12-1-2008

Platelet-Derived Growth Factor Receptor-Alpha-Expressing Cells Localize to the Alveolar Entry Ring and Have Characteristics of Myofibroblasts During Pulmonary Alveolar Septal Formation

Stephen E. McGowan
University of Iowa

Ruth E. Grossmann
University of Iowa

Patricia Kimani

Please see article for additional authors.

Material in the public domain. No restrictions on use.


Hosted by Iowa Research Online. For more information please contact: lib-ir@uiowa.edu.
Platelet-Derived Growth Factor Receptor-Alpha-Expressing Cells Localize to the Alveolar Entry Ring and Have Characteristics of Myofibroblasts During Pulmonary Alveolar Septal Formation

STEPHEN E. MCGOWAN,* RUTH E. GROSSMANN, PATRICIA W. KIMANI, AND AMEY J. HOLMES
Research Service, Iowa City Department of Veterans Affairs Medical Center, University of Iowa Carver College of Medicine, Iowa City, Iowa

ABSTRACT
Platelet-derived growth factor-A and its receptor, platelet-derived growth factor receptor-alpha (PDGF-Rα), are required for formation of the secondary pulmonary alveolar septa in mice. However, it remains unclear how these molecules direct the secondary septation process. We have examined the abundance, location, and the accumulation of alpha-smooth muscle actin (αSMA), neutral lipid droplets, and elastin in the proximity of PDGF-Rα-expressing alveolar cells during postnatal days 4 through 12 in the mouse. PDGF-Rα-expressing cells preferentially have characteristics of myofibroblasts and were more likely to contain αSMA than are alveolar cells that do not express PDGF-Rα. PDGF-Rα-expressing cells were preferentially located in the alveolar entry ring (AER) where αSMA and elastic fibers accumulate. In contrast, PDGF-Rα expression inversely correlated with neutral lipid accumulation, which was more prominent at the alveolar base, distant from the AER. PDGF-Rα-expressing alveolar cells accumulate in the AER where they may promote mechanical stability during respiration. In addition to defining how alveolar septa form, these findings may have implications for the treatment of diseases which involve alveolar effacement such as emphysema and pulmonary fibrosis. Anat Rec, 291:1649–1661, 2008. © 2008 Wiley-Liss, Inc.

Key words: myofibroblast; elastic fiber; lipid storage

Platelet-derived growth factor-A (PDGF-A) and its cognate receptor, platelet-derived growth factor receptor-alpha (PDGF-Rα), are required for pulmonary alveolar secondary septal formation. PDGF-A null mice have normal appearing terminal sacs and a full complement of PDGF-Rα expressing alveolar cells at birth, but thereafter, the sacs do not undergo septation. Failed septation is accompanied by a nearly complete absence of alveolar mesenchymal cells, which normally contain smooth muscle alpha-actin (αSMA) and produce elastin (Bostrom et al., 1996, 2002). Others have proposed that PDGF-Rα-expressing alveolar cells are precursors of alveolar mesenchymal cells which contain αSMA and produce elastin.

Grant sponsor: Department of Veterans Affairs Research Service.
*Correspondence to: Stephen E. McGowan, M.D., Division of Pulmonary, Critical Care, and Occupational Medicine, Department of Internal Medicine, C33B-GH, University of Iowa Hospitals and Clinics, 200 Hawkins Dr., Iowa City, IA 52242. Fax: 319-353-6406. E-mail: stephen-mcgowan@uiowa.edu
Received 11 March 2008; Accepted 26 June 2008
DOI 10.1002/ar.20764
Published online 2 October 2008 in Wiley InterScience (www.interscience.wiley.com).
Studies using PDGF-A null mice suggested that PDGF-A may exert one or more of four potential effects on alveolar MF (Lindahl et al., 1997; Bostrom et al., 2002). First, PDGF-A may increase αSMA expression in MF. Second, PDGF-A may promote the migration of PDGF-Rα-expressing MF from the septal base to the tip, where they produce elastin. Third, PDGF-A could promote the proliferation of MF. And fourth, PDGF-A may stimulate PDGF-Rα-expressing cells to assume an "activated" phenotype, diverting them from a lipid-storage to an αSMA-expressing phenotype (as is observed in hepatic stellate cells) (Davis et al., 1993). These effects are not mutually exclusive because in the hair follicles, PDGF-Rα-expressing mesenchymal cells in the dermal papilla do not incorporate BrdU, whereas PDGF-Rα-expressing mesenchymal cells in the papillary sheath both incorporate BrdU and express αSMA (Carlsson et al., 1999).

The seminal studies by Bostrom et al. and Lindahl et al. suggested that PDGF-Rα expressing alveolar cells require PDGF-A to ultimately occupy their characteristic position at the tips of the secondary septa (Bostrom et al., 1996; Lindahl et al., 1997). However several details need to be further elucidated in order to understand how this requirement is fulfilled. For example, is the PDGF-Rα gene transcribed concurrently during αSMA and elastin expression by MF, or is PDGF-Rα primarily required for establishing sufficient MF in the proper location? Once MF are established, PDGF-Rα may no longer influence the synthesis of αSMA and elastin, which are hallmarks of the alveolar MF. Second, do PDGF-Rα-expressing MF populate the septal tips because of a proliferative advantage, or do they migrate there from the base of the septa, or are both mechanisms involved? Third, does PDGF-Rα influence the propensity for the MF to assume a contractile, αSMA-producing instead of a lipid storage phenotype?

To address these questions, we hypothesized that if PDGF-A mediated signaling specifies a myofibroblastic phenotype, then spatial and temporal colocalization of its cognate receptor, PDGF-Rα, should coincide with specific myofibroblast products. We have studied the chronology and location of PDGF-Rα expression during secondary septal formation in mice with hemizygous deletion of the PDGF-Rα gene (Klinghoffer et al., 2002). We used mice that were heterozygous for the GFP insertion so one allele contained the wild-type PDGF-Rα coding region. These mice are phenotypically identical to mice bearing two copies of the PDGF-Rα allele including their postnatal generation of alveoli. The mice were housed in Thoren cages in a temperature-controlled environment with a 12-hr light–dark cycle and provided food and water ad libitum. The protocol was approved by the animal use committees at the Iowa City Veterans Affairs Medical Center and the University of Iowa.

Preparation of Lung Tissue for Confocal Microscopy

At postnatal days 4, 8, and 12 mice were euthanized with ketamine and xylazine and their lungs were inflated in situ to total lung capacity with 1% low melting point agarose in 0.5% paraformaldehyde, 15% picric acid, 0.1 M Na Phosphate pH 7.0, while the carcass was maintained at 4°C. After the agarose hardened, the lungs were removed and their volume was determined by displacement (Scherle, 1970). The lungs were sliced into 1-mm transverse sections with a McIlwain tissue chopper, and fixed for 6 hr at 4°C in 0.5% paraformaldehyde, 15% picric acid, 0.1 M Na Phosphate pH 7.0. Following fixation, the agarose was removed by incubating for 1 hr in PBS at 50°C. The tissues were stored at 4°C in 0.05 M NaPhosphate pH 7.2, 20% sucrose, 10% glycerol, 0.1% sodium-azide. To prepare cryosections for immunostaining, the external liquid was removed, the tissue was embedded in optimal cutting temperature (OCT) medium, quickly frozen and 100 μm sections were cut with a cryotome.

Immunohistochemistry and Confocal Microscopy

After removing the OCT, the tissue was permeabilized in 0.3% Triton X-100, washed with PBS containing 0.2%
bovine serum albumin (BSA), incubated overnight at 4 °C with Cy3-labeled mouse anti-αSMA (clone 1A4), and washed. The tissues were counterstained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD, Invitrogen Molecular Probes, Carlsbad, CA) which stains phospholipid-containing membranes and mounted. Sections were imaged using a Zeiss LSM 510 scanning laser confocal microscope (LSCM) with excitation at 488, 543, and 633 nm, and emission band pass filters 505–530 nm, or 560–615 nm, and a 650 nm long pass filter. Z-stacks were obtained at a 1024 × 1024 pixel density, using a 3 μm slice interval and a pin hole of 1 airy unit, which allowed resolution of individual nuclei. To identify neutral lipid droplets, fixed and permeabilized tissue slices were incubated with (E,E)-3,5-bis-(4-phenyl-1,3-butanediyl)-4,4-diﬂuoro-4-bora-3a,4a-diaza-s-indacene (BODIPY 665/676, Invitrogen Molecular Probes) (Bedner et al., 2000). Cells of mesenchymal origin were identified using mouse anti-vimentin, followed by goat-anti-mouse Alexafluor 543, which allowed us to localize lipid droplets that were in mesenchymal cells. The BODPY and vimentin-stained tissues were imaged using the Zeiss LSM 510 and the same parameters were used to image GFP and smooth muscle actin.

**Stereology**

Image stacks were analyzed using StereoInvestigator (MBF bioscience, Williston VT). The alveolar entry rings (AER) were identified by circular or oval collections of αSMA that led into an airspace (alveolus), which ended in a sheet of cells (identified by DiD staining) within the z-stack. Individual GFP+ nuclei within a core of tissue that included the entry ring (ending of the alveolar duct) and alveolar walls of a particular alveolus were enumerated using the optical fractionator probe and their distance from the AER was ascertained (Howard and Reed, 2005). Tissues were analyzed from mice at postnatal days 4, 8, and 12 and the number of GFP+ cells was expressed relative to the volume of the lungs, per mm³ of tissue, and was corrected for the V₉ of the alveolar walls (obtained by point counting). We also studied temporal changes in the colocalization of GFP+ nuclei and αSMA at AERs. To reduce the dependence of the measurements on the intensity of αSMA immunostaining, sets of P-4, P-8, and P-12 tissues were stained and imaged in parallel. Color information, which was collected in a RGB format was converted to HSI format to dissociate the yellow hue from the intensity of the red αSMA immunostain. The area of each AER was ascertained by manually tracing around the αSMA, which needed to occupy over 80% of the ring. The pixel area occupied by yellow (hue) within the entry ring (coalescence of green GFP and red αSMA-containing pixels) was calculated using IPLab (BD-Bioscience, Rockville, MD) and expressed relative to the pixels occupied by GFP+ nuclei, or to the entry ring-area.

**Isolation of Lung Fibroblasts**

Immediately after euthanaisa on postnatal day 12, lungs from PDGF-Rc-GFP mice were perfused with saline, removed, minced, and digested in a Hank’s balanced salt solution with 5 mM CaCl₂, containing 250 U trypsin, 75 μg DNAse I, and 300 μg collagenase per milliliter. The minced tissue was incubated at 37 °C, and at 10-min intervals the suspension of released cells was strained through 100 μm mesh, and made 10% in FBS. After complete dispersal, the cells were plated in 100-mm diameter tissue culture dishes and allowed to adhere for 1 hr at 37 °C. After washing to remove nonadherent cells, the adherent fibroblasts were released by brief trypsinization and gentle scraping, and collected by centrifugation. The fibroblast-enriched population was enumerated and resuspended in media containing 10% FBS and divided into aliquots of 5 or 10 × 10⁵ cells.

**Flow Activated Cell Sorting to Identify αSMA**

Two slightly different procedures were used to prepare freshly isolated LF for flow cytometry. Group A (N = 2): 1 × 10⁶ cells were fixed in 1% paraformaldehyde in 0.1 M sodium phosphate pH 7.2 for 10 min at room temperature. After extensive washing in PBS, the cells were permeabilized in PBS containing 0.1% saponin and then incubated with equal concentrations (1:100 dilution) of either monoclonal phycoerythrin (PE)-conjugated anti-human α-smooth muscle actin (R&D Systems, Minneapolis, MN) or the PE-conjugated-IgG₂A (isotype control) for 45 min at room temperature. The samples in Group B (N = 3) were processed in the same fashion with the following exceptions: 5 × 10⁵ LF were fixed in 2% paraformaldehyde for 20 min at 4 °C. After permeabilization, the LF were incubated in Mouse-on-Mouse blocking reagent (Vector Laboratories, Burlingame, CA) for 1 hr at 4 °C, followed by either anti-αSMA or the isotype control IgG for 45 min at 4 °C. After washing to remove bound antibody, the LF were resuspended in PBS. For both groups, flow activated cell sorting (FACS) analysis was performed at the University of Iowa Flow Cytometry Facility using Becton Dickinson LSR II or FACs DIVA instruments. After assessing forward and side scatter, using an unstained sample, gating parameters were set. Compensation for any overlap of the PE and GFP signals was carried out using either samples stained with the anti-αSMA or Becton Dickinson comp beads (BD Biosciences, San Jose, CA), consisting of anti-mouse IgG exposed polystyrene microparticles which bind mouse antibody, and a negative control of FBS-exposed particles. The beads were incubated with the anti-αSMA antibody, and thus were used to optimize for a positive and negative PE signal. An isotype control sample was used to correct for nonspecific binding of nonimmune IgG. Flow cytometry was performed using a LSR II or FACs DIVA (Becton Dickinson, Franklin Lakes, NJ) instrument. The data were analyzed using both CellQuest Pro software (BD Biosciences) and SigmaStat (Jandal Scientific, San Rafael, CA). Each “N” represents a separate experiment (litter of mice) and greater than 7000 gated events were analyzed for each “N.” After pooling the results from all five experiments, a two-way analysis of variance revealed no significant experimental differences between the Groups A and B (P = 0.265, Student-Neuman Keuls post-hoc test). Therefore, data from groups A and B were combined.
Evaluating the Purity of the Freshly Isolated Lung Fibroblasts

The putative fibroblast population was examined for contamination by endothelial and epithelial cells and macrophages using FACS. Cytotypic markers (CD31 for endothelial and F4/80 pan macrophage marker) were detected in an unfixed sample using a PE-conjugated monoclonal rat anti-mouse CD31 (BD Biosciences) or a monoclonal rat-anti mouse F4/80 (eBioscience, San Diego, CA). Isotype controls included PE-conjugated rat-IgG2a and nonimmune rat IgG, respectively. A preliminary step was included to block endogenous biotin (avidin/biotin blocking reagent, Vector Laboratories, Burlingame, CA) in the F4/80 staining procedure. Cell associated anti-F4/80 was detected using a biotinylated anti-rat IgG (Sigma-Aldrich, St. Louis, MO) followed by Alexafluor 660 streptavidin (Invitrogen Molecular Probes). The stained LF were postfixed with 0.5% paraformaldehyde. Epithelial cells were detected with monoclonal mouse-anti-cytokeratin 18 following fixation, permeabilization, and blocking with Mouse-on-Mouse blocking reagent, as we had done for aSMA. The mouse anti-cytokeratin was detected using AlexaFluor 647 conjugated goat anti-mouse IgG. Flow cytometry was performed using the LSR II instrument. The proportions of cells staining positive for CD31, F4/80, or cytokeratin 18 were corrected for background staining.

Definition of Circumferential Deposits of Elastic Fibers and aSMA in the AERs

Tissues from the lungs of mice at postnatal days 4, 8, and 12 were obtained and processed as previously described. After removing the OCT and permeabilizing with 0.1% Triton X-100, the tissue slices were incubated with 2.5 μM eosin Y at 25°C for 1 hr (Mergens et al., 2007). After rinsing, the slices were imaged using the Zeiss 510 confocal microscope to visualize GFP and eosin Y (excitation 514, 550 long pass emission filter). Background staining with Eosin Y was subtracted and contiguous pixels demonstrating linear deposits of elastin were highlighted using uniform algorithms defined in IPLab. These included a background subtraction with a roll ball radius of 15 and a 3 × 3 pixel median filter. The same algorithm was applied to all images which enhanced visualization of elastic fibers. All of the AERs were visualized within a particular field, which was chosen at random. A superimposing line was drawn over collections of bright red pixels (elastic fibers) that appeared in the z-slice that best depicted the entry ring or from z-slices position one level above or below the slice at the entry ring. Entry rings were defined by collections of GFP+ cells and by their junction with alveolar ducts. The alveoli that were analyzed had a discrete beginning and ending within the tissue section, and needed to branch from the alveolar duct at an approximately 90-degree angle. The circumference of the AER was also traced. The number of pixels occupied by the tracing overlying elastic fibers was expressed relative to the pixels occupied by the circumference of the alveolar duct. This adjusted the length of elastic fibers for the size of the respective AER. The same tracing protocol was applied to images that had been stained for aSMA. The images were obtained from lungs of the same mice that were used for imaging elastic fibers, but not from precisely the same microscopic fields. Deposits of elastin and aSMA were studied in 60 to 75 AERs for each lung and changes in the circumferential length of elastic fibers was compared to that of aSMA at the three different ages.

Enumeration of Proliferating Alveolar Cells

Anti-Ki67 antibody was used to identify cells that were progressing through the cell cycle (not in G0), which we have designated as "proliferating." After permeabilizing with Triton X-100, lung sections from mice at postnatal days 4, 8, and 12 were incubated for 90 min with a 1:100 dilution of anti-Ki67 (clone TEC-3, Dako, Carpinteria, CA). After washing, the tissues were incubated with 0.33 μM TO-PRO-3 (Invitrogen, Molecular Probes) and a 1:500 dilution of anti-rat-IgG-Alexafluor 546 for 1 hr at 25°C. After a final rinse, the tissue was mounted and examined using the Zeiss 510 confocal microscope with excitation wavelengths of 488, 543, and 633 to visualize GFP (PDGF-Rα-expressing nuclei), Ki-67 (nuclei of cells in S-phase), and TO-PRO-3 (all nuclei, Invitrogen Molecular Probes). Images were captured at 3 μm, z-stack intervals, using a 512 × 512 pixel density to allow a faster scan speed in order to avoid photobleaching of the TO-PRO-3. StereoInvestigator was used to analyze alveoli that had been transected so that at least 75% of the AER was visible in a single z-slice. A reference line was drawn around the AER, which demarcated the outer boundary of a cylinder of tissue that extended to the base of the alveolus. The optical fractionator probe was used to ascertain the volume density of all nuclei in the alveolar wall (TO-PRO-3), PDGF-Rα-GFP-expressing cells, and proliferating (Ki-67 containing nuclei). The volume of the cylinder served as the reference space and was normalized to the volume of the lung. Seventy-five alveoli from three lungs were examined at postnatal days 8 and 12, and from four lungs at postnatal day 4.

Localization of Lipid Droplets Within Alveolar Mesenchymal Cells

We first queried whether lipid-containing mesenchymal cells were more abundant at the AER or at the alveolar base. Because our prior studies showed that GFP containing cells were more abundant at the entry ring, and the BODIPY dye also served as a weaker counterstain for phospholipids, we could visualize the alveolar wall throughout its course from entry to base. The area of the AER was ascertainment by tracing the outer and inner perimeters, and calculating the areal difference between the two tracings. The area of the alveolar base was ascertainment as the area within the tracing, which outlined the column of alveolar airspace in the z-section closest to the base. A uniform segmentation algorithm was applied to both the entry ring and base to identify pixels with both red (vimentin) and blue (BODIPY 650/665) intensity that exceeded a particular threshold (magenta color). The threshold value was the same for both the entry ring and base. Segmentation was followed by an erosion to distinguish groups of pixels that were associated with lipid droplets from isolate pixels in the background. The pixel area of lipid droplets that were located...
at the entry ring was divided by the area of the entry ring, and a similar calculation was performed for the lipid droplets that were at the base. We avoided portions of z-stacks where there was a visible difference in the intensity of BODIPY staining at the AER compared to the base.

We also examined the pixel area of lipid droplets that were proximate to nuclei, which exhibited either a high or low level of PDGF-Rα gene expression (high or low intensity of GFP). Because the intensity of the BODIPY signal faded in the z-sections that were furthest from the laser beam, we examined a single slice that was 1/3 of the distance into each z-stack. All of the low and high-intensity GFP+ nuclei were examined if that particular slice was closest to the center of the nucleus. A uniformly sized circle (11.2 μm diameter) was placed around the centroid of the nucleus and lipid droplets that fell within the circle were identified. The pixel areas occupied by the droplets were ascertained for each nucleus that was examined. This allowed us to compare the area occupied by lipid droplets around either high or low intensity GFP cells within the same z-section. The mean lipid droplet pixel area for high PDGF-Rα expressing was calculated and subtracted from the area for both high and low level expressing cells. This reset the mean for the high expressing cells to zero, and provided a mean and variance of pixel area by which the low expressing cells exceeded that of the high expressing cells.

RESULTS

Is There a Temporal Correlation in the Abundance of PDGF-Rα-Expressing Alveolar Cells, aSMa and elastin?

We first examined the abundance of PDGF-Rα-expressing alveolar cells at postnatal (P) days 4, 8, and 12 (P-4, P-8, and P-12). A representative z-stack defining an alveolus at postnatal day 8 is shown in Fig. 1A. The AER is defined by the red αSMA in frame 1, whereas the base of the alveolus is defined by the continuous sheet of blue DiD stained cells in frame 5. The number of GFP+ alveolar cells increased from P-4 through P-12 when expressed relative to the volume of the entire lung parenchyma (mm3) of parenchyma: (*P < 0.01, P-4 compared to P-8 or P-12), Student-Neuman-Keuls post-hoc test.
not when compared to P-12 (Fig. 1B, right). These data indicate that the abundance of PDGF-Rα-expressing cells increases as the alveolar septa form.

To determine whether PDGF-Rα-expression correlates with a myofibroblastic phenotype, we isolated a subpopulation of alveolar cells with characteristics of lung fibroblasts (LF). Approximately 85% of the isolated cells on postnatal day 12 were characterized as fibroblasts because endothelial cells, macrophages, and epithelial cells comprised 1.6% ± 0.3% (N = 3), 6.6% ± 0.3% (N = 3), and 8.1% ± 4.1% (N = 2), respectively. The isolated LF were stained for αSMA and separated into GFP+ (express PDGF-Rα) and GFP− (do not or only express low levels of PDGF-Rα) populations using flow cytometry. A significantly larger fraction of the GFP+ LF contained αSMA (Fig. 2, right side) compared to LF which did not express the GFP transgene (Fig. 2, left side). This indicates that PDGF-Rα-expressing LF are more likely to contain αSMA, a distinguishing feature of MF, than are LF that are not expressing PDGF-Rα.

If PDGF-Rα-expression identifies a MF-phenotype, then PDGF-Rα-expression may persist as αSMA and elastin, which are produced by MF, accumulate in the AER. We analyzed collections of αSMA and elastic fibers in the AER. Exemplary AER at postnatal days 4, 8, and 12 are shown in Fig. 3A. The AER was identified as the opening of pockets of air that closed into a sheet of cells within the z-stack (alveolar base). The circumference of the AER was traced and a concentric line was traced over the immunoreactive αSMA filaments in the AER. This allowed us to assess the proportion of the AER circumference that was occupied by αSMA at P-4, -8, and -12. The proportion of the AER circumference that was occupied by elastic fibers was likewise determined using sections from the same mice that had been stained with eosin Y (white lines in Fig. 3A). Figure 3B shows that elastic fibers occupied a significantly lower proportion of the AER circumference at P-4 than at either P-8 or P-12. In contrast, αSMA occupied a similar proportion of the AER circumference at days P-4 and P-8, but decreased significantly by P-12. These findings indicate that PDGF-Rα-expression persists throughout the period when αSMA and elastin accumulate in the AER, and therefore could participate in the accumulation. These data also show that in the AER, αSMA is more abundant relative to elastin at P-4 than it is at P-8 and P-12.

Is There a Spatial Correlation Between PDGF-Rα Expression and the Accumulation of αSMA?

We hypothesized that if PDGF-Rα expression correlates with a MF-phenotype, then PDGF-Rα-expressing alveolar cells would be predominantly located in the AER where αSMA and elastin were most abundant. To assess whether PDGF-Rα-expressing alveolar cells are proximate to accumulating collections of αSMA, we evaluated the colocalization of the green (GFP, G) and red (cy3-αSMA) containing pixels (yellow, Y). We observed an age-related increase in the pixel area occupied by yellow relative to the pixel area occupied by green, within the area occupied by the AER (Fig. 4, left). This suggests that there is an age-related increase in αSMA in close proximity to GFP+ containing nuclei. We also calculated the area occupied by yellow pixels relative to the area of the AER (Fig. 4, right). This ratio also increased, which indicates that PDGF-Rα-expressing (GFP+) cells accumulated in the entry ring during the period from 4 to 12 postnatal days. Taken together with the data demonstrating that PDGF-Rα-expressing cells increase over this same period, these findings indicate that PDGF-Rα-expressing cells are accumulating in the same locale with αSMA. Albeit indirectly, these data further support the finding that PDGF-Rα-expressing cells, which accumulate in the AER, produce αSMA.

Do PDGF-Rα Expressing Progressively Colocalize with Accumulations of αSMA During Septal Elongation?

It is well established that septal elongation occurs at a maximal rate between postnatal days 4 and 12 (Burri, 2006). Therefore we compared the distribution of PDGF-Rα-expressing cells along an axis reaching from the AER to the base of the alveolus. We arbitrarily divided this axis into five segments (quintiles) so that we could compare data from saccular structures (terminal alveolar ducts or alveoli) at different ages (Figure 1A). At all three ages, the largest fraction of PDGF-Rα-expressing cells was found in the quintile located nearest the AER (quintile 1), ranging from approximately 37% to 48% at P-4 and P-12, respectively (Fig. 5). The majority of PDGF-Rα-expressing cells were found in the first two quintiles at all ages: 52%, 72%, and 68% at P-4, P-8, and P-12, respectively. The distribution at P-4 was unique in that the second most populated quintile was at the alveolar base, which contained the smallest fraction at P-8 and P-12. We analyzed the shape of the curves which fit a second-order polynomial distribution of the data at the three ages. The second moment of the polynomial varied significantly with age (one-way ANOVA on ranks, P < 0.05) indicating that the distribution of PDGF-Rα cells changed significantly after P-4, as a larger majority of the expressing cells were located at the AER. This could result from migration of PDGF-Rα-expressing cells from...
cells that had exited G₀ entered the proliferative portions of the cell cycle.

**PDGF-Rα-Expressing Alveolar Cells Increase with Age Whereas Proliferating Alveolar Cells Decrease**

PDGF-A stimulates mesenchymal cell proliferation, but PDGF-Rα-expression does not always predict cellular proliferative index (Karlsson et al., 1999, 2000). To examine whether PDGF-Rα-expression connoted a more proliferative phenotype, we determined the proliferative indices of all alveolar cells and of GFP⁺ expressing alveolar cells. A representative field from the lung at P-4 is shown in Fig. 6A and illustrates localization of Ki67 and GFP to the nucleus. The fractions of Ki67-staining cells relative to total alveolar cell (nuclear stain, Ki67⁻/nuclei) remained constant, whereas the fraction of Ki67-staining, PDGF-Rα-expressing cells (Ki67/GFP⁺) were maximal at P-4 and diminished with age (Fig. 6B). Therefore we observed an inverse correlation between the age-related changes in the fraction of proliferative PDGF-Rα-expressing cells and the increase in the number of PDGF-Rα expressing cells (Fig. 1B). These findings suggest that alveolar cells that express PDGF-Rα are not proliferating more rapidly than cells that do not express the receptor.

**PDGF-Rα-Expression Inversely Correlates with Lipid Droplet Accumulation**

Others have shown that the abundance of alveolar interstitial cells, which contain lipid droplets, decreases during the period of maximal septal elongation in mice and rats (Maksvytis et al., 1981; Awonusonu et al., 1999). This decrease coincides with the assumption of a more MF-like phenotype. We and others have shown that the lipid storage and MF phenotypes represent opposing poles of the spectrum of neonatal LF phenotype. A similar polarity has been observed in hepatic stellate cells are activated by PDGF-A, they store less retinyl ester and contain fewer lipid droplets (Moreira, 2007). Therefore we queried whether the abundance of lipid droplets varied along the axis extending from the AER to the base, and whether the distribution of...
droplets along this axis changed with age. We evaluated pixels that registered fluorescence from both BODIPY-650/665 and vimentin, to select for neutral lipids within mesenchymal cells. Figure 7A illustrates a representative field showing localization of blue BODIPY 650/655-stained lipid droplets with vimentin in some (*) but not other cells (†). We found that the pixel density of lipid droplets in mesenchymal cells was approximately 3.6 and 2.1 fold higher at the base on P-4 and P-8, which significantly differed from the distribution at P-12 when there was no difference between the pixel density of lipid droplets at the base and entry ring (Fig. 7B). This suggests that lipid droplets are predominantly located at and preferentially disappear from the base as the septa elongate. We also queried whether lipid droplet accumulation varied with the level of PDGF-Rα gene expression. To assess this, we analyzed the pixel area occupied by lipid droplets within a fixed radius around alveolar cells with high versus low levels of PDGF-Rα (high versus low intensity of GFP-emission). We compared lipid droplets around nuclei with high and low level GFP-signal, in one z-slice. The mean pixel area that surrounded cells with the brightest GFP-emission for a particular slice was subtracted from the individual pixel areas for all high and low level GFP-expressing nuclei in that slice. This enabled us to calculate a mean pixel area for the population of either high or low expressing nuclei in each slice that was analyzed, and to normalize for variance in the BODIPY signal intensity in different stacks (which differed somewhat in their total height). We found that the pixel area around cells with low level PDGF-Rα-expression significantly exceeded the area around cells with high level expression at P-4 and P-8, but not at P-12 (Fig. 8). When combined (Figs. 7 and 8), these data indicate that when lipid droplets are preferentially located at the alveolar base, they are also preferentially located around cells with a lower level of

Fig. 4. Colocalization of PDGF-Rα-expression and αSMA increase with age. Area of pixels that contained yellow (merge of red and green) and/or green was enumerated and expressed as ratio of yellow (Y) to green (G, left ordinate scale). The mean ± SEM area of pixels that recorded fluorescence for both GFP and αSMA (yellow) increased with age relative to the area of pixels that contained GFP. 12 AERs were analyzed for each of three lungs at all three ages. *P < 0.01, P-4 compared to either P-8 or P-12. **P < 0.01, P-12 compared to P-8. Area (mean ± SEM) of pixels that contained yellow was also expressed relative to the area occupied by the alveolar entry ring (AER, right panel).

Fig. 5. Alveolar distribution of PDGF-Rα-expressing (GFP+) cells changes with age. PDGF-Rα-expressing alveolar (GFP+) cells were enumerated using the optical fractionator and their location determined relative to an axis extending from the AER (quintile 1) to the base (quintile 5). The mean ± SEM fraction of GFP+ cells in each quintile are shown as bars. Line represents second order polynomial fit to distribution among the quintiles. Points illustrate closeness of fit to the equations; r² ranged from 0.94 to 0.98. The same 100 alveoli were analyzed for each age that was used for Figure 1.
PDGF-Rα expression. This suggests that lipid droplets are primarily lost from alveolar cells with the lowest level of PDGF-Rα expression by day 12.

**DISCUSSION**

The temporal course and stereology of alveolar septal formation have been extensively characterized using thin sections embedded in paraffin or plastic (Thibeault et al., 2003; Burri and Schittny, 2004). In contrast, relatively few studies have been conducted using optical slices obtained from confocal microscopy (Oldmixon et al., 2001). We opted to use confocal microscopy for several reasons. First, we desired to preserve the fluorescent emission of GFP and avoid autofluorescence induced by plastic embedding media. Second, because we relied on immunohistochemical identification of αSMA, Ki67, and vimentin we wanted to avoid alterations in antigens that may result from paraffin embedding or antigen retrieval. Third, dehydration, paraffin embedding, and dewaxing would have prohibited the detection of neutral lipid droplets. And fourth, optical sectioning is ideally suited to the optical fractionator probe, which allowed us to count nuclei using an unbiased stereological approach. Although it was advantageous for our objectives, confocal microscopy does have limitations. First, the stage of the Zeiss 510 microscope was only powered by a motor in the z-axis, so fields could not be randomly selected in an automated fashion. Therefore we intro-
duced randomness into the selection process by choosing areas using lower power so that the particular cellular features that we were studying were not sufficiently magnified to influence the decision. In all cases, fields that contained large airways or blood vessels, pleura, or were incompletely expanded were avoided. We chose the sizes of the pinhole and optical slice to limit optical distortion that would render it difficult to determine which section the cell nucleus primarily resided in. Finally, we used uniform criteria for selecting alveoli for analysis and avoided alveoli that arose from alveolar ducts at an obtuse angle.

Fig. 7. Lipid droplets occupied a larger portion of the alveolar base than that of the alveolar entry ring (AER). A: Bright blue (BODIPY 650/665) lipid droplets surround some GFP nuclei. Vimentin (red) colocalized with some GFP-containing cells that also contained lipid droplets (*). Other cells contained vimentin but lacked lipid droplets (t). Examples of nuclei exhibiting high (H, more intense) and low (L, less intense) levels of PDGF-Rα (GFP)-expression are shown. Left panel shows red, green, and blue merge, whereas right panel shows only the blue and green merge, to better visualize the lipid droplets. B: Mean ± SEM ratio of pixel area (pixel density) occupied by BODIPY-650/665 staining neutral lipids and vimentin was higher at the base than at the AER. The preferential localization of neutral lipids to the alveolar base was evident at P-4 and P-8, but not P-12. *P < 0.01 one-way ANOVA, Student-Newman-Keuls post-hoc test.
Several priorities were considered in our choice of stereological methods. First, we limited the use of staining intensity to quantify proteins or structures. Antibodies for αSMA, vimentin, and Ki67 were used to identify structures or cells: the AER, mesenchymal cells, and cycling cells, respectively. The AER was used to demarcate the junction with the alveolar duct, vimentin was used to identify lipid droplets that were associated with mesenchymal cells, and Ki67 was used to enumerate proliferating cells. To further limit the effects of variations in staining intensity, we always concurrently processed tissues from mice at all three ages. Although we did not use intensity to evaluate αSMA, Ki67, or vimentin, this parameter was involved in the quantification of BODIPY-650/665 fluorescence in neutral lipid droplets. In this instance, the intensity of the dye in lipid droplets was combined with uniform criteria for segmentation that allowed us to consistently measure the area of pixels meeting the criteria. Finally, we avoided counting structures in two dimensions by using the optical fractionator. This enabled us to avoid errors from counting structures with a centroid outside the plane of the section and allowed us to express the data relative to the volume of tissue.

Studies by Böstrom et al. and Lindahl et al. compared PDGF-A null and wild-type mice (Böstrom et al., 1996, 2002; Lindahl et al., 1997). They demonstrated that PDGF-Rα mRNA localized to blood vessels, airways, and scattered cells in the lung parenchyma after birth and the abundance of mRNA diminished after P-7. Alveolar septal elastic fiber formation was markedly diminished in the PDGF-A null mouse, which also exhibited a striking absence of PDGF-Rα in the postnatal alveolar sacs. However, these studies did not demonstrate a temporal or spatial association between PDGF-Rα-expressing cells and tropoelastin gene expression. The abundance of PDGF-Rα mRNA diminished prior to the decrease in tropoelastin mRNA. Whereas tropoelastin mRNA appeared most abundant at the septal buds or tips, PDGF-Rα mRNA localized in scattered cells that were removed from the vicinity of the septal buds and tips. Furthermore, αSMA was only observed in the airways and vasculature during the second postnatal week, and became most abundant after the maximal period of tropoelastin synthesis. These investigators observed a normal complement of PDGF-Rα containing cells in the PDGF-A null mice, which persisted until birth in the distal tubules and primitive sacs, but an absence of these cells by P-7. From these data, the authors reasoned that PDGF-A may be required for the “spreading” of PDGF-Rα-expressing cells into the distal portion of the alveolar septum.

Although our studies corroborated some of the findings of Böstrom et al. and Lindahl et al., we also observed important differences. Like these investigators, we observed that the distribution of PDGF-Rα-expressing cells shifted (“spread”) more distally from the alveolar base to the AER between postnatal days 4 and 8. However, unlike Böstrom et al. and Lindahl et al., we did not observe a decrease in PDGF-Rα-expressing cells by P-8, and instead the abundance of these cells was sustained at least until P-12 (Fig. 1). We found that the most prominent collections of PDGF-Rα-expressing cells, αSMA, and elastic fibers all localized to the AER, whereas the studies by Böstrom et al. and Lindahl et al. did not exhibit spatial or temporal colocalization of these components. We attribute these disparities to differences in the histological approaches that were used. Böstrom et al. and Lindahl et al. relied on in situ hybridization and immunohistochemistry using thin sections of tissue and quantitative stereology was not performed. Using GFP to localize PDGF-Rα expression allowed us to more precisely identify and quantify expressing cells. Another advantage of our approach was that it allowed us to localize proteins or expression patterns to identical or contiguous pixels, rather than general regions in sections that were obtained from somewhat different portions of the lung. The greater precision of our methods supports the significance of our finding that PDGF-Rα-expressing cells colocalize both spatially and temporally with αSMA. We also observed that newly isolated PDGF-Rα-expressing fibroblasts are more likely to contain αSMA than fibroblasts that are not expressing PDGF-Rα (Fig. 2). When combined, these findings indicate that cells, which are expressing the pdgf-Rα gene, concurrently produce αSMA and that they contribute to the denser accumulation of αSMA at the AERs. We also observed that a larger proportion AER circumference was occupied by αSMA than by elastic fibers at P-4 (Fig. 3). However by P-8 this disparity had disappeared and by P-12 αSMA occupied a smaller proportion of the circumference than at P-8. Because mechanical tension at the AER is required to prevent collapse during expiration, αSMA may play a more critical mechanical function at P-4 than at P-12 when elastic fibers are more abundant in the AER. This would be consistent with recent observations that tension stimulates αSMA gene expression (Zhao et al., 2007). As elastin becomes more abundant in the AER, the tension exerted on αSMA filaments would diminish and one would expect that αSMA may be less abundant, which is what we observed at P-12.

Although our studies have more precisely mapped the temporal and spatial locations of PDGF-Rα-expressing cells...
alveolar cells, they do not demonstrate that PDGF-A was directing these changes. However observations by others suggest that PDGF-A is a significant contributor. First in its absence, PDGF-Rα-expressing cells fail to populate the locations where we observed them (Bostrom et al., 2002). Second, although PDGF-C also requires PDGF-Rα for signaling, and PDGF-C is expressed in the lung in utero, abnormalities were not observed in the lungs of PDGF-C null mice at birth (Ding et al., 2004). These mice could not be studied postnatally because they are unable to suckle and die shortly after birth. Therefore PDGF-A is also likely to be the relevant PDGF-Rα ligand in our studies. Other soluble factors and extracellular components, which do not directly interact with PDGF-Rα, also likely impact the persistence and migration of the alveolar MF.

Our primary purpose for characterizing the abundance and location of PDGF-Rα-expressing alveolar cells was to map the temporal and spatial fate of the alveolar MF. Therefore the PDGF-Rα-GFP construct enabled us to address this intent. Others have used hemizygous PDGF-Rα gene insertions to map the temporal and spatial fate of the neural crest and pulmonary mesenchymal cells (Klinghofer et al., 2002; Mandeville et al., 2006). Zhang et al. demonstrated that the PDGF-Rα-LacZ transgene was expressed in the mesenchyme of the lung parenchyma during gestation and through postnatal day 7 (Zhang et al., 1998). The abundance of the LacZ gene product diminished after P-7 and was absent by P-20. More recently PDGF-Rα-GFP marker was used to show that Hox 5a gene deletion resulted in diminished migration and elastic fiber production by embryonic lung MF (Mandeville et al., 2006). Therefore a precedent exists that PDGF-Rα-expressing alveolar cells demonstrate a MF-like phenotype.

During the first 3 weeks of postnatal life in rats and mice, two LF subpopulations can be distinguished based on their abundance of neutral lipid droplets namely the lipid and nonlipid interstitial fibroblasts (LIF and NLIF). LIF predominantly found at the base of the septa (Burri, 1974) have a lower proliferative index than NLIF at P-4, and both LIF and NLIF proliferation diminishes significantly by P-10. The abundance of LIF decreases relative to that of NLIF between P-4 and P-12 and there is a precipitous decline in LIF after P-15. This decline results from apoptosis of the LIF and 51% of this subpopulation exhibits DNA fragmentation between P-15 and P-17. The LIF subpopulation has been further distinguished as having either a higher (LIF+) or lower (LIF−) abundance of lipid droplets. The LIF− retains insulin like growth factor 1-receptor mRNA and function and are more likely to avoid apoptosis (Srinivasan et al., 2002). Our findings corroborate those reported by Burri and by Kaplan and Brody demonstrating that LIF are more abundant at the base of the secondary septa. We have made the novel observation that alveolar cells with a lower intensity of GFP-fluorescence exhibit a greater areal density of surrounding lipid droplets. Because we analyzed pixel density of lipid droplets and the intensity of GFP in the same z-slice, it is unlikely that differences in excitation beam penetration or quenching of the emitted light account for our observation. Therefore differences in GFP-signal intensity most likely result from differences in the abundance of the GFP-protein, which is directly proportional to PDGF-Rα promoter activity.

Taken together these findings indicate that lipid droplet accumulation is most abundant in cells with lower PDGF-Rα gene expression. This is consistent with observations that others have made using hepatic stellate cells (Breitkopf et al., 2005).

In conclusion, we have shown that PDGF-Rα-expressing alveolar cells are preferentially located at the AER and produce αSMA. The proportion of alveolar PDGF-Rα-expressing cells that localize to the AER increases between postnatal days 4 and 12, whereas the proportion located at the alveolar base decreases. This is consistent with relocalization of PDGF-Rα-expressing cells from the alveolar base to the AER. The accumulation of αSMA in the AER occurs earlier after birth than the accumulation of elastin. Therefore, αSMA may serve an important mechanical function: to stabilize the AER during expiration, until elastic fibers accumulate. The level of PDGF-Rα expression is inversely correlated with lipid droplet accumulation which preferentially occurs at the alveolar base. Taken together, these findings indicate that PDGF-Rα-expressing alveolar cells are more likely to have myofibroblastic rather than lipid storage characteristics. Whereas the myofibroblast phenotype may be essential during secondary septation, it may be undesirable in the adult lung that is undergoing a fibrotic response. Strategies that modulate PDGF-Rα-expressing cells toward a lipid-storage phenotype may alter the outcome of fibrotic lung diseases (Rehan et al., 2006).

LITERATURE CITED


