Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth

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Sakao, Seiichiro, Laimute Taraseviciene-Stewart, Kathy Wood, Carlyne D. Cool, and Norbert F. Voelkel. Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth. Am J Physiol Lung Cell Mol Physiol 291: L362–L368, 2006.—We have previously hypothesized that the development of severe angioproliferative pulmonary hypertension is associated with not only initial endothelial cell (EC) apoptosis followed by the emergence of apoptosis-resistant proliferating EC but also with proliferation of vascular smooth muscle cells (VSMC). We have demonstrated that EC death results in the selection of an apoptosis-resistant, proliferating, and phenotypically altered EC phenotype. We postulate here that the initial apoptosis of EC induces the release of mediators that cause VSMC proliferation. We cultured EC in an artificial capillary CellMax system designed to simulate the highly efficient functions of the human capillary system. We induced apoptosis of microvascular EC using shear stress and the combined VEGF receptor (VEGFR-1 and -2) inhibitor SU-5416. Flow cytometry for the proliferation marker bromodeoxyuridine showed that serum-free medium conditioned by apoptosed EC induced proliferation of VSMC, whereas serum-free medium conditioned by nonapoptosed EC did not. We also show that medium conditioned by apoptosed EC is characterized by increased concentrations of transforming growth factor (TGF)-β1 and VEGF compared with medium conditioned by nonapoptosed EC and that TGF-β1 blockade prevented the proliferation of cultured VSMC. In conclusion, EC death induced by high shear stress and VEGFR1/2 blockade leads to the production of factors, in particular TGF-β1, that activate VSMC proliferation.

shear stress; vascular endothelial growth factor receptor; blockade; pulmonary microvascular endothelial cells

SEVERE PULMONARY HYPERTENSION is characterized by complex precapillary arteriolar lesions (2, 10, 28, 29) that contain phenotypically altered endothelial and smooth muscle cells (2). Histologically, the remodeled pulmonary arteries show various degrees of medial hypertrophy and endothelial cell (EC) growth, which ultimately lead to the obliteration of many of the precapillary arteries (29). Hyperplasia of pulmonary artery smooth muscle cells is a prominent feature of the so-called pulmonary vascular remodeling (20, 29, 30), and the initiation of myointimal thickening is thought to be caused by migration and accumulation of vascular smooth muscle cells (VSMC) within the intima. Interestingly, acquisition of resistance to apoptosis and increased rates of proliferation of VSMC appear to be necessary for neointima formation (3, 12, 14, 19, 23). Recently, it has been reported that VSMC express VEGF receptor (VEGFR)-1, VEGFR-2, and neuropilin-1 and that VEGF stimulation resulted in phosphorylation of VEGFR-1 (13). VEGF can also have a chemotactic effect on VSMC (11). Recent findings by Parenti et al. (17, 18) have demonstrated that autocrine production of VEGF-A can directly promote VSMC proliferation and migration. Together, these findings suggest that secreted VEGF can have differential effects on EC and VSMC.

We have previously hypothesized that the development of severe angioproliferative pulmonary hypertension may be caused by a two-step process: initial EC apoptosis followed by the emergence of apoptosis-resistant proliferating EC (21, 24). We have demonstrated that EC death results in the selection of an apoptosis-resistant, proliferating, and phenotypically altered EC phenotype (21) and postulate here that the initial apoptosis of EC also induces the release of mediators that cause VSMC proliferation. In the experiments considered here, we induced apoptosis of pulmonary microvascular EC by VEGFR1/2 blockade using the combined VEGFR-1 and -2 blocker SU-5416 in the CellMax artificial capillary system (21). The CellMax is a unique artificial capillary system designed to simulate the highly efficient, three-dimensional function of the human capillary system. The CellMax system houses viable cells and cell products. In the CellMax, cells thrive on and around a large surface provided by a network of artificial capillaries. Perfused with culture medium, these capillaries supply oxygen and nutrients to the cells while they carry away metabolic waste products and permit the accumulation of cell-secreted growth factors for optimal growth conditions.

To test whether apoptosed EC release mediators that cause VSMC proliferation, VSMC were incubated in serum-free medium conditioned by EC treated with SU-5416 in the CellMax. The aim of this study was to examine whether growth factors for VSMC were released into the medium conditioned by apoptosed EC. The importance of our studies lies in the in vitro demonstration that the death of pulmonary vascular EC can in principle affect VSMC growth. It has been well accepted that EC release vasoactivities that act on the adjacent contractile cells. Here, we provide another example of EC-VSMC interactions and show for the first time that EC-derived VEGF inhibits apoptosis of VSMC.

MATERIALS AND METHODS

Cell lines and reagents. Human pulmonary microvascular EC (HPMVEC) were from Clonetics (Baltimore, MD). Rat pulmonary...
arterial smooth muscle cells (RPASMC) were a gift from S. Walchak from the Cardiovascular Pulmonary Research Laboratory at the University of Colorado Health Sciences Center in Denver, CO. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Because both HPMVEC and RPASMC can be grown in the same culture medium, whereas human VSMC require a different, special medium, we decided to use rat and not human VSMC. The VEGFR antagonist SU-5416 was provided by SUGEN (South San Francisco, CA). The Vybrant Apoptosis Assay Kit was from Molecular Probes (Eugene, OR). The Bromodeoxyuridine (BrdU) Flow Kit was from BD Biosciences Pharmingen (San Diego, CA). The monoclonal transforming growth factor (TGF-β) neutralizing antibody and the monoclonal VEGF neutralizing antibody were from Sigma-Aldrich (St. Louis, MO).

**Pulsatile flow system.** HPMVEC (5 × 10⁵) were inoculated into the CellMax artificial capillary modules (FiberCell, Frederick, MD) and kept in a CO₂ incubator at 37°C. The CellMax Quad System used in this study can simultaneously operate up to four independent cultures at different flow rates. Every module contained 1% gelatin-coated, perfusable polyethylene capillaries, each with an internal diameter of 0.7 mm, length of 13 cm, pore size of 0.1 μm, and total module lumen surface area of 70 cm². The module and a reservoir (50 ml) were connected by silicone tubing. The culture medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) was circulated using a pump between the artificial capillary module and the reservoir at a desired flow rate. Shear stress (SS; dyn/cm²) was calculated using a pump between the artificial capillary module and the reservoir at a desired flow rate. Shear stress (SS; dyn/cm²) was calculated as 4Q/πr³, in which Q is flow rate within each capillary, η is viscosity of the culture medium (0.7 × 10⁻² Poise), and r is the fixed radius of the artificial capillary. Two distinct patterns of SS were generated using the CellMax system: steady flow of 1.9 dyn/cm², corresponding to pulmonary small artery-like flow pattern, and high SS of 10.9 dyn/cm², corresponding to flow in partially occluded pulmonary precapillary blood vessels. Initially, the HPMVEC were grown at a flow rate that provided a SS averaging 1.9 dyn/cm² in each capillary to adapt the cells to pulsatile flow conditions and to supply a continuous supply of CO₂/O₂. Subsequently, high SS of 10.9 dyn/cm² was applied at days 3–7 after inoculation of the cells. On day 7 after inoculation, the cells were incubated with medium alone or medium plus the VEGFR blocker SU-5416 for 24 h.

**Conditioned medium.** Twenty-four hours after SU-5416 addition or not, HPMVEC in the CellMax artificial capillary modules were washed three times using PBS and were incubated with serum-free medium for 48 h. After the incubation period, the medium was collected as the conditioned medium in the CellMax system.

The conditioned medium from the static HPMVEC cultures was generated by seeding HPMVEC at 5 × 10⁵ density in 75-cm² flasks; 24 h later, cells were washed three times using PBS and incubated with serum-free medium for 48 h. Then, medium was collected as conditioned medium from the culture dishes and was used for “no SS control” experiments.

**RPASMC in the conditioned medium.** RPASMC were seeded in 6-cm dishes at 1 × 10⁵ density and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. After 24 h, cultures were washed three times with PBS and incubated with serum-free medium for 48 h. Then, medium was collected as conditioned medium from the culture dishes and was used for “no SS control” experiments.

**Annexin V-propidium iodide binding assay.** The rate of apoptotic cells was detected in RPASMC by the Vybrant Apoptosis Assay Kit according to the manufacturer’s protocol. The cells were harvested and washed in cold PBS. The washed cells were centrifuged, and the supernatants were discarded. The cells were resuspended in annexin-binding buffer. After assessment of cell density, the cells were diluted in annexin-binding buffer to ~1 × 10⁶ cells/ml. Five microliters of FITC-annexin V and 1 μl of the 100 μg/ml propidium iodide working solution were added to each 100 μl of cell suspension. The cells were incubated for 15 min at room temperature. After the incubation period, 400 μl of annexin-binding buffer were added to each sample. After buffer addition, the samples were gently mixed and kept on ice. The stained cells were analyzed by flow cytometry, and the fluorescence emissions were measured at a wavelength = 530 nm (e.g., FL1) and a wavelength > 575 nm (e.g., FL3).

**BrdU-7-amino-actinomycin D binding assay.** The rate of DNA synthesis was detected in RPASMC by the BrdU Flow Kit according to the manufacturer’s protocol. Cells were incubated in the conditioned medium for 24 h, harvested, and washed in cold PBS. During the final 16 h of culture, BrdU was added to cartridges; cells were then fixed and permeabilized, and the DNA was denatured by treatment with fixative/denaturing solution. The detector fluorochrome-anti-BrdU monoclonal antibody (BD Biosciences) and 7-amino-actinomycin D (BD Biosciences), a fluorescent dye for labeling DNA, were then used for flow cytometric analysis. The cell-incorporated BrdU and total DNA content were analyzed using a flow cytometer.

**Cell proliferation assay.** RPASMC were plated on 96-well plates at 3 × 10⁴ cells/well and grown overnight. The next day, medium was replaced with conditioned medium plus or minus BQ-123 [endothelin (ET) receptor A blocker] or BQ-788 (ET receptor B blocker) or losartan (angiotensin receptor antagonist). After 24 h, the cells were washed three times with cold PBS, and the plates were stored at ~80°C for the cell proliferation assay. Cell proliferation was assessed using the CyQuant Cell Proliferation Assay Kit (Molecular Probes). Fluorescence measurements were made using a microplate reader Victor 1420 (Perkin Elmer, Boston, MA) with excitation at 480 nm and emission detection at 520 nm as described previously (9).

**ELISA.** TGF-β1 and VEGF were measured by sandwich ELISA techniques by ELISA Tech (Aurora, CO) utilizing reagents from R&D Systems (Minneapolis, MN). The samples were read in a spectrophotometer at 405 nm.

**Quantitative RT-PCR.** Quantitative RT-PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed to meet specific criteria by using Primer Express version 1.0 software (Applied Biosystems). Total RNA was extracted from the dish (no SS) and the CellMax artificial capillary modules on day 0 (high SS) and day 7 (high SS + SU-5416; see legend in Ref. 18) using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. One microgram of RNA was reverse transcribed using random primer and Multiscribe RT (High-Capacity cDNA Archive Kit; Applied Biosystems). Assay-on-demand gene expression probes for TGF-β1, VEGF, and β-actin were purchased from Applied Biosystems. PCRs were performed in 20-μl volumes containing 9 μl of cDNA, 10 μl of TaqMan Master Mix (Applied Biosystems), and 1 μl of assay-on-demand primer and probe. PCR amplifications (final volume 25 μl) were run in duplicate using the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers were used at a concentration of 200 nM in each reaction. Relative quantitation of gene expression was determined using GeneAmp 5700 SDS software (Applied Biosystems).

**Statistical analysis.** Three independent experiments were performed and subjected to statistical analysis. The results are expressed as means ± SE. Data were analyzed using Student’s t-test. P < 0.05 was considered significant for all comparisons.

**RESULTS** RPASMC proliferation is stimulated by HPMVEC-conditioned medium. Twenty-four hours after incubation in the conditioned medium, the proliferative state of the RPASMC was assessed microscopically. Conditioned medium from HPMVEC subjected to VEGFR SU-5416 blockade and high SS induced robust RPASMC proliferation (Fig. 1C) compared with
conditioned medium from cells without SU-5416 treatment (Fig. 1B) or serum-free medium alone (Fig. 1A).

**Gene and protein expression of TGF-β1.** Because TGF-β1 promotes VSMC (RPASMC) proliferation (4) and because of the observed hyperproliferation of RPASMC in the serum-free medium conditioned by HPMVEC in the CellMax system, gene expression of TGF-β1 in HPMVEC was measured by quantitative RT-PCR. The combination of SS plus VEGFR blockade induced higher TGF-β1 gene expression level when compared with high SS alone or no SS (Fig. 2A), suggesting that the gene expression of TGF-β1 was SS and VEGFR blockade dependent. Because HPMVEC secrete TGF-β1, the protein levels in the conditioned media were measured by ELISA. As shown in Fig. 2B, conditioned medium from the cells exposed to high SS and SU-5416 contained almost twofold higher TGF-β1 levels than the conditioned medium with SS alone or no SS (Fig. 2B).

**Gene and protein expression of VEGF.** VEGF is an obligatory survival factor for EC. Because HPMVEC treated with SU-5416 under high SS were shown to be hyperproliferative (21), gene expression of VEGF in HPMVEC and protein levels of VEGF in the conditioned medium were measured by quantitative RT-PCR and ELISA. SS and the combination of SS plus VEGFR blockade increased VEGF gene expression (Fig. 3A). HPMVEC conditioned medium contained VEGF, suggesting that VEGF was secreted by the HPMVEC. SS increased VEGF protein secretion, and the combination of VEGFR blockade plus SS increased VEGF protein secretion 1.5-fold more than SS alone (Fig. 3B).

**Quantitation of proliferating and apoptotic RPASMC.** To examine whether the initial apoptosis of HPMVEC induces the release of mediators that cause RPASMC proliferation, the RPASMC were counted after 24 h of incubation in the medium conditioned by HPMVEC with or without SU-5416. The numbers of RPASMC in the medium conditioned by HPMVEC with SU-5416 at high SS conditions were higher than in the medium conditioned by HPMVEC without SU-5416 in the culture dish or in the CellMax system or in fresh serum-free medium (Fig. 4A). The number of proliferating and apoptotic cells was assessed by flow cytometry. Cells that have synthesized DNA can be identified by immunofluorescent staining of incorporated BrdU and flow cytometric analysis. BrdU, an analog of the DNA precursor thymidine, is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle. We found a greater increase in the number of BrdU-positive RPASMC in the medium conditioned by HPMVEC with SU-5416 and high SS than in the medium without SU-5416 in the culture dish or high SS alone or in the fresh serum-free medium (Fig. 4B).

Apoptosis of RPASMC was assessed by annexin V staining. Annexin V is a 35- to 36-kDa Ca²⁺-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS). Annexin V labeled with a fluorophore can identify apoptotic...
cells by binding to PS exposed on the outer cell membrane leaflet. The number of annexin V-positive RPASMC was decreased in the medium conditioned by HPMVEC with and without SU-5416, independent of the SS (Fig. 4C). In contrast, in fresh serum-free medium, an increased number of apoptotic cells was observed, suggesting that the factors secreted by EC have a protective effect on RPASMC apoptosis (Fig. 4C).

**TGF-β1, but not VEGF, induces proliferation of RPASMC.** Because the protein levels of TGF-β1 (Fig. 2B) and VEGF (Fig. 3B) were increased in the medium conditioned by HPMVEC with SU-5416 under high SS conditions, we postulated that TGF-β1 or VEGF were the mediators that caused RPASMC proliferation. To examine this hypothesis, we incubated RPASMC with either TGF-β1 or VEGF neutralizing antibody in the medium conditioned by HPMVEC with SU-5416 under high-SS conditions. Figure 4 demonstrates that TGF-β1, but not VEGF, was the mediator of RPASMC proliferation in the medium conditioned by HPMVEC with SU-5416 under high-SS conditions because the effect was blocked by the TGF-β1, but not VEGF, neutralizing antibodies (Fig. 4, A and B).

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**Fig. 2.** Transforming growth factor (TGF)-β1 gene expression in HPMVEC and TGF-β1 protein levels in CM. A: TGF-β1 gene expression in HPMVEC cultivated under different SS conditions as assessed by quantitative RT-PCR. There is a significant difference between the no SS mode and the HSS + SU mode ($P < 0.05$; $n = 3$). B: protein levels of TGF-β1 in CM as measured by ELISA. TGF-β1 protein levels were significantly ($P < 0.05$) higher in HSS + SU CM compared with no SS or no SU medium. SFM, serum-free medium.

**Fig. 3.** VEGF gene expression in HPMVEC and VEGF protein levels in CM. A: VEGF gene expression in HPMVEC cultivated under different SS conditions as assessed by quantitative RT-PCR. There is a significant difference between the no SS mode and the HSS + SU-5416 mode ($P < 0.05$; $n = 3$). B: protein levels of VEGF in CM as measured by ELISA. The difference of VEGF levels in HSS CM ($n = 3$) and HSS + SU-5416 CM ($n = 3$) was significant ($P < 0.05$) compared with no SS or SFM.
VEGF, but not TGF-β1, is responsible for RPASMC apoptosis. To determine whether factors in the conditioned medium can have an antiapoptotic effect on smooth muscle cells, apoptosis was assessed by flow cytometry. As shown in Fig. 4C, conditioned medium from either static cultures or from high SS plus or minus SU-5416 cultures had a protective effect on RPASMC, reducing apoptotic events by 50%. Treatment with neutralizing anti-TGF-β1 antibody did not have an effect on RPASMC apoptosis. In contrast, treatment with neutralizing anti-VEGF antibody induced RPASMC apoptosis to the same level as observed in fresh serum-free medium (Fig. 4C), suggesting that VEGF, but not TGF-β1, plays a protective role against RPASMC apoptosis.

DISCUSSION

Over the last years, our knowledge of the cellular composition of the complex pulmonary vascular lesions that characterize the lung vascular abnormalities in severe pulmonary hypertension has grown considerably. Tuder et al. (27) used immunohistochemistry to demonstrate “exuberant” EC proliferation in the so-called plexiform lesions in the lungs from patients with severe pulmonary hypertension, with the presence of inflammatory cells, and with the expression of angiogenesis factors (25). In addition to angioobliterative lesions, muscularization of precapillary arterioles can be prominently observed in the lungs from patients with severe pulmonary hypertension. In fact, in pulmonary hypertension associated with idiopathic pulmonary fibrosis, impressive muscularization of the arterioles without lumen obliteration is the exclusive manifestation of lung vessel involvement (26). Whereas cell proliferation and growth factor involvement are concepts that have now been increasingly accepted and used to explain pulmonary vascular remodeling, the debate about the origin of the cells involved and the mechanisms underlying cell proliferation and pulmonary vascular muscularization continues (5, 6, 28). Here, we model in vitro a cell-cell interaction that could occur in vivo in the pulmonary arterioles after HPMVEC injury. We show that the medium conditioned by stressed and dying HPMVEC contains factors that promote RPASMC growth and inhibit RPASMC apoptosis (Fig. 5).

The serum-free medium conditioned by HPMVEC subjected to fluid SS, either treated with or without SU-5416 in the high SS (CellMax system), caused proliferation of RPASMC (Fig. 1). This indicated that it was the SS that caused HPMVEC to release factors that activate RPASMC proliferation, and this interpretation is supported by the results that show an effect of shear on the expression of the TGF-β1 and VEGF genes (Figs. 2, A and B, and 3, A and B). However, the combination of SU-5416 treatment and SS activated RPASMC proliferation more than SS alone (Fig. 1). Thus conditioned medium from HPMVEC apoptosed by SU-5416 caused a greater production of factors that activate RPASMC proliferation (Figs. 2, A and B, and 3, A and B). Apoptosis, in contrast to necrosis, of EC has recently been shown to stimulate VEGF and TGF-β production (9). For this reason, we believe that the growth signals for

Fig. 4. Quantitation of proliferating and apoptotic RPASMC. A: proliferation was assessed by cell count. Depicted is the RPASMC after 24 h of incubation in fresh SFM, no SS (dish condition), HSS, HSS + SU-5416, HSS + SU-5416 + TGF-β1-neutralizing antibodies (αTGF), and HSS + SU-5416 + VEGF-neutralizing antibodies (αVEGF). The addition of neutralizing TGF-β1 antibodies to HSS + SU-5416 significantly decreased RPASMC (*P < 0.05). In contrast, the addition of VEGF-neutralizing antibodies had no statistically significant effect on the RPASMC number. B: the number of proliferating cells was assessed by flow cytometry utilizing bromodeoxyuridine (BrdU) incorporation. The experimental conditions are the same as described for A. Flow cytometric analysis also revealed an inhibitory effect of neutralizing TGF-β1 antibodies on RPASMC proliferation (P < 0.05). C: the number of apoptotic cells was assessed by flow cytometry. Apoptosis was assessed by annexin V staining. The difference in the number of the apoptotic cells was statistically significant (*P < 0.05) when conditioned medium experiments with and without added VEGF antibodies were compared, suggesting that the addition of neutralizing VEGF antibodies to the CM prevented the reduction of apoptosis observed after addition of the CM. N = 3.
VSMC are being sent by apoptotic cells. Furthermore, we have recently demonstrated (22) that the addition of apoptotic EC was sufficient to increase the proliferation of the EC in the artificial capillary CellMax system (high-SS condition), which had not been challenged by VEGFR blockade (SU-5416).

The protein expression of TGF-β1 in the medium conditioned by HPMVEC without SU-5416 in the culture dish was comparable to that found under SS conditions without SU-5416 treatment (Fig. 2B). Although the total cell number per surface area was normalized at the time of seeding the cells, it may not be entirely meaningful to make this comparison since the unsheared dish and the artificial vessel system are different systems. HPMVEC treated with SU-5416 under SS released factors that caused RPASMC proliferation (Fig. 4, A and B), suggesting that apoptosis of HPMVEC induced by SU-5416 leads to the production of factors that activate RPASMC proliferation. We postulated that TGF-β1 was one of the mediators whose expression was induced by the initial apoptosis of HPMVEC and subsequently caused RPASMC proliferation since the effect of the conditioned medium on RPASMC growth was abolished by TGF-β1-neutralizing antibodies (Fig. 4, A and B). However, there was slightly more TGF-β1 produced by EC with no SS compared with cells with SS (Fig. 2B), yet while there was no significant effect of this conditioned medium on smooth muscle cell growth, there was a significant effect on smooth muscle cell proliferation by the conditioned medium retrieved from EC exposed to SS (Fig. 4A). This suggests that there might be other proliferation-inducing growth factors induced by SS and related to TGF-β1.

HPMVEC conditioned medium also caused a reduction of the apoptosis rate of RPASMC (Fig. 4C). The apoptosis-inhibiting factor (or factors) was (were) released into the medium conditioned by HPMVEC not only with SU-5416 in the CellMax system but also without SU-5416 in the culture dish. This indicated that the production of these factors was unrelated to the apoptosis of HPMVEC induced by SU-5416. We postulate that VEGF was one of these factors that caused reduction of the apoptosis rate of RPASMC because the effect of the conditioned medium, i.e., apoptosis reduction, was blocked by VEGF-neutralizing antibodies (Fig. 4C).

We recently discussed a thought model to conceptualize the restructuring of vessels in angioproliferative forms of severe pulmonary hypertension (25). This concept has in its pathogenetic center apoptosis of pulmonary arteriolar EC as the initiating event and EC proliferation as a consequence. The appearance of smooth muscle cells, often within clusters of abnormal EC, in the complex so-called plexiform lesions (15) raises the question of the origin of these smooth muscle cells and the possibility of these smooth muscle cells arising via EC smooth muscle cell transdifferentiation (1, 5, 6). Our observation that apoptosed EC secrete into the medium (their environment) growth and antiapoptosis factors (9) that alter the behavior of RPASMC provides an additional concept, i.e., that EC apoptosis may stimulate RPASMC growth. Although our growth factor blocking antibody experiments point toward participation of both TGF-β1 and VEGF, these experiments did not rule out the participation of other growth factors like ET (31) and angiotensin (8). To investigate whether angiotensin and ET play a role in vascular smooth muscle cell growth, we performed additional experiments using the ET receptor A and B blockers BQ-123 and BQ-788 and the angiotensin antagonist losartan. Data presented in Supplementary Fig. 1 demonstrate that the addition of these antagonists to the conditioned medium from the static HPMVEC cultures (no SS) with and without SU-5416 had no effect on PASMC proliferation, whereas SU-5416 alone inhibited smooth muscle cell proliferation by 50% (P < 0.01). (The online version of this article contains supplemental data.) A slight inhibition by BQ-788 was not statistically significant.

In conclusion, our experiments show that growth factors for VSMC were released into the medium conditioned by apoptosed EC and that the death of pulmonary vascular EC can in principle affect VSMC growth. We provide here another example of EC-VSMC interactions and show for the first time that EC-derived VEGF inhibits apoptosis of VSMC (Fig. 5). Our data also confirm data by others (16), namely that EC can produce and secrete significant amounts of VEGF proteins. This secreted VEGF can have both autocrine (on EC) effects and paracrine effects (on VSMC). Thus VEGF may, in the vessel wall, serve multiple homeostatic and adaptive functions,
not only as the obligatory EC survival factor (7) but also as an inhibitor of VSMC apoptosis.

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