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Matrix density alters zyxin phosphorylation, which limits peripheral process formation and extension in endothelial cells invading 3D collagen matrices

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ABSTRACT

This study was designed to determine the optimal conditions required for known pro-angiogenic stimuli to elicit successful endothelial sprouting responses. We used an established, quantifiable model of endothelial cell (EC) sprout initiation where ECs were tested for invasion in low (1 mg/mL) and high density (5 mg/mL) 3D collagen matrices. Sphingosine 1-phosphate (S1P) alone, or S1P combined with stromal derived factor-1α (SDF) and phorbol ester (TPA), elicited robust sprouting responses. The ability of these factors to stimulate sprouting was more effective in higher density collagen matrices. S1P stimulation resulted in a significant increase in invasion distance, and with the exception of treatment groups containing phorbol ester, invasion distance was longer in 1 mg/mL compared to 5 mg/mL collagen matrices. Closer examination of cell morphology revealed that increasing matrix density and supplementing with SDF and TPA enhanced the formation of multicellular structures more closely resembling capillaries. TPA enhanced the frequency and size of lumen formation and correlated with a robust increase in phosphorylation of p42/p44 Erk kinase, while S1P and SDF did not. Also, a higher number of significantly longer extended processes formed in 5 mg/mL compared to 1 mg/mL collagen matrices. Because collagen matrices at higher density have been reported to be stiffer, we tested for changes in the mechanosensitive protein, zyxin. Interestingly, zyxin phosphorylation levels inversely correlated with matrix density, while levels of total zyxin did not change significantly. Immunofluorescence and localization studies revealed that total zyxin was distributed evenly throughout invading structures, while phosphorylated zyxin was slightly more intense in extended peripheral processes. Silencing zyxin expression increased extended process length and number of processes, while increasing zyxin levels decreased extended process length. Altogether these data indicate that ECs integrate signals from multiple exogenous factors, including changes in matrix density, to accomplish successful sprouting responses. We show here for the first time that zyxin limited the formation and extension of fine peripheral processes used by ECs for matrix interrogation, providing a molecular explanation for altered EC responses to high and low density collagen matrices.

1. Introduction

Endothelial cells (ECs) respond to local environmental signals to develop rapidly into new blood vessels during angiogenesis in both physiological and pathological situations. ECs must navigate a three-dimensional (3D) extracellular matrix (ECM), which can vary widely in density and resistance. In the developing embryo, high levels of proteoglycans presumably enhance tissue hydration and decrease resistance, while adult tissues are stabilized via cross-links and exhibit higher overall resistance. In a tumor microenvironment, collagen content is frequently high, enhancing tissue stiffness (Netti et al., 2000; Brown et al., 2003). Thus, in many situations, the mechanical and cell adhesive cues provided by the ECM must be integrated with local biochemical signals to stimulate capillary outgrowth. These exogenous stimuli must be continually integrated by ECs to maintain homeostasis and initiate angiogenic responses. ECs also rapidly respond to biochemical factors, including vascular endothelial growth factor (Dvorak et al., 1991; Senger et al., 1993; Joukov et al., 1996; Olofsson et al., 1996), basic fibroblast growth factor (Friesel and Maciag, 1995), placental growth factor (Luttun et al., 2002), stromal-derived factor-1α (Mirshahi et al., 2000), phorbol ester (Montesano and Orci, 1985, 1987; Macalma et al., 1996; Ilan et al., 1998), and lysosphingolipids, such as sphingosine 1-phosphate (S1P) (Lee et al., 2001; Kluk and Hla, 2002). Thus, a multitude of pro-angiogenic factors act in concert with

Abbreviations: EC, endothelial cell; SDF, stromal-derived factor; TPA, phorbol ester; 3D, three-dimensional; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; S1P, sphingosine 1-phosphate.

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hemodynamic and mechanical signals from the ECM (Kaunas et al., 2011). In the current study, collagen matrix density was varied in the presence of various biochemical stimuli to investigate conditions that are optimal for vascular assembly.

ECs respond to changes in ECM composition, stiffness, and density (Discher et al., 2005), and various types of mechanical cues affect tubulogenesis. Tension between ECs can be sensed, and these forces regulate or guide capillary outgrowth and EC morphogenesis (Ingber and Folkman, 1989; Davis and Camarillo, 1995; Vernon et al., 1995; Korff and Augustin, 1999). Increased stiffness is associated with increased endothelial sprout formation (Vailhe et al., 1997; Vailhe et al., 1998; Sieminski et al., 2004; Yamamura et al., 2007; Sieminski et al., 2008; Shamloo and Heilshorn, 2010; Lee et al., 2013; Mason et al., 2013), while more compliant matrices allow longer sprout outgrowth in vitro (Vernon and Sage, 1999; Yamamura et al., 2007; Ghajar et al., 2008; Kniazeva and Putnam, 2009; Edgar et al., 2014a) and in vivo (Kniazeva et al., 2011). Tension between ECs can be sensed and can regulate or guide capillary outgrowth and EC morphogenesis (Ingber and Folkman, 1989; Davis and Camarillo, 1995; Vernon et al., 1995; Korff and Augustin, 1999; Krishnan et al., 2008). Sprout orientation can be regulated by tensional forces (Korff and Augustin, 1999; Krishnan et al., 2008), VEGF gradients (Shamloo et al., 2012), and matrix stiffness (Yamamura et al., 2007; Lee et al., 2013; Mason et al., 2013). The aforementioned studies show clearly that changing mechanical stimuli alter endothelial sprout morphology. Changing the matrix density in a 3D environment can alter stiffness, pore size, and confinement or restriction of cells (Doyle et al., 2013). Thus, various endothelial cell behaviors have been reported to vary with changes in matrix density. To fully understand the role of the 3D environment in guiding angiogenesis, the intracellular signals activated by changes in ECM composition, stiffness, and density need to be identified.

Cell interactions with the ECM occur through integrins. Integrins bind matrix substrates and assemble or aggregate when engaged. Focal adhesion assembly follows integrin engagement, and focal adhesions consist of aggregates of accessory proteins (Zaidel-Bar and Geiger, 2010). Well-studied focal adhesion proteins include talin, vinculin, paxillin, focal adhesion kinase, and zyxin, although approximately 200 proteins have been listed as part of the integrin adhesome (Zaidel-Bar and Geiger, 2010). Integrins are linked to mechanosensory signaling pathways (Littlewood Evans and Muller, 2000). It follows then that increased matrix stiffness, porosity, or integrin binding site availability would affect cell responses to various stimuli, and these signals are likely to be transduced through focal adhesions and associated proteins.

In this study, we examined whether ECs intercalate ECM-derived signals and biochemical stimuli by coordinately testing the combined effects of various pro-angiogenic factors and matrix density to determine conditions that promote assembly of capillary-like structures that are multicellular, contain lumens, and extend peripheral processes. We describe conditions that are optimal for vascular sprout initiation and assembly into multicellular structures containing lumens, which include 5 mg/mL collagen matrices that contain S1P and SDF, with stimulation by soluble VEGF and bFGF. While examining signaling pathways linked to mechanotransduction, we observed that levels of phosphorylated zyxin inversely correlate with matrix density and generation of small, extended filopodia-like processes that ECs used to interrogate the matrix, indicating that zyxin is integral in fine-tuning EC invasion responses in 3D collagen matrices.

2. Results

Known pro-angiogenic stimuli were used to test endothelial sprouting responses using an established, quantifiable model of endothelial cell (EC) sprout formation in low (1 mg/mL) and high (5 mg/mL) density 3D collagen matrices. In these experiments, we tested limited combinations of pro-angiogenic factors that activate various signaling pathways. We applied the well-known pro-angiogenic growth factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Senger et al., 1993; Murakami and Simons, 2008), as well as stromal-derived factor 1α (SDF-1α), which is also widely accepted as a pro-angiogenic molecule (Salcedo and Oppenheim, 2003). VEGF and bFGF are well-characterized to signal through receptor tyrosine kinase activation, while SDF-1α activates G-protein receptor (GPCR) signaling through the CXCR4 receptor. In addition, we added S1P, a sphingolipid that has been described previously to promote angiogenesis in vivo and in vitro (Kluk and Hla, 2002) by activating a distinct subset of GPCR, specifically $S1P_1$, and $S1P_3$ in endothelial cells. Finally, we utilized phorbol ester (TPA), which has previously been shown by Madri and others to promote angiogenic responses in 3D collagen and fibrin matrices (Ilan et al., 1998).

In all treatment groups, the media contained 40 ng/mL of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), because VEGF and bFGF were required for robust sprouting to occur in the presence of S1P and SDF-α (Supplemental Fig. 1). As shown in Supplemental Fig. 1, endothelial sprouting occurred in all conditions when VEGF and FGF are present (left panels). However, in the absence of growth factors (VEGF and FGF), no sprouting occurred with S1P and S1P + SDF treatment groups (right panels). These data warrant adding VEGF and FGF in all treatment groups to allow sprout morphology to be analyzed when matrix density is altered.

To induce endothelial sprouting, growth factor stimulation was combined with S1P, TPA, SDF, S1P + TPA, S1P + SDF, TPA + SDF, and S1P + TPA + SDF (ALL). Photographs of invasion responses for all treatment groups in both 1 mg/mL and 5 mg/mL collagen matrices are shown in Fig. 1A. Quantification of invasion responses are shown in Fig. 1B, and corresponding statistical analyses are shown in Supplemental Table I. Combining growth factor treatment with S1P, S1P + TPA, and S1P + SDF resulted in substantial invasion responses. Significantly lower invasion was observed with TPA and SDF treatments alone. When S1P, SDF, and TPA were combined (ALL), invasion was also significantly higher than SDF alone, TPA alone and TPA + SDF. In addition, EC invasion was significantly higher in 5 mg/mL versus 1 mg/mL collagen matrices in the S1P, TPA, S1P + TPA, TPA + SDF, and ALL treatment groups. The differences were not explained by changes in proliferation; Ki67 staining of cultures revealed no differences between S1P, S1P + TPA, and S1P + SDF in 1 mg/mL and 5 mg/mL collagen matrices (data not shown). These results show that the pro-angiogenic factors S1P, SDF, and TPA alone and in combination stimulated angiogenic sprouting, and the ability of these factors to enhance sprouting was more effective in higher density collagen matrices.

We observed a noticeable increase in invasion distance with S1P stimulation. Quantification of invasion distances are shown in Fig. 2, and corresponding statistical analyses are shown in Supplemental Table II. The highest invasion distances were observed in all groups containing S1P stimulation. Invasion distance decreased slightly with S1P + TPA and ALL treatment groups, while TPA + SDF, TPA alone, and SDF treatments stimulated the shortest invasion distances. With the exception of treatment groups containing TPA, invasion distance was higher in 1 mg/mL compared to 5 mg/mL collagen matrices, indicating that without TPA, ECs invaded farther in 1 mg/mL than 5 mg/mL collagen matrices.

A closer inspection of cell morphology revealed a distinct pattern of invading structures that consisted of both single and multicellular structures. Cells were stained with DAPI, and fluorescent images were overlayed with differential interference contrast (DIC) images to quantify the average number of nuclei within invading structures (Fig. 3A). Arrowheads indicate structures consisting of single cells while arrows indicate multicellular structures. The formation of multicellular structures was the lowest with S1P treatment in 1 mg/mL collagen matrices (Fig. 3B). The percentage of multicellular structures stimulated by S1P was increased in 5 mg/mL compared to 1 mg/mL matrix density. TPA and SDF treatments increased the percentage of invading structures that were multicellular (see arrows, Fig. 3A). In all groups except S1P