Vascular Morphogenesis of Human Umbilical Vein Endothelial Cells on Cell-Derived Macromolecular Matrix Microenvironment

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Extracellular matrix (ECM) is a highly organized network of proteins and other macromolecules that plays a critical role in cell adhesion, migration, and differentiation. In this study, we hypothesize that ECM derived from *in-vitro*-cultured cells possesses unique surface texture, topography, and mechanical property, and consequently carries some distinct cues for vascular morphogenesis of human umbilical vein endothelial cells (ECs). Cell-derived matrix (CDM) was obtained by culturing fibroblasts, preosteoblasts, and chondrocytes, respectively, on coverslips and then by decellularizing them using detergents and enzymes. These matrices were named fibroblast-derived matrix (FDM), preosteoblast-derived matrix (PDM), and chondrocyte-derived matrix (CHDM). Immunofluorescence of each CDM shows that some of the matrix components are fibronectin (FN), type I collagen, and laminin. Atomic force microscopy analysis presented that average fiber diameter ranged from 2 to 7 μm and FDM holds much larger fibers. The matrix elasticity measurements revealed that average Young's modulus of CHDM ($17.7 \pm 4.2 \text{ kPa}$) was much greater than that of PDM ($10.5 \pm 1.1 \text{ kPa}$) or FDM ($5.7 \pm 0.5 \text{ kPa}$). During 5-day culture, EC morphologies were dramatically changed on PDM and FDM, but those on CHDM and gelatin were rather stable, regardless of time lapse. Cell migration assay discovered quicker repopulation of the scratched areas on PDM and FDM than on gelatin and CHDM. A capillary-like structure (CLS) assembly was also notable only in the PDM and FDM, as compared with CHDM, gelatin, or FN that were very poor in CLS formation. Quantitative analysis of mean CLS branch points and branch lengths demonstrated much better angiogenic activity of ECs on PDM and FDM. Interestingly, CLS formation was closely associated with matrix remodeling by ECs and the matrix clearance on PDM with time was sharply contrasted with that on CHDM that majority of the matrix FN was reserved. It was notable that membrane type 1-matrix metalloprotease was deeply involved in the process of matrix remodeling. This study indicates that specific matrix microenvironments are very critical for vascular morphogenesis of ECs, and thus, provide a nice platform for angiogenesis study as well as vascular tissue engineering.

Introduction

NEOVASCULARIZATION is a fundamental biological process during embryonic development and tissue formation, and is also involved in wound healing, as well as cancer invasion, growth, and metastasis.¹ Neovascularization encompasses both angiogenesis and vasculogenesis. Angiogenesis is the process of branching and capillary sprouting from pre-existing blood vessels by mature and differentiated endothelial cells (ECs). Vasculogenesis involves the participation of endothelial progenitor cells that differentiate *in situ* into mature ECs.² The process of angiogenesis is regulated tightly by growth factors, cytokines, extracellular matrix

(ECM) cues, and integrins.³ Many studies suggest that the impact of growth factors and cytokines on ECs is closely associated with ECM, as cytokine effect is mostly dependent on EC adhesion to ECM.^{4,5} In fact, ECM serves as a growth factor reservoir, in which ECM can sequester numerous bioactive molecules, concentrate them locally, and release them in response to proteolytic degradation of ECM.⁶ The main growth factors that modulate EC migration, survival, proliferation, and morphogenesis *in vivo* are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).^{7–9} Several studies have demonstrated that VEGF and bFGF secreted or sequestered by fibroblast ECMs contribute to triggering a number of events of angiogenesis.^{10–13}

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Meanwhile it is also known that the extracellular environment plays a critical role in the regulation of EC behaviors. ECs adhere to laminin (LN)-rich basement membranes (BMs) that act as a structural support for maintaining the organization and functions of blood vessels. However, a great deal of vascular research has focused on the effects of angiogenic growth factors and their biological mechanisms. Therefore, studies that deal with the impact of ECM on ECs and angiogenesis are relatively weak. Matrigel, collagen, and fibrin gel are the typical biomaterials in the study of angiogenesis models and vascular tissue engineering. Matrigel is a solubilized protein extracted from Engelbreth-Holm-Swarm mouse sarcoma that is rich in BM proteins.¹⁴ Matrigel stimulates ECs to easily form a capillary-like structure (CLS), and is often preferred over other biomaterials. Biodegradable, fibrin-based hydrogels are also utilized wherein human microvascular ECs can develop highly organotypic capillaries in engineered tissue substitutes.¹⁵ Another study found that three-dimensional (3D) collagen gels provide a better reproduction of angiogenic process than that on two-dimensional (2D) Matrigel coats.¹⁶ Some investigations have employed cell-derived or tissue-derived ECMs for this purpose.^{17,18} Although those works utilize various ECMs in vascular tissue engineering, they pay little attention to the effects of biophysical and biomechanical properties of ECM on angiogenesis. Additionally, proper 2D microenvironments as an angiogenic platform are relatively very few.

The purpose of this study is to investigate angiogenic potential of human umbilical vein endothelial cells (HUVECs) on multiple cell-derived matrices (CDMs) that originated from different sources: fibroblast, preosteoblast, or chondrocyte. Our previous works have documented that these CDMs are excellent for cell adhesion, proliferation, and human mesenchymal stem cell differentiation into chondrogenesis and osteogenesis. Therefore, our hypothesis is that CDM may provide ECs with an angiogenic microenvironment, because CDM shares some physical characteristics with natural BM in terms of matrix compositions, thickness, and matrix elasticity. In this study, biophysical and biomechanical properties of CDMs are thoroughly investigated using atomic force microscopy (AFM)-nanoindentation method. The cellular responses, including EC adhesion, migration, and proliferation, on the CDMs were examined and compared with gelatin-coated substrate. More importantly, angiogenic activities of each CDM are carefully evaluated based on the CLS formation, along with the test of Matrigel, gelatin-, or fibronectin (FN)-coated microenvironments. Correlation between CLS assembly and matrix remodeling is also investigated. This study will significantly improve our understanding of the angiogenic responses of ECs on BM-like matrix microenvironments.

Materials and Methods

Preparation of CDM

NIH3T3 mouse fibroblasts, human chondrocytes, and MC3T3-E1 mouse preosteoblasts (ATCC) were cultured in Dulbecco's modified Eagle's medium or minimal essential alpha medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). Each cell type was separately seeded at a density of $2 \times 10^4/\text{cm}^2$ onto poly-L-lysine-coated glass

coverslips in 12-well plates, and was then cultivated for 5–8 days until confluence. The medium was changed every 2 or 3 days. The confluent cells were then treated briefly with a detergent solution containing 0.25% Triton X-100 and 10 mM NH_4OH (Sigma-Aldrich). After the samples were washed with phosphate-buffered saline (PBS), both 50 U/mL DNase I and 2.5 µL/mL RNase A (Invitrogen) in PBS were added and incubated at 37°C for 2 h, and the decellularized samples were then gently washed twice with PBS. Preosteoblast-derived matrix (PDM), fibroblast-derived matrix (FDM), and chondrocyte-derived matrix (CHDM) were used immediately or were stored at -70°C in 0.1 M glycine solution prior to use.

Compositional analysis of PDM

Some major components of each CDM after decellularization were identified by immunofluorescence (IFS) of FN, LN, and type I collagen (Col I), respectively. The samples were fixed and gently washed with PBS, and were then washed several times with PBS. Samples were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h, and were then incubated with primary antibodies overnight at 4°C: mouse anti-FN (SC-8422; Santa Cruz Biotechnology), goat anti-Col I (SC-25974), and goat anti-LN (SC-16588) diluted in 1% BSA (1:50), followed by the addition of Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) or Alexa Fluor 594-conjugated donkey anti-goat IgG (Jackson Immuno-research) in 1% BSA (1:200) for 1 h at room temperature. The immunostained specimens were further subjected to DNA-specific 4,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) staining for nucleic detection. The fluorescence image of the target proteins was observed using a confocal laser scanning microscope (Olympus FluoView FV1000).

Biophysical and biomechanical characterization of CDMs

For the surface and bulk matrix characterization, PDM, FDM, and CHDM were fixed using 4% paraformaldehyde in PBS (pH=7.4) for 30 min, and were dehydrated in a series of concentrations of ethanol. The air-dried samples on coverslips were placed onto aluminum stubs, platinum coated for 1 min, and observed by scanning electron microscope (SEM; Phenom G2 Pro Desktop). In addition, for AFM analysis, the surface topography of CDMs was acquired in a dry state at room temperature using a Nanowizard II bio-AFM (JPK Instruments), coupled with an inverted optical microscope (Nikon). The scan image was obtained in a tapping mode using 165.3 kHz and 48 N/m Tap190Al-G probes (Budget Sensors). Each sample was scanned at a $100 \times 100 \mu\text{m}^2$ scale and the average roughness and matrix thickness were collected from six different areas of individual CDM ($n=5$, each CDM). The average fiber diameter was determined from 12 different points on the matrix ($n=3$, each). Further, matrix elasticity of CDMs was also measured using AFM (JPK Instruments). Fresh CDM samples were prepared without the fixation process and tested in a liquid mode. Matrigel was also examined as a positive control. The combined inverted optical stage helps precise lateral positioning of the AFM tip over the region of interest in CDM. A sphere tip (5-µm diameter) on silicon nitride (Si_3N_4)

cantilever with the spring constant of 0.01 N/m was used (PT.SiO₂.AU.SN.5; Novascan Technologies, Inc.). Young's modulus (YM) measurements utilize the nanoindentation method. Indentation was carried out after scanning the 100×100 μm² area of each CDM using the same probe. The ramp size in this study was 1 μm, and the loading speed was 1 μm/s. The indentation forces that ranged from 0.5 to 10 nN were applied during the tests in order to find out a proper range of the indentation depths (50–500 nm). Both tip-sample separation curves and YMs were subsequently obtained using Hertz's contact model using JPK data processing software and the poisson ratio of the CDM was considered to be 0.5 (JPK Instruments). Followed by the JPK manual instruction, the average YM of each CDM is calculated from the average loads when the indentation depth reaches to 10–20% (shown in yellow box) of the matrix thickness.

Observation of HUVEC adhesion and morphology

HUVECs (C2517A; Lonza) were cultured in endothelial basal medium-2 (CC-3156; Lonza), supplemented with EGM™-2 Single Quots™ kit (CC-4156) at 37°C under a 5% CO₂ humidified atmosphere. The passaged ECs (P4–P6) were used in the present study. To investigate cell adhesion and morphology, HUVECs (5×10⁴) were seeded on the CDMs and gelatin-coated coverslips in 12-well plates. Cells were cultivated for 5 h and 5 days, respectively, using M199 added with 25 ng/mL VEGF or without VEGF and other supplements, including 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, 5 U/mL heparin sodium salt from porcine intestinal mucosa (H3149-100KU; Sigma-Aldrich), and 10 ng/mL human bFGF (100-18B; Peprotech). Each sample was fixed at different time points, dehydrated, and processed prior to SEM observation.

Cell proliferation with varying concentrations of VEGF

To compare the EC proliferation rate on different VEGF concentrations, PDM, FDM, and CHDM were employed, along with gelatin-coated coverslips as a control. For gelatin coating, gelatin type B from bovine skin (G-9391; Sigma-Aldrich) was dissolved in distilled water (0.5%, w/v) and was then sterilized in an autoclave at 120°C for 15 min. After the poly-L-lysine coating, coverslips (18-mm diameter, sterilized) in 12-well plates were immersed in the gelatin solution (1 mL), and then incubated for more than 15 min at 37°C, and washed twice with PBS before cell seeding. ECs (5×10⁴ cells/well) were seeded on each substrate and were cultured for 5 days using M199 with different VEGF concentrations (0, 10, 25, and 50 ng/mL). The cell proliferation rate of each group (*n*=3) was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo) assay at day 5. The samples were added with 10% CCK-8 solution, and were further incubated at 37°C for 2 h. An aliquot of each sample (100 μL) was transferred to 96-well plates and the absorbance was measured at 450 nm using a Multiscan microplate reader (Thermo Scientific).

Cell migration assay

To investigate cell migration, ECs (2×10⁵) were seeded onto CDMs and gelatin-coated coverslips in 12-well plates,

and then cultured in EGM-2 complete medium for 2 days until confluence. The confluent layers of ECs were carefully scratched using a pipette tip (1-mL volume) to create an artificial wound. The samples were then washed twice with PBS to remove the cell debris and cultivated for 3 days. Optical images were taken at different time intervals (0, 1, and 3 days) to monitor cell migration and repopulation in the scratched area. For the migrating cell numbers, six areas per sample (*n*=3, each group) were randomly selected from the scratched region and were then counted manually at days 1 and 3, respectively.

Evaluation of CLS formation

For the observation of CLS assembly on the 2D substrates, ECs (5×10⁴ cells/well) were seeded onto the CDMs in 12-well plates, along with some control groups: FN-coated (50 μg/mL FNs; Invitrogen), gelatin-coated, and Matrigel-coated coverslips. For the preparation of Matrigel-coated coverslips, BD Matrigel matrix (354234; BD Biosciences) was used following the manufacturer's protocol. Briefly, thawing Matrigel was loaded on coverslips (50 μL/cm²) and incubated at 37°C for 30 min before use. The CDMs were rinsed three times with PBS prior to cell seeding. The cells were cultured in M199 containing 25 ng/mL VEGF and the same supplements as mentioned before. The medium was changed once during the 5-day culture. To identify CLS formation on different 2D environments, multiple IFS were carried out. Primary antibody of rabbit polyclonal anti-CD31 (ab28364; Abcam) and secondary antibody of goat polyclonal rabbit IgG-H&L Dylight 488(ab96899) were employed to label ECs. Alexa Fluor 647-conjugated phalloidin (A22287; Invitrogen) was employed for F-actin filament staining. DAPI (46190; Thermo Scientific) staining was also applied for nucleic labeling. Beside the CLS formation, degradation of cell-adhesive matrix substratum by ECs was examined using mouse monoclonal anti-FN (SC-8422; Santa Cruz Biotechnology) and Alexa Fluor 488 F(ab')₂ fragment of goat anti-mouse IgG (ab96899), along with F-actin and DAPI staining. Further, to identify the lumen formation and to determine capillary tube thickness, consecutive Z-stack images (40 iterations) were acquired using confocal laser scanning microscopy (Olympus) and a 3D reconstruction of CLS was performed using PDM, FDM, CHDM, gelatin, and Matrigel, respectively.

Quantitative analysis of CLS branch point, branch length, and thickness

The ECs growing on the different substrates were subjected to the LIVE/DEAD® Viability/Cytotoxicity Kit (03224; Invitrogen) assay. The experimental groups were three CDMs, gelatin-, and Matrigel-coated coverslips. After a 5-day culture of ECs, 2 μM calcein AM and 4 μM EthD-1 solution were prepared in PBS, and ECs were incubated with the dye reagents for 30 min. Live cells were visualized as green under a fluorescent microscope (CKX-41; Olympus). To extract some quantitative data during the CLS formation, five different areas (×100 magnification) of the samples (*n*=3, each group) were obtained. The capillary branch points were counted manually and the Image J program (NIH) was utilized to quantify the mean CLS length, as previously described.¹⁹

Semiquantitative reverse transcription–polymerase chain reaction

For more analysis of matrix degradation mechanism, total mRNA was extracted from the ECs using Trizol[®] RNA isolation reagents (Invitrogen), strictly following the manufacturer's instruction. Genomic DNA digestion was not carried out at this time. Concentrations of the isolated RNA were determined at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The template RNA (1 µg) was added to Maxime RT PreMix Kit (25081; Intron) tubes to a total volume of 20 µL. They were then subjected to a cDNA synthesis reaction at 45°C for 60 min and RTase inactivation at 95°C for 5 min. The resulting cDNA product (1 µL) and 10 pmol of each reverse and forward primer were mixed in DW with Maxime PCR PreMix Kit (25165; Intron) to a total volume of 20 µL for polymerase chain reaction (PCR). Target genes and their primers were as follows: primers of membrane type 1-matrix metalloprotease (*MT1-MMP*): GTGCCTC TCGAATGTTAGCC (forward) and TCGTTTGTGTGCC TTCTCTG (reverse); matrix metalloproteinase-1 (*MMP-1*): TGTGGTGTCTCACAGCTTCC (forward) and TCCAGG TCCATCAAAAGGAG (reverse); intercellular adhesion molecule 1 (*ICAM-1*): CCAGAGGTTGAACCCACAG (forward) and ACAATCCCTCTCGTCCAGTC (reverse); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): GGCTCTCCAGAACATCATCC (forward) and TTTCTA GACGGCAGGTCAGG (reverse). The PCR products were separated electrophoretically using 1.5% agarose gel and were visualized under ultraviolet. In addition, nonreverse transcriptase control was also examined. Quantitative analysis of each band was carried out using a Gel dock system (ATTO E-graph, AE-9000; Takara) equipped with a CS Analyzer 2.0 program. The band intensity of target genes was normalized to that of GAPDH.

Statistical analysis

Statistical analysis of the data was performed using the unpaired Student's *t*-test. All of the data represent the mean values and standard errors. Statistical significance was determined as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001, respectively.

Results

From the optical microscopic observation, preosteoblasts, fibroblasts, and chondrocytes were confluent before decellularization, and their original cell morphologies were completely lost after detergent and enzyme treatments (Fig. 1A). For each CDM, some of the matrix proteins were FN, LN, and Col I, as identified by using IFS (Fig. 1B). Each matrix exhibited compositional homology but the intensity and distribution were greatly distinguished from each other. FDM displays the strongest fluorescent signals and uniform distribution of each protein. Further characterization of CDM revealed that surface morphology of each CDM showed a multilayered fibrous structure, with various sizes of fibers in the matrix (Fig. 2A). In addition, AFM images also exhibit the matrix fibers self-assembled in the CDM (Fig. 2B). The average fiber diameter ranged from 2 to 7 µm and FDM had much larger fibers compared with PDM and CHDM (Fig. 2C). The AFM analysis further presented that surface roughness of PDM, FDM, and CHDM was 211 ± 22 , 241 ± 24 , and 138 ± 5 nm, respectively (Fig. 2D) and average matrix thickness was 1200 ± 134 , 1700 ± 250 , and 860 ± 200 nm, respectively (Fig. 2E). Further, matrix elasticity measurements using AFM-nanoindentation showed that average YM of CHDM (17.7 ± 4.2 kPa) was much greater than that of PDM (10.5 ± 1.1 kPa) or FDM (5.7 ± 0.5 kPa) (Fig. 3). The YM of Matrigel (positive control) was 1.7 ± 0.4 kPa, which was much lower than those of CDMs. Average YM was determined from the each YM

FIG. 1. Optical images of *in-vitro*-cultured cells before and after decellularization and immunofluorescence (IFS) of matrix-derived macromolecules. Cell morphologies of all samples were photographed before (left) and after (right) decellularization (A). Scale bar = 200 µm. As the major compositions of each cell-derived matrix (CDM), fibronectin (FN), type I collagen (Col I), and laminin (LN) were identified by IFS after decellularization (B). Scale bar = 50 µm. CHDM, chondrocyte-derived matrix; FDM, fibroblast-derived matrix; PDM, preosteoblast-derived matrix. Color images available online at www.liebertpub.com/tea

FIG. 2. Biophysical characterization of CDMs. A mesh-like surface texture of preosteoblast-derived matrix (PDM), fibroblast-derived matrix (FDM), and chondrocyte-derived matrix (CHDM), as viewed by scanning electron microscope (SEM) ($\times 10K$) (A). The matrix deposition was the most abundant in the FDM. Scale bar = 10 μm . Atomic force microscope (AFM) also illustrates fibrous structure of each CDM in the scan area of $100 \times 100 \mu m^2$ (B). Quantitative analysis of AFM data found the average fiber diameter (C), surface roughness (D), and matrix thickness (E) of CDMs ($n=3$, each). Statistically significant difference between CHDM and the other groups was indicated as $**p < 0.01$, and $***p < 0.001$, respectively. Color images available online at www.liebertpub.com/tea

when the indentation depth reaches to 10–20% (as shown in yellow box) of the total matrix thickness. There were statistically significant differences between CHDM and the other two CDMs or Matrigel.

When EC adhesion and morphology were observed using SEM, cell morphology with or without VEGF was quite similar for each group at 5 h postseeding but the difference was obvious at day 5 (Fig. 4A). Some morphological changes were dramatic, especially with the VEGF treatment at day 5; ECs on PDM and FDM were connected to each other, forming a tube-like assembly, but

those on gelatin and CHDM were just confluent. It was interesting that the background matrix, as seen in nanofibers, was completely disappeared in PDM and FDM at day 5, whereas CHDM still reserved the background matrix. On the contrary, the test groups without VEGF treatment maintained the background matrix of CDMs at day 5 and showed no sign of tube-like assembly on PDM and FDM. Further, the EC proliferation rates on different VEGF concentrations showed that the number of ECs increased continuously with increasing VEGF dose, regardless of the type of substrate (Fig. 4B). Interestingly the growth rate of

FIG. 3. Determination of matrix elasticity of CDMs using AFM-nanoindentation method. Mechanical property of PDM, FDM, CHDM, and Matrigel was measured in a wet state using AFM (JPK Instruments). The combined inverted optical stage helps precise lateral positioning of the AFM tip over the region of interests in CDM. A sphere tip (5- μm diameter) on silicon nitride (Si_3N_4) cantilever with the spring constant of 0.01 N/m was used (PT-SiO₂-AU.SN.5; Novascan Technologies, Inc.). The indentation forces ranged from 0.5 to 10 nN during the tests in order to find out a proper range of the indentation depths (50–500 nm). Both tip-sample separation curves and Young's modulus (YM) were subsequently obtained using Hertz's contact model using JPK data processing software. Followed by the JPK manual instruction, the average YM of each CDM is calculated from the average loads when the indentation depth reaches to 10–20% (shown in yellow box) of the matrix thickness. When individual matrix elasticity was determined, there were statistically significant differences (***) ($p < 0.001$) between CHDM and the other CDMs or Matrigel. Color images available online at www.liebertpub.com/tea

ECs was much more competitive on CHDM than on PDM or FDM, even better than gelatin-coated coverslips, especially with 25 ng/mL VEGF treatment. It was notable that the proliferation rate of ECs was relatively slow on FDM and PDM, even though the VEGF concentration was doubled from 25 to 50 ng/mL. Meanwhile, the migration activity of ECs was also examined by wound healing assay. The results discovered that the migration of ECs was significantly better with PDM and FDM than with gelatin and CHDM (Fig. 5A). After 3 days, both migrating and proliferating ECs repopulated the area of artificial scratches, and especially, PDM exhibited the best performance among the test groups. The cell numbers that occupied wound scratch areas further demonstrated that both PDM and FDM could greatly improve the capability of EC migration (Fig. 5B). There were statistically significant differences between PDM and gelatin or CHDM.

For more evidences of angiogenic activity of ECs, vascular morphogenesis of ECs on the CDMs was directly compared with some control groups: FN, gelatin, and Matrigel. Matrigel exhibited a quick CLS formation in <20 h, whereas CLS formation on PDM was much slower (5 days), but more stable than that on Matrigel under the same con-

ditions (Fig. 6A). In addition, IFS of CD31 (green), F-actin (red), and DAPI staining (blue) located the ECs, and, more importantly, the merged photographs illustrated a very nice capillary-like network created on the PDM (Fig. 6B). The IFS images also clearly revealed the pattern of EC assembly on different 2D environments (Fig. 6C). The ECs on FN, gelatin, and CHDM maintained their proliferation activity, but failed to develop the similar CLS, even after 5 days of culture. It was very interesting to note that among the CDMs, while both PDM and FDM were very effective in the formation of capillary assembly comparable to Matrigel, CHDM only exhibited a full spreading of confluent ECs with many stress fibers, and the same phenomenon was witnessed on gelatin and FN. To quantitatively evaluate vascular morphogenesis of ECs on different 2D microenvironments, the angiogenic activity of ECs was determined using two parameters: CLS branch point and branch length. Live/dead staining of ECs at day 5 presented different patterns of EC assembly, where PDM, FDM, and Matrigel were very good in CLS formation but CHDM and gelatin found the confluent ECs (Fig. 7A). The capillary branch points were significantly higher for PDM and FDM than for any other groups (Fig. 7B). Few branch points were detected on

FIG. 4. Observation of human umbilical vein endothelial cell (HUVEC) morphology and cell proliferation on different vascular endothelial growth factor (VEGF) concentrations. The SEM images ($\times 500$) exhibit endothelial cell (EC) morphologies with or without VEGF on the CDMs and gelatin at 5 h and 5 days, respectively (A). A capillary-like assembly was observed only in PDM and FDM at 5 days with VEGF treatment. Scale bar = 260 μ m. In addition, the CCK-8 assay demonstrated that the cell proliferation rate increased with increasing VEGF dose, and that the rate was much improved in the case of CHDM (B). It was notable that the proliferation rate of ECs was relatively slow on FDM and PDM even though the VEGF concentration doubled from 25 to 50 ng/mL. Statistically significant differences between CHDM and the other groups were marked as *** $p < 0.001$ at 25 ng/mL VEGF dose. Color images available online at www.liebertpub.com/tea

FIG. 5. Cell migration assay on CDMs and gelatin-coated coverslips (control). (A) An artificial wound scratch was created in the middle of confluent EC layers (top), and EC migration was monitored at day 1 (middle) and at day 3 (bottom). Scale bar = 200 μ m. The cell numbers repopulating the scratched area were counted and compared (B). Statistically significant differences between PDM or FDM and the other groups are indicated as * p < 0.05, ** p < 0.01, and *** p < 0.001, respectively. Color images available online at www.liebertpub.com/tea

CHDM or on gelatin. Lower numbers of branch points on Matrigel were mainly due to the early collapse of CLS on Matrigel during the 5-day culture. In addition, mean capillary branch length ranged from 29 to 40 μ m for PDM and FDM, twice the length of CHDM and gelatin (15–17 μ m) (Fig. 7C). For further analysis of the lumen formation and capillary thickness, the merged images of CD31, F-actin, and DAPI were reconstructed into 3D configurations, adding the Z-stacks to 2D configuration. The cross-sectional images illustrated that 3D tubular structure was developed on PDM, FDM, and Matrigel, respectively, but they failed to exhibit that such a tube formation was undetectable on CHDM as well as gelatin (Fig. 8). The mean tube thickness of PDM and FDM ranged from 13 to 16 μ m and these values were very similar to that of Matrigel.

Further SEM images at higher magnifications ($\times 2000$ and $\times 5000$) exhibited the EC–matrix interactions (Fig. 9A). Highly extended lamellipodia (green arrows) of ECs were observed only in PDM and FDM at 5 days, which was an indicative of cell motility, as well as a tight interaction between ECs and matrix. The presence of lamellipodia suggests that cell migration was involved in the process of CLS formation. Interestingly, there was no such phenomenon observed with CHDM, which merely showed cell spreading on the matrix. It was notable that matrix degradation was apparent on the PDM and FDM as the blank matrix area (red star) developed. Further, matrix remodeling was also confirmed by IFS of the major matrix component FN that the initial pattern significantly changes with time (Fig. 9B). While the early PDM at 12 h reserves FN over the matrix, disappearance of FN signal becomes obvious at day 5 in a localized and large area (marked in white star) and this phenomenon is in accordance with active tube formation on PDM. In a comparison, CHDM that was poor in CLS formation also experiences some matrix degradation but the remodeling is occurring in a smaller area, which is greatly different when the extent of FN clearance was quantitatively

compared with PDM (Fig. 9C). As for further analysis of the matrix remodeling by ECs, the expression of molecular makers, such as *MT1-MMP* and *MMP-1*, was investigated, along with the angiogenic marker *ICAM-1*. The gene expression of *MT1-MMP* was significantly upregulated with PDM and FDM compared with that of gelatin or CHDM (Fig. 9D). *ICAM-1* was only detected for PDM and FDM. When the expression level was normalized to that of *GAPDH*, *MT1-MMP* expression of PDM and FDM was about 10-fold higher than that of gelatin, and was considerably greater than that of CHDM (Fig. 9E). The differences between PDM and gelatin or CHDM were statistically significant.

Discussion

Recovery of functional vasculature for tissue regeneration is still extremely challenging, as various parameters, such as signaling growth factors, physical cues, cell–cell, and cell–matrix interactions, are closely associated, and their interactions are therefore not yet fully understood. Primarily focused on the impact of the ECM environment on angiogenesis, this study utilized naturally derived CDMs obtained from different cell types, and investigated the angiogenic responses of ECs, including cell adhesion, proliferation, migration, vascular morphogenesis, and matrix remodeling, to diverse 2D microenvironments.

One of the most interesting findings in this work is that while angiogenic responses of ECs are very active on PDM and FDM, the same phenomenon was not observed with CHDM. Current results show that both PDM and FDM offer very strong angiogenic activities, such as capillary-like assembly and matrix remodeling, but CHDM retains a microenvironment that is supportive only for EC adhesion and proliferation. In fact, the difference is that PDM and FDM are derived from vascular-rich tissue cells and CHDM comes from avascular tissue cells. There is no comparative

FIG. 6. Capillary-like structure (CLS) formation on different surface environments. Optical images (×100) show that CLS is assembled on Matrigel within 20 h and PDM in 5 days (A). The arrows show elongated ECs, which are subsequently assembled into CLS. Scar bar=200 μm. The CLS developed on PDM was further analyzed using immunofluorescent staining of EC marker (CD31, green) and F-actin phalloidin (red), along with 4,6-diamidino-2-phenylindole (DAPI) staining (blue) (B). Scar bar=100 μm. (C) Representative images of DAPI, CD31, and F-actin staining demonstrate that ECs assemble into CLS after 5 days of culture on PDM, FDM, and Matrigel, and that they failed to do so on gelatin- and FN-coated coverslips or on CHDM (C). Scar bar=100 μm. Color images available online at www.liebertpub.com/tea

study on angiogenesis using a vascular tissue CDM and avascular tissue CDM. Soucy and Romer discovered that capillary tube networks were formed in 24 h after seeding HUVECs at a density of 2.1×10^5 cells/cm² on FDM.¹⁰ The present study employs a much smaller cell density (1.25×10^4 cells/cm²) and different culture medium conditions during 5-day culture. Another report prepared an ECM by coculturing different stages of breast cancer cells and primary fibroblasts, and demonstrated that tumor ECM can sequester cell-produced tumor necrosis factor- α , which in turn activates ECs and thus significantly increase CLS lumen diameters.²⁰ Even though there is a correlation between cell origin and angiogenic potential, it seems to be premature to determine whether the origin of tissue cells is a key regulator of angiogenic activity of ECs. The previous study of western blot and IFS showed that FN, LN, and Col I are the major components of CDMs, along with other proteins, such as Col II, Col IV, and vi-

tronectin.²¹ Quantitatively compared, PDM and FDM were much better in the production of those macromolecules than CHDM. Therefore compositional variations among the CDMs may be partly responsible for distinct angiogenic responses of ECs. The implications of multiple macromolecular components of CDM on angiogenesis should be addressed, because BM that is a complex of diverse macromolecules, including LN, Col IV, and heparan sulfate proteoglycan, is the best microenvironment for ECs.²² Single macromolecules tested in this study (FN and gelatin) failed to exhibit any signs of CLS formation and to proceed time-dependent matrix remodeling. Another issue is the chance of residual growth factors that might be embedded in the matrices. If there are some growth factors even in small amounts, then they can affect EC behaviors. It is more likely, however, that during decellularization, most of them were washed away or lost their activities. In our experiences, VEGF was barely detectable from the current

FIG. 7. Quantitative analysis of mean capillary branch point and branch length. Once ECs were cultured on different substrates for 5 days, Calcein AM was used to visualize EC assembly ($\times 100$) (A). Scar bar=200 μm . To extract some quantitative data, five different areas of the samples ($n=3$, each group) were randomly selected and capillary branch points were counted manually (B). Image J (NIH) was utilized to quantify the mean CLS tube length (C). Statistically significant differences between CHDM and PDM or FDM are marked as *** $p<0.001$. Color images available online at www.liebertpub.com/tea

FIG. 8. Determination of capillary tube thickness using three-dimensional (3D) reconstructed confocal images. To identify the lumen thickness, a consecutive Z-stack image was acquired using confocal microscopy, and a 3D reconstruction of CLS was realized for PDM, FDM, CHDM, Matrigel, and gelatin, respectively. In addition, mean capillary tube thickness was also quantified using 3D reconstructed confocal images and the results showed no statistical difference among PDM, FDM, and Matrigel. The cells were costained using F-actin (red), CD31 (green), and DAPI-stained nuclei (blue). Color images available online at www.liebertpub.com/tea

FIG. 9. Matrix remodeling and proteolytic enzyme expression during CLS formation. Cell morphologies on each CDM are seen from the SEM images under high magnification (top: 2K, bottom: 5K) after 5 days of culture (A). Highly extended lamellipodia (green arrows) were detected only in PDM and FDM, which indicated a sign of cell motility as well as a tight interaction between ECs and matrix (M: matrix; EC: HUVEC; red star: empty space). Confocal images of PDM and CHDM display the fluorescent FN signals at 12 h and 5 days, respectively, as ECs remodel the matrix with time in a different way (B). Significant loss of FN (marked in red star) was apparent in PDM at day 5. Scar bar=100 μ m. The degree of FN degradation at different time points was quantitatively compared between PDM and CHDM (C). Meanwhile, for the mechanism of matrix remodeling, gene expression levels of the related markers, membrane type 1-matrix metalloproteinase (*MT1-MMP*) and matrix metalloproteinase (*MMP-1*), were analyzed (D) and quantitatively compared (E). Especially *MT1-MMP* was significantly upregulated for both PDM and FDM compared with that of gelatin or CHDM. Statistically significant differences between PDM or FDM and the other groups were marked as $**p < 0.01$ and $***p < 0.001$, respectively. Color images available online at www.liebertpub.com/tea

matrices. Prewitz *et al.* obtained an extensive set of data with CDMs and demonstrated that no VEGF was present in the murine embryonic fibroblasts and dermal FDM.²³

For further speculations as to why CHDM downregulates the angiogenic activity of ECs, the issue can be the mechanical properties of surface microenvironments in which the ECs feel and we found out the meaningful differences of matrix elasticity among the CDMs (Fig. 3). In fact ECs are very sensitive to the mechanical property of underlying substrates as well as to surrounding 3D environments. ECs growing on compliant substrates prefer cell-cell connections for network formation, whereas on stiffer substrates, cells prefer to remain well-dispersed, without the formation of significant cell-cell connections.²⁴ We postulated that PDM and FDM might have the stiffness similar to that of

BM, and thus, support the vascular morphogenesis of ECs. Interestingly enough, present measurement of average matrix elasticity of PDM (10.5 kPa) matched well with that of BM ECM that surrounds striated muscle fibers (~ 10 kPa).²⁵ That of CHDM (~ 17.7 kPa) was also comparable with the elasticity of pericellular matrix of chondrocyte that was about 25 kPa.²⁵ Current result of matrix elasticity presents very important information regarding the upregulation or downregulation of the angiogenic activities on specific matrix microenvironments. Another biophysical factor, matrix thickness, was measured in a wet state and was found quite different from each CDM, where CHDM was much thinner than the other two CDMs. One study measured the thickness of BM from different sources of vessels, and revealed that the mean BM thickness of aorta was 506 ± 14 nm

and those of the rhesus macaque carotid and of the inferior vena cava were 319 ± 14 and 286 ± 8.2 nm, respectively.²⁶ The present data exhibit much thicker than those of natural BM. It is notable that the AFM test conditions affect the values of matrix thickness; matrix thickness in a wet state increased ~20%, 30%, and 40% for PDM, FDM, and CHDM, respectively, when compared with the matrix thickness determined in a dry state (data not shown).

Another interesting finding is that matrix remodeling *in vitro* was witnessed on certain matrix environments, and this was closely linked to the development of CLS formation. The apparent matrix degradation during capillary formation led us to investigate the expression level of matrix proteolytic enzymes. In fact, ECM degradation is a regulatory signal for initiating vascular morphogenesis and controlling vessel stabilization. *MT1-MMP*, also known as *MMP-14*, is a crucial regulator of ECM turnover (cleavage of large extracellular FN fibrils) in the process of angiogenesis²⁷ and this enzyme activity was remarkably upregulated on PDM and FDM compared with that on gelatin or CHDM (Fig. 9). Several studies have documented that *MT1-MMP* and *MMP-1* are actively involved in neovascularization, wherein they cause ECM degradation by migrating ECs both *in vivo* and *in vitro*.^{10,28,29} Researchers report that EC network formation is *MT1-MMP* dependent, which creates enough space for EC migration by degrading the surrounding matrix.³⁰ It is notable that the lack of CLS formation on gelatin and CHDM correlates well with the little expression of *MT1-MMP* on gelatin and weak expression on CHDM. In addition, we found that *ICAM-1* was activated during EC tube formation only for PDM and FDM. *ICAM-1* is a member of the immunoglobulin protein superfamily and its expression is induced by several angiogenic factors and likely to be a functional requirement for neovascularization. Kevil *et al.* showed that *ICAM-1* promotes the development of cell polarity and modulates EC migration via a signaling pathway of endothelial synthase activation and organization of the actin cytoskeleton.³¹ Therefore, *ICAM-1* expression, which was exclusive to PDM and FDM, is in very good agreement with the other results, such as those of the cell migration assay (Fig. 5) and the development of lamellipodia (Fig. 9A).

Taken together, current 2D matrix models need to be validated further using *in vivo* setting and 3D environment. A comparative study of 2D and 3D models would provide much valuable information regarding vasculogenesis of ECs. However, there are some issues in creating a 3D matrix, such as large-scale production of ECM and fabrication into a 3D construct. Reformulation of matrix into 3D platform may cause a significant turnover of intrinsic biophysical and biomechanical matrix properties. Nonetheless, the strength of our model is that current matrix reserves a strong proangiogenic activity, which is significantly different from single-protein-based model. And cell specificity of matrix is another benefit; while Matrigel shows a CLS formation with other cell types, that is, fibroblast (NIH3T3) and preosteoblast (MC3T3), FDM does not exhibit such a CLS with those cells at the same condition (data not shown).

Conclusions

Preparation of the CDMs from different cell sources is successful and they have different surface texture, topog-

raphy, matrix thickness, and matrix elasticity, as determined by using SEM and AFM. The EC culture on the CDMs shows that both PDM and FDM are very favorable 2D environments for EC vascular morphogenesis, and CHDM presents a microenvironment for EC adhesion and proliferation. Interestingly, while capillary network assembly occurred quickly on the Matrigel and was regressed in a short period of time, it was slow but rather stable on PDM and FDM. It was also clear that matrix remodeling was primarily noticed with PDM and FDM, and was closely associated with the proteolytic enzyme activity of ECs, as confirmed by the significant upregulation of *MT1-MMP* expression. This work demonstrates that *in vitro* vascular morphogenesis of ECs (CLS formation and matrix remodeling) is apparent on the specific 2D matrix microenvironments. To the best of our knowledge, the current study is the first report that demonstrates the significant difference of angiogenic potential of ECs between vascular-rich tissue cell and avascular tissue CDMs. Taken together, the present matrix microenvironments provide a nice platform for in-depth study of angiogenesis and EC-matrix interactions, and can find many applications in vascular tissue engineering.

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Disclosure Statement

No competing financial interests exist.

References

1. Wary, K.K., Kohler, E.E., and Chatterjee, I. Focal adhesion kinase regulation of neovascularization. *Microvasc Res* **83**, 64, 2012.
2. Isner, J.M., and Asahara, T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J Clin Invest* **103**, 1231, 1999.
3. Fam, N.P., Verma, S., Kutryk, M., and Stewart, D.J. Clinician guide to angiogenesis. *Circulation* **108**, 2613, 2003.
4. Davis, G.E., and Senger, D.R. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res* **97**, 1093, 2005.
5. Senger, D.R., and Davis, G.E. Angiogenesis. *Cold Spring Harb Perspect Biol* **3**, a005090, 2011.
6. Schultz, G.S., and Wysocki, A. Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair Regen* **17**, 153, 2009.
7. Ferrara, N., Gerber, H.P., and LeCouter, J. The biology of VEGF and its receptors. *Nat Med* **9**, 669, 2003.
8. Asahara, T., Bauters, T., Zheng, L.P., Takeshita, T., Bunting, S., Ferrara, N., *et al.* Synergistic effect of vascular endothelial growth-factor and basic fibroblast growth-factor on angiogenesis in-vivo. *Circulation* **92**, 365, 1995.
9. Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671, 1997.
10. Soucy, P.A., and Romer, L.H. Endothelial cell adhesion, signaling, and morphogenesis in fibroblast-derived matrix. *Matrix Biol* **28**, 273, 2009.

11. Newman, A.C., Nakatsu, M.N., Chou, W., Gershon, P.D., and Hughes, C.C. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell* **22**, 3791, 2011.
12. Berthod, F., Germain, L., Tremblay, N., and Auger, F.A. Extracellular matrix deposition by fibroblasts is necessary to promote capillary-like tube formation in vitro. *J Cell Physiol* **207**, 491, 2006.
13. Velazquez, O.C., Snyder, R., Liu, Z.J., Fairman, R.M., and Herlyn, M. Fibroblast-dependent differentiation of human microvascular endothelial cells into capillary-like 3-dimensional networks. *FASEB J* **16**, 1316, 2002.
14. Kleinman, H.K., and Martin, G.R. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol* **15**, 378, 2005.
15. Montano, I., Schiesti, C., Schneider, J., Pontiggia, L., Luginbühl, J., Biedermann, T., *et al.* Formation of human capillaries in vitro: the engineering of prevascularized matrices. *Tissue Eng Part A* **16**, 269, 2010.
16. Montanez, E., Casaroli-Marano, R.P., Vilaro, S., and Pagan, R. Comparative study of tube assembly in three-dimensional collagen matrix and on Matrigel coats. *Angiogenesis* **5**, 167, 2002.
17. L'Heureux, N., Dusserre, N., König, G., Victor, B., Keire, P., Wight, T.N., *et al.* Human tissue-engineered blood vessels for adult arterial revascularization. *Nat Med* **12**, 361, 2006.
18. Zhang, X., Yang, J., Li, Y., Liu, S., Long, K., Zhao, Q., *et al.* Functional neovascularization in tissue engineering with porcine acellular dermal matrix and human umbilical vein endothelial cells. *Tissue Eng Part C Methods* **17**, 423, 2011.
19. Guidolin, D., Vacca, A., Nussdorfer, G.G., and Ribatti, D. A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay in vitro. *Microvasc Res* **67**, 117, 2004.
20. Hielscher, A.C., Qiu, C., and Gerecht, S. Breast cancer cell-derived matrix supports vascular morphogenesis. *Am J Physiol Cell Physiol* **302**, C1243, 2012.
21. Bae, S.E., Bhang, S.H., Kim, B.S., and Park, K.D. Self-assembled extracellular macromolecular matrices and their different osteogenic potential with preosteoblasts and rat bone marrow mesenchymal stromal cells. *Biomacromolecules* **13**, 2811, 2012.
22. Kubota, Y., Kleinman, H.K., Martin, G.R., and Lawley, T.J. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* **107**, 1589, 1988.
23. Prewitz, M.C., Seib, F.P., Bonin, M.V., Friedrichs, J., Stibel, A., Niehage, C., *et al.* Tightly anchored tissue-mimetic matrices as instructive stem cell microenvironments. *Nat Methods* **10**, 788, 2013.
24. Califano, J.P., and Reinhart-King, C.A. A balance of substrate mechanics and matrix chemistry regulates endothelial cell network assembly. *Cell Mol Bioeng* **1**, 122, 2008.
25. Buxboim, A., Ivanovska, I.L., and Discher, D.E. Matrix elasticity, cytoskeletal forces and physics of the nucleus: how deeply do cells 'feel' outside and in? *J Cell Sci* **123**, 297, 2010.
26. Liliensiek, S.J., Nealey, P., and Murphy, C.J. Characterization of endothelial basement membrane nanotopography in rhesus macaque as a guide for vessel tissue engineering. *Tissue Eng Part A* **15**, 2643, 2009.
27. Shi, F., and Sottile, J. MT1-MMP regulates the turnover and endocytosis of extracellular matrix fibronectin. *J Cell Sci* **124**, 4039, 2011.
28. Robinet, A., Fahem, A., Cauchard, J.H., Huet, E., Vincent, L., Lorimier, S., *et al.* Elastin-derived peptides enhance angiogenesis by promoting endothelial cell migration and tubulogenesis through upregulation of MT1-MMP. *J Cell Sci* **118**, 343, 2005.
29. Hanjaya-Putra, D., Yee, J., Ceci, D., Truitt, R., Yee, D., and Gerecht, S. Vascular endothelial growth factor and substrate mechanics regulate in vitro tubulogenesis of endothelial progenitor cells. *J Cell Mol Med* **14**, 2436, 2010.
30. Stratman, A.N., Saunders, W.B., Sacharidou, A., Koh, W., Fisher, K.E., Zawieja, D.C., *et al.* Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices. *Blood* **114**, 237, 2009.
31. Kevil, C.G., Orr, A.W., Langston, W., Mickett, K., Murphy-Uilrich, J., Patel, R.P., *et al.* Intercellular adhesion molecule-1 (ICAM-1) regulates endothelial cell motility through a nitric oxide-dependent pathway. *J Biol Chem* **279**, 19230, 2004.

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