Role of IL-6 in systemic angiogenesis of the lung

Jessica Y. McClintock and Elizabeth M. Wagner
Department of Medicine, Johns Hopkins University, Baltimore, Maryland
Submitted 4 January 2005; accepted in final form 11 May 2005

McClintock, Jessica Y., and Elizabeth M. Wagner. Role of IL-6 in systemic angiogenesis of the lung. J Appl Physiol 99: 861–866, 2005.—The multifunctional cytokine interleukin (IL)-6 has been shown to modulate inflammation and angiogenesis. In a mouse model of lung angiogenesis induced by chronic left pulmonary artery ligation (LPAL), we previously showed increased expression of IL-6 mRNA in lung homogenates 4 h after the onset of pulmonary ischemia (31). To determine whether IL-6 influences both new vessel growth and inflammatory cell influx, we studied wild-type (WT) and IL-6-deficient C57Bl/6J (KO) mice after LPAL (4 h and 1, 7, 14 days). We measured IL-6 protein of the lung by ELISA, the lavage cell profile of the ischemic lung, and new systemic vessel growth with radiolabeled microspheres (14 days after LPAL) in WT and KO mice. We confirmed a 2.4-fold increase in IL-6 protein in the left lung of WT mice compared with right lung 4 h after LPAL. A significant increase in lavaged neutrophils (7.5% of total cells) was observed only in WT mice 4 h after LPAL. New vessel growth was significantly attenuated in KO relative to WT (0.7 vs. 1.9% cardiac output). In an additional series, treatment of WT mice with anti-neutrophil antibody demonstrated a reduction in lavaged neutrophils 4 h after LPAL; however, IL-6 protein remained elevated and neovascularization to the left lung (2.3% cardiac output) was not altered. These results demonstrate that IL-6 plays an important modulatory role in lung angiogenesis, but the changes are not dependent on trapped neutrophils.

interleukin-6; neovascularization; neutrophils; pulmonary ischemia

IN LUNG PATHOLOGICAL STATES, it is the systemic vasculature in and surrounding the lung that proliferates in response to growth factors that signal the need for neovascularization. Thus, in conditions such as asthma (18), cystic fibrosis (4), idiopathic pulmonary fibrosis (33), non-small cell lung cancer (1), and chronic pulmonary thromboembolism (8), the bronchial and/or intercostal arteries proliferate and send new vessels to ischemic lung tissue. Perhaps the most extensively characterized of these conditions in humans and in animal models is the process of new vessel growth that follows chronic occlusion of a pulmonary artery. Studies in humans (8), dogs (17), sheep (7), rats (37), and pigs (10) have all demonstrated the growth of bronchial and intercostal arteries to ischemic lung tissue. Our laboratory has shown previously that left pulmonary artery ligation (LPAL) in mice leads to rapid neovascularization of the lung from intercostal arteries that penetrate the visceral pleural surface (22). In this model, angiogenesis always develops in the upper left lung adjacent to the chest wall space recovering from thoracotomy by 4–5 days after ligation (35). Gene array profiling has shown upregulation of several cytokines in the upper, proangiogenic left lung relative to the lower lung where new vessels do not develop. Specifically, interleukin-IL-6, IL-1β, and three CXC chemokines [macrophage inflammatory protein (MIP)-2, keratinocyte-derived chemokine, lipopolysaccharide inducible CXC chemokine] were increased by two- to sixfold within 24 h after LPAL (31). Each of these factors has been shown in other models to be involved in systemic neovascularization (11, 20, 32).

IL-6 is a multifunctional cytokine that can regulate various immune and inflammatory responses, although it is usually absent from the lung under normal circumstances (6). It has been shown to be released by activated T cells (36), B cells (19), monocytes (36), macrophages (14), fibroblasts (27), epithelial cells (14), and endothelial cells (30). Several studies have suggested a crucial role for IL-6 in angiogenesis. Increased IL-6 expression was measured in vivo during the formation of the vascular system that accompanies development of ovarian follicles after embryo implantation (23). In a wound-healing model, Mateo and colleagues (21) reported increased IL-6 protein in wound fluid and serum within 12 h after wounding. Additional studies by Lin et al. (20) confirmed the presence of IL-6 protein and neutrophils, macrophages, and fibroblasts within wound sites after skin incisions. Both tumor growth and systemic inflammation were reduced in a mouse cancer model treated with IL-6 antibodies (5). In sum, these results suggest that both IL-6 and inflammation are part of the angiogenic process. Furthermore, our initial screening demonstrated that IL-6 might play a role in lung angiogenesis after ischemia. Thus we designed this study to determine the functional importance of IL-6 for new vessel growth to the lung after left pulmonary artery obstruction and its relation to the inflammatory cell profile of the ischemic lung after pulmonary artery obstruction.

METHODS

Left pulmonary artery ligation. Our protocol was approved by the Johns Hopkins Animal Care and Use Committee. Five- to six-week-old male C57Bl/6J mice (WT; Charles River, Wilmington, MA) and IL-6-deficient (−/−) homozygote mice raised on C57Bl/6J background (KO; Jackson Laboratories, Bar Harbor, ME) were used in this study. Mice were anesthetized (2% isoflurane in oxygen) and 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; the left pulmonary artery was located, separated from the airway, and ligated using 6-0 silk suture. The thoracotomy was closed with a suture while the animal was placed on positive end-expiratory pressure (1 cmH2O). A drop of 2% lidocaine was applied for analgesia, and the skin incision was closed using methyl acrylamide adhesive. The animal was removed from the ventilator, extubated, and allowed to recover. Sham surgical control mice were treated the same as experimental mice in all respects except for LPAL. No surgical procedures were performed on naive mice.

Address for reprint requests and other correspondence: E. M. Wagner, Johns Hopkins Asthma and Allergy Center, Division of Pulmonary and Critical Care Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (E-mail: wagnerem@jhmi.edu).

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After specified times after LPAL, anesthetized mice were killed by cervical dislocation.

IL-6 and MIP-2. After specific time points after LPAL (4 h or 1 or 7 days), mice were euthanized, and the upper thirds of the left lung and the right lung were dissected and excised. Lung samples were weighed, homogenized (Fastprep Bio101; Thermo Savant, Holbrook, NY), and aliquoted for ELISA and bicinchoninic acid assays. The samples were processed according to the Quantikine Mouse IL-6 or MIP-2 ELISA kits (R & D Systems, Minneapolis, MN). Total protein measurements were made according to the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Bronchoalveolar lavage. Immediately after death, the right lung was ligated and the left lung was washed (3 × 0.3 ml of 0.2% BSA/PBS). Bronchoalveolar lavage fluid was gently aspirated, the total recovered volume recorded, and total cell count (Bright Line Hemacytometer; Horsham, PA) and differential cell counts (Cytospin 4; Shandon, Pittsburgh, PA) were determined. Circulating white blood cell counts were also obtained in antibody-treated mice from a blood sample taken from direct cardiac puncture immediately before bronchoalveolar lavage. The blood sample was processed using the Unopette system (Becton Dickinson, Franklin Lakes, NJ), and total and differential cell counts were determined.

Blood flow determination. To determine the extent of neovascularization, systemic blood flow to the left lung was measured 14 days after LPAL in WT (n = 6) and KO mice (n = 6) using radiolabeled microspheres. Diethylenetriamine pentaacetic acid-coated polystyrene microspheres (10–μm diameter; Kisker Products, Steinfurt, Germany) were bound to technetium-99m radioligand (Cardinal Health; Dublin, OH). Fourteen days after LPAL, mice were anesthetized and ventilated as described above. The carotid artery was cannulated (PE 10) and 150,000 microspheres (stock = 1.5 million spheres/ml) were infused (0.1 ml at 0.04 ml/min; Harvard Apparatus, Holliston, MA). Mice were killed by exsanguination, and the left lung, kidneys, liver, heart, and brain were excised. Gamma emissions from lodged radiolabeled microspheres in individual organs were immediately counted in the Hidex Triathler (Bioscan, Washington, DC). Organ activity was normalized to whole body activity counted in a Capintec counter (Capintec Products, Ramsey, NJ), which had been calibrated to the Bioscan instrument. Organ blood flow was expressed as percent of total measured blood flow (carcass + all organs), i.e., % cardiac output.

Anti-neutrophil antibody. To deplete mice of circulating neutrophils, methods described by Ribeiro-Gomes and colleagues (26) were followed. Naïve C57Bl/6J mice were given intraperitoneal injections of anti-mouse granulocytes (Ly-6G, Ly-6C, Gr-1, Myeloid), purified whole molecule (Leinco Technologies, St. Louis, MO), or purified rat anti-mouse IgG1 monoclonal antibody (BD Biosciences, Pharmingen, San Diego, CA). Each mouse received 100 μg (200 μl) of 0.5 mg/ml stock of antibody or IgG control 1 day before LPAL. Bronchoalveolar lavage fluid and blood samples were collected 4 h (n = 6) and 24 h (n = 3–4) after LPAL to confirm neutrophil reduction. In additional mice (n = 6), IL-6 protein level after neutrophil depletion was determined. The upper left lung was excised, rinsed in saline, and frozen in liquid nitrogen 4 h after LPAL for subsequent IL-6 protein detection by ELISA. In a third group of animals, mice were treated with either anti-neutrophil antibody (n = 6) or IgG control (n = 6) the day preceding LPAL and every other day for 14 days. After 14 days, neovascularization was determined using labeled microspheres.

Statistics. All data are presented as means ± SE. The time course of changes in inflammatory cells was evaluated using a two-way ANOVA. Changes in IL-6 protein were evaluated with ANOVA. Group comparisons of statistically significant differences were made using Bonferroni’s test of multiple comparisons. Unpaired t-tests were used to compare WT with KO single time point measurements or paired t-tests for left lung comparisons to right lung. A P value of <0.05 was accepted as significant.

RESULTS

IL-6 protein after LPAL. Figure 1 shows the time course of changes in expression of IL-6 protein in lung homogenates. Both the upper left lung and the right lung were evaluated in naive WT (n = 6) and KO (n = 3) mice and in WT mice 4 h, 1 day, and 7 days (n = 5/time point) after LPAL. IL-6 protein was significantly greater in the left lung than in the right lung at 4 h and compared with all other left lung time points and naive lungs (*P < 0.05).

Table 1. MIP-2 protein after left pulmonary artery ligation (pg/μg total lung protein)

<table>
<thead>
<tr>
<th>WT</th>
<th>IL-6 KO</th>
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<tr>
<td>Left upper lung</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td>Right lung</td>
<td>0.04±0.01</td>
</tr>
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Mean ± SE macrophage inflammatory protein (MIP-2) protein in wild-type (WT) and IL-6-deficient (KO) mouse lungs (n = 8/group). No difference in MIP-2 protein was observed between left WT and IL-6 KO lungs or right WT and IL-6 KO lungs. However, all left lungs showed increased MIP-2 protein expression relative to right lungs (P = 0.005).
operated animals and WT mice with LPAL (n = 3 mice/time point) at 4 and 8 h and 1, 2, 3, 5, 7, and 14 days after surgery. This series demonstrated a significant increase in total lavaged cells in mice after LPAL relative to sham animals (Fig. 2; P < 0.05). Based on this preliminary information, we selected specific time points (4 h and 1, 7, and 14 days) to study left lung lavage cell profiles in a larger series of WT (n = 6–10 mice/time point) and IL-6 KO (n = 6–8 mice/time point) mice after LPAL. There was no difference in the total number of lavaged cells recovered in naive WT and naive KO (4 × 10^4 cells/ml). After LPAL, the total number of lavaged inflammatory cells overall was significantly decreased in IL-6 KO relative to WT as observed in Fig. 2 (P = 0.02). Specifically, lavaged neutrophils were different between the two groups of mice. Figure 3 shows these results. Naive WT and IL-6 KO mice did not differ in neutrophils as a percent of total cells (P = 0.58). However, after LPAL, WT mice demonstrated a significant increase in the percent of neutrophils in left lung lavage compared with IL-6 KO mice (P = 0.001). Specifically, 4 h after LPAL, the percent neutrophils was significantly greater in WT than IL-6 KO mice (P < 0.01) and greater than at all other left lung time points (P < 0.05). Further cell differential analysis showed no difference in the percent of monocytes/macrophages in naive WT and KO mice (average: 94%; P = 0.84) or lymphocytes (average: 2–3%; P = 0.64). After LPAL, a small decrease in the percent monocytes/macrophages was observed in WT mice at 4 h relative to KO (average: 90 vs. 96%; P < 0.01). The percent lavaged lymphocytes did not differ between WT and KO mice at any of the measured time points. Lavage volume recovered did not differ between WT (842 ± 10 µl) and KO (817 ± 17 µl; P = 0.19).

**Blood flow.** New systemic vessel growth to the left lung after LPAL was quantified using radiolabeled microspheres injected into the systemic circulation. Fourteen days after LPAL, blood flow to the left lung of WT mice averaged 1.9 ± 0.4% of cardiac output. New systemic blood flow to the left lung of IL-6 KO mice was significantly reduced (0.7 ± 0.1% cardiac output) relative to WT (P = 0.004). Figure 4 shows the blood
flow measurements in each mouse studied. There were no significant differences in blood flow to any of the other organs evaluated between WT and KO mice (P > 0.05).

Anti-neutrophil antibody treatment. Anti-neutrophil treatment had a significant effect on reducing total circulating leukocytes (3.1 × 10⁴ vs. IgG control: 4.8 × 10⁴, P = 0.05) in the animals overall. The treatment effectively reduced the number of lavaged neutrophils (Fig. 5). After 4 h of LPAL, mice treated with anti-neutrophil antibody showed a significant reduction in lavaged neutrophils compared with IgG control mice (P = 0.003). Similar to the previous groups, 4 h after LPAL, IgG control mice showed an increase in lavaged neutrophils relative to treated sham (n = 2; P < 0.01) and 1-day-treated mice (P < 0.001). Combining the results from mice from both time points after LPAL for the two treatment groups (n = 9 mice/group), no difference in total lavaged leukocytes was observed in anti-neutrophil antibody-treated mice (3.6 × 10⁴ vs. 4.6 × 10⁴ in IgG controls; P = 0.39) or lavaged lymphocytes (0.5 vs. 0.8% in IgG controls; P = 0.26). However, a small difference was observed in the number of lavaged monocytes/macrophages (99 vs. 97% in IgG controls; P = 0.01).

In mice undergoing either IgG control treatment or anti-neutrophil antibody before LPAL, a significant increase in IL-6 protein expression was measured after 4 h relative to right lungs (P < 0.01; Fig. 6). This increase did not differ in the left lungs of these two treatment groups from the original series of WT left lungs presented in Fig. 1 (P = 0.76). Furthermore, the right lungs of each of the three groups did not differ in their IL-6 protein expression 4 h after LPAL (P = 0.39). New systemic blood flow to the left lung 14 days after LPAL in WT mice treated with either anti-neutrophil antibody (n = 6 mice) or IgG (n = 6 mice) did not differ from WT mice (P = 0.49). Average blood flow for mice treated with anti-neutrophil antibody was 2.3 ± 0.8% cardiac output and IgG control mice averaged 2.9 ± 0.7%.

**DISCUSSION**

Systemic vascular angiogenesis in the lung after chronic pulmonary artery obstruction is a well-documented phenomenon. Numerous animal models as well as human pathological conditions demonstrate a robust neovascularization of ischemic lung by bronchial, intercostal, and other systemic thoracic arteries after pulmonary artery obstruction (8). However, the molecular mechanisms responsible for this neovascularization have not been determined. We have previously reported increased gene expression of several inflammatory cytokines with purported proangiogenic potential in the lung, early after LPAL in the mouse (31). Significant among these is IL-6. In the present series of experiments, we confirmed an increase in IL-6 protein expression only in the left ischemic lung 4 h after LPAL. The use of mice deficient in IL-6 (−/−) demonstrated a critical role for this protein since neovascularization assessed by systemic blood flow to the left lung 14 days after LPAL was significantly reduced relative to WT animals after LPAL. These results suggest an important cell regulatory role for IL-6 protein early after lung ischemia. Furthermore, these observations are consistent with those of others showing a role for IL-6 in angiogenesis. IL-6 has been shown to cause proliferation and migration of systemic endothelial cells in culture (29). Hernandez-Rodriguez and colleagues (12) demonstrated that IL-6 caused a dose-dependent stimulation of tube formation and microvessel sprouting of cultured aortic rings in vitro as well as an increase in vascular density in the chick chorioallantoic membrane model. Tube formation was inhibited by pretreatment with soluble IL-6 receptor, thus suggesting a direct effect of IL-6 on endothelial cell proliferation and migration. Interestingly, the soluble receptor was not effective at reducing microvessel sprouting or the in vivo increase in vascular density, indicating differences in binding affinity and/or signaling in more complex angiogenesis systems.

The results of other studies suggest that IL-6 can cause the release of additional growth factors from endothelial cells such as vascular endothelial growth factor (2) and CXC chemokines (28) to promote new vessel growth. We have previously shown in this model that the CXC chemokines, specifically MIP-2, are increased early after LPAL (31). Thus, in the present series, we also measured MIP-2 protein in WT as well as IL-6 KO mice. We confirmed an increase in MIP-2 protein expression in the left lung early (4–24 h) after LPAL in both WT and KO mice. Thus the decrease in neovascularization observed in IL-6 KO mice 14 days after LPAL appears not to be related to a...
reduction in the early MIP-2 protein expression. The overall requirement for IL-6, coupled with only a brief, early expression of IL-6 protein after LPAL, implies an indirect regulatory role of this protein. However, within the complex environment of the lung, additional studies are needed to determine the specific role of IL-6 in subsequent systemic neovascularization.

Tissue acquisition for the measurement of IL-6 protein in the upper left lung was consistent with previous tissue sampling for gene array studies (31). In those experiments, we demonstrated clear differences in gene expression of the left upper lung (ischemic and proangiogenic) compared with the lower left lung (ischemic but no neovascularization). In this model, it appears that the ischemic lung requires proximity to the site of thoracic wound healing for systemic neovascularization since mice undergoing double left thoracotomies after LPAL develop blood vessels from both thoracic sites (unpublished observations) and sham thoracotomy in mice with normal pulmonary perfusion do not develop systemic perfusion to the lung (22). Although IL-6 protein from the upper-left lung was elevated after LPAL, suggesting a role for lung IL-6 early after the induction of pulmonary ischemia, our results cannot rule out the possibility that, in IL-6-deficient mice, thoracic wound healing and/or circulating progenitor cells might also be altered to prevent ischemia-induced neovascularization of the lung.

The cell source responsible for the increase in lung IL-6 protein expression during pulmonary ischemia is, as of yet, undetermined. IL-6 has been shown to be released by most cells within the lung including T cells (36), B cells (19), monocytes (36), macrophages (14), neutrophils (15), fibroblasts (27), epithelial cells (14), and endothelial cells (23, 30). Therefore, a second overall goal of the present series of experiments was to determine whether there was an association between IL-6 and the inflammatory cell profile of the lung. Angiogenesis and inflammation have been shown to be dependent in many models (16). In an endometrial angiogenesis model, evidence was presented showing that neutrophils secrete VEGF, which regulates angiogenesis (13). Another model of angiogenesis directly correlated blocking neutrophils with inhibition of angiogenesis (3). We observed an increase in lavaged neutrophils from the left lung after LPAL that followed a similar time course as the increase in IL-6 protein with a significant elevation 4 h after LPAL. Interestingly, Ericson and colleagues (9) demonstrated that activated neutrophils provided an important source of IL-6. Therefore, we performed additional experiments to try to disassociate the early increase in lavaged neutrophils with IL-6 protein. Pretreating mice with anti-neutrophil antibody reduced the number of circulating and lavaged neutrophils after LPAL. However, IL-6 protein was still markedly elevated in the upper left lung relative to control right lung 4 h after LPAL. Blood flow in the antibody-treated animals after 14 days of LPAL was not different from WT controls after LPAL. The results of these experiments suggest that neutrophils are unlikely to play a predominant role in neovascularization in this model. Furthermore, the primary cellular source for IL-6 in this model appears not to be trapped neutrophils.

The importance of circulating inflammatory cells in this model, in which the left pulmonary artery is permanently ligated, requires additional comment. There is no forward blood flow through the left lung until after a new systemic vasculature is established 5–7 days after LPAL (22). Early after LPAL, lavaged cells are likely those that have migrated into the intra-alveolar compartment after being trapped by LPAL. It is also possible that retrograde pulsatile flow from pulmonary veins could also recruit circulating inflammatory cells to the left lung (25). We suggest this possible mechanism since histological evaluation of the left lung shows pulmonary capillaries filled with red blood cells throughout the early time course after LPAL.

The time course selected for these experiments was based on previous work that demonstrated important molecular signaling occurred by 4 h after LPAL (31) and the appearance of new blood vessels developing along the visceral pleural surface of the left lung occurred by 4–5 days after LPAL (35). However, the ability to consistently measure blood flow by circulating systemic microspheres occurs somewhat later after ~6–7 days of LPAL (34). Because we hypothesized a decrease in neovascularization with IL-6-deficient mice, we selected a later time point for blood flow measurement. Regarding this end-point measurement, we believe that blood flow through an organ is the relevant physiological end point to confirm that new, patent, perfusing vessels are present in vivo. We are unaware of any technique that would provide better substantiation of neovascularization in the complex setting of the lung. Counting endothelial lined structures could not discern new from existing vessels nor could it confirm structures that are involved in forward flow. Thus we reported the number of microspheres lodged in the lung vasculature normalized to the total number of injected spheres that lodged throughout the body (i.e., % cardiac output). The results of this study describe the entire time course from 4 h through 14 days, with an emphasis on signaling events early in the time course and measurement of a functional vasculature later in the time course.

In summary, we have confirmed an increase in IL-6 protein expression in the left lung early after LPAL that is coincident with an increase in lavaged neutrophils. Furthermore, IL-6 protein is essential for neovascularization since systemic blood flow is significantly reduced in mice deficient in this protein. However, the early neutrophilia is likely unrelated and not required for subsequent neovascularization.

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