CALL FOR PAPERS | Stem Cells in Lung Biology

Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells


Developmental Lung Biology and Division of Pulmonary Sciences, University of Colorado Health Sciences Center and Denver Veterans Medical Center, Denver, Colorado 80220; California National Primate Research Center, University of California, Davis, California 95616; and Division of Hematologic Malignancies, Johns Hopkins Oncology Center, Baltimore, Maryland 21231

Submitted 11 April 2003; accepted in final form 7 May 2003

Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells. Am J Physiol Lung Cell Mol Physiol 286: L668–L678, 2004. First published May 16, 2003; 10.1152/ajplung.00108.2003.—Information is rapidly emerging regarding the important role of the arterial vasa vasorum in a variety of systemic vascular diseases. In addition, increasing evidence suggests that progenitor cells of bone marrow (BM) origin may contribute to postnatal neovascularization and/or vascular wall thickening that is characteristic in some forms of systemic vascular disease. Little is known regarding postnatal vasa formation and the role of BM-derived progenitor cells in the setting of pulmonary hypertension (PH). We sought to determine the effects of chronic hypoxia on the density of vasa vasorum in the pulmonary artery and to evaluate if BM-derived progenitor cells contribute to the increased vessel wall mass in a bovine model of hypoxia-induced PH. Quantitative morphometric analyses of lung tissue from normoxic and hypoxic calves revealed that hypoxia results in a dramatic expansion of the pulmonary artery adventitial vasa vasorum. Flow cytometric analysis demonstrated that cells expressing the transmembrane tyrosine kinase receptor for stem cell factor, c-kit, are mobilized from the BM in the circulation in response to hypoxia. Immunohistochemistry revealed an increase in the expression of c-kit+ cells together with vascular endothelial growth factor, fibronectin, and thrombin in the hypoxia-induced remodeled pulmonary artery vessel wall. Circulating mononuclear cells isolated from neonatal calves exposed to hypoxia were found to differentiate into endothelial and smooth muscle cell phenotypes depending on culture conditions. From these observations, we suggest that the vasa vasorum and circulating progenitor cells could be involved in vessel wall thickening in the setting of hypoxia-induced PH.

Vascular remodeling: bone marrow

Chronic pulmonary hypertension (PH) is a debilitating disease characterized clinically by an increased and sustained pulmonary arterial pressure with concomitant increases in pulmonary vascular resistance, frequently leading to right ventricular failure and death (37). The pathogenesis of PH has been ascribed to two mechanisms, vasoconstriction and vascular remodeling. Characteristic features of vascular remodeling include vessel wall thickening resulting from proliferation and migration of resident vessel wall cells together with accumulation of specific extracellular matrix (ECM) proteins (33). Although the precise mechanisms mediating the remodeling process remain unresolved, previous work from our laboratory has demonstrated that the adventitial fibroblast is critically involved in these vascular changes in a neonatal bovine model of hypoxia-induced PH (9, 10, 44). These findings are in accordance with substantial experimental data, which suggests that the cellular constituents of the adventitial compartment play a pivotal role in the remodeling process in a wide spectrum of vascular diseases (38, 41, 48).

The adventitial compartment includes within its borders a source of oxygenated blood to the outer wall of arteries, the vasa vasorum. This microcirculatory network has historically been assigned a passive role of providing O2 and nutrients to the outer regions of the vessel wall. Recently, however, an increasing body of experimental data have suggested that the vasa vasorum may play a significant role in maintaining vessel integrity and may contribute to the initiation and progression of certain types of vascular disease of the systemic circulation (4, 5, 18, 19, 23–25). To our knowledge, no previous studies have directly examined changes in the volume density of the pulmonary artery vasa network in response to hypoxia.

In addition to the dogma that proliferation and migration of resident vascular wall cells are the sole contributors to vessel wall thickening, a nonresident source of cells within the adventitial compartment of systemic vascular lesions has recently been postulated (48). In support of this, a number of studies in recent years have demonstrated that, in postnatal tissue, including bone marrow (BM; see Ref. 36) and connective tissue (49), stem-like cells reside that exhibit the potential to differentiate into multiple lineages, including vascular cell phenotypes (i.e., vascular smooth muscle cells and endothelial cells). Additionally, BM-derived hematopoietic stem cells (HSC) have been shown to differentiate into vascular cells that participate in the development of atherosclerotic lesions (39) as well as organ regeneration (30). There is also accumulating evidence of the...
existence of both BM-derived circulating endothelial progenitor cells (EPC; see Ref. 2), circulating smooth muscle progenitor cells (43), and circulating fibrocytes (1), which may contribute to the process of vascular remodeling. Indeed, EPC have been shown to participate in postnatal vasculogenesis in physiological and pathophysiological neovascularization (2) and to differentiate into cells that are indistinguishable from luminal endothelial cells in culture (21). Currently, there is no definitive consensus on the single best marker for BM-derived vascular progenitor cells. However, a generally accepted marker for BM-derived HSC (although not exclusive to this cell type) is c-kit, a transmembrane tyrosine kinase receptor for stem cell factor (29). Previous studies have reported that c-kit positive (c-kit+) cells are mobilized from the BM and track to atherosclerotic lesions (39) and sites of cardiac infarct (30). In addition, c-kit + cells have previously been isolated from both bovine (20) and mouse (29) BM and peripheral blood. No previous studies have investigated the effects of hypoxia on the mobilization, trafficking, and incorporation of BM-derived progenitor cells in the setting of PH.

Accordingly, the aim of the present investigation was to test the following hypotheses: 1) that hypoxia increases the density of the pulmonary artery vasa vasorum network, 2) that hypoxia stimulates mobilization of c-kit + cells from the BM into the circulation, 3) that hypoxia creates an environment in the pulmonary artery vessel wall that facilitates vasa expansion and circulating cell adhesion, 4) that hypoxia increases the expression of c-kit + cells in the remodeled pulmonary artery adventitia, and 5) that circulating progenitor cells from hypoxic animals exhibit the potential to differentiate into endothelial or smooth muscle-like phenotypes in culture.

**METHODS**

**Neonatal bovine model of hypoxia-induced PH.** Male Holstein dairy calves were obtained within 24 h of birth (day 1) and transported to the Department of Physiology, Colorado State University, Fort Collins, Colorado. The calves were randomly assigned to normoxic (n = 5) or hypoxic (n = 5) groups. The normoxic group was housed indoors at ambient altitude (1,500 m, barometric pressure = 640 mmHg). The hypoxic group was placed in a hypobaric chamber that simulated an altitude of 4,570 m above sea level with a barometric pressure of 430 mmHg. Pulmonary artery catheterization was performed on both normoxic and hypoxic calves at day 14, and pressures were determined by pressure-wave contours using a Statham P23 transducer (Gould, Valley View, OH), as previously described (45). The calves were killed by pentobarbital sodium overdose and exsanguinated via the carotid artery. All animal procedures were undertaken using standard veterinary care and institutional guidelines at the Department of Physiology, School of Veterinary Medicine, Colorado State University.

**Quantitative morphometric analyses.** Commencing at the level of the lobar bronchus, specific airway generations (5, 9, 12, 17, 20, 24, and 26) and their associated pulmonary arteries were identified and isolated from formalin-fixed tissue using microdissection techniques (32). Isolated pulmonary arteries and portions of their associated airways were paraffin-embedded, and sections were cut transversely across the vessel at 7 μm and stained with Movat’s pentachrome (6). This staining technique facilitated demarcation of the boundaries between the tunica adventitia and tunica media of pulmonary arteries associated with airways. Small endothelial cell-lined vessels with or without smooth muscle in their walls within the tunica adventitia and the outer region of the tunica media of pulmonary arteries were defined as vasa vasorum. With the use of tissue dissected from normoxic (n = 5) and hypoxic (n = 5) calves, the volume density of vasa vasorum along with other pulmonary arterial wall components was estimated using point-count estimation and stratified sampling with a random start to achieve a minimum of 200 points/feature block (14). The points in the 25-point lattice system that hit vasa vasorum divided by the points that hit tunica media or adventitia were used to estimate the volume-to-volume ratio (volume density). Point and intersect counting was used at a magnification of ×100 to estimate the surface to volume (Sv) of the endothelial elastic lamina (el) to the internal diameter (D) of the vessel. The equation for Sv is \[ Sv = \pi D^2 / 4 \] where D is the diameter of the vessel. The number of intersections with the arterial elastic lamina, I el, is the length per point of the lattice test system, and P el or P v are the points that hit the reference space of media or adventitia and the cross section of the artery assumed to be the shape of a cylinder in cross section (6). At each airway generation, the volume density of the adventitial vascularity was expressed as a ratio between the vasa vasorum, the combined volume of media and adventitia, or normalized to the surface of the internal elastic membrane of the artery. In addition, linear regression analysis was performed to identify the direction and magnitude of the possible correlation between the density of the vasa vasorum and the arithmetic mean thickness of the vessel wall. The arithmetic mean thickness of the adventitia or media of the vessel wall was estimated by the volume of the adventitia or media to the internal elastic membrane of the artery. Pulmonary veins were not evaluated in this present study.

**Antibodies, nuclear stains, and cytokines.** The following primary and secondary antibodies were used as per the manufacturer’s instructions. Primary antibody was as follows: fetal liver kinase-1 (Flk-1; 1:1,000), vascular endothelial (VE)-cadherin (1:1,000), tyrosine kinase receptor-1 (Tie-1; 1:1,000), and platelet endothelial cell adhesion molecule-1 (PECAM-1; 1:000 for Westerns and 1:200 for tissue sections; all from Santa Cruz Biotechnology, Santa Cruz, CA); endothelial nitric oxide synthase (1:1,000 eNOS; Transduction Laboratories, San Jose, CA); vascular endothelial cell growth factor (VEGF; 1:100) and fibronectin (1:100; abcam; Cambridge); α-smooth muscle actin (α-SMA; 1:1,000 for Westerns and 1:600 for tissue sections) and β-actin (1:10,000; Sigma, St. Louis, MO); von Willebrand factor (vWF; 1:100 and c-kit 1:50 (Dako, Carpenteria, CA); phycocyanin (PE)-conjugated c-kit (1:100) and thrombin (1:100; all from Research Diagnostics, Flanders, NJ); hypoxia-inducible factor-1α (1:100; HIF-1α; Transduction Laboratories); and a noncommercially available smooth muscle myosin antibody (22) used at 1:100. Secondary antibody was as follows: biotinylated anti-mouse or anti-rabbit IgG (1:300; Dako), FITC-conjugated anti-mouse or -rabbit IgG (1:300; Sigma), Cy5 (1:300; all from Jackson Laboratories, West Grove, PA) and horseradish peroxidase (HRP)-conjugated goat anti-mouse or -rabbit IgG (1:10,000; Santa Cruz). Cell nuclei were counterstained with hematoxylin (Vector, Burlingame, CA) or Hoechst 33342 (5 μg/ml; Molecular Probes, Eugene, OR). Human recombinant transforming growth factor-β (TGF-β); 1 ng/ml and retinoic acid (RA; 10−7 M) were purchased from R&D Systems (Minneapolis, MN). Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL; 4 μg/ml) was purchased from Biomedical Technologies (Stougham, MA).

**Immunohistochemistry and immunofluorescence.** Indirect immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections. Sections were dewaxed, placed in antigen unmasking solution (Vector), microwaved for 10 min at 180°C, incubated in hydrogen peroxide for 30 min, and incubated with primary antibodies overnight at 4°C. After a brief wash in PBS, sections were incubated with appropriate secondary antibodies for 1 h at room temperature. Antigenic sites were localized using an ABC kit (Vector) or immunofluorescence secondary antibodies, as appropriate. Indirect immunohistochemistry was performed on peripheral blood mononuclear cells (MNC). Cells were fixed in ice-cold methanol for 10 min, washed in PBS, and incubated overnight in primary antibodies. Antigenic sites were localized using immunofluorescence secondary
antibodies. The specificity of immunostaining (antibody control) was demonstrated by omission of the primary antibody and incubation with appropriate nonimmune serum. Images of antigenic sites were captured using transmitted light and epifluorescence microscopy using a Zeiss microscope linked to a personal computer.

Isolation of MNC from peripheral blood. Peripheral blood was collected from the internal jugular vein of systemically heparinized 14-day-old normoxic and hypoxic calves, as previously described (45). The MNC fraction was obtained by centrifugation over a Histopaque 1077 density gradient (Sigma). The cell pellet was washed in 1× PBS with 5 mM EDTA and resuspended in EBM-2 medium (Biowhittaker, Walkersville, MD) with 20% FCS (Gemini Bio-products, Woodland, CA), plated on fibronectin (5 μg/ml; Invitrogen, Carlsbad, CA)-coated plates, and cultured as previously described (21).

Flow cytometry. Flow cytometric analysis was performed on cells isolated from femoral BM and peripheral blood collected from 14-day-old normoxic and hypoxic calves using a FACSscan flow cytometer (Becton-Dickenson, Franklin Lakes, NJ). Cellular material from BM was flushed with PBS supplemented with 5% FCS and filtered over a 100 μm mesh to remove fat and BM debris. The MNC fraction of BM was obtained by centrifugation over a Histopaque 1077 density gradient (Sigma). The MNC suspension was isolated, and the pellet was resuspended in PBS supplemented with 5% FCS. The erythrocytes from peripheral blood were lysed, pelleted by centrifugation, and resuspended in PBS supplemented with 5% FCS. The cell suspensions were incubated with a PE-conjugated monoclonal antibody against c-kit (Research Diagnostics) for 1 h at 4°C, and ~1 × 10^6 cells/sample were analyzed. The emission fluorescence was monitored at 488 nm with an argon laser for PE. Nonspecific background
emission (incubation of cells with appropriate isotype-matched non-immune IgG) was subtracted from antibody emission, and data were analyzed using CellQuest software (Becton-Dickenson).

Western-blot analysis. Protein homogenates were separated by SDS-PAGE using the NuPAGE electrophoresis system (Invitrogen) and transferred to a Hybond-P membrane (Amersham Pharmacia, Buckinghamshire, UK). Membranes were probed with primary antibodies overnight in Tris-buffered saline + 0.05% (vol/vol) Tween 20 together with 5% (wt/vol) dried milk at 4°C. Membranes were washed and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized using the Western Lightening detection system (Perkin-Elmer Life Sciences, Boston, MA).

Statistical analyses. All data are expressed as means ± SD. Statistical analysis was performed using SAS Statistical Software (Cary, NC) and GraphPAD Prism, version 3 (GraphPad Software, San Diego, CA). ANOVA followed by Duncan’s multiple-comparison test or Pearson’s correlation coefficient was used, as appropriate.

RESULTS

Hypoxia-induced changes in the density of pulmonary artery adventitial vasa vasorum. We subjected neonatal calves to hypobaric hypoxia for 14 days to induce severe PH (45). Mean pulmonary artery pressures increased from 25 ± 2 mmHg in normoxic calves to 100 ± 12 mmHg in hypoxic calves (P < 0.001). Evaluation of hematoxylin- and eosin-stained tissue sections demonstrated marked thickening of the proximal (Fig. 1A) and distal (Fig. 1B) pulmonary artery vessel walls in hypoxic animals compared with proximal (Fig. 1C) and distal (Fig. 1D) arteries from normoxic animals. This vascular remodeling was associated with a prominent increase in the density of blood vessels that were predominantly localized within the adventitial compartment and at the adventitial-medial interface (Fig. 1, A and B). Indirect immunohistochemistry demonstrated that vascular structures in both proximal and distal pulmonary arteries express immunoreactivity for PECAM-1 (Fig. 1, E and F, respectively) and α-SMA (Fig. 1, G and H, respectively). In light of these observations, we went on to quantitatively test the hypothesis that chronic hypoxia increases the density of adventitial vasculature throughout the pulmonary arterial tree.

With the use of microdissection techniques and quantitative morphometric analyses, we demonstrated that, at each airway generation evaluated, the volume density of adventitial vasculature (expressed as a ratio of the combined medial plus adventitial thickness) was significantly (P < 0.001) greater in pulmonary arteries from hypoxic animals compared with control (Fig. 2A). Furthermore, the magnitude of increased vasa density was greater in more distal, resistance vessels compared with more conduit, proximal vessels (Fig. 2A). Increased volume density of the adventitial vasculature in hypoxic animals was associated with increased adventitial (Fig. 2B) and medial (Fig. 2C) thickness compared with control. Linear regression analysis identified a significant (P < 0.001, r² = 0.9355) correlation between the density of adventitial vasculature and the combined (adventitial plus medial) vessel wall thickness (Fig. 2D).

Erythrocytes in the vessel wall of the hypoxia-induced pulmonary artery vessel wall. Newly formed vessels are thought to be intrinsically “leaky” (15). An indirect method for evaluating leakiness of blood vessels is the presence of extravasated erythrocytes close to these vessels. Commonly observed was the presence of erythrocytes in the pulmonary artery vessel wall in tissue sections from chronically hypoxic animals that were not present in control vessels. These erythrocytes were predominantly localized in the adventitia of proximal (Fig. 3, A and B) and distal (Fig. 3C) vessels and in the extravascular adventitial compartment contiguous to vasa vasorum (Fig. 3D).

Hypoxia-induced mobilization of c-kit+ cells from the BM into the circulation. Next, we tested the hypothesis that hypoxia stimulates mobilization of BM-derived progenitor cells into the circulation. Using a monoclonal PE-conjugated antibody against c-kit receptor and flow cytometry, we identified significantly (P < 0.001) greater numbers of c-kit+ cells [when normalized to white blood cell (WBC) counts] in the circulating blood of hypoxic animals compared with blood from normoxic animals. As illustrated in Fig. 4A, ~9,400 c-kit+ cells/10⁶ WBC were identified in peripheral blood isolated from hypoxic animals compared with ~3,800 c-kit+ cells/10⁶.
WBC in peripheral blood from normoxic animals. We next analyzed the numbers of c-kit<sup>+</sup> cells in BM samples from normoxic and hypoxic calves. We identified significantly (P < 0.001) fewer c-kit<sup>+</sup> cells (when normalized to BM MNC count) in BM isolated from hypoxic animals compared with BM isolated from normoxic animals.

Identification of c-kit<sup>+</sup> cells in the hypoxic pulmonary artery vessel wall and vasa vasorum. We sought to evaluate whether hypoxia would increase the expression of c-kit<sup>+</sup> cells in the remodelled pulmonary artery. Immunohistochemistry revealed a greater number of c-kit<sup>+</sup> cells in the adventitia of distal (Fig. 5A) and proximal (Fig. 5B and C) pulmonary arteries from hypoxic animals compared with control (Fig. 5D and E). The c-kit<sup>+</sup> cells in pulmonary arteries from hypoxic animals were frequently identified contiguous to vasa vasorum (Fig. 5B and C) and within the vessel walls of vasa vasorum located in the adventitia (Fig. 5F). The c-kit<sup>+</sup> cells in the adventitia exhibited either round (Fig. 5B) or elongated (Fig. 5C) morphology.

Identification of proteins that may facilitate vasa vasorum expansion and circulating cell adhesion. We next examined the effects of hypoxia on the expression of proteins within the pulmonary artery vessel wall microenvironment that may facilitate vasa vasorum expansion and circulating cell adhesion. We initially focused on VEGF because of its critical role in several aspects of vascular cell function, including both physiological and pathological angiogenesis, regulation by hypoxia, and as a mediator of vascular permeability (12). In ~90% of hypoxia-induced remodelled pulmonary arteries, we observed immunoreactivity for VEGF in cells localized to the endothelium, media, adventitia, and endothelium of adventitial vasa at different regions of the vascular tree (Fig. 6, A–C). Although HIF-1α is known to promote VEGF expression (28), we did not detect HIF-1α protein by immunostaining in the vessel wall of the chronically hypoxic animals (data not shown). We also investigated the expression profiles of two additional proteins, fibronectin and thrombin. Fibronectin is well documented to support adhesion, migration, division, and differentiation of multiple cell types and represents a provisional ECM protein expressed during tissue remodeling (8). Supplementary to its pivotal role in coagulation and platelet function, thrombin is
mitogenic and chemotactic for vascular cells, is a potent stimulus of VEGF expression in endothelial cells (47), and may contribute to vascular lesion formation (15). Both fibronectin (Fig. 6, D–F) and thrombin (Fig. 6, G–I) immunoreactivity was detected in hypoxia-induced remodeled pulmonary arteries at different regions of the vascular tree. Weak, or negative, VEGF, fibronectin, and thrombin immunoreactivity was detected in pulmonary arteries from normoxic animals (Fig. 6, J–L).

Circulating MNC differentiate into vascular cells in culture. We sought to determine whether circulating progenitor cells isolated from hypoxic animals exhibited the potential to differentiate into vascular cell phenotypes. To test this hypothesis, we used a fibronectin-enriched culture system in an attempt to mimic the remodeled in vivo microenvironment of the hypoxic vessel wall. First, we isolated the MNC fraction of peripheral blood (21) from both normoxic and hypoxic animals. As demonstrated in Fig. 7A, when cultured in an endothelial-specific growth medium (EBM-2), MNC from hypoxic animals formed colonies within 21 days and exhibited “cobblestone” morphology after 4 wk in culture. MNC from normoxic animals formed colonies, but at a much slower rate, and exhibited a lower proliferative index compared with MNC from hypoxic animals, especially after subculture (data not shown). MNC isolated from hypoxic animals and cultured until a subconfluent monolayer was observed (~4 wk) were immunoreactive for vWF (Fig. 7B), VEGF (Fig. 7C), and eNOS (Fig. 7D), markers previously used to characterize circulating EPC (21). In addition to these markers, MNC from hypoxic animals expressed immunoreactivity for c-kit receptor (Fig. 7E). Cultured MNC incorporated Dil-Ac-LDL (Fig. 7F) and formed capillary-like networks when plated on basement membrane proteins (Fig. 7G). Furthermore, cells released endothelin-1 in response to serum and platelet-derived growth factor-BB stimulation (data not shown).

Separate isolates of MNC cultured under identical conditions from normoxic and hypoxic animals revealed an expression profile of specific endothelial cell markers indistinguishable from resident luminal pulmonary artery endothelial cells (RECs). MNCs expressed Flk-1, PECAM-1, VE-cadherin, Tie-1, and ENOS and were negative for α-SMA (Fig. 7H). For a control cell phenotype, cultures of pulmonary artery smooth muscle cells were used that expressed α-SMA but did not express any of the endothelial-specific markers (Fig. 7H). MNC isolated, cultured, and expanded under these conditions meet the previously reported criteria for circulating EPC (21).
We used MNC-derived EPC isolated from hypoxic animals to investigate the “plasticity” of these cells in culture in response to two versatile biological modifiers of vascular smooth muscle cell differentiation, TGF-β1 and RA. Distinct morphological changes were observed when EPC, plated on fibronectin and cultured in EBM-2 (Fig. 8A), were stimulated with EBM-2 supplemented with 1 ng/ml TGF-β1 (Fig. 8B) or 10⁻⁷ mol/l RA (Fig. 8C) for 7 days and 4 wk. EPC changed from their “cobblestone” morphology to a more elongated, spindle-shaped morphology characteristic of a smooth muscle cell in culture. Immunoblotting revealed that the morphological changes in TGF-β1-stimulated cells were associated with decreased expression of endothelial markers Flk-1, VE-cadherin, and Tie-1 by day 7 and decreased expression of PECAM-1 and eNOS by day 14, with an increase in α-SMA expression (Fig. 8D). Interestingly, REC from hypoxic animals also decreased their expression of endothelial cell markers when stimulated with TGF-β1 for 4 wk but did not gain expression of α-SMA (Fig. 8D). EPC stimulated with RA for 4 wk also lost expression on endothelial markers but did not gain expression of α-SMA (Fig. 8E). REC were relatively nonresponsive to RA stimulation (Fig. 8E).

DISCUSSION

The first principal finding from this study was that hypoxia stimulates an increase in the density of pulmonary artery adventitial vasa vasorum. Furthermore, extravascularized erythrocytes were present in the adventitial compartment of hypoxia-induced remodeled pulmonary arteries,
consistent with the idea that newly formed blood vessels are prone to leakiness (3).

With the use of a well-established technique for quantitative morphometric analysis of tissue sections, this study demonstrated that chronic hypoxia leads to a significant increase in the volume density of adventitial vasa vasorum along the longitudinal axis of the pulmonary arterial tree. Moreover, the magnitude of this increase was greater in more distal regions of the pulmonary artery tree, where the structural reorganization of the vessel wall is thought to have its greatest impact. The vasa vasorum of the pulmonary circulation is the distal vascular bed of the bronchial circulation, which serves to provide oxygenated blood and nutrients to the outer regions of the vessel wall. Thus “pulmonary artery adventitial neovascularization” may be, at least in part, a compensatory mechanism aimed at providing nourishment to the hypoxia-induced thickened vessel wall. Alternatively, expansion of the vasa network could be the result of a specific angiogenic process induced in the bronchial circulation in response to hypoxia-induced increases in blood flow. Indeed, increased blood flow through the pulmonary artery vasa network after hypoxic exposure has been reported previously (17). In addition, the upstream conduit of the vasa network in the pulmonary artery, the bronchial circulation, is modified under certain pathophysiological con-
ditions. For example, systemic hypoxemia and hypercarbia have been shown to increase bronchial blood flow in anesthetized dogs (7). Furthermore, the bronchial circulation is enlarged in newborn calves with high altitude-induced PH (35) and in rats with monocrotaline-induced PH (40). Thus it is possible that several mechanisms could be operating to cause adventitial neovascularization in the chronically hypoxic pulmonary circulation.

We commonly found erythrocytes in the extravascular adventitial compartment, close to vasa, in the hypoxic pulmonary artery vessel wall. These findings are in accordance with studies describing newly formed tumor blood vessels (16) and are consistent with the notion that newly formed vasa are incomplete and fragile nutrient channels with a propensity to leak or rupture (3). These findings raise the possibility that expansion of the vasa vasorum may increase the transmural flux of blood cells, plasma proteins, and circulating progenitor cells into the vessel wall (3). It is possible, therefore, that hypoxia-induced pulmonary artery neovascularization may facilitate trafficking and incorporation of circulating cells into the adventitial compartment of the vessel wall.

We demonstrated by flow cytometric analysis that chronic hypoxia significantly increases the number of c-kit+ cells in the circulation compared with control. To our knowledge, this is the first report directly linking systemic hypoxia with mobilization of BM-derived stem cells. Reduced oxygen has, however, been shown to favor self-renewal of both human and murine CD34+ stem cells in culture (11). In addition, previous studies have reported that local tissue ischemia mobilizes BM-derived progenitor cells that home to sites of wound healing (42, 46). Of more relevance may be the finding that genes that are regulated by hypoxia, including erythropoietin and VEGF, have been implicated in the generation and differentiation of hemangioblasts, the precursor cells of both HSCs and primitive endothelial cells (34). Moreover, VEGF has been shown to mediate mobilization of BM-derived HSCs and EPCs, which promotes tumor blood vessel growth (27) and postnatal corneal neovascularization (2). The precise mechanisms mediating hypoxia-induced mobilization of BM progenitor cells from their stromal cell/ECM anchor are not fully understood but may involve a complex interaction between adhesion molecules, chemokines, and proteolytic enzymes (26). Although we do not provide direct evidence in our animal model, an increase in VEGF within the BM compartment (either from outside or within) may be a mechanism by which progenitor cells are mobilized from the BM. This area of investigation is the subject of an ongoing study in our laboratory.

We used a commercially available antibody and a well-characterized immunohistochemical technique to evaluate if hypoxia increases the number of c-kit+ cells in the pulmonary artery vessel wall. Indeed, we identified a greater number of c-kit+ cells in the remodeled adventitia and vasa vasorum of hypoxic pulmonary arteries compared with the relatively thin-walled, acellular adventitia of normoxic animals. These observations raise several possibilities. The most challenging of

![Fig. 8. Distinct morphological changes were observed when EPC from hypoxic animals (A) were stimulated with transforming growth factor (TGF)-β1 (B) or retinoic acid (RA; C). The morphological changes after TGF-β1 stimulation were associated with a decrease in endothelial-specific proteins and a concomitant increase in smooth muscle-associated proteins after 4 wk in culture (D). REC from hypoxic animals exhibited a marked decrease in endothelial-specific proteins with no gain of α-SMA expression after TGF-β1 stimulation (D). EPC stimulated with RA exhibited a marked decrease in endothelial-specific proteins with no gain of α-SMA expression (E). REC were nonresponsive to RA stimulation (E). PASMC were used as a control. Scale bar represents 200 μm.](http://ajplung.physiology.org/doi/10.1152/ajplung.00228.2003)
these, based on our flow cytometric data, is that chronic hypoxia mobilizes c-kit+ cells from the BM into the circulation and that these cells traffic to the lung and incorporate into the pulmonary artery adventitia. This suggestion is supported, at least in part, by the identification of circulating cells (blood lakes) within the hypoxic vessel wall and by previous studies reporting that BM-derived c-kit+ cells give rise to vessel wall thickening in atherosclerotic lesions (39).

An alternative interpretation of our observations would be that hypoxia stimulates expression of c-kit in resident adventitial cells, notably fibroblasts. It is also possible that within or close by the adventitial compartment exists resident c-kit+ progenitor cells that are activated in response to injury to subserve a repertoire of unique cellular functions. This possibility is consistent with the observations of resident stem cells in muscle and other normal organs (13, 31), which are thought to contribute to normal cell turnover and/or tissue repair after injury.

We demonstrated by immunohistochemistry that the hypoxic pulmonary artery vessel wall in the neonatal calf is abundant in the expression of proteins that serve critical roles in several aspects of vascular cell function under both physiological and pathological settings. VEGF is a potent stimulator of angiogenesis, vasculogenesis, and vascular permeability (12). Fibronectin supports adhesion, migration, division, and differentiation of multiple cell types and represents a provisional ECM protein expressed during tissue remodeling (8). Thrombin is a multifunctional serine protease and G protein-coupled receptor ligand that is mitogenic and chemotactic for vascular cells, as well as a potent stimulus of VEGF expression in endothelial cells (47). Accordingly, these proteins may act independently, or in concert, to facilitate neovascularization of the pulmonary artery vasa vasorum in response to hypoxia. Furthermore, the microenvironment of the hypoxic pulmonary artery vessel wall, with increased expression of VEGF, fibronectin, and thrombin, might be conducive for attraction, adhesion, and differentiation of circulating progenitor cells.

Finally, we isolated, cultured, and expanded MNC-derived EPC from hypoxic calves that exhibited the potential to differentiate into endothelial and smooth muscle-like phenotypes and in vitro plasticity, depending on culture conditions. These findings are pursuant to previous studies that have demonstrated that BM-derived HSC (39) and MNC-derived EPC (21) differentiate into vascular cells in culture. It is noteworthy that, in this present study, MNC-derived EPC isolated from hypoxic animals formed colonies faster, exhibited a greater proliferative index, and maintained these characteristics after subculture compared with cells isolated from normoxic animals (data not shown). Furthermore, within aggregate isolates of EPC from hypoxic animals was a significant population of cells that expressed immunoreactivity for c-kit receptor. Although no comparative evaluation was performed in this study, we suggest that the phenotype of “hypoxic” EPC may be distinctly different from that of “normoxic” EPC.

Collectively, the findings of the present study provide further evidence in support of the concept that the adventitial compartment is critically involved in the development of hypoxia-induced vascular remodeling in the setting of PH. We fully acknowledge that our observations do not provide “proof of principle” that progenitor cells are mobilized from the BM and incorporate into the pulmonary artery vessel wall in response to hypoxia. A limitation of using this large animal model is that it precludes us from using labeled BM cells and tracking them, since they are mobilized from their BM compartment in response to hypoxia. This type of experiment, using recipient mice after labeled BM transplants, is currently in progress within our laboratory. Unfortunately, the degree of pulmonary artery vascular remodeling after hypoxic exposure in mice is markedly less than that observed in our bovine model of PH. At this point, we can only speculate that the vasa vasorum and circulating progenitor cells may contribute to vessel wall thickening in the setting of hypoxic PH. Circulating progenitor cells, mobilized by hypoxia, may be delivered to the pulmonary artery adventitia via the vasa vasorum. These cells may proliferate and migrate into the vessel wall and/or fuse with resident vascular cells to affect structural changes in the vessel wall. These findings establish hitherto unidentified factors that may contribute to vascular remodeling in a neonatal bovine model of hypoxia-induced PH. It has yet to be determined if expansion of the vasa network and circulating progenitor cells contributes to the vascular remodeling process in patients with PH.

ACKNOWLEDGMENTS

We acknowledge Sandy Walchak, Jennifer Dreith, and Stacey Schomberg for technical assistance and Peter Lloyd Jones for critical reading of the manuscript.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Specialized Center of Research Grant HL-57144 and Program Project Grant HL-14985. J. T. Crossno, Jr., was supported by an Associate Investigatorship from the Veterans Administration.

REFERENCES


