Sustained hypoxia leads to the emergence of cells with enhanced growth, migratory, and promitogenic potentials within the distal pulmonary artery wall

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Frid MG, Li M, Gnanasekaran M, Burke DL, Fragoso M, Strassheim D, Sylman JL, Stenmark KR. Sustained hypoxia leads to the emergence of cells with enhanced growth, migratory, and promitogenic potentials within the distal pulmonary artery wall. Am J Physiol Lung Cell Mol Physiol 297: L1059–L1072, 2009. First published September 18, 2009; doi:10.1152/ajplung.90611.2008.—All forms of chronic pulmonary hypertension (PH) are characterized by structural remodeling of the pulmonary artery (PA) media, a process previously attributed solely to changes in the phenotype of resident smooth muscle cells (SMC). However, recent experimental evidence in both systemic and pulmonary circulations suggests that other cell types, including circulating and local progenitors, contribute significantly to this process. The goal of this study was to determine if hypoxia-induced remodeling of distal PA (dPA) media involves the emergence of cells with phenotypic and functional characteristics distinct from those of resident dPA SMC and fibroblasts. In vivo, in contrast to the phenotypically uniform SMC composition of dPA media in control calves, the remodeled dPA media of neonatal calves with severe hypoxia-induced PH comprised cells exhibiting a distinct phenotype, including the expression of hematopoietic (CD45), leukocytyc/monocytic (CD11b, CD14), progenitor (cKit), and motility-associated (S100A4) cell markers. Consistent with these in vivo observations, primary cell cultures isolated from dPA media of hypertensive calves exhibited not only differentially expressed SMC, but also smaller, morphologically rhomboidal (thus termed here “R”) cells that transiently expressed CD11b, constitutively expressed the mesenchymal cell marker type I procollagen, expressed high mRNA levels of progenitor cell markers cKit, CD34, CD73, as well as for inflammatory mediators, IL-6 and MCP-1, and, with time in culture, gained expression of a myofibroblast marker, α-SM-actin. R cells exhibited highly augmented proliferative, migratory, invasive, and potent promitogenic capabilities, which were due, at least in part, to the production of PDGFs, SDF-1/CXCL12, and S100A4. These data suggest that the cellular mechanisms of dPA remodeling include the emergence of cells with phenotypic and functional characteristics markedly distinct from those of resident dPA cells.

pulmonary hypertension; vascular remodeling; progenitor cells; inflammation; S100A4

CHRONIC PULMONARY HYPERTENSION (PH) is characterized by profound remodeling of the pulmonary artery (PA) wall (29, 64, 73). The remodeling is complex, and all three vascular layers (intima, media, and adventitia) can be involved, albeit in different ways and degrees depending on the location of the vessel studied, the injurious stimulus involved, and the age of the patient. In neonatal forms of PH, remodeling of the PA tunica media is prominent and was traditionally believed to be the result of dedifferentiation and subsequent hyperplasia of arterial smooth muscle cells (SMC) (31, 66). However, the additional possibility that cells other than resident differentiated SMC contribute to medial remodeling has been raised in a variety of experimental vascular injury settings. These possibilities include, but are not limited to: 1) recruitment and differentiation of circulating progenitor cells (6, 9, 17, 22, 24, 41, 52, 57, 61, 63, 70, 75, 79), 2) activation and differentiation of resident vascular progenitor cells (13, 26, 28, 32, 47, 81), 3) activation and migration of adventitial fibroblasts to the media and even intima (56, 60, 62, 80), and 4) transdifferentiation of endothelial-like cells into mesenchymal cells (1, 20). At present, it remains unclear if any of these cellular mechanisms contribute to PA media remodeling in PH, but a number of studies have recently emerged in both humans and animal models documenting the presence of cells expressing hematopoietic, progenitor-like, and motility-associated antigens in the intima and media of remodeled pulmonary vessels (21–23, 38, 42, 74).

The goal of the present study was to, first, evaluate in vivo, in the setting of neonatal hypoxia-induced PH, whether remodeling of the distal PA (dPA) media is associated with emergence of cells exhibiting phenotypic characteristics distinct from those of resident dPA cells. If so, our goal was to compare, in vitro, their functional capabilities to those of resident dPA wall cells. As the animal model of neonatal hypoxic PH, we used neonatal calves, which develop severe PH and marked PA medial thickening in response to chronic hypoxia (17, 64, 65). We focused our analysis on distal elastic PAs of 600–1,500 μm in diameter, since we have previously shown that the tunica media of these PAs is normally composed of a uniform population of differentiated SMCs (67), and, therefore, any changes in the cellular composition of the vessel wall (such as emergence of cells phenotypically distinct from resident PA cells) would be noticeable. We performed immunostaining of dPA tissues from control and hypertensive calves to detect the appearance of hematopoietic, leukocytic, and progenitor-like cells. Ex vivo, cell cultures of dPA media from the same animals were isolated, and subsequent morphological, immunohistochemical, RT-PCR, protein, and functional (proliferative, promitogenic, and migratory) assessments were performed.

METHODS

Animal model. The neonatal calf model of severe hypoxic pulmonary hypertension has been described previously (1, 15, 20) and includes the development of PA pressure equal to, or exceeding, systemic pressure as well as remarkable PA remodeling with medial
and adventitial thickening being prominent along with periadventitial inflammation, resembling that of human neonatal PH (22, 29, 31, 73). One-day-old male Holstein calves were purchased from Laluna Dairy Farm (Fort Collins, CO). The experimental group (n = 7) was exposed to hypobaric hypoxia (Ppa = 445 mmHg) for 2 wk, whereas age-matched controls (n = 6) were kept at ambient altitude (Ppa = 640 mmHg) (64). Standard veterinary care was used following institutional guidelines, and the procedure was Institutional Animal Care and Use Committee approved (Dept. of Physiology, School of Veterinary Medicine, Colorado State Univ., Fort Collins, CO). Animals were euthanized by overdose of pentobarbital sodium (160 mg/kg body wt).

**Immunofluorescent analysis.** Immunofluorescent staining was performed as previously described (18). Antibodies (Abs) against the Cytometer (Hialeah, FL).

**Isolation of cells.** Isolation of cells was performed from dPAs of an external diameter of 670 ± 1,340 µm (means 994 ± 25 µm) as previously described (65) from both control normotensive and chronically hypoxic hypertensive calves using explant techniques (65). Briefly, the bronchus entering the lung lobe was cut open and followed (under the dissecting microscope) through several branching points all the way to the tip of the lung. Next, the bronchus was removed and the underlying PA was exposed. The smaller PA branches were followed until the desired size (~1 mm in diameter) was identified. These dPAs were dissected out and placed into PBS for further size evaluation and processing. The external diameter was measured under the microscope, and the selected dPAs were pinned to the bottom of a polymer-coated Petri dish for further cleaning of tunica media. First, all the remaining pieces of lung parenchyma were removed. Next the adventitia was thoroughly removed so that the artery appeared “smooth” on the outside. These dPAs were cut into pieces of ~1/16 in length, and each piece was placed on the bottom of a well into a 24-well culture plate for the explant technique method of cell isolation (15). Briefly, after arterial pieces appeared adhered to plastic, growth medium was gently added, and explants were left undisturbed in the incubator (5% CO₂) for a period of 5–10 days (cells from the dPAs of hypertensive calves were found to migrate out from the tissue pieces faster than those from control animals). When a substantial number of cells had migrated out, the tissue piece was removed, and cells were allowed to expand until ready for passaging. Cells were expanded in complete DMEM (Sigma-Aldrich) with 10% calf serum (CS; HyClone Laboratories, Logan, UT). Colonies of cells morphologically distinct from dPA-SMC were selectively isolated using Teflon “cloning” rings (15). All experimental assays were performed on cells at passages 4–6. 

**Cell size analysis.** Cell size analysis was performed by forward scatter expressed as channel number (33). Cell cultures were analyzed in a log phase of growth (10% CS) and under growth arrest (0.1% CS, 72 h). Cells were diluted in Isoton reagent (Beckman-Coulter), and relative size distribution was measured on a Coulter EPICS Flow Cytometer (Hialeah, FL).

**Cell proliferation analyses.** Cell proliferation analyses were performed either in the format of a growth curve (16) or as assessment of BrdU incorporation. Generation of a growth curve was performed as previously described (16). Briefly, cells were plated onto 24-multwell plates at initial density of 20 × 10³ cells per well in DMEM supplemented with 10% CS. For the growth curve under serum-supplemented conditions, cells were maintained in 10% CS throughout the course. For the growth curves under serum-free conditions, cells, plated in 10% CS-DMEM on day 0, were rinsed in PBS, and medium was replaced with DMEM supplemented with 0.2% plasma-derived serum (PDS; Cocalico Biologicals, Reamstown, PA), which is depleted of growth factors present in serum via charcoal stripping. During the 2-wk period, with the intervals of 2–4 days, cells in four wells were triplicated and counted in a standard Spotlite hemacytometer (Baxter). Data were expressed as cell number × 10³ per well. All experiments were repeated with cells derived from five to seven calves in both control and hypoxic groups.

**ELISA assay of conditioned medium samples.** To collect conditioned medium, cells were plated in 100-mm Petri dishes in serum-containing DMEM and grown until confluent. Next, cells were rinsed with PBS, and growth medium was replaced with serum-deprived (0.2% PDS) DMEM. In 72 h, conditioned medium was collected, aliquoted and snap-frozen for ELISA analysis.

**Immunoreactive PDGF-BB was quantified using a commercially available ELISA kit (900-K04 PeproTech) according to the manufacturer’s instructions, using Nunc Maxisorp plates. Capture antibody was bound overnight at 1 µg/mL washed 4× in PBS/0.05% Tween 20, and blocked with 1% BSA/PBS for 1 h. Samples and standards were applied in triplicate for 2 h. Detection antibody (0.25 µg/mL) was incubated for 2 h. Avidin-HRP conjugate (1:2,000) was applied for 30 min and washed, and ABTS substrate was applied until color developed. Plates were immediately read at 405 nm in an ELISA plate reader. Wavelength correction was set at 650 nm.

**Inhibition assays.** Inhibition assays were performed to test a potential role of PDGF, SDF-1 and S100A4 in autocrine growth of dR" cells and in paracrine effect of dS"-SMC and/or dPA adventitial cells, whose conditioned medium is to be tested. For testing the potential role of these mediators on autocrine growth of dR" cells, the latter were plated at 10 × 10³ cells/well onto a 24-well plate in complete 10% CS-DMEM. The next day, cells were washed with PBS, and serum-deprived (0.2% PDS) DMEM was added for 10 days with or without antibodies. On days 4 and 8, one-half of culture medium was replaced, and antibodies were re-added. The following antibodies were used in these experiments: goat anti-human PDGF-BB/AB neutralizing IgG, goat anti-human SDF-1/CXCL12 neutralizing IgG (both from R&D Systems, Minneapolis, MN), rabbit anti-S100A4 IgG (Thermo Fisher Scientific). Concentrations of antibodies/reagents are specified in the figure legends. For assaying paracrine effects of R-CM, target cells (d"S"-SMC and/or dPA adventitial fibroblasts) were plated at 20 × 10³ cells per well onto 24-well culture plates in complete 10% CS-DMEM. The next day, cells were washed with PBS and growth arrested in serum-deprived (0.2% PDS) DMEM for 72 h. Next, R-CM was added with or without “inhibitory” agents or antibodies to be tested. BrdU was added 24 h before termination of the experiment. Cell numbers were acquired at time points specified in the figure legends.

**Coculture experiments.** Coculture experiments were performed using a “source/target” design (16) to determine if a certain cell population secreted either growth-promoting or growth-inhibitory factors. Briefly, target cells (in which the effect of the conditioned medium from the source cells was tested) were plated inside a 15-mm plastic ring, which was greased to the center of a 35-mm Petri dish. The source cells (whose conditioned medium is to be tested for production of paracrine factors) were plated outside the plastic ring (with target cells inside the ring). When cell cultures displayed sufficient confluence, culture medium for both cell types was replaced with 0.1% CS-DMEM for 72 h. All the medium of the target cells was then withdrawn, and the ring separating the two cell types was removed, allowing the medium, conditioned by the source cells for 72 h, to spread over the target cells. After 16 h, BrdU was added to culture medium, and, after additional 24 h, cells were fixed, immu-
nostained for BrdU, and counterstained with hematoxylin, and BrdU nuclear incorporation was quantified by a manual count and normalized per total number of cell nuclei.

**DNA synthesis analysis.** DNA synthesis analysis was determined by measuring [3H]thymidine incorporation in growth-arrested cells isolated from control or hypertensive calves and exposed in vitro to either normoxic (21% O2) or hypoxic (3% O2) conditions as previously described (15). Briefly, cells were plated at 20 × 10^4 cells/well onto 24-multwell plates in 10% CS-DMEM and rinsed with PBS within 24 h, and growth medium was replaced with serum-deprived (0.1% CS) DMEM for 72 h. Cells were then placed in air-tight sealed humidified modular incubator gas chambers (Billups-Rothenberg, Del Mar, CA), which were purged for 20 min with the defined gas mixture of either 21% O2 or 3% O2 and with 5% CO2 balanced with N2 (AirGas, Denver, CO) and placed in the 37°C incubator for 48 h. pH of the culture medium was monitored by the color of phenol red. Next, chambers were opened, [3H]thymidine (0.5 μCi/ml) was added to plates, and chambers were sealed and gassed again. After an additional 24 h incubation, measurement of [3H]thymidine incorporation was performed and expressed as dpm per cell.

**Cell migration.** Cell migration was assessed using a “ scrape” assay in complete DMEM supplemented with 5 μM hydroxyurea to inhibit cell proliferation. Briefly, in subconfluent cell cultures, growth medium was replaced with 0.2% PDS-DMEM for 72 h, and hydroxyurea (5 μM, Sigma) was added for 2 h to inhibit cell replication, after which a 2-mm scrape was performed (that time point was considered 0 h). The borders of the wound (scrape) were labeled with a permanent marker (on the bottom of the Petri dish), and the number of cells migrating past the drawn line (into the “scrape area”) was counted at 24 and 48 h. Cell migration analysis was also performed in the presence of human recombinant S100A4 (2.5 μg/ml) and soluble RAGE (5 μg/ml) as well as with antibodies against S100A4 (1.25 μg/ml) and against RAGE (6 μg/ml). All these reagents were purchased from Abcam (Cambridge, MA).

**Quantitative real-time PCR.** Total cellular RNA from each sample was extracted using RNasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized from 1 μg of total cellular RNA using iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Quantitative real-time RT-PCR was performed in triplicate with the iCycler My iQ with IQ SYBR Green Supermix (BioRad). Primers were designed using Primer 3 Software (53).

Genes were normalized to the housekeeping gene hypoxanthine-xanthine phosphoribosyl transferase (HPRT). The sequences for primers were as follows: PGDF-B (fwd 5′-AGTGAACACTCCATCGCTCC-3′, rev 5′-TCAGGCCTGTCCTCATTAC-3′), PGDF-A (fwd 5′-CCACAGCAGATTGAGGTGAA-3′, rev 5′-CTTGGCAGTAGAACCACGGTACA-3′), SFDF-I/CXCL12 (fwd 5′-CCTTGCGCATTGTCTTGGAG-3′, rev 5′-CTGAAAGGAGCACTTTGGAG-3′), CXCR4 (fwd 5′-TCAAGGAOGTGGATGAGAAGG-3′, rev 5′-AGGATGACGATACCACACGAC-3′), S100A4 (fwd 5′-CAAGGAGGAGGTGCAACGT-3′, rev 5′-CTGTTGTCTGTTCTGTCAGTGC-3′), RAGE (fwd 5′-TGATGGAACAGAGTTTCAACG-3′, rev 5′-CAAGGAGGAGGTGCAACGT-3′), eCKIT (fwd 5′-TTGATGTTGACCCTCCTCTG-3′, rev 5′-TCTCATACTCTGTTGGAGGACAG-3′), CD34 (fwd 5′-AACACCTTCCCTGATATCCC-3′, rev 5′-TCCGGGGAACCTTAAGAGGAGCA-3′), CD73 (fwd 5′-GTGCTGGTGCGCCATAGTAT-3′, rev 5′-AACTCCTGCTACACACATGC-3′), IL-6 (fwd 5′-GATGAAAGCAGCAAGAGGAGCA-3′, rev 5′-ATCCGTCTCCTCATTCCATCA-3′), MCP-1 (fwd 5′-CCCTCTGTCTCTACAATCAG-3′, rev 5′-ACACTGTCGCTGTGGACTC-3′), Flt-1 (fwd 5′-CTCCCGGCTAATGCAATCA-3′, rev 5′-ACACTGTCGCTGTGGACTC-3′), PGDF-B (fwd 5′-CTTGGCAGTAGAACCACGGTACA-3′, rev 5′-CTTGGCAGTAGAACCACGGTACA-3′), Tie-2 (fwd 5′-CTTGCTTGCGCCATTTG-3′, rev 5′-CTTGGCAGTAGAACCACGGTACA-3′), PECAM-1 (fwd 5′-GAAGGGCTTCAAGAAGGAGCA-3′, rev 5′-CTTGGCAGTAGAACCACGGTACA-3′), VE-cadherin (fwd 5′-GTATCCTCCCAAATGCTCTGAAG-3′, rev 5′-CTTGGCAGTAGAACCACGGTACA-3′). Results are presented either as relative expression to HPRT using delta CT method or as fold increase relative to S cells using ΔΔCT method (59).

**RESULTS**

*In vivo, phenotypically distinct cells emerge within the remodeled dPA media.* Two-week exposure of neonatal calves to hypobaric hypoxia consistently resulted in marked PA media thickening (Fig. 1 and Refs. 64 and 65). As previously reported (67), in control normoxic calves, the media of dPAs appears composed of a phenotypically uniform population of differentiated SMC, expressing α-SM-actin, SM-myosin, calponin, and meta-vinculin (α-SM-actin is shown) in Fig. 1A. In contrast, the dPA media of chronically hypoxic hypertensive calves consistently contained a number of cells that either did not express any SM-related markers (Fig. 1B, staining for α-SM-actin is shown) or expressed very low levels of α-SM-actin only, i.e., exhibited a “myofibroblast” phenotype (Fig. 1C).

Interestingly, cells were observed within the dPA media of hypertensive, but not control, animals, which expressed CD45 (not shown). No coexpression of CD45, and/or S100A4 (Fig. 1). **Cell invasion.** Cell invasion capacity was tested in a Matrigel (BD Pharmingen) assay as per the manufacturer’s instructions with or without the general MMP inhibitor GM6001 (20 μM).

**Gelatin zymography.** Gelatin zymography was performed as described elsewhere (35). Briefly, cultured cells were lysed in cold lysis buffer (50 mM Tris, 250 mM NaCl, 0.5% Triton X-100, 0.1% SDS). Lysates were concentrated depending on the protein amounts (determined by Bradford and BCA assay) using Centricron-10 centrifugal filter devices. Samples were mixed with 2× sample buffer (63 mM Tris·HCl, 10% glyceroi, 2% SDS, 0.0025% bromphenol blue, pH 6.8), and protein (10.5 μg/lane) was separated by electrophoresis under nonreducing conditions, using precast gelatin-embedded 10% polyacrylamide gels (Novex) in zymogram running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3). Gels were renatured at room temperature for 30 min with gentle agitation in zymogram-renaturing buffer [25% (vol/vol) Triton X-100] followed by 30-min incubation in zymogram-developing buffer [50 mM Tris base, 40 mM HCl, 20 mM NaCl, 5 mM CaCl2, 0.02% (wt/vol) Brig 35-Novex]. Gels were then incubated overnight at 37°C in fresh developing buffer. To confirm the presence of MMPs, duplicate gels were incubated in developing buffer containing 20 mM EDTA to chelate the zinc ions and inactivate the MMPs. Gels were rinsed, stained with Simply Blue SafeStain, and destained until clear bands, indicative of gelatinase activity, were evident. Precision Plus Dual Color Protein Standards (BioRad Laboratories) were used on every gel.

**Data analysis.** Data are presented as means ± SE. Student’s t-test and one-way ANOVA were used for statistical analysis; significance was accepted at *P* < 0.05.
Phenotypically distinct cells emerge in cultures from dPA media of hypertensive calves. Primary cell cultures isolated from the dPA media of control calves were consistently composed of a morphologically and phenotypically uniform population of large spindle-shaped SMC that grew in a “hill-and-valley” pattern (Fig. 2A) and expressed a number of SM-related markers, α-SM-actin, calponin, SM-myosin, metavinculin, findings consistent with those previously described by us (67). In contrast, cell cultures isolated from the remodeled dPA media of hypoxic hypertensive calves consistently expressed CD45 (as shown in D), small arrows pointing to cells stained in red; large arrows point to cells that do not express either α-SM-actin or CD45. There are a few cells that express cKit (E, green) either within the outer media (large arrows) or at the medial-adventitial border (small arrows). Cell nuclei are labeled with DAPI (blue). AW, airway. Several cells within the dPA media (M) express S100A4 (F, large arrows pointing to brown cells; small single arrows point to S100A4-positive cells at the medial-adventitial border; small double-arrows point to S100A4-positive cells within the adventitia (A)). Control arteries lacking cKit+ and/or S100A4+ cells within the media are shown in Supplemental Fig. 1, available online at the AJP-Lung web site.

Fig. 2. In primary culture, dPA media of chronically hypoxic calves yields phenotypically distinct cell populations. A: primary cultures isolated from the dPA media of control calves consistently yield a morphologically uniform population of large spindle-shaped SMC that grew in a “hill-and-valley” pattern (Fig. 2A) and expressed a number of SM-related markers, α-SM-actin, calponin, SM-myosin, metavinculin, findings consistent with those previously described by us (67). In contrast, cell cultures isolated from the remodeled dPA media of hypoxic hypertensive calves consistently
hsp47 is shown), but initially did not express E

gen-associated enzyme heat shock protein 47 (Hsp47) (Fig. 3, B and C), which were phenotypically similar to SMC obtained from control calves, yet significantly hypertrophied, as analyzed by forward scatter (Supplemental Fig. 2). Another population (Fig. 2D) was composed of very small cells (as analyzed by forward scatter, Fig. 2E) of a rhomboidal morphological appearance that were growing without cell-cell contact inhibition. They always demonstrated a delayed appearance in primary culture, but subsequently expanded rapidly and formed dense cell colonies. For reference purposes, we termed the larger spindle-shaped SMC as dS-SMC (“d” for distal PA), and the small rhomboidal cells as dR cells. Importantly, adventitial fibroblast isolated from the same vessel were morphologically (Fig. 2F) and functionally (see below) different from the dR cells.

In primary culture, some dR cell colonies transiently expressed CD11b (Fig. 3D) and consistently expressed mesenchymal cell markers, type I procollagen, and the type I collagen-associated enzyme heat shock protein 47 (Hsp47) (Fig. 3E, Hsp47 is shown), but initially did not express α-SM-actin. With time in early subculture (passage 1), cells gradually lost expression of CD11b and gained immunoreactivity for α-SM-actin (Fig. 3F). The acquisition of α-SM-actin reactivity was also confirmed by analysis of clonally derived R cells (all obtained from single-cell clones) that gained expression of α-SM-actin and calponin (Fig. 3, G and H). Importantly, no phenotypic switch of dPA SMC into R cells was observed under any conditions/stimuli tested, including hypoxia, PDGF, M-CSF, EBM-2 medium, MSC medium, coculture with endothelial cells (EC), etc. (not shown).

To further characterize dR cell phenotype, we compared these cells to EC isolated from main pulmonary artery (MPA-EC) and from dPA (1,000 μm in diameter, dPA-EC) of control calves. dR cells expressed markedly lower mRNA levels of endothelial markers, Tie2, PECAM-1/CD31, and VE-cadherin than MPA-EC and dPA-EC. The exception was observed for Flt-1 mRNA, which was expressed by dR cells and adventitial fibroblasts (Fibs) at levels comparable to those of dPA-EC, yet lower than those of MPA-EC (Supplemental Fig. 3).

dR cells express mRNA for progenitor cell markers and for inflammatory mediators. Notably, dR cells expressed high mRNA levels for antigens commonly expressed by progenitor-like cells cKit, CD34, and CD73 (68.03 ± 13.79-fold, 20.59 ± 2.35-fold, 42.02 ± 13.28-fold, respectively, compared with dS-SMC) (Fig. 3F). Furthermore, dR cells exhibited higher than dS-SMC mRNA expression levels for inflammatory mediators IL-6 and MCP-1 (11.54 ± 2.29-fold and 2.29 ± 0.14-fold, respectively, Fig. 3J).

dR cells exhibit augmented proliferative capabilities. dR cell and dS-SMC populations exhibited markedly different growth

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**Fig. 3.** Cultured dR cells express an immunophenotype distinct from that of dS-SMC and mRNA for progenitor-associated markers and inflammatory mediators. A: morphological differences between dS-SMC and dR cells are apparent in primary culture of dPA media. B and C: dS-SMC express SM-related markers, α-SM-actin (B) and SM-myosin heavy chains (C), not only in primary culture but also at subsequent passages in subculture (passage 5 is shown). D and E: dR cell colonies express CD11b in early primary culture (D) and consistently (at all passages in culture) express type I procollagen-associated enzyme Hsp47 (E). F: in early subculture (passage 1), CD11b expression (large arrows, red staining) is gradually lost, whereas expression of α-SM-actin is gradually acquired (small single arrows point at cells that coexpress both antigens, whereas small triple arrows point at cells that express only α-SM-actin). G and H: clonally derived dR cells express myofibroblast-related markers α-SM-actin (G) and calponin (H). I: dR cells express high levels of mRNA for progenitor cell-associated antigens cKit, CD34, and CD73. Fold-change dR vs. dS: cKit 68.03 ± 13.79; CD34 20.59 ± 2.35; CD73 42.02 ± 13.28. J: dR cells express higher levels of mRNA for inflammatory mediators IL-6 and MCP-1 compared with dS-SMC. Fold-change dR vs. dS: IL-6 11.54 ± 2.29-fold and MCP-1 2.29 ± 0.14-fold, respectively.
capabilities under both serum-stimulated (10% calf serum) (Fig. 4A) and serum-deprived (Fig. 4, B and C) conditions. dR cells proliferated far more rapidly and plateaued at higher cell densities upon serum stimulation (Fig. 4A), as well as exhibited augmented DNA synthesis under serum-deprived (0.2% PDS) conditions (Fig. 4B). Interestingly, hypoxia (3% O2) further augmented DNA synthesis in dR cells (Fig. 4B), which was far more significant than the response of adventitial fibroblasts to hypoxia. Notably, DNA synthesis of dS-SMC was not increased and was rather attenuated in response to hypoxia. Furthermore, dR cells exhibited serum-independent autocrine growth (Fig. 4C), thus suggesting secretion of promitogenic factors that contributed to this growth pattern.

Autocrine growth of dR cells is due, in part, to production of PDGFs and SDF-1. To test the hypothesis that dR cells secreted promitogenic factors, we first performed RT-PCR analysis of “candidate” mitogens, PDGFs and SDF-1/CXCL12. dR cells exhibited high mRNA expression levels of PDGF-B and -A compared with dS-SMC (PDGF-B 26.4 ± 1.6-fold; PDGF-A 13.2 ± 1.3-fold, respectively) (Fig. 5A). ELISA assay of serum-deprived medium conditioned by dR cells (R-CM) for 72 h demonstrated PDGF-BB protein at concentrations of 21.84 ± 7.92 pg/ml/10^7 cells (Fig. 5B). Furthermore, dR cells expressed higher mRNA levels of SDF-1/CXCL12 and its receptor CXCR4 compared with dS-SMC (SDF-1/CXCL12: 10.0 ± 1.2-fold; CXCR4: 80.84 ± 19.19-fold, respectively) (Fig. 5, D and E).

We then tested if autocrine, serum-independent growth of dR cells was due, at least in part, to production of PDGFs and SDF-1. Neutralizing antibodies against PDGF-BB, -AB and/or against SDF-1/CXCL12 inhibited autocrine growth of dR cells in a dose-dependent manner (Fig. 5, C and F, respectively).

dR cells exhibit augmented migratory capabilities due to S100A4/RAGE axis. Findings on high proliferative potential of dR cells led us to test the migratory capabilities of these cells. “Wound/scrape” assay (Fig. 6A), using hydroxyurea (5 μM) to exclude contribution of cell proliferation demonstrated that, under normoxic (21% O2) conditions, dR cells exhibited six-fold higher migratory capabilities than the dS-SMC and migrated into the scrape even faster than the adventitial fibroblasts (Fig. 6B). Hypoxia (3% O2) further augmented the migratory potential of dR cells (up to 9-fold compared with dS-SMC) (Fig. 6B) but not that of dS-SMC.

Since S100A4 has been shown to be associated with enhanced cell motility (5, 71), we analyzed mRNA expression of S100A4 and of its receptor, RAGE (receptor for advanced glycation end products), and found that dR cells expressed higher than dS-SMC mRNA levels of S100A4 (2.27 ± 0.21-fold) and of RAGE (72.88 ± 13.36-fold, respectively) (Fig. 7A). At the protein level, S100A4 was expressed by dR cells at markedly higher levels than by dS-SMC (8.98 ± 2.12-fold) or even dPA adventitial fibroblasts (5.29 ± 1.25-fold) (Fig. 7, B–D).

To test the potential role of S100A4 and RAGE in conferring high migratory capabilities to dR cells, we utilized several inhibitory strategies and found that antibodies against S100A4 and/or against RAGE, as well as soluble RAGE, all markedly attenuated high migratory capabilities of dR cells (Fig. 7E). Interestingly, autocrine, serum-independent growth of dR cells was partially inhibited by S100A4 Abs (Fig. 7F), thus suggest-
ing a potential role of S100A4 not only in cell motility but in cell replication as well.

We also assessed the invasive capabilities of dR cells using a Matrigel assay. dR cells demonstrated a robust outgrowth and sprouting in Matrigel, whereas dS-SMC remained as compact cell clusters, as has been reported for SMC (Fig. 8A). Since the ability of cells to form sprouts in Matrigel has been attributed, in part, to MMP production (72), we tested the effect of the general MMP inhibitor GM6001 (20 μM) and observed a substantial inhibition of dR cell outgrowth in Matrigel (Fig. 8B), thus suggesting a role for MMPs in this process. Assessment of MMP activity in gelatin zymography assay showed that dR cells produced a pattern of gelatinolytic activity markedly distinct from that of dS-SMC and adventitial fibroblasts (Fig. 8C). Western blot analysis demonstrated that the observed enhanced gelatinolytic activity in dR cell extracts was specific for the active (68-kDa isoform) MMP-2 (Fig. 8D).

dR cells produce potent paracrine promitogenic activity, which is due, in part, to production of PDGFs, SDF-1, and S100A4. To test if dR cells produced factors that could exert paracrine mitogenic effects on dPA wall cells, we performed coculture experiments (see METHODS) using dR cells as the source of conditioned medium and as target cells, dPA SMC and adventitial fibroblasts. As shown in Fig. 9, serum-deprived (0.2% PDS) medium conditioned by dR cells for 72 h markedly augmented BrdU incorporation in target dS-SMC (3-fold) and target adventitial fibroblasts (2-fold). Importantly, serum-deprived medium conditioned by SMC and adventitial fibroblasts had no to minimal effect, respectively, on the target cells. Of interest, when dR cells were exposed to hypoxia (3% O2, 24 h) the promitogenic effect of their conditioned medium was further augmented compared with normoxic conditions (Fig. 9).

We then tested if potent paracrine promitogenic activity of dR cells was due to production of PDGFs, SDF-1/CXCL12,
and S100A4. Neutralizing antibodies against PDGF-BB/-AB, against SDF-1/CXCL12, and against S100A4 all inhibited paracrine promitogenic effect of dR cell-conditioned medium on dS-SMC (Fig. 10, A–C, respectively) and adventitial fibroblasts (not shown). Fibroblast-conditioned medium did not significantly stimulate BrdU incorporation in S-SMC (Fig. 10A), and, importantly, PDGF-BB (10 ng/ml)-stimulated BrdU incorporation in dS-SMC was not affected by either SDF-1 or S100A4 neutralizing Abs.

DISCUSSION

Based on emerging experimental data demonstrating that, in addition to phenotypic modulation of resident SMC, other cellular mechanisms contribute to remodeling of vascular tu-

nica media in response to injury, we sought to determine, using both in vivo and ex vivo approaches, if the cellular composition of the remodeled dPA media in calves with severe hypoxia-induced PH has been altered. In vivo, we observed that dPA media of chronically hypoxic hypertensive calves contained, in addition to differentiated SMC, phenotypically distinct cells that expressed hematopoietic (CD45), leukocytic/moноcytic (CD11b, CD14), progenitor (cKit), and motility-associated (S100A4) cell markers, which were not observed within the dPA media of control animals. Ex vivo experiments with cell cultures obtained from dPA media of hypertensive calves showed the emergence of cells (termed dR), which exhibited phenotypic and functional characteristics distinct from those of resident SMC, including augmented growth, migratory and invasive potentials, production of potent promitiogen activity, and expression of mRNA for inflammatory mediators and for progenitor-like cells. No phenotypic switch of dPA SMC into R cells was observed under any conditions/stimuli tested. Thus the data of the present study demonstrate a significant alteration in the cellular composition of the remodelled dPA media and suggest that the newly emerging cells could exhibit profound modulatory effects on the vascular microenvironment.

Our in vivo data, showing the emergence of cells expressing antigens not usually observed in differentiated SMC (cKit, CD45, CD11b, CD14), are consistent with observations in human and animal models of PH. In human PH patients, the presence of cells, expressing hematopoietic and inflammatory markers, has been documented both within and around the pulmonary vessel wall (22, 23, 42, 74). In PAs of infants and children with PH, expression of S100A4 (also known as mts1), a calcium-binding protein associated with invasion, metastasis of cancer cells, and with augmented cell motility (5, 45, 54, 71), has been documented, and the numbers of S100A4-expressing cells markedly increased with the severity of pulmonary vascular lesions (21, 22). Recent studies in adults with idiopathic pulmonary arterial hypertension and familial pulmonary arterial hypertension have reported the presence of cells expressing hematopoietic and progenitor cell markers within the vessel wall, and cells exhibiting mesenchymal progenitor characteristics have been reported in remodeled PAs of patients with COPD (2, 38, 39, 48, 49, 75). We have previously reported a marked increase in accumulation of CD45+, CD11b+, CD68+, and cKit+ cells in the adventitia of chronically hypoxic hypertensive calves (9, 17). Others have also found increased numbers of cKit+ cells at the medial-adven
titial border of chronically hypoxic mice, and demonstrated that, in vitro, these cells were capable of differentiating into α-SM-actin-expressing cells (58, 79). Bone marrow-derived cells have also been shown to accumulate in the PAs of chronically hypoxic chimeric mice and to differentiate into α-SM-actin-expressing cells (24, 79). Thus our findings regarding the presence of cells expressing hematopoietic/leukocytic (CD45/CD11b), progenitor-like (cKit), and motility-associated (S100A4) markers in the dPA media of hypertensive calves support and extend the idea that multiple cell types and/or cellular mechanisms may contribute to PA medial remodeling in the setting of hypoxia-induced PH.

Our ex vivo findings demonstrated consistently the outgrowth of at least two cell populations (dS-SMC and dR cells)
exhibiting distinct and stable phenotypic differences in primary cultures obtained from dPA of hypertensive, but not control, calves. dR cells exhibited augmented growth capabilities under both serum-stimulated and serum-deprived conditions, as well as augmented migratory and invasive potentials. Autocrine (serum-independent) growth of dR cells was due, at least in part, to secretion of PDGFs, SDF-1/CXCL12, and S100A4. Vascular cells with autocrine growth capabilities, obtained from injured adult systemic vessels, have been previously reported, and several factors, including those described here, have been suggested as conferring autocrine growth capabilities to those cells (34, 43, 44, 76, 77). Our study also indicates that the augmented migratory capabilities of dR cells were mediated, at least in part, through the S100A4/RAGE pathway (11, 14, 25). These findings are compatible with a recent report in systemic circulation where diseased porcine and human

![Fig. 7](image-url)
coronary arteries yielded in culture, besides traditional differentiated SMC, smaller morphologically rhomboid cells that exhibited augmented growth and migratory capabilities, partially due to high expression levels of S100A4 (5). Other studies have shown that S100A4 expression may confer invasive properties to the cell by inducing MMP expression (19, 54, 55, 69), which is consistent with our data demonstrating that dR cells express S100A4 and produce active MMP-2, and that both play a role in augmented growth and/or invasive capabilities of dR cells. Factors participating in regulation of S100A4/RAGE axis in mesenchymal cells have not been delineated, but serotonin (5-HT), frequently implicated in PH remodeling, has been shown to upregulate S100A4 mRNA/protein expression and to stimulate release of S100A4 (36). Hypoxia has also been shown to upregulate RAGE and S100A4 (8, 27, 37, 40). It is possible that other factors implicated in pulmonary hypertension could also operate through S100/RAGE signaling axis.

The present study also demonstrates that dR cells produce potent paracrine promitogenic activity for resident PA wall cells (medial SMC and adventitial fibroblasts), which is due, at least in part, to secretion of PDGFs, SDF-1/CXCL12, and S100A4. Moreover, dR cells have high mRNA expression levels of inflammatory cytokines, IL-6 and MCP-1. Release of these growth factors and inflammatory mediators would have major effects on the pulmonary vascular microenvironment, and the data thus suggest that dR cells can contribute to pulmonary vascular remodeling by modulating the proliferative and inflammatory status of resident PA wall cells. Recent reports have shown that mesenchymal precursor cells, recruited to the site of tissue injury, exert major effects on tissue remodeling through release of paracrine factors, thus altering the local microenvironment (6, 12, 30, 44, 50). For instance, circulating fibrocytes, being critical in the wound healing process, have been reported to secrete both growth-promoting and proangiogenic factors (4, 51). PDGFs are well known to be secreted by monocytes and progenitor cells and to stimulate proliferation of SMC and fibroblasts (3, 46). SDF-1/CXCL12 is secreted by several cell types in response to hypoxia and has been reported to stimulate SMC proliferation (7, 45). Consistent with this observation, we found that hypoxia further induces the potency of promitogenic activity of dR cells. Of relevance to the potential importance of SDF-1 production by the dR cells is the report by Young et al. (79), which demon-
strated that inhibition of SDF-1/CXCR4 axis attenuated neonatal hypoxia-induced PH in mice.

The emergence of dR cells, markedly distinct from the resident PA wall cells, raises questions regarding the origin/source of these cells as well as other phenotypically distinct cells that accumulate in the remodeled blood vessels in both pulmonary and systemic circulations. Besides the traditional hypothesis of phenotypic modulation of vascular medial SMCs, possibilities that have been recognized include fibroblasts, local tissue progenitor cells, circulating (hematopoietic or non-hematopoietic) mesenchymal progenitors (including fibrocytes), or even transdifferentiation of endothelial cells (1, 13, 28, 32, 41, 47, 56, 57, 60, 62, 63, 80). Our in vivo data demonstrate that some of the newly emerging cells within the dPA media of hypertensive calves express the hematopoietic/ leukocytic markers CD45, CD11b, and CD14, which could potentially be ascribed to the recruitment of circulating hematopoietic cells as described by others (57, 58, 61, 70). We have recently documented the robust recruitment of circulating fibrocytes to the remodeled pulmonary perivascular adventitia of chronically hypoxic calves and rats (17). Circulating fibrocytes are monocyteic cells that are recruited to the site of tissue injury where they acquire a mesenchymal (collagen-producing) and even a myofibroblast (α-SM-actin+) phenotype (4, 51, 75). Although we were unable to identify CD45+/collagen+ or CD11b+/collagen+ fibrocyte-like cells in the dPA media in vivo, dR cell colonies in primary culture transiently expressed CD11b, and consistently expressed type I procollagen and mRNA for CXCR4 (receptor for SDF-1/CXCL12), an expression pattern consistent with a fibrocyte phenotype. As suggested by others, this may be due to rapid differentiation (loss of hematopoietic/leukocytic antigens) of fibrocytes into mesenchymal cells under specific pathological environments (68). We also documented the presence of cKit+ (CD45+) cells within the media (notably, closer to the medial-adventitial border). Recent reports in chimeric mice suggest that the appearance of cKit+ cells in the PAs of chronically hypoxic animals is likely due to recruitment of bone marrow-derived cells, which are capable of differentiating into mesenchymal α-SM-actin+ cells (24, 58, 79). In fact, cKit function may be necessary for myogenic differentiation of bone marrow-derived cells (78). Yet another possibility, raised by the findings of Zengin et al. (81) and Passman et al. (47), suggests that there is a subset of resident vascular progenitor cells that reside at the medial-adventitial border (similar to the pattern of cKit+ cells reported here) and have the potential of differentiating into mesenchymal (endothelial-like or SMC-like) cells. Ex vivo data of the present study demonstrate that dR cells exhibited high mRNA expression levels of progenitor-associated cell markers cKit, CD34, and CD73 and had the potential of differentiating into mesenchymal (endothelial-like or SMC-like) cells. Ex vivo data of the present study demonstrate that dR cells exhibited high mRNA expression levels of progenitor-associated cell markers cKit, CD34, and CD73 and had the potential of differentiating into mesenchymal (endothelial-like or SMC-like) cells. In addition, the possibility that cKit may be expressed under certain conditions by activated fibroblasts, which can migrate into the media and even intima in response to injury, needs to be considered (10). Future studies are necessary to determine the origin(s) of the cells described here within.

In conclusion, in vivo and in vitro experiments of the present study demonstrate that chronic hypoxia induces the emergence of cells within the remodeled dPA media, that are phenotypically and functionally distinct from medial SMC, exhibit augmented proliferative (including autocrine), migratory and invasive capabilities, and secreted potent paracrine promitogenic factors for resident PA wall cells. These data suggest that accumulation of dR cells could contribute to the remodeling process not only directly through differentiation into mesenchymal/myofibroblast-like cells, but also indirectly through secretion of promitogenic and inflammatory mediators, which can modulate the pulmonary vessel wall microenvironment and the proliferative status of resident PA wall cells.
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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES

FUNCTIONALLY DISTINCT CELLS IN ARTERIAL REMODELING


