Coregulation of VEGF-A And PLGF in Vascular Endothelial and Smooth Muscle Cells

Nabil A. Rashdan
Louisiana State University Health Sciences - Shreveport

Pamela C. Lovem (✉ pamela.lovem@okstate.edu)
Oklahoma State University

Article

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Abstract

Placental growth factor (PLGF) and vascular endothelial growth factor-A (VEGF-A) are important regulators of both physiological and pathological vascular remodeling. We previously reported that in monoculture human coronary EC primarily expresses PLGF, while human coronary smooth muscle cells (SMC) primarily express VEGF-A. However, in the vasculature, EC and SMC are in close proximity. Thus, in this study we sought to 1) determine whether this cell-specific expression pattern is maintained when EC and SMC are cocultured, and 2) test the hypothesis that EC and SMC co-regulate the expression of VEGF-A and PLGF. EC and SMC were cultured on either side of a porous Transwell insert and media PLGF and VEGF-A levels were measured by ELISA. We confirmed that the cell type-specific expression of PLGF and VEGF-A we observed in monocultures remains evident in the coculture model. Interestingly, coculture of EC and SMC increased media PLGF relative to EC monoculture, but decreased media VEGF-A compared to SMC monoculture. Coculture conditions also increased VEGFR2 levels on the surface of EC but decreased VEGFR1 levels on the surface of SMC. Inhibition of VEGFR2 tyrosine kinase activity decreased PLGF and increased VEGF in both EC and cocultures but not in SMC monocultures. We conclude that PLGF and VEGF-A exert both paracrine and autocrine regulatory effects on each other and that these effects are mediated by VEGFR2 (in EC) and VEGFR1 (in SMC). Based on these results, we recommend that researchers investigating VEGFR signaling consider the use of coculture models when designing studies.

1 Introduction

Despite advances in pharmacological interventions, ischemic cardiovascular disease (CVD) remains a leading cause of death in the United States (Mozaffarian et al. 2015) and is likely to remain so with the increased incidence of metabolic syndrome and diabetes, which favor the development of ischemic CVD. The development of novel therapeutic agents for the treatment of ischemic CVD with increased efficacy requires (among other things) improved in vitro models of the vessel wall.

Vascular endothelial growth factor-A (VEGF-A) is the archetypal member of the VEGF family of growth factors and is a ligand for the tyrosine kinase VEGF receptors 1 and 2 (VEGFR1, VEGFR2) and neuropilin-1 (de Vries et al. 1992, Joukov et al. 1996, Terman et al. 1992, Soker et al. 1998). VEGF-A plays an important role in the development of the vasculature during embryogenesis and during both physiological and pathological vascular remodeling later in life (Roy, Bhardwaj and Yla-Herttuala 2006). Mice under-expressing VEGF-A exhibit a decrease in the number of preexisting collateral vessels, whereas mice overexpressing VEGF-A have a higher density of preexisting collateral vessels (Clayton, Chalothorn and Faber 2008). Adenoviral mediated overexpression of VEGF-A is associated with increased atherosclerotic plaque in apolipoprotein (apo) E knockout mice (Heinonen et al. 2013). Placental growth factor (PLGF) is another member of the VEGF family, which only signals through VEGFR1 (Maglione et al. 1991, Cao et al. 1997). PLGF expression is necessary for collateral remodeling, and mice lacking PLGF exhibit impaired reestablishment of flow following femoral ligation (Pipp et al. 2003). PLGF is chemotactic for monocytes, which express VEGFR1 but not VEGFR2 (Sawano et al. 2001). It is through the recruitment of monocytes
that PLGF stimulates collateral remodeling (Pipp et al. 2003). Consistent with PLGF's role in monocyte chemotaxis, mice deficient in both ApoE and PLGF have smaller atherosclerotic lesions with less macrophages compared to single knockout apoE mice. While adenoviral-mediated overexpression of PLGF induced increased neointimal thickening and macrophage accumulation in hypercholesterolemic rabbits (Khurana et al. 2005). Interestingly, PLGF binding to VEGFR1 elicits a downstream response distinct from the response that occurs when VEGF-A binds to VEGFR1 (Autiero et al. 2003b), and despite the similarity in the structure of the two cytokines, they are not interchangeable and exhibit different physiological effects (Pipp et al. 2003, Luttun et al. 2002, Autiero et al. 2003a, Autiero et al. 2003b). In clinical trials, therapies focusing on the administration of a single growth factor have fallen short of their intended goals (Schirmer et al. 2009). Vascular wall remodeling is a complex process involving multiple chemokines and cytokines, and there is a significant overlap in these pathways between physiological and pathological remodeling (Epstein et al. 2004). An understanding of the interactions between these factors is required to reveal the underlying mechanisms governing the remodeling process.

We previously reported that endothelial cells are the predominant source of PLGF in the vessel wall and that smooth muscle cells are the predominant source of VEGF-A (Xiang et al. 2014). Recently, we demonstrated that elevated fluid shear stress increases PLGF expression in an endothelial cell/vascular smooth muscle cell coculture model and intact vessels (Rashdan and Lloyd 2015) via a heme oxygenase-1 and iron-dependent pathway (Rashdan, Zhai and Lovern 2021). Importantly, coculturing endothelial cells with smooth muscle cells was necessary for these effects of fluid shear stress on PLGF to be observed in vitro, emphasizing the importance of cross talk between these cell types when modeling the vessel wall. In the present study, we sought to further characterize the influence of endothelial cell and smooth muscle cell interaction on the expression of PLGF and VEGF-A, as well as their receptors VEGFR1 and VEGFR2.

2 Materials And Methods

2.1 Reagents

Human recombinant VEGF-A and PLGF were purchased from R&D Systems (Minneapolis, MN). VEGF-E was purchased from Prospec (East Brunswick, NJ). The VEGFR2 tyrosine kinase inhibitor SU 1498 was purchased from Cayman Chemicals (Ann Arbor, MI). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless stated otherwise.

2.2 Cell culture

Primary human coronary artery endothelial cells (EC) and smooth muscle cells (SMC) were purchased from Lonza (Walkersville, MD). Cells were grown in 5% CO₂ in a humidified incubator at 37°C. SMC were grown in smooth muscle basal medium (SmBM, Lonza) supplemented with a smooth muscle growth medium kit (SmGm2, Lonza). The cell donors were a 56-year-old female and a 30-year-old male. EC were grown in endothelial basal medium (EBM, Lonza) supplemented with an endothelial growth medium kit
The cell donors were a 21-year-old male and a 30-year-old male. All experiments were carried out between passage 4 and 6.

Endothelial cell/smooth muscle cell cocultures were established as we previously described (Rashdan and Lloyd 2015). Briefly, Corning Costar (Corning, NY) 24 mm TRANSWELL® polyester membrane inserts with a pore size of 0.4 µM were inverted and coated with 0.1% gelatin diluted in growth medium. Inserts were incubated for 1 h at 37°C in a humidified incubator. Gelatin was aspirated off the insert and SMC were seeded onto the inverted inserts (~ 10^5 cells/insert) and incubated for 4 h in 5% CO₂ at 37°C in a humidified incubator. Inserts were then inverted into 6 well plates containing growth medium and incubated for 48 h, after which 0.1% gelatin diluted in growth medium was added to the apical side of the membrane and incubated as above for 1 h. The gelatin was then aspirated and EC (2x10^5 cells/insert) were seeded on the apical side of the insert and incubated as above for 24 h. Cocultures were subjectively checked for confluence using an inverted light microscope. All experiments (monoculture and coculture) were carried out at 80%-90% confluence. Prior to experiments, cells were serum-starved by replacing culture medium with reduced (2%) serum medium for 24 h. Reduced serum medium was made by diluting the corresponding medium in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Pittsburgh, PA).

2.3 Real-Time PCR

Cells were rinsed with 37°C PBS (Hyclone), then incubated in TrypLE Express (Gibco, Waltham, MA) for 5 min at 37°C. Following cell detachment, 2 volumes of complete medium were added to the cells and the cell suspension was centrifuged at 300g for 5 min. Total RNA was isolated from the pellet using RNeasy Mini columns (Qiagen, Valencia, CA) per manufacturer's instructions. The concentration and quality of the RNA was determined using a Take 3 plate in a Synergy HT multimode plate reader (BioTek, Winooski, VT). Both genomic DNA removal and cDNA synthesis were carried out using the QuantiTect reverse transcription kit (Qiagen). Real-time PCR reactions were carried out in an ABI 7500 Fast instrument (Applied Biosystems, Carlsbad, CA) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems, Gaithersburg, MD). Primers were designed using Primer Express software and custom-synthesized by Invitrogen (Carlsbad, CA). Primer sequences were as previously described (Xiang et al. 2014, Shaw et al. 2011). To avoid confounding effects of housekeeping gene selection when making comparisons between different cell types, PLGF and VEGF-A mRNA expression were normalized to the geometric average of the housekeeping genes β-actin and GAPDH and relative gene expression was quantified using the ΔΔCt method.

2.3 ELISA

Secreted PLGF and VEGF-A concentrations in culture media were determined using the human PLGF and VEGF-A DuoSet ELISA development kits (R&D Systems, Minneapolis, MN) according to manufacturer's protocol. Data were normalized to total protein concentration, as determined by BCA assay (Pierce, Rockford, IL). Samples were treated with protease inhibitor cocktail (1 mM PMSF, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM benzamidine-HCl, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A) before analysis.
2.4 Flow cytometry

To measure the cell surface expression of VEGFR1 and VEGFR2, cells were collected as above and resuspended in PBS containing 1% BSA, then incubated for 45 minutes at room temperature in the dark with a 1:500 dilution of both Dylight 650-conjugated rabbit anti-human VEGFR1 polyclonal antibody (NB100527, Novus Biologicals, San Diego, CA) and Dylight 488-conjugated rabbit anti-human VEGFR2 polyclonal antibody (NB1002382G, Novus Biologicals). After incubation, labeled cells were resuspended in 1% BSA-PBS and the mean fluorescence intensity (MFI) was determined by flow cytometry (Accuri C6, Ann Arbor, MI).

2.5 Transfection

Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) following manufacturer’s instructions. Five nM siRNA was precomplexed with Lipofectamine RNAiMAX for 20 min in Opti-MEM media (Gibco), after which 4 volumes of DMEM (Hyclone) containing 10% FBS (Gibco) were added, making complete transfection medium. siRNA consisting of the following sequences was used to knock down PLGF: sense, 5’-AGGUGGAAGUGGUACCCUU-3’, overhang dTdT; antisense, 5’-AAGGGUACCACUUCCACCU-3’, overhang, dCdT (Invitrogen). For VEGF-A knockdown, a predesigned siRNA was used (Silencer Select s460, Invitrogen). Silencer Select negative control no.1 siRNA (Invitrogen) was used as a negative control. Cells were seeded into 6 well plates and grown to 90% confluence, after which cells were rinsed with DMEM 10% FBS and the medium replaced with transfection medium. After 18 h in transfection medium, the cells were switched to 2% serum medium for a further 24 h. For cell-specific knockdown in cocultures, SMC were transfected directly on the insert prior to the addition of EC, whereas EC were transfected in a T75 flask then trypsinized and seeded onto the apical side of the insert. The effectiveness of the siRNA treatment was determined by real-time PCR, as outlined above.

2.6. Statistical analysis

All data are presented as mean ± SEM. Experiments were replicated at least five times. Data were analyzed by either student’s T-test, or one-way ANOVA followed by Tukey’s range test, as appropriate. The level of significance was set at p < 0.05.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 Results

Consistent with our previously reported results (Xiang et al. 2014), we found that PLGF was highly expressed in monocultured EC (38.38 ± 4.17 pg/µg total protein) but was expressed at much lower levels in monocultured SMC (below detection limit). Interestingly, coculture of EC and SMC significantly increased the PLGF protein concentration in media (63.78 ± 1.82 pg/µg total protein), compared to media from EC monocultures (Fig. 1A, p < 0.01). The opposite pattern was observed for VEGF-A, which was found at low levels in monocultured EC (5.28 ± 1.73 pg/µg total protein) but was highly expressed by
monocultured SMC (VEGF-A, 228.13 ± 9.57 pg/µg total protein). Additionally, the opposite effect of coculture was observed for VEGF-A, which was significantly lower in coculture media (38.90 ± 2.98 pg/µg total protein) compared to media from SMC monocultures (Fig. 1B, p < 0.001).

PLGF mRNA levels were consistent with the PLGF protein results described above and were significantly higher under coculture conditions in both EC (2.14 ± 0.26-fold of monoculture; p < 0.01; Fig. 1C) and SMC (1.78 ± 0.10-fold of monoculture, p < 0.01; Fig. 1D). However, VEGF mRNA levels were not consistent with the previously observed VEGF protein levels. Coculture significantly increased VEGF mRNA in EC (1.67 ± 0.22-fold of monoculture; p < 0.01; Fig. 1E) and had no effect on VEGF-A mRNA in SMC (Fig. 1F).

Next, we evaluated the expression of VEGFR1 and VEGFR2. In EC, coculture had no significant effect on VEGFR1 surface expression (Fig. 2A), but significantly increased surface VEGFR2 expression (Fig. 2B; 1.36 ± 0.07-fold MFI; p < 0.05). In contrast to this finding, coculture significantly decreased SMC VEGFR1 surface expression (Fig. 2C; 0.74 ± 0.02-fold MFI; p < 0.001), with no significant effect on surface VEGFR2 expression (Fig. 2D).

We hypothesized that SMC-derived VEGF-A could be a factor contributing to the increased production of PLGF by EC in cocultures, and vice versa. Therefore, we next tested the effect of supplementing EC monocultures with exogenous VEGF-A (100 ng/mL). Indeed, the mixed VEGFR1/VEGFR2 ligand VEGF-A significantly increased the concentration of PLGF in media of monocultured EC (1.86 ± 0.01-fold of control; p < 0.001) (Fig. 3A). Treatment of monocultured EC with the VEGFR2 specific ligand VEGF-E (100 ng/mL) also significantly increased secreted PLGF, but to a lesser extent (Fig. 3B; 1.30 ± 0.06-fold of control; p < 0.01). Treatment of monocultured SMC with PLGF, a VEGFR1 specific ligand (100 ng/mL) significantly reduced media VEGF-A (0.81 ± 0.04-fold of control; p < 0.01). In contrast, the VEGFR2 ligand VEGF-E (100 ng/mL) had no significant effect on SMC media VEGF-A (Fig. 3C).

We next tested the effect of knocking down either endogenous VEGF-A or PLGF on the level of the other cytokine. In EC monocultures, knockdown of VEGF-A significantly decreased media PLGF protein levels (0.54 ± 0.02-fold of control; p < 0.001; Fig. 4A). Additionally, supplementing media with VEGF-A (100 ng/mL) concurrently with VEGF knockdown rescued PLGF expression and led to a significant increase in media PLGF compared to control (1.28 ± 0.03-fold of control; p < 0.001; Fig. 4A). Interestingly, despite its effect on PLGF protein levels, knockdown of VEGF-A in monocultured EC had no significant effect on PLGF mRNA (Fig. 4B). VEGF-A knockdown was confirmed by real-time PCR (Fig. 4C).

In SMC monocultures, knockdown of PLGF significantly increased media VEGF-A, compared to control (1.63 ± 0.05-fold; p < 0.01; Fig. 5A). PLGF knockdown also strongly and significantly increased VEGF-A mRNA (3.84 ± 0.83-fold of control; p < 0.05; Fig. 5B). PLGF knockdown was confirmed by real time PCR (Fig. 5C).

To further assess the role of VEGFR2 in regulating PLGF and VEGF-A, we treated both monocultures and cocultures with the VEGFR2 specific tyrosine kinase inhibitor SU1498 (10 nM). Inhibition of VEGFR2 tyrosine kinase activity significantly decreased media PLGF protein in monocultured EC (Fig. 6A; 0.60 ±
VEGFR2 inhibition also decreased PLGF protein levels in cocultures (control coculture, 4.81 ± 0.22-fold of control EC monoculture; SU1498-treated coculture, 1.80 ± 0.09-fold of control EC monoculture). This result, in agreement with our other findings, suggests a positive role for VEGFR2 signaling in regulation of PLGF. PLGF was not detected in SMC monoculture media either with or without SU1498 treatment (Fig. 6A). Interestingly, SU1498 significantly increased media VEGF-A in monocultured EC (Fig. 6B; 3.15 ± 0.39-fold of control EC monoculture; p < 0.05), and to a lesser extent in cocultures. Consistent with the results of our experiments with VEGFR-selective ligands (which suggested that VEGFR1, but not VEGFR2, contributes to regulation of VEGF-A in SMC), SU1498 had no significant effect on VEGF-A in SMC monoculture media (Fig. 6B).

4 Discussion

Coculturing EC with SMC significantly increased PLGF protein in culture media (as compared to media from EC monocultures). EC produced much more PLGF than SMC, consistent with previous reports by our group (Rashdan and Lloyd 2015). Along with increased PLGF protein, coculture resulted in a significant increase in PLGF mRNA in both EC and SMC, suggesting that the increase in PLGF protein with coculture is at least partly due to increased gene transcription. These findings suggest that it is likely that PLGF production by endothelial cells in vivo is underestimated by in vitro studies of endothelial cell monocultures. Furthermore, PLGF levels may be reduced in vessels in which EC/SMC contact and/or communication is dysfunctional due to disease or injury, relative to expression in healthy quiescent vessels.

In contrast to its effects on PLGF, coculture of EC and SMC significantly reduced VEGF-A protein in media. This effect occurred without a significant effect of coculture on VEGF-A mRNA in SMC, and in the face of a significant increase in VEGF mRNA in EC. The most likely explanation for the reduction in VEGF-A protein in cocultures without a corresponding effect on VEGF-A mRNA in SMC (the primary source of VEGF-A in the coculture model) is that VEGF-A was rapidly removed from the media by endothelial cell VEGF receptor binding and uptake. Indeed, we previously demonstrated that VEGF-A decreases in the presence of endothelial cells (Xiang et al. 2014), while others have demonstrated active uptake (endocytosis) of VEGF-A by endothelial cells via VEGFR2 (Santos et al. 2007) and other pathways which lead to nuclear accumulation (Li and Keller 2000, Wang et al. 2002). In agreement with this possibility, we found that coculture increased EC cell surface VEGFR2 levels. Another mechanism that may contribute to the effects we observed is post-transcriptional regulation of VEGF expression. Coculturing endothelial cells and smooth muscle cells has been demonstrated to result in trafficking of miRNA from endothelial cells to smooth muscle cells via micro-particles (Zhou et al. 2013, Hergenreider et al. 2012). These miRNA include miR-145, which has been shown to inhibit VEGF translation (Fan et al. 2012). Although VEGF-A mRNA increased with coculture in EC, EC are a relatively minor source of VEGF-A in the coculture system and thus this effect on overall VEGF-A protein levels was negligible.

Exogenous VEGF-A has been shown to upregulate PLGF production in several primary endothelial cell lines (Zhao, Cai and Boulton 2004, Fujii et al. 2008). In the present study, we found that
exogenous VEGF-A increased PLGF production in monocultured EC. Conversely, knockdown of VEGF-A in EC monocultures resulted in a significant reduction in media PLGF. This is consistent with the *in vivo* reduction of PLGF expression that has been reported in mice treated with VEGF-A neutralizing antibodies (Fujii et al. 2008). Concurrent treatment of EC with exogenous VEGF-A and knockdown of VEGF-A not only rescued the reduction in media PLGF, but significantly increased media PLGF, demonstrating that the effects of the knockdown are directly due to a lack of VEGF-A.

Interestingly, we found that VEGF-E also increases PLGF production in monocultured EC. VEGF-E exclusively binds VEGFR2, while VEGF-A binds both VEGFR1 and VEGFR2. Therefore, these data suggest that VEGFR2 activation alone is sufficient to enhance PLGF expression. Similarly, it has previously been reported that blocking VEGFR2 *in vivo* attenuates the VEGF-A induced increase in PLGF (Zhao et al. 2004). In keeping with these results, our present study found that inhibiting VEGFR2 tyrosine kinase activity with the small molecule inhibitor SU1498 decreased PLGF protein expression in both EC monocultures and in cocultures. In contrast, VEGF-A protein expression was increased by SU1498 in both EC monocultures and in cocultures. We conclude that VEGF-A acts as a positive regulator of PLGF expression in EC via VEGFR2.

Treating SMC monocultures with exogenous PLGF resulted in a significant decrease in VEGF-A protein in culture media, while knockdown of PLGF in SMC significantly increased both VEGF-A protein and mRNA. However, the VEGFR2 specific ligand VEGF-E had no effect on SMC-produced VEGF-A, and no significant effect of VEGFR2 inhibition on PLGF or VEGF-A expression was observed in SMC. PLGF exclusively signals through VEGFR1 (Cao et al. 1997, Maglione et al. 1991), and we found SMC surface VEGFR1 to be significantly higher than VEGFR2 (not shown). Combined, these findings suggest that PLGF negatively regulates VEGF-A expression in SMC via a VEGFR1-dependent mechanism. This conclusion is supported by other reports in the literature. Homozygous VEGFR1 knockout is embryonically lethal in mice, and disorganized vasculature resulting from uncontrolled endothelial overgrowth is the primary defect causing lethality (Fong et al. 1995). Similarly, blocking VEGFR1 results in increased angiogenesis in adipose tissue (Xue et al. 2009) while infantile hemangiomas have markedly reduced expression of VEGFR1(Jinnin et al. 2008). Conversely, PLGF has been demonstrated to induce vascular normalization and decrease sprouting (Hedlund et al. 2009). Similarly, several studies have demonstrated inhibition of VEGF-A induced angiogenesis by PLGF (Cao 2009, Bjorndahl et al. 2004, Eriksson et al. 2002, Schomber et al. 2007, Xu et al. 2006). These observations suggest that PLGF/VEGFR1 signaling is a negative regulator of endothelial proliferation. Our findings suggest that one mechanism for this effect of PLGF is negative regulation of SMC VEGF-A expression via VEGFR1.

Our current study also showed that coculturing EC with SMC significantly increased VEGFR2 expression, compared to EC monocultures. Several studies have demonstrated the role of endothelial VEGFR2 in mechanosensing of fluid shear stress. VEGFR2 tyrosine kinase activity is required for fluid shear stress-induced activation of eNOS and vasodilation (Jin et al. 2003). Likewise, fluid shear stress increases VEGFR2 phosphorylation, and inhibiting VEGFR2 tyrosine kinase activity attenuates the antiapoptotic effects of fluid shear stress (dela Paz et al. 2012). Consistent with these findings, we previously reported
that coculturing EC with SMC results in an increase in sensitivity to fluid shear stress (Rashdan and Lloyd 2015). Taken together, these data suggest that accurate in vitro examination of the effects of flow on the vasculature requires a coculture system, since VEGFR2 signaling is a key component of the endothelial response to shear.

In contrast to the effect of coculturing cells to increase EC VEGFR2 expression, we observed decreased cell surface VEGFR1 expression in cocultured SMC, compared to monocultured SMC. Interestingly, this finding is consistent with results reported in models of vascular injury (which presents the opposite situation to coculture, in the sense that EC/SMC interactions are disrupted, with corresponding effects on VEGFR1). As an example, smooth muscle VEGFR1 expression is increased in rat carotid arteries following balloon injury (Orlandi et al. 2010). Likewise, wire injury of mouse carotid arteries increases smooth muscle VEGFR1 expression (Pruthi et al. 2014). Together, our data and these reports suggest that normal EC-SMC communication has an inhibitory effect on SMC VEGFR1 expression.

In conclusion, the results of this study demonstrate that crosstalk between vascular smooth muscle cells and vascular endothelial cells regulates the expression of both PLGF in EC (through VEGFR2) and VEGF-A in SMC (through VEGFR1) summarized in Fig. 7. These findings suggest that cocultures of these two cell types better recapitulate the signaling environment of stable quiescent vessels than do monocultures. Studies of endothelial cell biology may provide more meaningful results that better translate to clinical applications when the endothelial cells being studied are grown in coculture with smooth muscle cells. Therefore, the vascular coculture model is an essential tool in studying vascular physiology. Considering that the regulation of PLGF and VEGF-A has overarching implications for arteriogenesis, atherosclerosis, and tumor angiogenesis, greater insight into the molecular and cellular interactions of these factors in the vessel wall will contribute greatly to the understanding of these pathophysiological conditions.

Declarations

5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Author Contributions

P.C.L. supervised the project, N.A.R. and P.C.L. conceived and designed experiments and wrote the manuscript, N.A.R. performed experiments and analyzed the data. All authors read, discussed, and edited the manuscript.

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8. Acknowledgments

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Data Availability Statement

The datasets generated for this study are available from the corresponding author upon request.

References


Figures
Figure 1

PLGF and VEGF expression in EC and SMC monoculture and coculture. (A) PLGF protein is predominantly produced by EC and is significantly increased when EC and SMC are cocultured (n=5, *p<0.001 compared to EC monoculture). (B) VEGF-A protein is mostly produced by SMC, and is decreased when EC and SMC are cocultured (n=5, *p<0.001 compared to EC monoculture, †p<0.001 compared to SMC monoculture). Coculture significantly increased PLGF mRNA in both EC (C) and SMC (D). Coculture significantly increased VEGF-A mRNA in EC (E), but not SMC (F) (n=5, *p<0.001 compared to monoculture).
Figure 2

**VEGFR-1 and VEGFR-2 expression in EC and SMC monoculture and coculture.** (A) For VEGFR-1 no difference in MFI was detected between EC in monoculture and coculture. (B) MFI was significantly increased for VEGFR-2 in cocultured EC compare to monoculture EC. (C) SMC VEGFR-1 was significantly decreased in coculture compare to monoculture. (D) no significant change MFI was detected between SMC monoculture and coculture (n=5, *p<0.05 compared to monoculture)
Figure 3

Effect of exogenous VEGFR ligands on VEGF-A and PLGF production in monocultures. (A) The VEGFR-2 specific ligand VEGF-E (100 ng/mL) significantly increased PLGF protein in HCAEC culture media. The mixed VEGFR-1/VEGFR-2 ligand VEGF-A had no significant effect. (B) The VEGFR-1 ligand PLGF (100 ng/mL) significantly decreased VEGF-A protein in HCASMC culture media, whereas the VEGFR-2 ligand VEGF-E had no effect (n=5, * p<0.05).
Figure 4

**Effect of knockdown of VEGF-A in HCAEC monoculture**  (A) Knockdown of VEGF-A in HCAEC monoculture significantly decreased PLGF protein. Concurrent treatment with exogenous VEGF-A rescued PLGF protein expression and led to an increase relative to untreated cells. (B) In contrast to its effect on PLGF protein, knockdown of VEGF-A in HCAEC monocultures had no significant effect on PLGF mRNA expression. (C) siVEGF reduced VEGF-A mRNA levels to ~50% of control in HCAEC monocultures (n=5, *p<0.05 compared to both control and siSCR).
**Figure 5**

**Effect of knockdown of PLGF in HCASMC monoculture.** (A) Knockdown of PLGF in HCASMC monocultures increased VEGF-A protein in culture media. (B) siPLGF decreased PLGF mRNA to ~25% of control in HCASMC monocultures (n=5, *p<0.05 compared to both control and siSCR). (C) PLGF knockdown in HCASMC monoculture significantly increased VEGF-A mRNA (n=5, *p<0.05 compared to both control and siSCR).
**Figure 6**

**SU1498 inhibits PLGF expression and induces VEGF expression.** (A) SU1498 significantly reduced PLGF protein in the media of both EC monocultures and cocultures. PLGF was below the limit of detection for SMC monocultures. (B) VEGF protein was significantly increased in SU1498 treated EC and coculture culture media. No significant difference was observed in SMC monocultures (n=5, *p<0.05 compared to DMSO)
Figure 7

VEGF-A and PLGF have opposing regulatory effects on each other. Our data suggest SMC derived VEGF-A is a positive regulator EC derived PLGF, while PLGF itself is a negative regulator of SMC VEGF-A. Figure created with BioRender