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Chemokines Accumulate in the Lungs of Rats with Severe Pulmonary Embolism Induced by Polystyrene Microspheres

John Zagorski,1 Jacob Debelak, Michael Gellar, John A. Watts, and Jeffrey A. Kline

Pulmonary thromboembolism (PEm) is a serious and life threatening disease and the most common cause of acute pulmonary vascular occlusion. Even following successful treatment of PEm, many patients experience long-term disability due to diminished heart and lung function. Considerable damage to the lungs presumably occurs due to reperfusion injury following anti-occlusive treatments for PEm and the resulting chronic inflammatory state in the lung vasculature. We have used a rat model of irreversible PEm to ask whether pulmonary vascular occlusion in the absence of reperfusion is itself sufficient to induce an inflammatory response in lungs. By adjusting the severity of the vascular occlusion, we were able to generate hypertensive and nonhypertensive PEm, and then examine lung tissue for expression of CXC and C-C chemokine genes and bronchoalveolar lavage (BAL) fluid for the presence of chemokine proteins. Hypertensive and nonhypertensive PEm resulted in increased expression of both CXC and C-C chemokines in lung tissues. Hypertensive PEm was also associated with a 50–100-fold increase in protein content in lung BAL fluid, which included the CXC chemokines cytokine-induced neutrophil chemoattractant and macrophage-inflammatory protein 2. The presence of chemokines in BALs was reflected by a potent neutrophil chemoattractant activity in in vitro chemotaxis assays. Abs to cytokine-induced neutrophil chemoattractant blocked the in vitro neutrophil chemoattractant activity of BAL by 44%. Our results indicate that the ischemia and hypertension associated with PEm are sufficient to induce expression of proinflammatory mediators such as chemokines, and establish a proinflammatory environment in the ischemic lung even before reperfusion. The Journal of Immunology, 2003, 171: 5529–5536.

Pulmonary vascular obstruction in humans can result from multiple disease processes, including venous embolism of aggregated carcinoma cells (1), erythrocyte and bone marrow fat embolism secondary to sickle cell disease (2, 3), massive blood transfusion (4), or surgical repair of fractures (5, 6) and secondary to fibrin deposition in the setting of acute respiratory distress syndrome (3, 7, 8). In the clinical setting, the most commonly recognized cause of acute pulmonary vascular occlusion is pulmonary embolism (PEm)2 of the thrombosis containing fibrin and platelets formed in the deep venous system. The incidence of PEm exceeds 1 in 1000 per year in outpatient (9, 10) and hospitalized populations (11, 12), making PEm the third most common cardiovascular disease in the United States (13).

In addition to causing pulmonary hypertension by mechanical vascular occlusion by thrombus (14), persistent PEm can induce intimal hyperplasia leading to luminal occlusion of thrombosed and nonthrombosed pulmonary arteries (15, 16). Patients with pulmonary hypertension secondary to chronic PEm often exhibit in-exorable clinical deterioration characterized by impaired pulmonary gas exchange (17–19), increased pulmonary vascular resistance (20), and biventricular cardiac dysfunction (21, 22). The majority of patients with chronic PEm present clinically with chronic dyspnea, exercise intolerance, or death (23–25).

PEm produces multiple stimuli that are capable of inciting an inflammatory response, including pulmonary artery hypertension (26, 27), ischemia (28), hypoxia (29), and expression of emobilized material with vascular endothelium (30). A common feature of these stimuli is the potential to induce expression and release of additional proinflammatory molecules and to initiate a cascade of events resulting in a state of chronic inflammation and tissue remodeling. The evidence that has implicated pulmonary inflammation with PEm includes the observation of increased neutrophil content in the bronchoalveolar lavage (BAL) samples from patients with PEm (31). A hallmark of all inflammatory responses is the recruitment of leukocytes from the peripheral circulation into the site of the disease pathology (32). A key step regulating the extravasation of PBLs is the controlled secretion of chemotactic factors from inflammatory sites. The most important known leukocyte chemotactic factors include leukotriene B4, complement fragment C5a, TGFβ, and chemokines (33–36).

Chemokines are an attractive target for immunosuppressive therapy of inflammation because chemokines demonstrate selectivity for limited subsets of leukocytes and lymphocytes. We have described recently a model of PEm that uses venous infusion of polystyrene microspheres in rats (37). This model produces pulmonary vascular occlusion with physiological alterations similar to those observed in humans with PEm, and allows precise dosing of the insult. In this study, we report the use of this model of PEm to test whether fixed pulmonary vascular occlusions, and the pulmonary hypertension and lung ischemia that result from these occlusions, induce a chemokine-mediated inflammatory response in rat lungs.

Materials and Methods

Animal care

Experiments were done on male Sprague Dawley rats weighing between 375 and 500 g. All experiments were approved by the Institutional Animal Care and Use Committee. Animals were housed in the institutional animal facility on a 12-h light/12-h dark cycle, with food and water available ad libitum. All surgical procedures were performed under halothane anesthesia.

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Care and Use Committee of the Carolinas Medical Center. Before use, rats had ad lib access to food and water.

**Induction of PEm**

This study used a model of PEm in rats induced by intrajugular vein injection of polystyrene microsphere beads (mean diameter, 24 ± 1 μm, 7525A; Duke Scientific, Palo Alto, CA) that has been previously described (37). Before use, microspheres were sterilized with 70% ethanol, washed extensively with sterile 0.015% Tween 20, and carefully resuspended in 0.01% Tween 20 to a 10% concentration (13 million beads/ml). Based upon prior work, rats were injected with either 1.3 or 1.95 million beads/100 g body weight (0.10 or 0.15 ml bead suspension/100 g) to produce either moderate or severe PEm. Two types of sham groups were employed. The vehicle sham group was treated identically to the PEm groups, except that 0.01% Tween 20 at 0.15 ml/100 g rat weight was injected in the jugular vein; the surgical sham group of rats were anesthetized and left jugular vein was ligated and no injection was given.

**Right ventricular pressure measurements**

Rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (3 mg/kg) injected i.p. Rats were placed on a warming pad filled with recirculating water, warmed to 105°F (solid-state T-pump; Gaymar, Orchard Park, NY). The rat’s neck was shaved and a tracheostomy was performed by cannulating the trachea with PE-240 tubing. The left carotid artery and right internal jugular vein were dissected and cannulated with Millar Micro-Tip micromanometer catheter transducers (Millar Instruments, Houston, TX). A 2 French Millar (SPR-249-A) catheter monitored arterial blood pressure in the carotid artery. A 2 French bent Millar catheter (SPR-513) was advanced through the internal jugular vein into the right ventricle to measure pressure, as we have previously described (38). The right femoral artery was dissected and cannulated with PE-50 tubing filled with saline for arterial blood sampling. Rats were injected with either vehicle (0.01% Tween 20, 0.15 ml/100 g rat weight, n = 6), moderate PE dose (1.3 million microspheres/100 g body weight, n = 6), or severe PE dose (1.95 million microspheres/100 g body weight, n = 6). Pressures were continuously monitored for 90 min.

**Isolation of tissues, pleural effusions (Peffs), and BAL**

**Peffs.** Peffs were collected from pleural cavities by creating a 3-mm window through the diaphragm, with a hand-held cauterizing pen, and aspirating the effusions with a disposable propylene transfer pipette, using care to not induce bleeding. Pleural cavities were then lavaged with 10 ml sterile saline. Control rats without recoverable Peffs were lavaged to collect cells. Effusions and pleural lavages were anticoagulated with EDTA and centrifuged at 500 × g for 5 min to collect cells. Peff supernatants were removed and stored frozen at −20°–70°C. Cell pellets from Peffs and pleural lavages were pooled and subjected to one or two rounds of hypotonic lysis, followed by centrifugation, cleared of erythrocytes by hypotonic lysis, counted and stained using Polymorphprep cell separation medium (Accurate Chemical and Scientific, Westbury, NY), and stored at −70°C until use. RNA was isolated from powdered lungs by the method of Chromczynski and Sacchi (42). RNA was added to a small volume of sample buffer (20 mM sodium phosphate, pH 7.0, 50% formamide, 6% formaldehyde), electrophoresed through 1.5% agarose containing 20 mM sodium phosphate and 6% formaldehyde, and transferred to Hybond-N nylon (Amersham Pharmacia, Piscataway, NJ) by capillary action.

**Chemokine cDNA probes**

**Study of cDNAs and primer sequences.** Cytokine-induced neutrophil chemotactic receptor (CINC) (GenBank/EMBL D11444) and macrophage-inflammatory protein (MIP)-2 (X65647) cDNA clones have been previously described (40). The other chemokine cDNA probes used for this study were originally cloned by one of the authors for an unrelated project while a Senior Staff Fellow in the Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health (J.Z., unpublished observations). Briefly, the CXC chemokines CINC-2 (D21985) and inflammatory protein (IP)-10 (U22520) and the C-C chemokines monocyte chemotactic protein (MCP)-1 (M57441), RANTES (U06436), MIP1α (U06435) and MIP1β (U06434), and GAPDH (M17701) were amplified by RT-PCR as previously described (40), blunted-end cloned into pBluescript SK+ (Stratagene, La Jolla, CA) or pMalC2 (New England Biolabs, Beverly, MA), and verified by sequencing. RNA sources for these amplifications were as follows: CINC2, streptococcal cell wall-induced granulomatous rat liver (43); MCP-1, cultured rat synovial fibroblasts (J. Zagorski, unpublished observations); IP-10, normal rat spleen; RANTES, MIP1α, MIP1β, and peritoneal exudate cells from rats with chronic-phase streptococcal cell wall-induced granulomatous liver/ polyanethyrl (43, 44). RT-PCR was done using a commercially available kit as described (40), with a modified cDNA template procedure. Final recovery of lavage was typically 5.0 ml, which was ligated and no injection was given.

**Northern blot hybridizations.** Probes for Northern blot hybridizations were obtained by amplifying cDNAs from chemokine plasmid clones by PCR, using the primers and conditions previously listed. Hybridizations were done in 12.5 ml of a solution containing four parts pre-hybridization buffer (50% formamide, 5× SSC, 10× Denhardt’s reagent), with 5× SSC, 0.1% SDS at 60°C. Membranes were washed to a final stringency of 0.2× SSC, 0.1% SDS at 60°C. Between uses, membranes were stripped with 500-ml washes of 0.1% SDS (95–100°C).

**Pulmonary water content measurements**

The total water content of lungs was determined by comparing the weights of aliquots of powdered lung tissues before and after desiccation. Frozen lungs were finely weighed on a digital balance pieces of aluminum foil and then dried for at least 24 h at 60–80°C and re-weighed. Lung water content was calculated with the formula: 1 − (dry weight/wet weight) × 100 = percentage of water.

**Chemotaxis assays**

Neutrophils were purified using Polymorphprep cell separation medium (Accurate Chemical and Scientific, Westbury, NY) and chemotaxis assays done using 24-well Corning-Costar Transwell migration chambers as previously described (40, 45). Each experiment was performed using Polymorphprep cell separation medium (43–45°C). Matrigel (BD Biosciences, San Jose, CA) was included in the lower chamber and cytokine-induced neutrophil chemotaxis was performed using one-way ANOVA, with individual means being compared by Student-Neuman-Keuls tests in which data were normally distributed, with equal variance. When data failed the tests of equal variance or normality, comparable nonparametric tests were employed. These tests included Kruskal-Wallis ANOVA on ranks, followed by Dunn’s method for pairwise comparisons. Analysis of chemotactic activity in BAL samples (Table II) was performed by one-way ANOVA, with comparison of individual
treatment groups to control being made with Dunnett’s method. Changes in right ventricular systolic pressure (RVSP) over time (Fig. 1) were made using two-way repeated-measures ANOVA to compare changes in each PE group with the vehicle group. Significance was determined as \( p < 0.05 \).

**Results**

**Physiological consequences of moderate and severe PEm**

In pilot survival studies, we observed a sharp decrease in 18-h survival as the dose of i.v. microspheres was increased from 1.3 to 1.95 million microspheres/100 g rat weight (hence referred to as PE1.3 and PE2.0). Survival among rats in the control and PE1.3 groups was 100% (8/8 and 9/9, respectively), whereas survival in the PE2.0 group dropped to 62.2% (23/37). Fig. 1 demonstrates that PE1.3 did not significantly increase the RVSP above that of rats treated with vehicle, whereas PE2.0 caused a significant increase in RVSP. PE2.0 had minimal effect on mean arterial blood pressure (data not shown). Rats with PE2.0 developed hypoxemia (arterial \( \text{PO}_2 \) = 70 ± 4.3 torr) at 18 h, whereas rats with PE1.3 did not develop significant hypoxemia (arterial \( \text{PO}_2 \) = 80 ± 3.0 torr) compared with controls (arterial \( \text{PO}_2 \) = 84 ± 2.5 torr). We interpret these data to indicate that PE2.0 induced “severe PEm,” whereas PE1.3 resulted in “moderate PEm.”

**Table I. Characteristics of BAL and Peff**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell Count (10^6/Peff)</th>
<th>MPO (mU)</th>
<th>Cell Count (10^5/BAL)</th>
<th>MPO (mU)</th>
<th>Protein − acetone</th>
<th>Protein + acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.2 ± 1.4</td>
<td>4931 ± 2199</td>
<td>4.4 ± 3.3</td>
<td>52 ± 17.3</td>
<td>0.12 ± 0.06</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>PE1.3</td>
<td>9.0 ± 1.1</td>
<td>5744 ± 904</td>
<td>11.2 ± 5.6</td>
<td>47 ± 20.5</td>
<td>0.12 ± 0.02</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>PE2.0</td>
<td>9.4 ± 0.6</td>
<td>9536 ± 2128</td>
<td>4.8 ± 2.7</td>
<td>280 ± 135</td>
<td>7.10 ± 1.88</td>
<td>7.16 ± 2.10</td>
</tr>
</tbody>
</table>

* Pleural effusion cell counts and MPO are expressed as total cells and total cell-associated MPO activity recovered per Peff. BAL cell counts and MPO are expressed as extrapolated total cells and total cell-associated MPO per lung (extrapolated total cells or MPO per lung = cells or MPO recovered × 8 ml × lavage volume recovered). Protein concentrations were determined by the bicinchoninic acid method using a commercially available kit, and are expressed as milligram per milliliter total protein in BAL. All data are presented as mean ± SD.

**Table II. Chemotactic activity in BAL**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Migrating Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe PE</td>
<td>250 ± 40</td>
</tr>
<tr>
<td>HBSS</td>
<td></td>
</tr>
<tr>
<td>CINC, 100 ng/ml</td>
<td>84,085 ± 1,015</td>
</tr>
<tr>
<td>Control BALs</td>
<td>690 ± 397</td>
</tr>
<tr>
<td>PE2.0 BALs</td>
<td>96,864 ± 16,734</td>
</tr>
<tr>
<td>PE1.3 vs PE2.0</td>
<td></td>
</tr>
<tr>
<td>HBSS</td>
<td>353 ± 121</td>
</tr>
<tr>
<td>CINC, 100 ng/ml</td>
<td>92,965 ± 2,805</td>
</tr>
<tr>
<td>PE1.3 BALs</td>
<td>3,198 ± 756</td>
</tr>
<tr>
<td>PE2.0 BALs</td>
<td>157,695 ± 15,851</td>
</tr>
<tr>
<td>PE2.0* + Abs</td>
<td></td>
</tr>
<tr>
<td>HBSS</td>
<td>247 ± 19</td>
</tr>
<tr>
<td>CINC, 20 ng/ml</td>
<td>91,450 ± 10,024</td>
</tr>
<tr>
<td>CINC + anti-CINC</td>
<td>707 ± 148</td>
</tr>
<tr>
<td>MIP2, 2.5 ng/ml</td>
<td>29,707 ± 7,262</td>
</tr>
<tr>
<td>MIP2 + anti-MIP2</td>
<td>1,393 ± 74</td>
</tr>
<tr>
<td>PE2.0 BAL Pool</td>
<td>103,363 ± 12,173</td>
</tr>
<tr>
<td>BAL + anti-CINC</td>
<td>58,133 ± 4,473*</td>
</tr>
<tr>
<td>BAL + anti-MIP2</td>
<td>91,680 ± 18,925</td>
</tr>
<tr>
<td>BAL + anti-CINC/MIP2</td>
<td>55,897 ± 9,620*</td>
</tr>
</tbody>
</table>

* Chemotactic activity is expressed as the number of cells that migrated from apical to basal transwell chambers. Apical chambers were initially loaded with 300,000 purified rat neutrophils. All data are presented as mean ± SD.

**Lung histology in rats with PEm**

Histology of lungs from rats after 18 h of moderate and severe PEm, as well as a vehicle control are shown in Fig. 2. Polystyrene microspheres in the lungs of rats with PE1.3 and PE2.0 are indicated with arrows in Fig. 2, B and C. The most obvious difference between lungs with moderate or severe PEm is the dense eosinophilic staining throughout the alveolar spaces in Fig. 2C, suggesting massive protein accumulation in these lungs.

**Characteristics of BAL and Peff**

Table I shows some of the characteristics of rats with moderate and severe PEm. A total of 21/23 surviving rats in the PE2.0 group developed straw-colored Peff after 18 h, which ranged from 0.2 ml to 12.5 ml (5.5 ± 3.6 ml, mean ± SD). In contrast, only 1 of 9 rats in the PE1.3 group had a measurable Peff of 0.4 ml. Pleural cavities of control rats contained <0.1 ml of fluid. Peffs from rats with severe PEm, as well as pleural lavages from rats with moderate PEm, had a modest <2-fold increase in total cell count compared with pleural lavages from control rats. Similarly, a <2-fold increase in pleural cavity neutrophils was observed in rats with severe PEm, using cell-associated MPO activity as a marker for neutrophils. These data indicate that minimal cell trafficking into the pleural cavity occurred in this model of PEm. Total BAL cell increased nearly 3-fold after 18 h of moderate PEm, but not severe PEm. However, BAL-associated neutrophils showed an almost 6-fold increase in rats with severe PEm compared with controls or rats with moderate PEm, indicating that chemotactic signals were present in the lungs during PEm. The BAL neutrophilia was not a significant difference from control.
result of an increase in total cell count. Neutrophils normally account for a small fraction of the total alveolar cellular pool, and changes in alveolar neutrophil counts are not necessarily reflected by an increase in total cell count (31).

The diffuse pink staining in the alveoli of PE2.0 lungs shown in Fig. 2 suggested the accumulation of excess protein. To verify this, we measured protein concentrations in cell-free BAL supernatants recovered after 18 h of PEm. Protein concentrations were determined for crude BALs and for BAL constituents precipitated with acetone, which served to eliminate background due to phospholipids (46), which constitute 70–90% of surfactant (47, 48). As shown in Table I, rats with severe PEm had a 50-fold higher concentration of protein in their BALs (7.10 mg/ml) than control rats (0.12 mg/ml). Significantly, rats with moderate PEm had no more protein in their BALs than control rats. Following acetone precipitation, the concentration of protein in BALs after severe PEm was essentially unchanged (7.16 mg/ml), whereas the protein in control and moderate PEm BALs was significantly reduced (0.08 and 0.07 mg/ml, respectively). The increase in BAL protein content was apparently not associated with pulmonary edema, because the total water content of sham control lungs and lungs with severe PEm were indistinguishable (78.7 ± 0.9%, n = 5 vs 79.2 ± 0.5%, n = 4, mean ± SD).

Protein patterns in BALs from rats with severe PEm

We compared the composition of proteins accumulated in the alveoli of rats with severe PEm to the proteins present in normal control rats using SDS-PAGE (49). As shown in Fig. 3A, the protein constituents of BAL from rats with severe PEm were remarkably similar to the proteins seen in BAL from sham or vehicle-treated rats. In all samples, one ∼60-kDa protein, consistent in size with albumin predominated. Moderately abundant proteins of ∼45 and 80 kDa were also apparent. The overall similarity of proteins found in severe PEm and control BALs indicated that the ∼100-fold increase in total BAL protein during severe PEm was caused by accumulation of the same proteins normally present in BAL. As shown in Fig. 3B, all of the abundant BAL proteins co-migrated with proteins present in rat plasma. However, many abundant plasma proteins were not prominent in BAL, suggesting some selectivity in protein transfer into the alveoli.

Neutrophil chemotactic activity is present in BALs from rats with severe PEm

The significant increase in alveolar neutrophils in PE2.0 BALs indicated to us that neutrophil chemotactic activity was elevated in these lungs. This was verified by performing in vitro neutrophil chemotaxis assays on BALs recovered after 18 h of moderate and severe PEm. In Table II, BALs from rats with severe PEm were compared with control BALs. BALs were diluted 1:2 with chemotaxis buffer before use. PE2.0 BALs had >100-fold higher neutrophil chemotactic activity than controls, whereas BALs from rats with PE1.3 (Table II) had neutrophil chemotactic activity that was elevated above buffer alone, but greatly reduced relative to rats with PE2.0. When subjected to size fractionation, all the chemotactic activity in the PE2.0 BALs was retained by a 3-kDa molecular mass cut-off ultrafiltration membrane (Centricon-3; Amicon, Beverly MA) and no activity was detected in the <3 kDa filtrate.

![FIGURE 2. Lung histology in rats with PEm. A, 18 h vehicle, 18 h PE1.3 (B); 18 h PE2.0 (C) are shown. Tissues were fixed with Z-Fix fixative, embedded in paraffin, sectioned, and stained with H&E. Photographs were taken at ×240 magnification.](http://www.jimmunol.org/)

![FIGURE 3. Protein patterns in BALs from rats with severe PEm. A, Control BALs (two sham controls and three vehicle controls, in order of loading) containing 25 μg of total protein were precipitated overnight with 3 volumes of acetone and resuspended in 20 μl of 1× Laemmli sample buffer (see Ref. 49). Aliquots of BALs from rats with PE2.0 containing 25 μg of total protein were added directly to sample buffer. Samples were separated by SDS-PAGE (12.5% acrylamide) under reducing conditions and stained with Coomassie blue. B, 5 μg of protein each from five separate BAL (B) and plasma (P) samples from rats with PE2.0 were pooled and electrophoresed as previously described.](http://www.jimmunol.org/)
These data eliminate chemotactic leukotrienes such as B4 as the source of the neutrophil chemotactic activity. CXC chemokines contribute to the chemotactic activity in PE2.0 BALs. CXC chemokines are important neutrophil chemoattractants during acute inflammation. We used ELISAs to test for the presence of the rat CXC chemokines CINC and MIP-2 in 18 h BALs from control, PE1.3 and PE2.0 rats (n = 5 BALs per group). CINC was detected in all PE2.0 BALs tested (19.8 ± 8.6 ng/ml, mean ± SD), but was undetectable in control BALs or PE1.3 BALs (detection limit was 0.625 ng/ml using a previously described CINC ELISA; Refs. 40 and 45). MIP-2 was assayed using a commercially available ELISA (BioSource International, Camarillo, CA). Control and PE1.3 BALs contained 165 ± 44 pg/ml and 234 ± 13 pg/ml MIP-2, respectively, whereas PE2.0 BALs contained 2500 ± 580 pg/ml MIP-2 (mean ± SD). These data indicate that severe PE, but not moderate PE, is associated with a 10- to 20-fold increase in CXC chemokines in BAL.

The presence of two CXC chemokines in PE2.0 BAL suggested that they contributed to the neutrophil chemotactic activity in these BALs. To test this, we used anti-CINC (40, 45) and anti-MIP-2 (Santa Cruz Biotechnology, Santa Cruz CA) Abs to block the neutrophil chemotactic activity in a pool of PE2.0 BALs in vitro. The BAL pool was diluted 1:3 with chemotaxis buffer before use. Sufficient amounts of Ab were used to block the concentrations of each chemokine present in undiluted PE2.0 BAL (20 ng/ml CINC and 2.5 ng/ml MIP-2), which resulted in a 3-fold excess of Ab for buffer-diluted BAL. As shown in Table II, 10 µg/ml anti-CINC completely blocked the activity of 20 ng/ml CINC, whereas 20 µg/ml anti-MIP-2 blocked 2.5 ng/ml MIP-2 by 95%. When added to PE2.0 BAL, the same amount of anti-CINC blocked neutrophil chemotaxis by 44%. Anti-MIP-2 did not significantly block chemotactic activity. The combination of both Abs blocked activity no better than anti-CINC alone. These data indicate that CINC accounts for a substantial fraction of the total neutrophil chemotactic activity in BAL from rats with severe PE, but that other chemoattractant factors also contribute to this response.

Chemokine gene expression in lung tissues

The accumulation of CINC and MIP-2 proteins and chemotactic activities in BAL after 18 h of severe PE led us to examine the
expression of these and other chemokine genes in lung tissue by Northern blot analysis. Lung RNA was prepared from rats after 2, 6, or 18 h of severe PEm. As shown in Fig. 4, both CINC and MIP-2 genes were rapidly up-regulated after 2 h and remained elevated for at least 6 h, as were CINC2 and IP-10. Among C-C chemokines, MCP-1, MIP1α, and MIP1β were also induced by PEm. These data indicate that PEm induces expression of chemokines that are chemotactic for both neutrophils (CINC, CINC2, MIP-2) and for monocytes and T lymphocytes (MCP-1, MIP1α, MIP1β, IP-10).

The observations that moderate PE1.3 did not cause accumulation of total protein or neutrophils (Table I), chemokine proteins (previously noted) or neutrophil chemotactic activity (Table II) into BALs suggested that chemokine gene expression might require a threshold of ischemia and/or hypertension, and only be elevated at the higher dose of PE2.0. However, as shown in Fig. 5, when we compared chemokine gene expression in lungs from rats with moderate and severe PEm, significant CINC2 and MCP-1 expression was observed in the lungs of rats with moderate PEm. However, CINC expression was clearly dose-dependent at 2 and 18 h, and MIP2 expression was dose-dependent at 18 h. These data indicate that the increases in chemokine gene expression alone are not sufficient to account for the different characteristics of BALs from rats with moderate and severe PEm.

Effect of polystyrene microspheres on rat endothelial cells in vitro

We next sought to determine whether the increased chemokine gene expression in the lungs of rats with PEm was caused by direct interaction of pulmonary endothelial cells with the polystyrene microspheres. To accomplish this, we treated a rat pulmonary artery endothelial cell line (MPA cells) (50) with polystyrene microspheres in vitro and then compared chemokine expression in microsphere-treated MPA cells with chemokine expression in lungs from rats with PEm by Northern blot analysis. These results are shown in Fig. 6. To help interpret these results, we performed calculations to compare the dose of microspheres that were delivered per total mass of RNA in vivo and in vitro. For MPA cells in vitro (lane 5, 1 million microspheres/100 mm dish), the total yield of RNA was 224 μg, or 89,000 microspheres/20 μg RNA loaded. For pooled RNA from rat lungs in the PE2.0 group (Fig. 6, lane 3), we first estimated the yield of RNA per milligram of lung tissue (0.56 μg/mg) and multiplied for the total amount of RNA per lung in this group, or 1904 μg/lung (average lung weight after BAL, 3.4 g). Using this number and the known number of microspheres injected into rats in this group we extrapolated that the 20 μg loaded in Fig. 6, lane 3 is representative of 90,000 microspheres in vivo. Thus, when adjusted for RNA mass, the dose of beads delivered to the endothelial cells in vitro was approximately equal to the dose of beads delivered to the lungs in vivo. Yet, for all chemokines examined, expression in vivo with PE2.0 lungs (Fig. 6, lane 3) was considerably greater than expression in vitro with MPA cells (Fig. 6, lane 5). This effect was particularly apparent for CINC2, because the basal level of expression in vitro was detectable and was not elevated after treatment with microspheres. A positive control for the responsiveness of MPA cells to LPS is also shown (Fig. 6, lanes 8 and 16). These data provide evidence that physical contact of polystyrene microspheres with endothelial cells does not strongly induce chemokine gene expression.

Discussion

We have previously described a model of PEm in rats initiated by infusion of 25-μm polystyrene microspheres into the jugular vein, which results in irreversible pulmonary vascular occlusions (37). An important characteristic of this model of PEm is the ability to manipulate the severity of the PEm by adjusting the dose of microspheres infused into rats. In this current study, we were able to reproducibly generate two types of PEm, a severe PEm characterized by significant right ventricular hypertension, systemic hypoxemia and high incidence of 18 h mortality, and moderate PEm without hypertension, hypoxemia or excess mortality. Severe PEm with secondary pulmonary hypertension also resulted in a high incidence of pleural effusions whereas moderate PEm without hypertension did not. In addition, although ischemia was not directly measured during this study, we have previously documented by angiography that severe PEm in our model is associated with reduced blood flow into the periphery of the pulmonary arteries (37), as would be expected from a vascular occlusive disease.

Hypertensive PE2.0 differed most notably from nonhypertensive PE1.3 by the accumulation of proteins and neutrophils in the alveolar spaces. Comparison of BAL proteins from control rats and rats with severe PEm indicated that the increase in protein content of PE2.0 lungs was due to accumulation of normal bronchoalveolar proteins. Many of these proteins were also abundant in plasma and were presumably plasma-derived, as has been previously suggested (51, 52). The flow of proteins across the alveolar-capillary wall is bidirectional in normal lungs: plasma proteins enter the alveoli down a steep pressure gradient, while lung-specific surfactant proteins, present in high concentrations on the alveolar epithelial lining surface, normally diffuse across the capillary wall down their own steep concentration gradients and can be detected in blood (53, 54). Under conditions of pulmonary artery hypertension, the in-flow of plasma proteins across the capillary wall would likely be increased while the rate of absorption of surfactant into the blood or lymph would be reduced. Increased synthesis of surfactant proteins may also occur during severe PEm, but we have observed that after only 2 h of severe PEm, total BAL protein is increased by over 13-fold relative to controls (data not shown). It seems unlikely that secretion of new proteins accounted significantly for this increase.

It is well known that ischemia-reperfusion injury involves a potent inflammatory response and that expression of proinflammatory cytokines, including chemokines, is strongly increased in tissues insulted with ischemia-reperfusion (55, 56). However, we have discovered that PEm in the absence of reperfusion also results in expression of chemokine genes, indicating a nascent inflammatory response resulting in neutrophil recruitment into alveoli. The recruitment of neutrophils into the lungs of rats with PE2.0, though significant, was almost certainly limited by the restricted blood supply through the occluded pulmonary artery vasculature. This ischemia presumably resulted in a relative scarcity of PBLs exposed to chemotactic signals present in the lungs and thus available for recruitment into the alveoli. Our model suggests that the ischemia associated with PEm primes the lungs for a more significant influx of PBLs into the alveolar compartment by causing accumulation of chemotactic proteins. Following reperfusion, PBLs circulating through the pulmonary vasculature would encounter a ready pool of chemokines, independent of new chemokine expression induced by reperfusion injury, and recruitment into the alveoli would be rapid and severe.

Chemokine expression was increased during both moderate and severe PEm although CINC expression, at least, was greater during severe PEm. Because treatment of endothelial cells with microspheres in vitro did not strongly induce chemokine expression, we do not believe that direct interaction of microspheres with luminal endothelia constituted a significant stimulus for chemokine expression in our model. Several other possible stimuli may be responsible for chemokine expression during PEm. The likely stimulus...
for increased chemokine expression during moderate PE1.3 was ischemia. The transcription factors early growth response gene-1 and hypoxia-inducible factor 1 are important terminal mediators of the signaling pathways induced by hypoxic ischemia (57). However, lung ischemia associated with PEm, in which the lungs are fully ventilated, is presumably (and uniquely) normoxic (55). It seems likely that different signaling pathways are activated during normoxic, as opposed to hypoxic ischemia, perhaps initiated by metabolism-sensitive (58, 59) or hypotension-sensitive (60) signal transduction pathways activated in capillary beds distal to the microsphere emboli. Increased expression of CINC during severe PE2.0 compared with moderate PE1.3 presumably reflected the additional stimuli of hypertension, which is known to activate numerous signal transduction pathways (61, 62).

Increased expression of chemokines was evident 2 h after microsphere infusion, indicating that the processes of inflammatory injury begin early in PEm. This finding has major importance in the treatment of PEm. Current therapy is to initiate systemic anticoagulation and wait for resolution of vascular occlusion, which may take a week or longer. Our data emphasize the importance of rapid diagnosis and shows the basis for aggressive treatment to ameliorate pulmonary vascular obstruction as quickly as possible to forestall the rapid expression in proinflammatory genes. The action of the chemokines on neutrophil trafficking may exacerbate injury, known to occur during reperfusion with treatment for PEm (63, 64). Blockade of the induction of gene expression, or the synthesis or action of chemokines might help reduce injury from PEm and from reperfusion after PEm. However our data show that the chemokines tested accounted for only part of the chemotactic response of PEm. Other chemokines and chemotactic factors may play a partial role.

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