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Effects of vascular endothelial growth factor on endothelin-1 production by human lung microvascular endothelial cells in vitro

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ABSTRACT

Aims: Increased endothelin-1 (ET-1) is a hallmark of pulmonary arterial hypertension (PAH), and contributes to its pathogenesis. The factors controlling ET-1 in PAH are poorly understood. Combined with other stimuli, vascular endothelial growth factor (VEGF) blockade results in PAH-like lesions in animal models, and has been associated with PAH in humans. The effects of VEGF on ET-1 production by human lung blood microvascular endothelial cells (HMVEC-LBl) are unknown.
Main methods: We exposed HMVEC-LBl in-vitro to human VEGF-121 (40 ng/mL) in serum-free medium for 7 h, in the absence or presence of the VEGF receptor antagonist, SU5416 (3 and 10 μM). ET-1 production was measured in the supernatant. Phosphorylation of VEGF receptor 2 (VEGFR2) was measured by Western blotting after exposure to VEGF without or with SU5416 for 5 and 10 min.
Key findings: VEGF effectively caused VEGFR2 phosphorylation, which was blocked by SU5416. VEGF decreased ET-1 production by at least 29%. In the absence of VEGF, SU5416 increased ET-1 production, by 16% at 10 μM, and SU5416 was able to completely abolish the VEGF effect on ET-1 production.
Significance: VEGF may promote vascular health by decreasing ET-1 production in HVMEC-LBl. Blockade of VEGF signaling by SU5416 increases ET-1 levels. The role of VEGF in modulating endothelin production in PAH deserves further study.

Introduction

The pathogenesis of pulmonary arterial hypertension (PAH) involves microvascular endothelial proliferation and dysfunction, with formation of plexiform lesions (Morrell et al., 2009; Tuder et al., 2009). One manifestation of this dysfunction is increased production of the deleterious vasoconstrictor and mitogen, endothelin-1 (ET-1) (Giaid et al., 1993; Stewart et al., 1991). It has been proposed that in PAH a variety of injurious stimuli result in endothelial apoptosis, with emergence of apoptosis-resistant endothelial clones that narrow the vascular lumen, restricting blood flow and ultimately causing right heart failure and death (Teichert-Kuliszewska et al., 2006; Tuder et al., 2001a).

Vascular endothelial growth factor (VEGF) is important in endothelial homeostasis, controlling differentiation, mitogenicity and endothelial survival (Ferrara, 2004). VEGF increases nitric oxide and prostacyclin production. Loss of VEGF signaling results in endothelial apoptosis. However, the role of VEGF in PAH is complex. Levels of VEGF and its receptors are increased in PAH (Hirose et al., 2000; Tuder et al., 2001a). Confounding the issue is the finding that VEGF blockade in animals, combined with other stimuli such as hypoxia, causes pulmonary hypertension and has provided a useful model of PAH (Taraseviciene-Stewart et al., 2002; Tuder et al., 2001b). Loss of VEGF might contribute to the endothelial apoptosis that is thought to be an initiating event in PAH (Teichert-Kuliszewska et al., 2006). Indeed, PAH has been reported in humans after treatment with bevacizumab, a VEGF blocker (Liotta et al., 2009). Thus, VEGF may play a role in the pathogenesis of PAH.

The stimuli for increased ET-1 in PAH are unknown. It is not known how ET-1 synthesis by pulmonary microvascular endothelium is affected by VEGF. We therefore explored the effects of VEGF on ET-1 production. The bone morphogenic protein (BMP) and transforming growth factor-β (TGFβ) receptor pathways are highly involved in the development of heritable PAH (Morrell et al., 2009). BMP-9 is a strong activator of endothelial activin receptor-like kinase 1 (David et al., 2007). We have previously demonstrated that BMP-9 stimulates ET-1 production by lung microvascular endothelial cells in vitro (Star et al., 2010). We

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therefore also studied the interactions of BMP9 and VEGF, in terms of effects on ET-1 production.

Materials and methods

Cell cultures

Human microvascular endothelial cells of the lung-blood (HMVEC-LBl, Lonza, Walkersville, MD) or human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD) were plated into 24-well plates and grown to confluence in EGM-2MV medium (Lonza) containing 2.5% fetal bovine serum (FBS) plus supplements. Only cells in passage 5 were used, with n = 12 wells per experimental condition.

Experimental design

To confirm in HMVEC-LBl the appropriate signaling of VEGF via its receptor, phosphorylation of the VEGF receptor 2 (VEGFR2) was measured by Western blotting after exposure to VEGF-121 (0 or 40 ng/mL, Peprotech, Rocky Hill, NJ) in serum-free medium with 0.1% bovine serum albumin (BSA), with or without the VEGF receptor antagonists, SU5416 (3 μM, CID 5329098, Cayman Chemical, Ann Arbor, Michigan) or (E)-FeCP-oxindole (1 μM, CID 57369962, Tocris, Minneapolis, MN), for 5 and 10 min. The cells were then immediately lysed in SDS sample buffer. The cell lysates were separated on a polyacrylamide gel and then transferred to a PVDF membrane. Afterwards, the samples were blocked in 5% fat free milk in TBST for 1 h. The membranes were subsequently incubated overnight at 4 °C in TBST with 5% BSA containing antibodies to either rabbit anti-P-VEGFR2 (Cell Signaling, Danvers, MA) or the housekeeping gene mouse anti-GAPDH (Fitzgerald, Concord, MA). All membranes were then washed in TBST and incubated with anti-rabbit (Cell Signaling, Danvers, MA) or anti-mouse (Abcam, Cambridge, UK) antibodies. After a final washing the membranes were incubated with Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA) and exposed to film.

To study the effects on ET-1 production and to search for a dose response, after confluence of HMVEC-LBl or HUVEC, the medium was replaced with medium containing VEGF-121 (0–40 ng/mL) in serum-free medium for 7 h. The supernatants were collected at the end of the study period, and frozen at −70 °C. To examine interactions between VEGF and BMP9 with reference to ET-1 production, the HMVEC-LBl and HUVEC were exposed to VEGF-121 (40 ng/mL) and/or BMP9 (2.5 ng/mL, R&D Systems, Minneapolis, MN) in serum-free medium for 7 h. The supernatants were then collected and frozen as above. In another experiment, HMVEC-LBl were exposed to VEGF-121 (40 ng/mL) in serum-free medium for 7 h, with or without the VEGF receptor antagonist SU5416 (0–10 μM). The supernatant was collected and frozen. In all these experiments, the supernatant was subsequently thawed and immunoreactive ET-1 levels were measured by ELISA (Enzo Life Sciences, Ann Arbor, Michigan) and expressed as pg/mL.

Statistics

Data are expressed as mean ± SD. To compare groups, analysis-of-variance was performed and followed, where appropriate by the Tukey–Kramer multiple comparison test.

Results

Effects of VEGF and its blockers on VEGF receptor phosphorylation (Fig. 1)

Using Western blotting, in the HMVEC-LBl and in serum-free medium, there was no evidence of background VEGF receptor-2 phosphorylation. Addition of the VEGF receptor blockers, (E)-FeCP-oxindole and SU5416, did not affect the background phosphorylation.

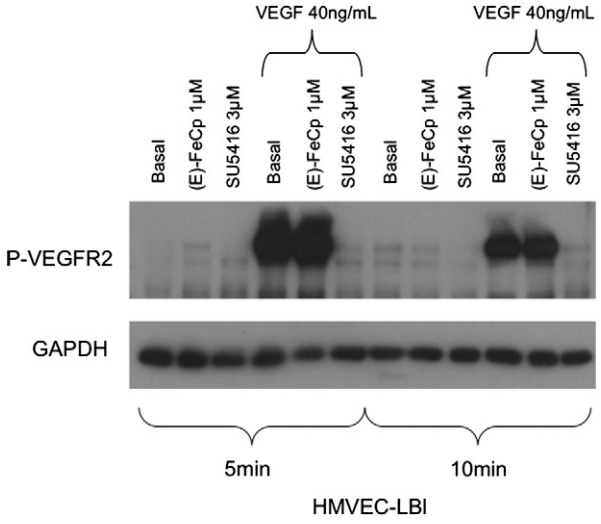

Fig. 1. Western blot showing levels of phosphorylated VEGF receptor-2 (P-VEGFR2), in HMVEC-LBl in basal medium and after 5 and 10 minute exposure to VEGF (40 ng/mL). The VEGF receptor blockers (E)-FeCP-oxindole and SU5416 were added in the indicated lanes. GAPDH is used as a loading control.

Addition of VEGF caused rapid phosphorylation as detected after 5 min, and this was sustained although slightly diminished at 10 min. Attempted blockade with (E)-FeCP-oxindole at the manufacturer's recommended dose had no effect on the phosphorylation. However, SU5416 blocked the effects of VEGF, completely preventing VEGFR2 phosphorylation.

Effects of VEGF on ET-1 production (Fig. 2)

In both HMVEC-LBl and HUVEC, exposure to VEGF significantly decreased ET-1 production, as assessed by ET-1 levels in the supernatant, after 7 h. This reduction occurred in a concentration-dependent manner, with the greatest effect in each case seen at VEGF 40 ng/mL. At that concentration, ET-1 levels were decreased by 63% in HMVEC-LBl and by 48% in HUVEC. Within the range studied, the dose response was more evident in HMVEC-LBl.

Effects of VEGF receptor blockade on ET-1 production (Fig. 3)

In HMVEC-LBl not exposed to VEGF, and in serum-free medium, addition of the VEGF receptor blocker, SU5416, significantly increased basal ET-1 production, by up to 21% at 3 ng/mL, $p < 0.02$. Addition of VEGF alone significantly decreased ET-1 production by 29% versus no VEGF, $p < 0.02$. In the presence of VEGF, addition of SU5416 completely blocked the inhibitory effects of VEGF on ET-1 production, with an increase in ET-1 levels of 82% as compared to VEGF alone. Furthermore, in the presence of SU5416, the ET-1 levels even surpassed those of the baseline control (no VEGF) by 30%, $p < 0.02$. At 3 ng/mL of SU5416, the ET-1 levels in the presence of VEGF were also slightly higher than those with SU5416 (3 ng/mL) in the absence of VEGF, $p < 0.02$.

Effects of VEGF and BMP-9 on ET-1 production (Fig. 4)

In HMVEC-LBl, VEGF (40 ng/mL) significantly reduced ET-1 production by 29%. As has been previously described, addition of BMP-9 (2.5 ng/mL) greatly stimulated ET-1 production, by 81% over baseline. Combination of VEGF and BMP-9 resulted in lower ET-1 levels as compared to BMP-9 alone, but they were still 35% higher than baseline and 90% higher than with VEGF alone. A similar pattern was observed when HUVEC were studied.

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