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Type I collagen, fibrin and Puramatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks

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Abstract

The field of tissue engineering seeks to create metabolically demanding, functional tissues, which will require blood vessel networks capable of forming rapidly in a variety of extracellular matrix (ECM) environments. We tested whether human endothelial progenitor cells (EPCs) and mesenchymal progenitor cells (MPCs) could form microvascular networks in type I collagen, fibrin, and an engineered peptide hydrogel, PuraMatrix, in 7 days in vivo in immune-deficient mice. These results are compared to those previously published, based on the Matrigel ECM.

Perfused blood vessels formed in all three types of ECM within 7 days. Collagen at 5 and 6 mg/mL and 10 mg/mL fibrin supported vessel formation at 30–60 vessels/mm^2, and PuraMatrix enabled vessel formation to 160 vessels/mm^2, significantly greater than collagen or fibrin. Vessels were composed of EPCs with perivascular cells on their abluminal surfaces. EPCs injected alone formed a low density of blood vessels in collagen and PuraMatrix, while MPCs injected alone resulted in sparse vessel networks in all ECMs tested.

A rheometer was used to determine whether the ECMs which supported vascularization had bulk physical properties similar or distinct from Matrigel. Collagen and fibrin were the stiffest matrices to support extensive vascularization, with storage moduli from 385–510 Pa, while Matrigel, at 80 Pa, and PuraMatrix, at 5 Pa, were far more compliant. Thus, EPCs and MPCs were capable of vasculogenesis in environments having disparate physical properties, although vascular density was greater in more compliant ECMs. We propose that EPC/MPC-mediated vascularization is a versatile technology which may enable development of engineered organs.

Keywords

Vascularization; endothelial progenitor cells; tissue engineering; regenerative medicine; collagen; fibrin; PuraMatrix; Matrigel

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7. Author Disclosure Statement

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1. Introduction

A widely-cited challenge in the field of tissue engineering is to provide a blood vessel network to facilitate oxygen, nutrient, biochemical and waste exchange for thick tissues beyond the range of diffusion. In the absence of a blood vessel network, necrosis will quickly occur beyond the surface of any implanted, metabolic tissue (Jain *et al.*, 2005). Indeed, efforts at clinical implementation of tissue engineering therapies, such as skin substitutes, have been restricted by a lack of blood vessel development (Egana *et al.*, 2009). Microfabrication techniques have been explored as a means of forming vascular networks (Cabodi *et al.*, 2005, Golden and Tien 2007) yet these methods have thus far yielded networks of only limited complexity. Any practical artificial organ will need to be complex over multiple length scales and be able to grow and reform in response to physiological demands from the host, which will be impossible without an emergent, cell-mediated microvasculature. We and others have demonstrated the formation of vascular networks, de novo, from human endothelial and mesenchymal progenitor cells (EPCs and MPCs, respectively) suspended as single cells in an extracellular matrix (ECM) and injected or implanted subcutaneously in immune-deficient mice.

Research from several groups suggests that two cell types are required for optimal vessel network formation, and many groups have been successful combining EPCs with a variety of perivascular cell types in extracellular matrix. Our lab demonstrated that coinjection of EPCs and smooth muscle cells subcutaneously into an immune-deficient mouse resulted in human blood vessels anastomosed with the host circulation within 7 days (Melero-Martin *et al.*, 2007). Jain's group combined EPCs with 10T1/2 cells in collagen in vitro for an overnight pre-incubation step, then implanted this construct into a cranial window model and showed rapid, robust blood vessel formation (Au *et al.*, 2008a). This was soon repeated using human umbilical vein endothelial cells (HUVECs) combined with bone marrow MPCs (Au *et al.*, 2008b). Hughes and George reported blood-perfused networks formed by EPCs with human dermal fibroblasts (Chen *et al.*, 2009a, Chen *et al.*, 2009b), while March's group coimplanted EPCs with stromal cells from human adipose tissue (Traktuev *et al.*, 2009), both of which specified preincubation in vitro. In 2008 our group demonstrated creation of microvascular networks using human adult bone marrow MPCs with EPCs from adult blood or umbilical cord blood. When directly injected in Matrigel, a vascular network formed and became perfused by the host circulation within one week, proving that a functional vessel network can be formed rapidly, from injectible constituents, using clinically accessible adult cells (Melero-Martin *et al.*, 2008). In all the studies described above, blood vessels were organized with perivascular cells closely surrounding EPC-lined lumens, and both cell types were required for greatest vascularization.

Matrigel has served as a provisional matrix which supports blood vessel formation, as demonstrated by our own group as well as others (Melero-Martin *et al.*, 2007, Melero-Martin *et al.*, 2008, Alajati *et al.*, 2008, Mammoto *et al.*, 2009), but it is not a desirable material for tissue engineering. Matrigel is a proprietary substance whose complete composition, including trace angiogenic factors, is variable and not publicly known (BD-Biosciences 2008), and since it is derived from a murine tumor, its translational potential is limited. Furthermore, it may be desirable for future applications to have a versatile method of generating vascular networks in a variety of environments. For example, a number of groups (Battista *et al.*, 2005, Engler *et al.*, 2006) have found that stem cell differentiation can be mediated by the stiffness of the ECM, and thus future therapies may dictate the need to direct stem cell differentiation in situ with a variety of ECM types suited to particular applications. Thus it is essential to demonstrate a method of forming vessels in matrices having a wide range of biochemical and physical properties. Other groups have demonstrated blood vessel formation in collagen/fibronectin (Au *et al.*, 2008a, Traktuev *et*

al., 2009, Enis *et al.*, 2005, Critser *et al.*, 2010a, Schechner *et al.*, 2000) and fibrin (Chen *et al.*, 2009a, Chen *et al.*, 2009b), but in each study constructs were formed in vitro then surgically implanted. While this demonstrates that bio-engineered vessels can be created in multiple ECM types, it was not known whether an injection-based model would support vascularization in ECMs other than Matrigel.

For these reasons, we have investigated vascularization in ECMs whose composition is fully defined, which are mechanically and chemically tunable, and therefore may have potential for clinical translation. Here we report that EPCs and MPCs formed perfused blood vessels when suspended and injected in mechanically and chemically diverse ECMs: type I collagen, fibrin, and PuraMatrix, a hydrogel-forming synthetic peptide.

2. Materials and Methods

2.1. Cell Sourcing and Culture

All experiments used human umbilical cord blood-derived endothelial progenitor cells (EPCs), human bone marrow-derived mesenchymal progenitor cells (MPCs), or both, which were isolated as detailed previously (Melero-Martin *et al.*, 2007, Melero-Martin *et al.*, 2008). Briefly, for these experiments EPCs were isolated and expanded from the mononuclear cell fraction of umbilical cord blood. Mononuclear cells were plated and expanded on human fibronectin-coated tissue culture plates (1 μg/cm^2, Millipore) using complete media: endothelial growth medium (EGM-2, Lonza) with 20% fetal bovine serum (Hyclone), with bFGF, VEGF, insulin-like growth factor (IGF), ascorbic acid, epidermal growth factor (EGF), gentamycin sulfate and amphotericin-B (GA-1000), and heparin, (all from Lonza SingleQuots), and 1× glutamine/penicillin/streptomycin (Cellgro). Nonadherent cells were removed and endothelial-like cell colonies grown to confluence, then trypsinized (Cellgro) and purified with anti-CD31-coated magnetic beads (Dynal). This population was subsequently cultured in complete media on fibronectin-coated plates.

MPCs were isolated by culturing the mononuclear fraction of adult bone marrow on uncoated tissue culture dishes in media identical to complete media, except lacking VEGF, bFGF, and heparin. Nonadherent cells were removed and the adherent population cultured with no further purification.

2.2. In Vivo Vasculogenesis

For all in vivo experiments, 9.4 million cells were used per milliliter of ECM, with EPCs and MPCs dispersed as individual cells in a 40:60 ratio, or 100% EPCs or 100% MPCs for cell-type control experiments. Cells were used between passages 5 and 9. In vivo experiments using the Matrigel ECM were carried out as detailed previously (Melero-Martin *et al.*, 2007, Melero-Martin *et al.*, 2008). Briefly, EPCs and MPCs were first harvested from confluent monolayer cultures using trypsin, which was quenched using DMEM/10% FBS (D10). Cells were counted and combined in a centrifuge tube for each experimental group, then centrifuged to a pellet, from which the media was aspirated. The cell pellet was loosened using 80 μL of D10 media. The cells were then suspended in Phenol Red-Free Matrigel (Becton Dickinson, thawed on ice in advance), then loaded into 1 mL syringes, fitted with needles, and stored on ice until injection. 200–300 μL subcutaneous injections were then made into the shaven dorsal flanks of severe combined immune-deficient (SCID) mice (Massachusetts General Hospital), upon which the Matrigel immediately formed a gel. Some animals were injected on one side (right) with matrix plus cells, and on the other side (left) with Matrigel without cells, as a carrier control. All in vivo experiments were carried out using 6–8 week old SCID mice under approval of Children's Hospital Boston's Institutional Animal Care and Use Committee.

2.3. Type I collagen

For in vivo experiments with type l collagen, the ECM was prepared before cell harvesting. On ice under aseptic conditions, high concentration rat tail type I collagen (Becton Dickinson) was combined with 10× PBS, 1M NaOH, and distilled water according to the manufacturer's instructions for a final concentration of 5, 6, or 7 mg/mL collagen, and the pH was titrated to 7.4 using NaOH; pH was monitored by blotting onto pH paper. Cells were suspended in this ECM and injected as described for Matrigel, and the injected matrix formed a gel immediately.

2.4. Fibrin

To inject EPCs and MPCs in a fibrin ECM, a stock solution of bovine plasma fibrinogen (Sigma) at 25 mg/mL was generated by suspending fibrinogen in pH 7.7 PBS and passing through a 0.2 μm sterile filter. After harvesting and centrifuging the cells needed for each experimental group, the cells were resuspended in the fibrinogen solution with any additional pH 7.7 PBS required for the final dilution, along with recombinant bovine aprotinin (Sigma) for a final concentration of 3 TIU/mL. The EPC/MPC/fibrinogen/aprotinin/PBS mixture was then distributed into one tube per injection, on ice. Thrombin (Sigma) at 0.25 U/mg of fibrinogen was added and the animal was immediately injected, and the injected matrix formed a gel immediately.

2.5. PuraMatrix

For injection of cells in PuraMatrix, EPCs and MPCs are first resuspended from a pellet in a mixture of PBS and 25 mM pH 9 HEPES (Sigma) stock (making up 25% and 50% of the final injection volume, respectively). This mixture was then distributed to one tube per injection. PuraMatrix stock solution (Becton Dickinson, stock concentration 10 mg peptide/mL) was added as 25% of the final volume and the animal immediately injected, after which the matrix formed a gel. Thus, the final peptide concentration was 2.5 mg/mL, dissolved in 0.25× PBS and 12.5 mM HEPES, which resulted in a final gel pH of 7.4.

2.6. Histology and Quantification of Blood Vessel Networks

7 days after injection, each animal was euthanized by CO_2 asphyxiation. Upon dorsal incision, each construct was easily recognized as a circular mass 3–5 mm in diameter and 2–3 mm thick, differentiated from any subcutaneous fat by its more solid consistency and white to pink color, depending on vascularization. The construct was excised, fixed overnight in 10% neutral buffered formalin, then embedded in paraffin and sectioned along its broad plane. Two 5 μm sections, roughly 160 μm apart, were taken from near the middle of the construct and hematoxylin and eosin (H&E)-stained. Through a 40× objective, 10 random images were taken of each stained slide, for 20 images per construct. The area and quantity of blood vessels appearing in each image were then manually measured using ImageJ software, counting vessels as endothelialized luminal structures containing red blood cells.

2.7. Immunohistochemistry

Immunohistochemical staining was performed on 5 μm thick sections fixed and embedded in paraffin as described above. Antigen retrieval (AR) buffer contained Trizma base, EDTA, and Tween-20, at pH 9. Antigen retrieval was performed at 95 °C for 15 minutes followed by a 20 minute cool-down, also in AR buffer. Primary antibodies were mouse anti-human CD31 (Dako, 1:20 dilution, for DAB only), goat anti-human CD31 (Santa Cruz, 1:20 dilution, for fluorescence only), mouse anti-αSMA (1:750 dilution, Sigma), and goat anti-VE-Cadherin (Santa Cruz, 1:50 dilution). Secondary antibodies were HRP-anti-mouse, biotin-anti-goat, and FITC-anti-mouse, all from Vector at 1:200. HRP- and Texas Red-

conjugated streptavidin were also used, also from Vector at 1:200. All antibodies were diluted in 5% serum/PBS of the secondary antibody production species, and incubated for 1 hour each. HRP-antibodies were visualized using the ImmPACT DAB Peroxidase Substrate from Vector.

2.8. Rheological Characterization of ECMs

The physical properties of each ECM were assessed using an AR2000 rheometer (TA Instruments), fitted with a 40 mm 4° cone. Each ECM sample was reconstituted on ice, then 800 μL was pipetted onto the stage of the rheometer. The cone was lowered to its 51 μm geometery gap, and the temperature set to 37 °C. Upon reaching full temperature, gels were allowed to polymerize for 10 minutes for fibrin, 15 minutes for collagen and Matrigel, and 20 minutes for PuraMatrix gels. This polymerization time was dictated by the time required for G′ and G″ of each material to stabilize (data not shown). Storage modulus, G′, and loss modulus, G″, as well as phase shift δ (such that $\tan \delta = G''/G'$) was assessed for each gel between 10^{-3} and 10^{-1} strain units at 0.5 Hz oscillation frequency.

2.9. Statistical Analyses

All values are presented as average ± standard deviation of each group, where appropriate, and differences are taken as significant for $P < 0.050$ between groups, by Student's two-tailed T-test. In vivo results presented are for representative experiments of 4–6 animals per group, and experiments for each ECM type occurred on separate days. Rheological characterization of gels is based on a minimum of 3 independent gel preparations.

3. Results

3.1. EPCs and MPCs formed vessels in collagen, fibrin, and PuraMatrix

In order to determine whether EPCs and MPCs would form vessels in ECMs other than Matrigel, we developed methods for suspending and injecting these cells in type l collagen, fibrin, and PuraMatrix. Collagen was incorporated into the model by buffering a stock solution of rat tail type I collagen with PBS, diluting with distilled water, and adjusting the pH to 7.4, and cells were suspended in this solution then injected. The collagen spontaneously formed a gel at physiological temperature. We adapted the model for a fibrin ECM by suspending the cells in a bovine fibrinogen/aprotinin solution, then added thrombin immediately before injection, triggering fibrillogenesis and thus gel formation immediately after injection. We tested PuraMatrix by suspending the cells in PBS and HEPES buffer, then adding PuraMatrix stock solution immediately before injection. PuraMatrix spontaneously formed a gel a physiological salt concentration.

We explanted each cell/ECM construct after 7 days in vivo, and found that collagen, fibrin, and PuraMatrix each supported blood vessel development. Figure 1A shows macro images of representative collagen, fibrin, and PuraMatrix tissues, vascularized as indicated by their pink color, as compared to pale or translucent cell-deficient tissues shown in Supplementary Figure 1A. Figure 1B shows H&E-stained microscopic images of blood vessels formed in each ECM, displaying erythrocytes within endothelial cell-lined lumens. The presence of erythrocytes indicated that the nascent vessels within the construct had anastomosed with host vessels. We found that in collagen, the most patent blood vessels occurred in ECM concentrations of 5, 6, or 7 mg/mL, while lower concentrations resulted in excessive hemorrhage within the constructs. Fibrin showed similar hemorrhage at concentrations below 10 mg/mL, but showed patent vessel formation at 10, 15, and 20 mg/mL. PuraMatrix was injected at 25% stock concentration, and this matrix also supported development of perfused, patent blood vessels. It was not possible to increase the concentration of PuraMatrix in our model due to premature gelling. Regardless of ECM type, when matrix

was injected without cells, blood vessels were never observed within the construct, confirming that vascularization is a result of the activities of the injected human cells, and not just a response of the animal to the insult of an injection. Collagen and fibrin supported very little migration of mouse cells into the matrix, but PuraMatrix appeared to elicit significant cellular infiltration, however, we never observed blood vessels. H&E stained images of collagen, fibrin, and PuraMatrix injected without cells and explanted after 7 days are shown in Supplementary Figure 1B.

In order to determine whether the endothelial cells lining the blood vessels within each construct were human or murine, we stained tissue sections with a human-specific antibody against CD31. As shown in Figure 1C, the vast majority of vessels in each construct were formed by human cells, not invading murine endothelial cells. Because MPCs do not express CD31, we concluded that the injected human EPCs formed the luminal lining of these vessels.

In order to determine whether EPCs alone formed these blood vessels, or whether perivascular cells were also participating in vasculogenesis, we fluorescently stained tissue sections with a human-specific CD31 antibody, and a non-species-specific antibody against smooth muscle α-actin (αSMA), a marker of pericytes and smooth muscle cells. In each type of ECM, we observed numerous blood vessels consisting of EPCs forming a lumen, with αSMA-expressing perivascular cells in close contact on the abluminal surface of the lumen, as shown in Figure 1D.

We quantified the density of the observed blood vessel networks by counting the number of vessels per area of histological sections, and by measuring the fraction of sectional area occupied by vessels. The density of blood vessels in each concentration of collagen, fibrin, and PuraMatrix is shown in Figure 1E. For injections using 5 and 6 mg/mL collagen, we measured vessel density of 57 vessels/mm^2, and average vascular area fraction of 1.3%. At 7 mg/mL, the highest collagen concentration tested, we measured 8 vessels/mm^2 and area fraction of 0.2%, a significant difference compared to 6 mg/mL collagen. In 10 mg/mL fibrin, we found that vessel density reached 38 vessels/mm^2 and 1% vascular area. As in collagen gels, the cells formed fewer than 10 vessels/mm^2, with a vascular area of 0.1%, at the higher fibrin concentrations of 15 and 20 mg/mL. Finally, PuraMatrix supported an average density of 160 vessels/mm^2 and 3.4% vascular area, which was significantly higher than that of any collagen or fibrin ECMs tested. For comparison, the average microvessel density supported by Matrigel is shown in Figure 1E as a dashed line at 100 vessels/mm^2, and Matrigel has a vascular area fraction of 2.3% (not shown). This is significantly greater than 7 mg/mL collagen, and 15 and 20 mg/mL fibrin. Average microvessel density pooled for all experiments in each ECM formulation is displayed in Supplementary Figure 2.

3.2. EPCs injected alone formed vessels in collagen and PuraMatrix

To determine whether both EPCs and MPCs were required for blood vessel formation, we injected EPCs alone suspended in 5 mg/mL collagen, 10 mg/mL fibrin, and PuraMatrix. Contrary to prior observations in Matrigel, we found that EPCs alone were capable of forming blood vessels in collagen and PuraMatrix. H&E-stained microscopic images of these vessels are shown in Figure 2A. Fibrin supported vessel development by EPCs alone in only one out of 10 constructs; an H&E stained image of this construct is shown in Supplementary Figure 4, left.

We stained these constructs with a human-specific CD31 antibody to determine whether human EPCs or endothelial cells from the host formed the vessels. As shown in Figure 2B, most vessels stained for the human CD31 antigen, indicating that human EPCs formed the majority of perfused lumens in each ECM injected with EPCs alone. For the one fibrin

construct in which EPCs alone formed vessels, CD31-stained lumens were observed, as shown in Supplementary Figure 4, center.

Since EPC-alone constructs were injected without perivascular cells, we stained these tissues with human-specific CD31 and non-species-specific αSMA to determine whether the blood vessels formed in vivo had perivascular cells. As shown in Figure 2C, EPCs injected alone in collagen and PuraMatrix formed lumens, with αSMA-expressing perivascular cells about their abluminal surfaces. Because EPCs do not express αSMA and have never been known to undergo endothelial-to-mesenchymal transition, this suggests that the perivascular cells we observed were of murine origin and were recruited by the human EPCs. This was also observed for the fibrin construct in which vessels were noted, as shown in Supplementary Figure 4, right.

We quantified the density of the vascular networks formed by EPCs injected alone in ECM, shown in Figure 2D. In collagen, we observed 12 vessels/mm^2 on average, filling 0.3 % vascular area, while PuraMatrix constructs resulted in 50 vessels/mm^2 and 1 % vascular area, a significant difference. Constructs containing EPCs alone in fibrin did not develop blood vessels, except in one construct which formed 24 vessels/mm^2, as indicated in Supplementary Figure 4. In all cases, the microvessel density was less than what was achieved when EPCs and MPCs were co-injected (Figure 1). Significantly, no vessels were observed when EPCs alone in Matrigel were injected, consistent with our previous results showing a requirement of both cell types to build bio-engineered vessels in Matrigel (Melero-Martin *et al.*, 2008). Average microvessel density pooled for all EPC-only experiments in each ECM type is shown in Supplementary Figure 3.

3.3. Vessels were formed when MPCs were injected alone in collagen, fibrin, and PuraMatrix

To determine if MPCs alone could trigger development of blood vessels, we injected MPCs in 5 mg/mL collagen, 10 mg/mL fibrin, and PuraMatrix. We observed sparse blood vessel networks in all ECMs, as shown in the H&E stained microscopic images in Figure 3A.

To determine whether the endothelial cells forming these vessels were recruited from the host, or whether the injected human MPCs differentiated to become endothelial cells, we stained with antibodies against CD31 and VE-Cadherin. The endothelial cells forming these vessels did not stain with human-specific CD31 antibodies, as shown in Supplementary Figure 5, indicating that they are of murine, not human origin. These vessels stained using a non-species-specific VE-Cadherin antibody, as shown in Figure 3B. Perivascular cells expressing αSMA were also observed forming these vessels, based on double staining with VE-Cadherin and αSMA, as shown in Figure 3C. Thus, these vessels consisted of murine endothelial cells and αSMA-expressing perivascular cells of either murine or human origin.

The density of vessel networks which resulted from injection of MPCs alone was quantified, as shown in Figure 3D. Each ECM supported less than 20 vessels/mm^2 and 0.4% or less vascular area. The average microvessel density which resulted from injecting MPCs alone in Matrigel is displayed as a dashed line at 19 vessels/mm^2, with 0.4% vascular area (not shown). Supplementary Figure 6 shows average microvessel density pooled for all MPC-only experiments in each ECM type.

3.4. ECM Physical Characterization

To learn more about the gels which enabled microvessel formation by endothelial and mesenchymal progenitor cells, we assessed the physical stiffness of collagen, fibrin, PuraMatrix, and Matrigel using a rheometer. The goal of this approach was to determine whether those gels which supported vascularization had bulk mechanical properties similar

to Matrigel, or whether blood vessels had formed in gels having a range of bulk physical properties.

The storage modulus, G′, and loss modulus, G″, was measured for each gel between 10^{-3} and 10^{-1} strain units at 0.5 Hz oscillation frequency. G′ represents the elastic contribution to the gel's resistance to deformation, while G″ represents the viscous contribution. A representative example of raw data is shown in Figure 4A, showing changes in G′ over the range of strain tested for individual preparations of fibrin, collagen, Matrigel, and PuraMatrix gels. Fibrin at 10 and 15 mg/mL showed little difference in stiffness from 10^{-3} to 10^{-1} strain units, nor does Matrigel or PuraMatrix. Fibrin at 20 mg/mL, however, showed a drop in stiffness above 10^{-2} strain units, as did collagen at 5, 6, and 7 mg/mL. These trends are qualitatively mirrored in the raw data of G″ versus strain (data not shown). Thus, collagen and high concentration fibrin exhibit strain softening at high deformations.

To compare the average stiffness of each gel formulation at low strains, the raw data for modulus of each individual ECM preparation was averaged from 10^{-3} to 10^{-2} strain units, shown in Figure 4B. For this range of strain, Matrigel was found to be highly compliant, as expected, with a G′ value of 80 Pa, and G″ of 7 Pa. Collagen at 5 mg/mL was found to have an average G′ value of 410 Pa, while 7 mg/mL was significantly stiffer at 560 Pa. 6 mg/mL collagen gels were in between at 500 Pa. Results for G″ were qualitatively similar, with values of 75, 90, and 100 Pa for 5, 6, and 7 mg/mL collagen. Fibrin ranged from 390 Pa at 10 mg/mL to over 1070 Pa at 20 mg/mL, with a step-like increase between, and G″ between 50 and 170 Pa. PuraMatrix was far more compliant than any of the protein-based ECMs, barely stiff enough to register on the rheometer at G′ of 5 Pa, and G″ of 1 Pa.

In order to describe the relative contributions of the elastic and viscous components of gel stiffness, we considered G″ and G′ as orthogonal vectors related by angle δ, the phase shift. Thus, $\tan \delta = G''/G'$, and a small δ value indicates predominance of elastic effects while high δ indicates a relatively viscous gel. We plotted δ, averaging among the gel preparations, as shown in Figure 4C. Matrigel had angle δ of approximately 5° among all samples and over all strains tested, indicating dominance of elastic behavior of this gel. Fibrin at 10, 15, and 20 mg/mL had δ value of roughly 7°, greater than Matrigel but still indicating predominantly elastic behavior over this strain range. Lower concentrations of fibrin showed greater elasticity, with δ between 6 and 7° (data not shown). This greater viscous behavior at higher concentrations is most likely due to increasing interference between adjacent fibrils as total protein concentration goes up, having an effect similar to friction within the gel. All concentrations of collagen gels had δ around 10°. PuraMatrix, by far the least stiff ECM investigated, also had unique elastic properties among this group. PuraMatix showed δ around 8°, but this rapidly increased beyond 4×10^{-3} strain units, most likely due to disturbance of the hydrogel structure at this level of strain and thus destruction of the network of elastic fibrils.

5. Discussion

We undertook the present study to determine whether EPCs and MPCs are capable of creating vascular networks in vivo in biochemically-defined ECMs following delivery by injection. Our results demonstrate the versatility of EPCs and MPCs, as they are capable of in situ vasculogenesis in four different ECM types, having a wide range of mechanical characteristics.

This study was motivated by the question of whether in situ vasculogenesis is dependent on chemical or physical cues particular to Matrigel, and whether the vasculogenic behavior observed in Matrigel could be replicated in less complex matrix environments. It was not

known whether blood vessel formation in Matrigel was due to the presence of trace growth factors, including bFGF, or to some chemical or physical property unique to this blend of ECM proteins and proteoglycans. Other groups, including the labs of Jain, March, Ingram, Yoder, and Augustin, have demonstrated successful blood vessel formation in other ECMs, including collagen with fibronectin (Au *et al.*, 2008a, Traktuev *et al.*, 2009, Enis *et al.*, 2005, Schechner *et al.*, 2000, Critser *et al.*, 2010b), fibrin (Chen *et al.*, 2009a, Chen *et al.*, 2009b), and Matrigel supplemented with fibrin (Alajati *et al.*, 2008). However, these models involved polymerization of the construct in vitro, incubation in a nutrient-rich environment overnight or for several days, and then surgical implantation into a sub-cutaneous pouch, as opposed to direct injection of cells and ECM with subsequent vessel development in situ. The Augustin group delivered HUVEC spheroid constructs by injection, however, these constructs included Matrigel in the ECM, as well as exogenous growth factors VEGF and bFGF and thus did not address the question of vasculogensis in a minimal provisional matrix. It may be desirable to deliver vasculogenic therapies by direct injection, without exogenous growth factors, yet the subcutaneous environment is more challenging than the cell culture incubator for implanted cells due to the reduced availability of oxygen. For this reason, we tested whether EPCs and MPCs can form blood vessels in ECM environments unsupplemented by exogenous growth factors and delivered by injection.

Collagen, fibrin, and PuraMatrix were selected for incorporation into this model because they exist in FDA-approved forms, as for collagen and fibrin, or are biocompatible and have potential as future therapeutic matrices, as for PuraMatrix (Narmoneva *et al.*, 2005). Bovine and porcine collagen, and human collagen derived from fibroblast culture, are FDA-approved in a variety of applications. Because bovine, porcine, and human collagen were not commercially available in adequately high concentrations, we chose to substitute rat tail type I collagen to study the behavior of EPCs and MPCs in this matrix type. Human fibrin products are FDA-approved for clinical use as surgical sealants; because human fibrin was not available we substituted bovine fibrin. We felt that demonstrating rapid in situ vascularization in matrices closely related to FDA-approved products would be an important validation of this approach for future therapeutic applications. Furthermore, collagen and fibrin were of interest because their concentration can be adjusted to create gels having a range of physical stiffness. PuraMatrix, the only synthetic ECM studied, is a 16 amino-acid oligopeptide which self-assembles into nanometer-scale fibers, resulting in a gel to which cells adhere, but lacks the canonical integrin-binding sequence RGD (arginine-gylcine-aspartic acid) (Zhang *et al.*, 1995). The ability of PuraMatrix to support vasculogenesis in vivo was not known. Matrigel was included in rheological analyses to compare its physical properties to the other ECMs. Thus, this study encompasses diverse provisional matrix formulations: three distinct types of fibrillar protein ECM and a short peptide hydrogel.

As displayed in Figure 1, all four ECM types supported the formation of patent, perfused blood vessels by EPCs and MPCs. Injections of ECM without cells never caused blood vessels to form, indicating that cells are required for vasculogenesis, and blood vessel development is not merely a nonspecific response to the insult of the injection, or an inflammatory reaction to the ECM. PuraMatrix allowed greater cellular influx than collagen or fibrin (Supplementary Figure 1B), possibly because it has the lowest peptide concentration, or because it is much less stiff than the others, as shown in Figure 4B. When EPCs were included in the ECM, the blood vessels which formed stained for human-specific endothelial marker CD31 (Figure 1C), indicating that the injected human EPCs line the majority of the resulting blood vessels. We verified this in a previous study by transforming EPCs to express firefly luciferase, and we then injected these cells with MPCs in Matrigel. After 7 days, as well at 4 weeks, these EPCs luminesced upon injection of the substrate luciferin, thus indicating functional perfusion, while constructs which contained EPCs

without MPCs (and thus could not form blood vessels) did not luminesce (Melero-Martin *et al.*, 2008).

As seen in Figure 1D, each ECM facilitated mature vessels overlain by perivascular cells expressing αSMA. This “anatomical” organization of luminal EPCs with perivascular cells on their abluminal surface indicates that EPCs are able to recruit perivascular cells, perhaps via platelet-derived growth factor (PDGF)/PDGF receptor-beta signaling (Hirschi *et al.*, 1999, Gaengel *et al.*, 2009). Presence of perivascular cells about microvessels is associated with stability and longevity of these vessels, chiefly through perivascular cell-secreted angiopoietin-1 (Ang1), and its receptor on endothelial cells, Tie2, which provides pro-survival signals (Jones *et al.*, 2001), serves to stabilize the association between endothelial and perivascular cells (Suri *et al.*, 1996, Foubert *et al.*, 2008), and contributes to organization and barrier function of the vasculature (Uemura *et al.*, 2002). Furthermore, perivascular cells have been shown to stabilize networks of endothelial cell lumens in vitro by supplying tissue inhibitor of metalloproteinase-3 (Saunders *et al.*, 2006) and promoting basement membrane deposition by endothelial cells (Stratman *et al.*, 2009a). Although we did not track the identity of these cells in collagen, fibrin, and PuraMatrix, our previous studies in Matrigel incorporated fluorescently tagged MPCs into the model and determined that the majority of αSMA-expressing perivascular cells were human MPCs (Melero-Martin *et al.*, 2008).

We found that in both collagen and fibrin, the ability of EPCs and MPCs to form blood vessels was reduced at very high concentrations. In collagen, ample blood vessel formation was observed from 1.5 to 6 mg/mL, however, leaky vessels were prevalent below 5 mg/mL. Patent vessel formation was maximal at 5 mg/mL and above, while vessel formation was significantly inhibited at 7 mg/mL. Fibrin showed a similar trend, with vessel formation occurring readily up to 10 mg/mL, at which patent vessel density was greatest, while 15 and 20 mg/mL fibrin supported fewer vessels. The reason for this reduction is not known, though it is possible that at these high protein concentrations, the proteases necessary to break down the ECM to facilitate vascular lumen development are simply not available in adequate amounts. Conversely, PuraMatrix, with a final peptide concentration of only 2.5 mg/mL, formed significantly more blood vessels than collagen or fibrin. Matrix metalloproteases critical for such lumen formation in vitro in collagen were detailed by Davis’s group, though his work does not describe a relationship between collagen concentration and the density or rate of lumen formation (Stratman *et al.*, 2009b).

EPCs injected alone, without MPCs, formed blood vessels in 5 mg/mL collagen and PuraMatrix ECMs, but never in Matrigel, and only once out of ten injections in 10 mg/mL fibrin. Examples of these networks are shown in Figure 2, and Supplementary Figure 3 for the exceptional fibrin case. In each ECM, vessels stained for human CD31 and recruited αSMA-expressing perivascular cells, displaying a degree of maturity comparable to the vessels resulting from EPC/MPC injections. Since EPCs do not express αSMA, these are cells which have migrated into the construct from the host. As shown in Figure 2D, PuraMatrix supported significantly higher vessel density, possibly because it is the least rigid matrix studied (Figure 4B), has the lowest peptide concentration, or is most permissive for host cell migration into the matrix itself (Supplementary Figure 1B). These results suggest that at the concentrations tested, collagen and PuraMatrix are inherently more hospitable than fibrin or Matrigel to vasculogenesis by human endothelial cells alone.

MPCs injected alone in collagen, fibrin, and PuraMatrix resulted in blood vessel formation, similar to MPCs alone in Matrigel. These sparse vessels, presented in Figure 3, did not stain for human-specific CD31, ruling out transdifferentiation of the human MPCs or accidental cross-contamination with EPCs; thus these vessels are lined with mouse endothelium, and,

similar to vessels resulting from EPC injection alone and two cell type co-injections, are composed of both endothelium and αSMA-expressing perivascular cells, as seen in Figure 3C. Vascular density resulting from injections of MPCs alone were uniformly low in each ECM (Figure 3D).

After learning that ECMs which were chemically distinct from Matrigel supported blood vessel development by EPCs and MPCs, we wanted to know whether these ECMs were distinct in their physical properties as well. To address this, we evaluated the bulk physical properties of each gel using a rheometer. The "vasculogenic" ECMs reported here encompass a wide range of physical environments, as shown in Figure 4. Fibrin gels ranged from 400 to over 1000 Pa in storage modulus among 10, 15 and 20 mg/mL gels, but only the softest, lowest concentration supported a high degree of vessel development. 5 and 6 mg/mL collagen gels, which supported similar degrees of vascularization, also had similar physical stiffness, with G′ of 400 to 500 Pa, while 7 mg/mL collagen showed a significantly greater stiffness of 550 Pa, accompanied by significantly reduced vascular density. Conversely, Matrigel's storage modulus was measured at roughly 80 Pa, the second most compliant gel we report, yet here and in previous studies, has shown greater average microvessel density than collagen and fibrin matrices. PuraMatrix was dramatically less stiff than the other ECMs at less than 10 Pa, and had the greatest vascular density. Thus, we observed a weak inverse correlation between the stiffness of each gel and the density of vasculogenesis supported therein. Most interestingly, a 40-fold difference in G′ was measured between PuraMatrix and 10 mg/mL fibrin, yet EPCs and MPCs formed vessel networks in each environment.

Although we have observed a tendency of EPCs and MPCs to form higher density vascular networks in more compliant ECMs, it must be acknowledged that the physical stiffness of the provisional matrix is not the only determinant of cell behavior. Two-dimensional in vitro models of endothelial cell behavior have been developed which introduce substrate stiffness as an independent variable (Mammoto *et al.*, 2009, Deroanne *et al.*, 2001). In three-dimensional culture, however, parameters such as fibril diameter, porosity, and cross-linking or other chemical differences among different ECM proteins or peptides most likely contribute to the observed differences in blood vessel density, in addition to bulk material stiffness. In our own model, and in nearly all three-dimensional in vivo models described in the literature, these parameters are intrinsically coupled. Indeed, in creating gels having a range of physical stiffness, we may have simultaneously altered fibril diameter and pore size, as reported for lower concentrations of collagen (Yang *et al.*, 2009), and we have changed the mass of ECM protein per volume, making it impossible to claim that differences in blood vessel density are a result of physical stiffness alone. Similarly, although there are differences in the bulk material stiffness of PuraMatrix, Matrigel, and collagen, we cannot claim that this causes the observed differences in vascularization because there are differences in chemical composition among these gels.

There is a great deal we do not understand about the interaction between EPCs, MPCs and the extracellular matrix. Specifically, although we have suspended cells in gels ranging widely in stiffness and elasticity, we describe the properties of these gels only on the bulk scale, not the micro-scale at which cells interact by pulling, stretching, and traveling through the ECM. Nevertheless, although the rheological data reported here are bulk properties of the gels, differences in these data are an expression of micro- and nano-scale structural differences between ECMs. This structure can be altered by changing protein concentration (as in the current work), or such factors as polymerization conditions or chemical treatments. Thus, our finding that EPCs and MPCs can form comparable networks in provisional matrices having such disparate rheological properties suggests they have great potential for either coping with microstructural differences or remodeling them to their liking.

We do not know the degree to which EPCs and MPCs remodel the extracellular matrix over the course of 7 days in vivo, nor what effect the in vivo environment itself has on the ECM. Davis's group has described the essential nature of matrix metalloproteases to vascular network formation in vitro (Stratman *et al.*, 2009b), and it is reasonable to suppose that such protease secretion could affect the physical properties of the matrix, both immediately adjacent to vascular lumens and in general. Furthermore, our group demonstrated that myeloid cells from the host pervade our Matrigel model in the first 1–2 days in vivo. Depletion of Gr-1-positive cells, which includes granulocytes and monocytes, reduced vascular density in EPC/MPC/Matrigel implants. The infiltrating myeloid cells produce matrix metalloproteases -9 and -2 and therefore may have an effect on the mechanical or structural characteristics of the ECM (Melero-Martin *et al.*, 2010). It is possible that during the in vivo period the large physical differences among provisional matrices will be partly abrogated by the actions of the human and host cells. It remains significant that qualitatively similar vessel networks result from radically different provisional matrices, all within the relatively short 7 day period in vivo.

At present, the two-cell type system developed in our laboratory is unique as the sole model demonstrating sub-7 day vascularization, in situ, of multiple ECM formulations, using clinically accessible cells, without reliance on exogenous growth factors, delivered by injection and not surgery. In addition to being a possible pathway to vascular regeneration and tissue engineering therapies, this model is becoming a testbed to study the effects of stem cells, genetic manipulations, and drugs on vasculogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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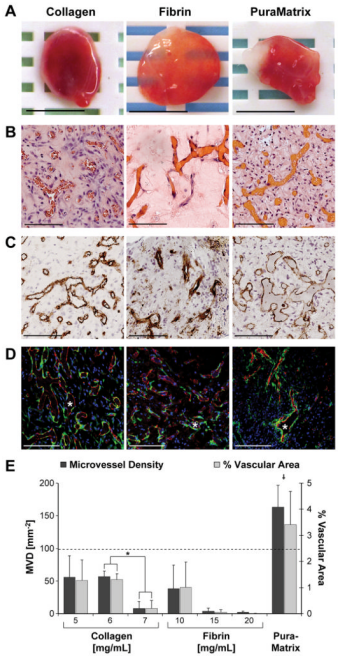

Figure 1.

Images of vascularized constructs containing human EPCs and MPCs, explanted after 7 days in vivo. Left column, collagen, center column, fibrin, right column, PuraMatrix. (A) Macro images of vascularized constructs. Bar, 5 mm. (B) H&E stained images of vascularized constructs, showing patent, erythrocyte-filled lumens. Bar, 100 μm. (C) Construct sections antibody-stained for human CD31, showing erythrocyte-filled lumens lined with EPCs. Bar, 100 μm. (D) Construct sections antibody-stained for CD31 (red) and αSMA (green) showing EPC-lined vessels (one example per image marked with *) overlaid with αSMA-expressing perivascular cells. Bar, 50 μm, nuclei blue. (E) Quantification of blood vessel density in three concentrations of collagen and fibrin ECMs, and PuraMatrix ECM containing EPCs and MPCs. Dashed line indicates average value of microvessel density in Matrigel containing EPCs and MPCs. Microvessel density on left axis, dark bars, % vascular area on right axis, light bars. * indicates significance for microvessel density and % vascular area between 6 and 7 mg/mL collagen groups. † indicates significance between PuraMatrix and all concentrations of collagen and fibrin for microvessel density and % vascular area.

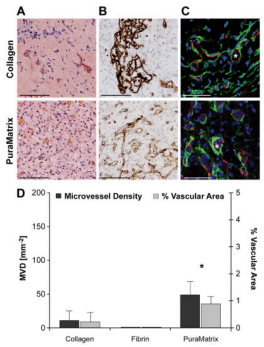

Figure 2.

Images of vascularized constructs containing only human EPCs explanted after 7 days in vivo. (A) H&E stained images of vascularized constructs, showing patent, erythrocyte-filled lumens. Bar, 100 μm. (B) Construct sections antibody-stained for human CD31, showing erythrocyte-filled lumens lined with EPCs. Bar, 100 μm. (C) Construct sections antibody-stained for CD31 (red) and αSMA (green) showing EPC-lined vessels (one example per image marked with *) overlaid with αSMA-expressing perivascular cells. Bar, 50 μm, nuclei blue. (D) Quantification of blood vessel density in 5 mg/mL collagen, 10 mg/mL fibrin, and PuraMatrix ECMs containing EPCs. Microvessel density on left axis and dark bars, % vascular area on right axis and light bars. * indicates significance of PuraMatrix microvessel density and % vascular area compared to collagen and fibrin.

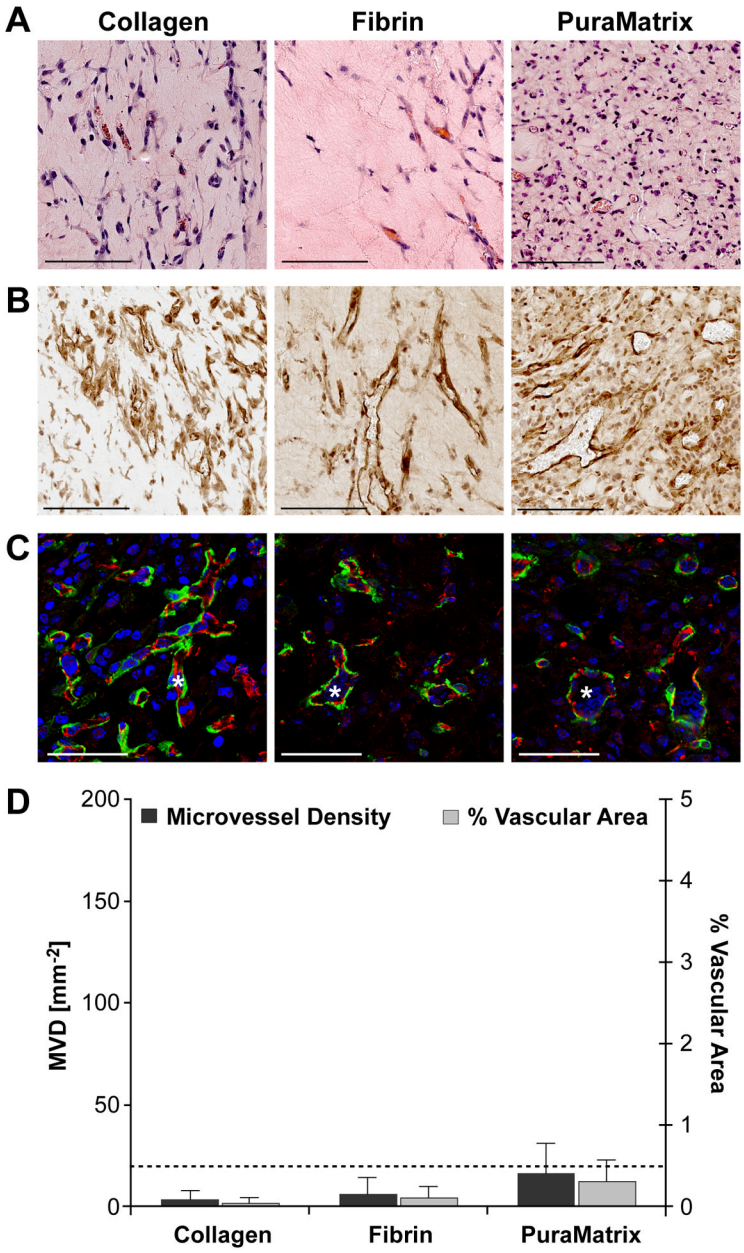

Figure 3.

Images of vascularized constructs containing only human MPCs explanted after 7 days in vivo. (A) H&E stained images of vascularized constructs, showing patent, erythrocyte-filled lumens. Bar, 100 μm. (B) Construct sections antibody-stained for endothelial marker VE-Cadherin, showing erythrocyte-filled, endothelialized lumens. Bar, 100 μm. (C) Construct sections antibody-stained for VE-Cadherin (red) and αSMA (green) showing vessels (one example per image marked with *) overlaid with αSMA-expressing perivascular cells. Bar, 50 μm, nuclei blue. (D) Quantification of blood vessel density in 5 mg/mL collagen, 10 mg/mL fibrin, and PuraMatrix ECMs containing MPCs. Microvessel density on left axis and dark bars, % vascular area on right axis and light bars.

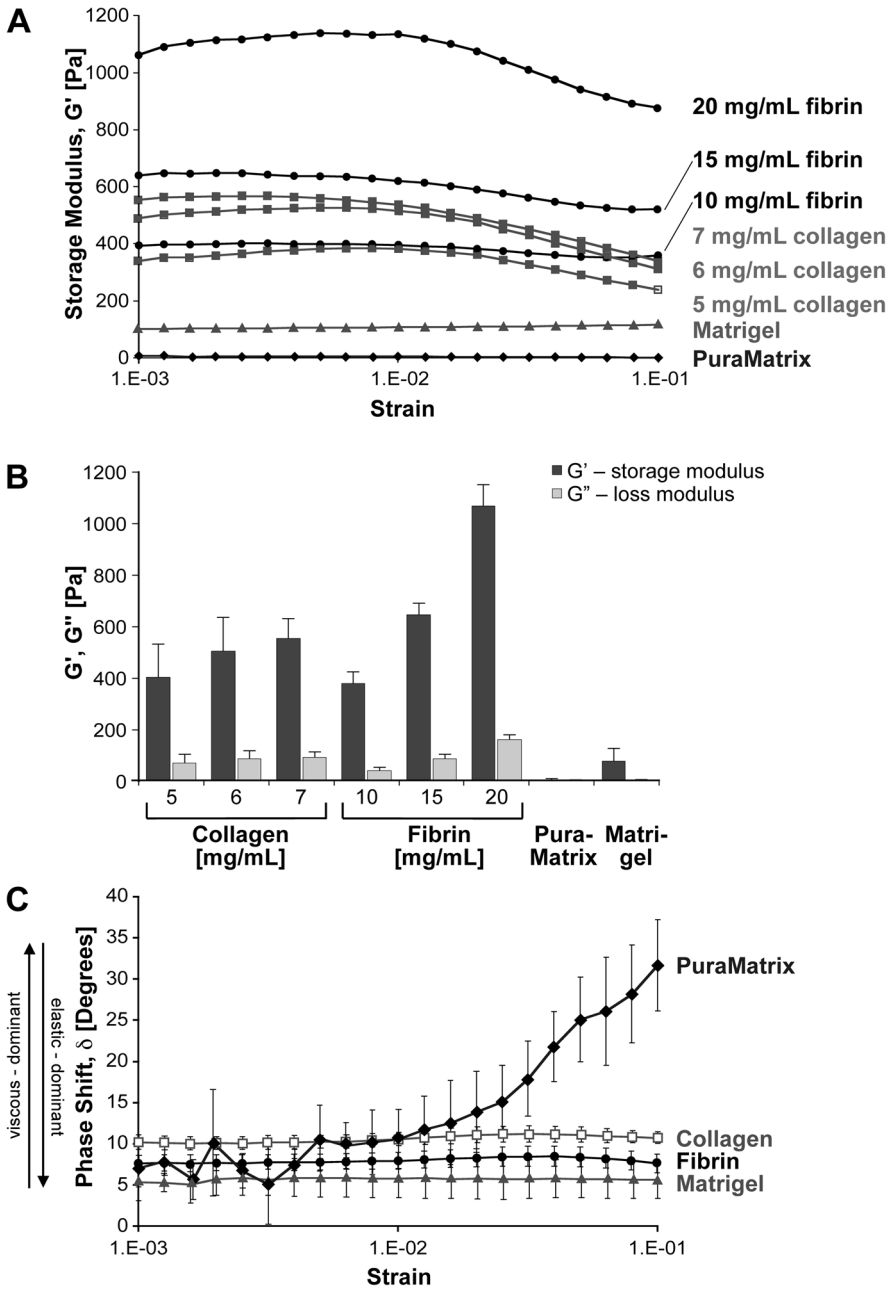

Figure 4.

Rheological characterization of collagen, fibrin, and PuraMatrix ECMs. (A) Representative raw data showing storage modulus, G′, from 10-3 strain units to 10-1, for three concentrations of collagen and fibrin ECMs, and Matrigel and PuraMatrix. (B) Average values of storage modulus, G′ (grey bars), and loss modulus, G″ (white bars), for each ECM, average of multiple preparations, average value from 10-3 to 10-2 strain units. (C) Phase shift, δ, for each ECM from 10-3 to 10-1 strain units, average of multiple preparations. Collagen and fibrin data are average of multiple preparations from 5, 6, and 7 mg/mL, and 10, 15, and 20 mg/mL, respectively.