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Endothelial cell adhesion, signaling, and morphogenesis in fibroblast-derived matrix

Patricia A. Soucy [a,b], Lewis H. Romer [a,b,c,d,e,*]

[a] Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, United States
[b] Department of Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States
[c] Department of Cell Biology, JHU-SOM, United States
[d] Department of Pediatrics, JHU-SOM, United States
[e] Center for Cell Dynamics, JHU-SOM, United States

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ABSTRACT

Extracellular matrix plays a critical role in cellular development by providing signaling cues that direct morphogenesis. In order to study both the cues that natural matrix provides and endothelial cell responses to that information, human fetal lung fibroblasts were used to produce a fibrous three-dimensional matrix. Following the removal of the fibroblasts by detergent extraction, protein and proteoglycan constituents of the remaining matrix were identified by immunofluorescence and immunoblotting. Matrix components included fibronectin, tenascin-C, collagen I, collagen IV, collagen VI, versican, and decorin. Colocalization analysis suggested that fibronectin was a uniquely distributed matrix protein. Morphology, three-dimensional matrix adhesions, and integrin-mediated signaling during vasculogenesis were then studied in human endothelial cells seeded onto the fibroblast-derived matrix. Elongated morphology and decreased cell area were noted, as compared with cells on fibronectin-coated coverslips. Cell-matrix adhesions contained vinculin, pY397-FAK, and pY410-p130Cas, and all of these colocalized more with fibronectin than tenascin-C, collagen I, or collagen VI. Additionally, the endothelial cells remodeled the fibroblast-derived matrix and formed networks of tubes with demonstrable lumens. Matrix adhesions in these tubes also predominantly colocalized with fibronectin. The pattern of membrane type 1 matrix metalloprotease expression in the endothelial cells suggested its involvement in the matrix remodeling that occurred during tubulogenesis. These results indicated that information in fibroblast-derived matrix promoted vasculogenic behavior.

1. Introduction

The extracellular matrix (ECM) is a network of proteins and proteoglycans that provide a microenvironment for cellular development, homeostasis, and regeneration (Goh et al., 1997; Joshi et al., 1993). The mechanical properties and biochemical composition of substrates have a major impact on patterns of cell adhesion to matrix and growth behavior (Discher et al., 2005; Engler et al., 2004, 2006; Georges and Janmey, 2005; Pelham and Wang, 1998). In order to design effective biomimetic materials for tissue engineering, it is necessary to more fully define and decode the morphogenic signals that the ECM provides (Vogel and Baneyx, 2003).

Environmental cues are transduced into signals that regulate cell behavior at cell-matrix adhesion sites, which are mediated by transmembrane integrins (Hynes et al., 2002; Romer et al., 2006). Cell-matrix adhesions have been classified as focal adhesions, focal complexes, fibrillar adhesions, and 3D-matrix adhesions. They differ from each other in their molecular composition, the forces they exert, and the extracellular substrate required for their formation (Cukierman et al., 2001, 2002; Geiger and Bershadsky, 2001; Geiger et al., 2001; Pankov et al., 2000). One signaling event that occurs at the cell-matrix connection is the discrete tyrosine phosphorylation of specific proteins in cell-matrix adhesions in response to extrinsic stretch and strain. Two mechanically responsive proteins that are found in cell-matrix adhesions are focal adhesion kinase (FAK) and p130Cas, which are phosphorylated at tyrosine 397 and at tyrosine 410 respectively (Sawada et al., 2006; Wang et al., 2001). Such biochemical signals can activate signaling pathways for proliferation, migration, and morphogenesis (Giannone and Sheetz, 2006; Parsons, 2003; Sawada et al., 2006; Schlaepfer and Mitra, 2004; Wang et al., 2001). These cell adhesion mechanosensors and their role in specific downstream regulatory pathways have mostly been studied on tissue culture plastic — a static and rigid surface that does not accurately model the *in vivo* microenvironment.

Three-dimensional scaffolds for investigational platforms have been designed to mimic the fibroblast-produced ECM found *in vivo*. *In vitro* studies have detailed the secretion of matrix constituents by fibroblasts, including fibronectin, collagens, and tenascin-C, and also the importance of fibronectin fibrillogenesis for matrix assembly and stability (Chung and Erickson, 1997; Dzamba and Peters, 1991; Sabatelli et al., 2001; Sottile and Hocking, 2002). In addition,

* Corresponding author. 904 Blalock, Pediatric Anesthesiology and Critical Care, 600 North Wolfe Street, Johns Hopkins University School of Medicine, Baltimore, Maryland, 21287-4904, United States. Tel.: +1 410 955 7610; fax: +1 410 502 5312.
E-mail addresses: parauz1@jhmi.edu (P.A. Soucy), lromer@jhmi.edu (L.H. Romer).

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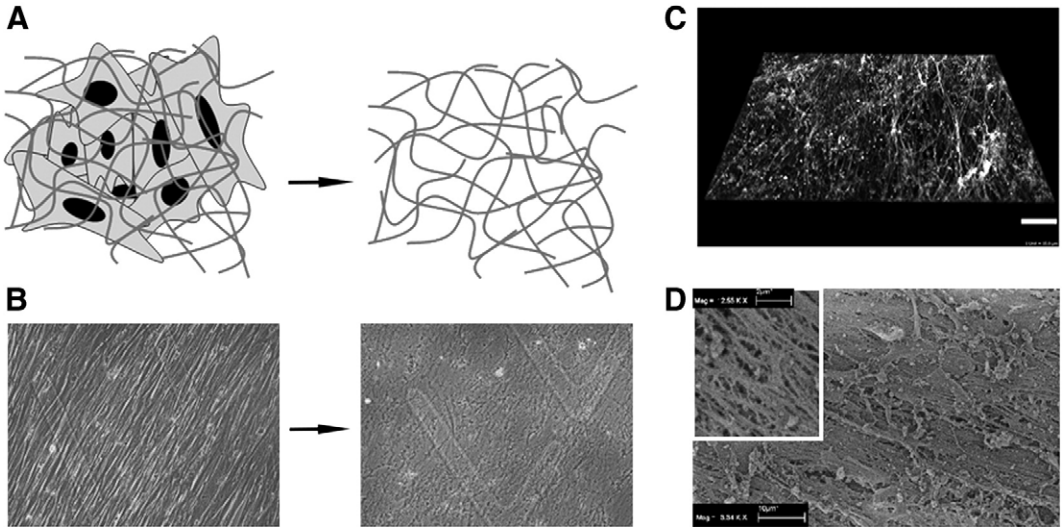

Fig. 1. Production of fibroblast-derived ECM. A) Schematic of ECM production (left, matrix is depicted as lines) and the ECM after the extraction procedure (right). B) Phase contrast images of WI-38 fibroblast cultured for 9 days after confluence, before (left) and after extraction (right). Location "77" marked the identical field, line thickness of these numbers = 20 μm. C) Three-dimensional reconstruction of an amine-labeled (using Alexa Fluor 488 carboxylic acid, succinimidyl ester) fibroblast-derived ECM. Scale bar = 15 μm. D) The fibrous structure of the ECM was also visualized with SEM. Scale bar = 10 μm, and 2 μm in the inset.

fibroblast-derived matrices may include inactive, latent growth factors such as TGF-β1 (Koli et al., 2005; Wipff et al., 2007). Previous studies have explored the effects of fibroblast behavior on three-dimensional ECM organized by fibroblasts (Cukierman et al., 2001; Mao and Schwarzbauer, 2005). Fibroblast morphology and three-dimensional adhesions to the decellularized matrix resembled the *in vivo* environment and were notably different from those seen in typical two-dimensional culture conditions (Cukierman et al., 2001; Mao and Schwarzbauer, 2005). These investigations laid the groundwork for further study of fibroblast-derived matrices as templates for the regulation of tissue morphogenesis.

The role of ECM in angiogenesis and vasculogenesis has been examined in a number of contexts. Various single component matrices (including collagen, fibrin, and Matrigel™) have been prepared as three-dimensional scaffolds, and have been shown to support endothelial tube formation in the presence of specialized growth factors (Chalupowicz et al., 1995; Kubota et al., 1988; Montesano et al., 1986; Sieminski et al., 2004; Zhou et al., 2008). In addition, *in vitro* tubulogenesis has been demonstrated in various fibroblast-endothelial cell co-cultures, as fibroblasts have been shown to produce matrix and growth factor components that facilitate vessel formation (Berthod et al., 2006; Bishop et al., 1999; Montesano et al., 1993; Sorrell et al., 2007; Villaschi and Nicosia, 1994).

In the current work, we have focused on the cues for endothelial morphogenesis that may be provided by a natural, multi-component, decellularized matrix. Human fetal lung fibroblast-derived matrix was voided of fibroblasts and used as a scaffold to guide endothelial cell adhesion and growth. We identified multiple proteins and proteoglycan components of this fibroblast-derived matrix, and demonstrated the influence of this microenvironment on endothelial cell tube formation. Human umbilical vein endothelial cells (HUVEC) displayed a different morphology on three-dimensional fibroblast-derived matrices compared with typical two-dimensional cultures. This observation illustrated the impact of the three-dimensional matrix and its unique signals. The endothelial cell-matrix adhesions incorporated mechanically sensitive tyrosine phosphorylation events. These adhesions were preferentially colocalized with fibronectin, the protein with a distinct spatial distribution in the fibroblast-derived matrix. Additionally, endothelial cells remodeled the ECM during tube formation and membrane type 1 matrix metalloprotease (MT1-MMP) expression localized near sites of cell-matrix interaction. These data demonstrated that the ECM organized by lung fibroblasts was sufficient to induce HUVEC tubulogenesis, thereby providing a system for the examination of the intracellular signaling events that drive this process.

2. Results

2.1. Production of fibroblast-derived matrix

Fibroblast-derived ECM was produced by WI-38 human fetal lung fibroblasts during 7–10 days of culture. Except where indicated, studies were conducted with matrices that had been voided of fibroblasts using the extraction protocol detailed in the Experimental procedures. Fig. 1A schematically illustrates ECM production and fibroblast extraction. Serial phase contrast images of the same field show fibroblasts cultured for 9 days after confluence and the remaining extracellular matrix after extraction (Fig. 1B). Discrete, thin ECM fibrils were clearly discernible by phase contrast following extraction. To study the overall structure of this fibroblast-derived matrix, an Alexa Fluor 488 amine-reactive probe was used to label most of the ECM proteins. The three-dimensional reconstruction of a deconvolved z-stack displayed the dense fibrous nature of this fibroblast-derived matrix (Fig. 1C). The average thickness of these labeled matrices was 4.97 ± 0.37 μm and the average mesh size was 1.98 ± 0.04 μm. The distribution of measured mesh sizes is shown in Supplemental Fig. 1. Scanning electron microscopy (SEM) corroborated the observation that the fibroblast-derived matrix contained a dense and heterogeneous fibrous structure (Fig. 1D).

2.2. Matrix composition

Immunoblotting and immunofluorescence defined the identity and distribution of the matrix constituents. Western blots demonstrated the presence of tenascin-C, fibronectin, collagen I, and versican (Fig. 2A). The two major size variants reported elsewhere for tenascin-C were identified at 220 kDa and 320 kDa (Savarese et al., 1996). Immunoblots for collagen I reproducibly showed 3 bands as seen in Fig. 2A (3 different experiments). These three bands likely corresponded to (in order of increasing molecular weight): α1(I), pro α1C^{-}/N^{-} (cleaved at the C or N terminus), and pro α1(I), as reported by others (Bernstein et al., 1996; Rocnik et al., 2002). Notably, vitronectin was not found to be a component of these matrices via immunoblotting. However, it was noted in the positive control (data not shown).