouts formed by the CEC in response to peptide treatment. The sprouts were counted manually on the merged low power images of large wounds 3 days post-injury and plotted as the mean $\pm$ standard error. * - $p < 0.05$ compared to serum-stimulated control.

Figure 10. Proposed mechanism of wound healing activity of bacterial collagenase: the role of ECM fragments

Bacterial collagenase stimulates both endothelial and epithelial responses to injury via the following mechanisms. Degradation of the ECM in close proximity to the cells the enzyme allows for efficient cell migration. Release of growth factors and liberation of biologically active matrix fragments that can interact with and activate cellular receptors increases motogenic and mitogenic potential of the cells within the wound bed promoting the healing responses. Similarly to naturally occurring ECM fragments released by bacterial collagenase in vivo, synthetic peptides identified and tested in this study can enhance cellular responses to injury. Therefore we propose that the peptides could be used in combination or as an alternative to the bacterial product for promote wound healing in vivo.

Table 1

Structure and function of extracellular matrix-derived pro-angiogenic peptides

Peptide name	Sequence	Origin	Effects on endothelial cells in vitro	
			Endothelial proliferation	In vitro angiogenesis
Col1-Hyp	GSAGPXGATGFXGAAGR X=hydroxyproline	collagen I alpha 1 chain, aa 704-720	—	—
Col1-Leu	GSAGPLGATGFLGAAGR	collagen I alpha 1 chain, aa 704-720	—	—
Col4-1	MFRKPIPTVKA	collagen IV alpha 3 chain, NC1 domain, aa 1282-1353	+	++
Col4-2	IISRCQVCMKMRP	collagen IV alpha 3 chain, C-terminus, aa 1359-1371	—	+
Col4-C	MFRKPIPTVKAPPIISRCQ VCMKMRP	combination between Col4-1 and col4-2	—	—
Fibr1	DINECELSANL	fibrillin 1, modified EGF-like calcium binding domain 13	—	—
Fibr2	DIDCESSPCINGV	fibrillin 1, EGF-like domain 13, aa 807-846	+	+
Fibr3	DEDECEEKGHDCAE	fibrillin 1, EGF-like calcium-binding domain 39, aa 2247-2290,	—	+
Ten1	GVRSCPRGCSQKGRCED	tenascin X, EGF-like domain, aa 306-322	+	—
Ten2	GEETEVTVEGLEPG	tenascin X, fibronectin III-like domain, combination between aa2425-2430 and aa 3103-3110	—	++
Ten3	CVCWPGYTGRD	tenascin X, EGF-like domain rich fragment, aa360-370	—	—
Ten4	CGTRACPGDC DINECEIGAPAGEETEVTV	tenascin X, EGF-like domain rich fragment, aa490-500	—	+
Comb1	EGLEPG	combination between fibr1 and ten2	+	++

— no effect; + - peptide induces significant increase in cellular responses; ++ - peptide induce a two-fold increase in