Pulmonary ischemia induces lung remodeling and angiogenesis

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Submitted 10 January 2005; accepted in final form 1 October 2005

Wagner, Elizabeth M., Irina Petrache, Brian Schofield, and Wayne Mitzner. Pulmonary ischemia induces lung remodeling and angiogenesis. J Appl Physiol 100: 587–593, 2006. First published October 6, 2005; doi:10.1152/japplphysiol.00029.2005.—Cellular remodeling during angiogenesis in the lung is poorly described. Furthermore, it is the systemic vasculature of the lung and surrounding the lung that is proangiogenic when the pulmonary circulation becomes impaired. In a mouse model of chronic pulmonary thromboembolism, after left pulmonary artery ligation (LPAL), the intercostal vasculature, in proximity to the ischemic lung, proliferates and invades the lung (12). In the present study, we performed a detailed investigation of the kinetics of remodeling using histological sections of the left lung of C57Bl/6j mice after LPAL (4 h to 20 days) or after sham surgery. New vessels were seen within the thickened visceral pleura 4 days after LPAL predominantly in the upper portion of the left lung. Connections between new vessels within the pleura and pulmonary capillaries were clearly discerned by 7 days after LPAL. The visceral pleura and the lung parenchyma showed intense tissue remodeling, as evidenced by markedly elevated levels of both proliferating cell nuclear antigen and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling positive cells. Rapidly dividing cells were predominantly macrophages and type II pneumocytes. The increased apoptotic activity was further quantified by caspase-3 activity, which showed a sixfold increase relative to naive lungs, by 24 h after LPAL. Because sham surgeries had little effect on measured parameters, we conclude that both thoracic wound healing and pulmonary ischemia are required for systemic neovascularization.

the process of new vessel growth induced by tissue ischemia requires an orchestrated series of local cellular events. New vessel formation follows an organized progression of changes, including matrix dissolution, cell migration, and proliferation (3). Pulmonary ischemia resulting from chronic pulmonary embolism or other forms of pulmonary artery obstruction lead to proliferation of the systemic circulation within and surrounding the lung (4, 7, 8, 18). The bronchial and intercostal vasculatures respond to pulmonary ischemia due to pulmonary artery obstruction with rapid neovascularization. It is interesting to note that the pulmonary vasculature appears not to be responsive in this setting. In general, the pulmonary artery is not proangiogenic, except in rare circumstances, as a secondary source of perfusion in some lung carcinomas (11). Unlike other organs, the lung is unique in that pulmonary ischemia is not accompanied by tissue hypoxia. Our laboratory previously developed a mouse model of lung angiogenesis and quantified new systemic perfusion of the lung following left pulmonary artery ligation (LPAL) (12). Rapid neovascularization occurs, and systemic perfusion of the lung can be measured 5 days after pulmonary artery ligation. However, unlike most other species, new vessel growth appears to be exclusively from intercostal arteries and not the bronchial vasculature, which is poorly developed in the mouse (17). The gross anatomic changes in this model show intercostal vessels bridging the pleural space (12). However, the cellular and morphological changes involved in lung neovascularization have not been evaluated. In this study, we determined the rate and extent of cellular remodeling in the lung parenchyma and the visceral pleura following chronic LPAL. Furthermore, we determined cell proliferation and apoptosis in the lung parenchyma and pleura induced by pulmonary ischemia following LPAL. Our results demonstrate a robust vascular proliferation that develops within the rapidly thickening visceral pleura with subsequent connections to existing pulmonary vessels.

METHODS

LPAL. Our experimental protocols were approved by the Johns Hopkins Animal Care and Use Committee. Mice (5- to 6-wk-old male C57Bl/6J; Jackson Laboratories, Bar Harbor, ME) were anesthetized (2% isoflurane in oxygen), intubated, and ventilated at 120 breaths/min, 0.2 ml/breath, with the anesthetic/gas mixture. A left thoracotomy was performed at the third intercostal space to expose the left lung, and the animal was placed on positive end-expiratory pressure. The left pulmonary artery was located, separated from the airway, and ligated using 6-0 silk suture. The thoracotomy was closed with a silk suture. A drop of 2% lidocaine was applied for analgesia, and the skin incision was closed by using methyl acrylamide adhesive. The animal was removed from the ventilator, extubated, and allowed to recover. Sham surgical control mice were subjected to the same procedures, except for LPAL. Mice were killed with an overdose of pentobarbital sodium at selected times after LPAL.

Blood vessels. To detect the appearance of new blood vessels, mice (n = 3–6/time point) were killed 1, 4, 7, 14, and 20 days after LPAL or sham thoracotomy. Lungs were fixed at this relatively low liquid pressure serves not only to keep the lung well inflated (14) but also to leave blood vessels filled with red blood cells, thereby enabling their identification. The lungs and heart were removed from the thoracic cavity en bloc and washed in water overnight, and the left lung was cut transversely into three consecutive 2-mm-thick slices, beginning 2 mm below the apical surface. Slices were immersed in Weigert’s iodine, followed by sodium thiosulfate to remove mercury precipitate, and embedded in glycol methacrylate. Three-micrometer-thick sections were stained with Eosin Ponceau Acid Fuchsin solution (Poly Scientific, Bay Shore, NY) and 0.01% methylene blue (Electron Microscopy Science, Fort Washington, PA). Sections were visualized and photographed by using an Olympus BX60 microscope and Cool Snap digital camera (Media Cybernetics, Silver Spring, MD).

Proliferating cell nuclear antigen. To determine the number and site of actively dividing cells, mice were killed 4 h, 12 h, and 1, 2, 3,
Fig. 1. Time course of lung vascularization after left pulmonary artery ligation (LPAL) compared with sham-operated mice (×10 objective). A: At 1 day post-LPAL, there is slightly increased subpleural tissue density, but little change after sham thoracotomy (representative of n = 5 LPAL, n = 5 sham). B: At 4 days post-LPAL, there are numerous enlarged pleural vessels. After sham thoracotomy, there is slight visceral pleural thickening in the lung region opposite the thoracotomy wound, but with no increased vascularization. (representative of n = 4 LPAL, n = 5 sham). C: At 7 days post-LPAL, there is extensive subpleural vascularization, with some breaks in the pleural membrane. After sham thoracotomy, the pleural thickening seen earlier has regressed substantially (representative of n = 6 LPAL, n = 5 sham). D: At 14 days post-LPAL, there appears to be a well-established subpleural circulation with many openings and tears in the pleura, where vessels would have connected to the chest wall. After sham thoracotomy, the localized pleural thickening has almost returned to normal (representative of n = 5 LPAL, n = 3 sham). E: At 20 days post-LPAL, the new systemic circulation to the lung has stabilized with many vascular connections through the visceral pleura. The ×20 magnification shows these vessels more clearly. Large vessels are seen in the lung periphery and appear to connect to preexisting pulmonary vessels. At this time point after sham thoracotomy, the pleura has returned to a normal appearance (representative of n = 5 LPAL, n = 4 sham).
5, 7, and 14 days after LPAL or sham thoracotomy, and the left upper lung was processed for evaluation of proliferating cell nuclear antigen (PCNA) positive cells. Mice were intubated, and agarose (0.8 ml of 7.5%) was infused through the endotracheal tube. After infusion, the tracheas were tied off to prevent fluid leaking. The abdominal and thoracic cavities were opened. The entire lung was removed and placed into a 50-ml tube. The agarose in the lung was allowed to cool on ice for 10 min. After cooling, the left lung was cut transversely into three 2-mm-thick sections, beginning 2 mm below the apical surface and fixed in methacarn for 6 h. Six-micrometer paraffin sections were subsequently immunostained with a PCNA antibody kit from ZYMED Laboratories (San Francisco, CA), following the manufacturer’s instructions. The numbers of cells with PCNA-expressing nuclei were counted in 10 high-power (×20 objective). The fold change in caspase-3 activity relative to the average of naive lungs was calculated at each time point.

**RESULTS**

**Blood vessels.** Figure 1 shows the time series of the appearance of blood vessels within the visceral pleura and peripheral left lung parenchyma. These sections reveal thickened visceral pleura in both LPAL mice and sham animals, which formed during the first 24 h after surgery and persisted through day 14, although isolated thickened areas were seen at day 20 also. In LPAL mice only, enlarged vessels filled with erythrocytes within the pleural surface tissue were observed by day 4 and persisted until day 14. After day 14, these enlarged vessels disappeared, yet localized areas of high red blood cell density were seen near the pleural surface. Breaks in the pleural surface seem to coincide with areas of dense red cell concentration and reflect the location where vascular connections to the chest wall were torn by the lung removal. These vessels can be seen to connect to preexisting pulmonary vessels. By day 20, in the LPAL lungs, these large connecting vessels in the lung periphery become more established.

**TUNEL assay.** To detect the presence and location of apoptotic cells, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was applied to lung sections fixed 4 h, 12 h, and 2, 3, 5, 7, and 14 days after LPAL and sham thoracotomy (1–2 mice/time point) by airway instillation of 4% paraformaldehyde, followed by 24-h fixation at 4°C in the same fixative, and then embedded in paraffin. The in situ labeling of apoptotic cells in paraffin sections was performed by using a terminal deoxynucleotidyl transferase apoptosis detection kit from Oncogene Research Products (Cambridge, MA), according to the manufacturer’s directions. This method is based on the principle that apoptotic endonucleases generate free 3′-OH groups at the end of cleaved DNA fragments, labeled with fluorescein-conjugated deoxyxynucleotides. Apoptotic cells were identified by their strong, green fluorescence using a 465- to 495-nm filter. The total number of nuclei was detected by staining with 4′,6-diamidino-2-phenylindole (DAPI) visualized as blue, using a 330- to 380-nm filter. The positive control slides were a mixture of HL-60 cells incubated with 0.5 μg/ml actinomycin D for 19 h to induce apoptosis. The control slides were treated similarly as the lung tissue slides at the time of the experiment. Pictures were taken using an Eclipse TE300 inverted microscope (Nikon, Melville, NY) connected to a digital camera linked to an image processor. A semiquantitative assessment was performed by counting 8–10 separate fields in each lung. All positive apoptotic cells per field were counted. A qualitative assessment of fluorescent intensity of the pleura was assigned a score of 0 to 3+. The fold change in caspase-3 activity relative to the average of naive lungs was calculated at each time point.

**Caspase-3 activity.** Caspase-3 activity was determined to provide a quantitative estimate of apoptotic activity. In a separate group of animals, lungs from naive mice and mice 1, 4 (n = 2), and 14 days after LPAL (n = 4/all other time points) were harvested and snap frozen. Caspase-3 activity was measured in lung homogenates of the upper left lung using a colorimetric assay (Clontech Laboratories, Palo Alto, CA), according to the manufacturer’s instructions. Caspase-3 activity was normalized to total lung protein (Bradford assay). The fold change in caspase-3 activity relative to the average of naive lungs was calculated at each time point.

**Data analysis.** Average data are presented as the mean ± SE measurement. The statistical analysis of PCNA positive cells and caspase-3 activity between groups was by one-way ANOVA and Bonferroni post hoc test for multiple comparisons. P < 0.05 was accepted as a significant difference.
Fig. 4. Kinetics of apoptosis induced by LPAL as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. A: TUNEL positive cells stain intensely green in the pleura and parenchyma 4 h after LPAL (right, arrows), but not after sham surgery (×10 objective). Morphological detail with concomitant 4’,6-diamidino-2-phenylindole staining of all nuclei at right is provided in B for pleural staining (×20 objective) and in C for parenchymal apoptosis. D: extensive pleural TUNEL signal (arrows, ×20; inset ×100) 12 h after LPAL. E: quantitative assessment of TUNEL is provided by a score ranging from 0 (as no TUNEL positive cells) to 3 (intense staining for pleura and >10 cells per field for parenchymal apoptosis). Compared with sham, there was a peak of pleural apoptosis at 12 h after LPAL and sustained apoptosis in the parenchyma at all time points assessed after LPAL.
Figure 2 shows a representative example of PCNA staining of the nuclei of several types of lung cells 5 days after LPAL. Note the intense brown staining of many nuclei of cells within the thickened pleura. Although we did not quantify each stained cell type, the majority of PCNA positive cells were macrophages and alveolar type II cells. We also found a small number of positive bronchial epithelial cells. However, endothelial cells in all slides were PCNA negative. Numerous PCNA positive cells, predominantly macrophages, were also present within the thickened visceral pleura. Figure 3 shows the time course of PCNA expression and its distribution within the pleura and parenchyma. By 3 days after LPAL, there was a statistically significant difference \((P < 0.001)\) in the number of PCNA positive cells in the lung parenchyma relative to lungs from sham mice. This difference was maintained when maximum expression was observed at 5 days in both regions that were evaluated. However, the density of proliferating cells was much greater within the visceral pleura, as can be seen in Fig. 2. By 7 days after LPAL, the number of proliferating cells was similar to those in lungs from sham-operated mice.

Figure 4 shows the kinetics of apoptosis induced by LPAL as detected by TUNEL assay. TUNEL positive cells stain intensely green in the visceral pleura and parenchyma 4 h after LPAL (right panel, arrows), but not after sham surgery (Fig. 4A; \(\times 10\) objective). Morphological detail with concomitant DAPI staining of all nuclei in the right panels is provided for visceral pleura staining and for parenchymal apoptosis. Twelve hours after LPAL, an extensive pleural TUNEL signal is present within the thickened visceral pleura. Figure 3 shows PCNA positive cells, predominantly macrophages, were also present within the thickened visceral pleura. Figure 3 shows the time course of PCNA expression and its distribution within the pleura and parenchyma. By 3 days after LPAL, there was a statistically significant difference \((P < 0.001)\) in the number of PCNA positive cells in the lung parenchyma relative to lungs from sham mice. This difference was maintained when maximum expression was observed at 5 days in both regions that were evaluated. However, the density of proliferating cells was much greater within the visceral pleura, as can be seen in Fig. 2. By 7 days after LPAL, the number of proliferating cells was similar to those in lungs from sham-operated mice.

Figure 4 shows the kinetics of apoptosis induced by LPAL as detected by TUNEL assay. TUNEL positive cells stain intensely green in the visceral pleura and parenchyma 4 h after LPAL (right panel, arrows), but not after sham surgery (Fig. 4A; \(\times 10\) objective). Morphological detail with concomitant DAPI staining of all nuclei in the right panels is provided for visceral pleura staining and for parenchymal apoptosis. Twelve hours after LPAL, an extensive pleural TUNEL signal is observed (Fig. 4D). Figure 4E shows the results of the quantitative assessment of TUNEL, with scores ranging from 0 (no TUNEL positive cells) to 3 (intense staining for pleura and >10 cells per field for parenchymal apoptosis). Compared with sham mice, there was a peak of pleural apoptosis at 12 h after LPAL and sustained apoptosis in the parenchyma at all time points assessed after LPAL. Confirmation of apoptosis data was obtained by measurement of caspase-3 activity, which also provided quantitative assessment of whole lung apoptosis. Lung samples measured included both the left upper lung parenchyma and the attached visceral pleura. By 1 day after LPAL, greater than a sixfold increase in caspase-3 activity was measured compared with naive lung (Fig. 5). Caspase-3 activity remained significantly elevated throughout the measured time course \((P < 0.01)\). Thus temporally, within the left lung, the number of PCNA positive cells and apoptotic cells (TUNEL positive and/or caspase-3 activity) increased in concert over the first 24 h after LPAL. However, apoptotic activity remained at this maximum over the subsequent time course, whereas the number of PCNA positive cells continued to increase for up to 5 days after LPAL, after which time the numbers decreased.

**DISCUSSION**

The gross anatomical consequences of LPAL and/or chronic pulmonary thromboembolism have long been recognized. In human disease, as well as in animal models, obstruction of the pulmonary vasculature results in rapid and sustained systemic neovascularization of the lung \((4, 6, 7, 10, 18)\). Both bronchial and intercostal arteries participate by proliferating and perfusing lung vascular networks. However, the temporal cellular sequence of events leading to neovascularization of the lung has not been well documented. We have shown that LPAL in mice results in a rapid, systemic neovascularization of the lung, with intercostal arteries bridging the pleural space and perfusing the left lung \((12)\). Using labeled microspheres injected into the left ventricle in vivo, we showed new systemic perfusion to the lung 5–6 days after LPAL. Systemic blood flow reached a maximum of ~6% of cardiac output by 28 days after ligation, whereas no systemic perfusion of the left lung was detected in sham-operated mice. Additionally, unlike other animal models, no obvious bronchial vascular proliferation was noted. This result is consistent with observations of Verloop that mice lack a bronchial vasculature beyond the main stem bronchi \((17)\).

Because of the extremely thin airway walls and consequent short diffusion distances for oxygen and nutrients, it seems likely that a subcarinal airway vasculature is not physiologically necessary and that pulmonary capillaries adjacent to airways provide nutritive perfusion for intraparenchymal airways.

In this model, the entire left lung is ischemic after LPAL. Our laboratory previously showed, by intravascular casting, that a dense vascular plexus originated from the thoracic wall near the site of thoracotomy \((12)\). In the present study, histological sections of lungs 1–20 days after LPAL demonstrated neovascularization predominantly in the upper left lung, in immediate proximity to the thoracic wall where the thoracotomy was performed. New vessels were seen in the pleura predominantly in this limited region. Furthermore, the upper, proangiogenic left lung was shown to express a unique molecular signaling profile compared with the lower left lung \((15)\).

The results of the present study predict a local upregulation of transcriptional activity and growth factors within the interstitial matrix of the thickened visceral pleura. These molecular changes serve not only to induce vascular growth and enlargement in the visceral pleura, but also to signal nearby intercostal vessels to bridge the pleural space and connect with the new vessels in the visceral pleura. That pulmonary ischemia is required is confirmed by studies of sham-operated mice, which showed limited pleural thickening (likely a result of events associated with the thoracic wound healing), but no observable neovascularization. Thus both lung ischemia and thoracic wound healing appear to be required in this lung angiogenesis model.

Although, in our previous study, blood flow measurements with systemic microsphere infusion were able to detect systemic perfusion by day 5–6 after LPAL, current histological...
assessment demonstrated well-established large blood vessels within the visceral pleura by 4 days after LPAL (Fig. 1B). Whether these large vessels were fully connected to a functional vascular bed that could trap 10- to 15-μm microspheres is not clear. However, the visceral pleura is the site of intense tissue remodeling, as evidenced by increased PCNA positive cells (Fig. 2 and 3B) and TUNEL positive cells (Fig. 4), as early as 12 h after LPAL. Large blood vessels formed in the visceral pleura by 4 days only in LPAL lungs. These vascular structures eventually connect to capillary networks, a finding seen more easily at later time points when these communications are well established (Fig. 1, C–E). Although the number of actively dividing cells and cells undergoing apoptosis are increased within lung parenchyma (Figs. 3A and 4), it is not clear from these studies whether new vascular networks are developing or whether preexisting pulmonary capillaries are remodeling to accommodate perfusion from the new systemic source. To date, we have been unable to find a unique endothelial cell marker that discriminates between vascular structures within the pleural space and pulmonary capillaries.

The time course of changes in the number of PCNA positive cells suggests that most cell division occurs within the first 5 days after LPAL. Differences are seen between sham-operated animals and those with LPAL, both on the pleural surface in proximity to the site of thoracotomy and within the parenchyma. Based on blood flow data, this is the time period when new functional perfusion pathways are established. The identification of replicating cells using PCNA labeling demonstrated that these cells were largely macrophages and alveolar type II cells. Among many chemical mediators, macrophages secrete CXC chemokines, cytokines, and metalloproteinases, which all have been shown to play a prominent role in angiogenesis in other models (5). Strieter and colleagues have shown that the proangiogenic ELR+ (Glu-Leu-Arg) CXC chemokines play an essential role in lung angiogenesis in nonsmall cell lung cancer (1) and idiopathic pulmonary fibrosis (9). Our laboratory’s previous work confirmed increased mRNA and protein expression of the three mouse CXC chemokines, macrophage inflammatory protein-2, keratinocyte-derived chemokine, and lypopolysaccharide-inducible CXC chemokine in the upper left lung relative to the lower left lung (15). The current histological demonstration of an increase in the number of replicating macrophages is consistent with our previous observations showing increased macrophage-derived products early after LPAL. Thus it appears likely that the replicating macrophage, stimulated by pulmonary ischemia, plays a central role in tissue remodeling and vessel recruitment to the ischemic lung.

We demonstrated augmented apoptosis in response to LPAL by measuring caspase activity in whole lung lysates and TUNEL staining by immunohistochemistry. The latter approach allowed for localization of apoptotic cells. The pleural cells themselves exhibited pronounced TUNEL staining at a time preceding neovascularization, suggesting a permissive effect of apoptosis on future neocapillary penetration. Although both the pleural surface and lung parenchyma show increased apoptotic activity at the earliest time points, parenchymal apoptosis remained elevated throughout the time course studied. This observation was supported by measurement of sustained caspase-3 activity (Fig. 5), although this measurement could not distinguish between activity in pleural vs. parenchymal locations. Overall, the results suggest that ongoing apoptosis is associated with lung remodeling and may be essential to maximize perfusion pathways. Programmed endothelial cell death is an integral part of both lung development and postnatal lung remodeling (2, 16). Segura and colleagues (13) demonstrated that caspase inhibition blocked vascular formation at the endothelial alignment step. Furthermore, they showed that endothelial cell apoptosis is important for precise vascular tissue arrangement. Our present results cannot distinguish between cell types undergoing apoptosis, since prolonged ischemia could trigger endothelial cell, epithelial cell, or inflammatory cell apoptosis, and thereby contribute to the sustained apoptotic signal. Additional studies are needed to elucidate which pulmonary cells are undergoing apoptosis in this model and whether the increases in cellular apoptosis observed are a required step of the angiogenic signal induced by prolonged ischemia or whether they are merely a marker of cellular stress and/or remodeling.

In summary, we have shown the time course of neovascularization of the lung after LPAL in the mouse. Our histological assessment demonstrates new vessel formation within the thickened visceral pleura of the upper portion of the left lung as early as 4 days after LPAL. These results are consistent with our previous published data demonstrating that new vessels arise from intercostal arteries at the site of thoracic wound healing and invade the visceral pleura of the ischemic, upper left lung. The visceral pleura is a region of intense tissue remodeling, as evidenced by markedly elevated levels of both PCNA and TUNEL positive cells. Parenchymal cells also exhibit increased proliferation (predominantly macrophages and type II pneumocytes) and apoptosis, indicating enhanced turnover and/or remodeling that precedes the connection of new vessels with preexisting capillaries. Because sham surgeries had little effect on measured parameters, it is clear that both thoracic wound healing and pulmonary ischemia are required for systemic neovascularization. Future studies are needed to determine mechanisms by which molecular signaling bridges the pleural space to induce this neovascularization.

ACKNOWLEDGMENTS

We appreciate the careful histological processing of tissue and technical support of Sandra Gallagher, Lisa Kostura, Richard Rabold, and Sathyavathi Munirayana.

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