Functional contribution of CXCR2 to lung injury after aspiration of acid and gastric particulates

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ASPIRATION OF GASTRIC CONTENTS is a potentially severe complication in patients with altered consciousness due to anesthesia, trauma, intoxication, or metabolic disease (12, 15). The associated lung injury is characterized by pulmonary inflammation, capillary leakage, and oxidative damage (8, 10). This results in variable outcomes, ranging from mild, rapidly resolving pneumonitis to fulminate acute respiratory distress syndrome and death. In fact, aspiration has been recognized as a complication in 20% of anesthetic-related deaths (13, 28). The outcome after aspiration of gastric contents may be related to the volume, pH, and composition of the aspirated material (8). Recent experimental studies have shown that, compared with simple acid aspiration, aspirates containing particulate gastric material dramatically increase lung injury. This increase in pulmonary injury is accompanied by increases in proinflammatory cytokine levels, surfactant dysfunction, and neutrophil recruitment to the lungs (4, 7, 9, 24).

Neutrophil recruitment is a hallmark of the response to aspiration of gastric contents. Once localized to the lung, neutrophils are primarily responsible for lung injury through the release of oxygen radicals and proteases (10). Experimental studies have shown that aspiration-induced neutrophil recruitment to the lung is mediated by chemokines (9, 16, 17, 24). Specifically, the neutralization of human CXCL8/IL-8 (6) as well as functional counterparts in rodents, mCXCL1/KC or CXCL2/MIP-2α (16, 17, 25), significantly decreased neutrophil recruitment and lung injury after acid aspiration. In addition, there is evidence that increased severity of the aspirate will produce greater chemokine concentrations in the lung. The CXCL1/CINC concentration in bronchoalveolar lavage (BAL) fluid of rats is an early indicator of lung injury, and relative concentrations of CXCL1/CINC may be diagnostic for the type of aspirate (soluble vs. particulate) (7). However, the impact of the CXC chemokines on aspiration lung injury has not been fully defined. Experimental studies have not addressed the redundancy of chemokine function by evaluating outcome after simultaneously neutralizing multiple chemokines. Likewise, the relative impact of neutralizing the function of the CXC chemokines after aspiration of particulate material has not been examined.

Although chemokine concentrations increase and may be predictive after aspiration, the relative expression of receptors for ELR+ CXC chemokines has not been examined extensively. There are two receptors for ELR+ CXC chemokines in humans. CXCR1 is a selective receptor, binding only to CXCL8/IL-8 and CXCL6/GCP-2, whereas CXCR2 binds to all of the ELR+ CXC chemokines (3). Although murine CXCR1 was recently cloned (5), CXCR2 is currently considered to be the primary functional receptor for the rodent ELR+ CXC chemokines, including mCXCL1/KC (CINC), CXCL2/MIP-2α, and CXCL5/LIX. Therefore, it is possible that blocking CXCR2 will have a greater effect on lung injury induced by acid aspiration than did the neutralization of individual chemokines discussed in previous reports. In addition, our previous work presented immunohistochemistry of lung tissue that revealed a qualitative increase in CXCR2 expression after aspiration, the relative impact of neutralizing the function of the CXC chemokines has not been examined.

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Materials and Methods

Study design. To examine lung inflammation and injury after aspiration of gastric contents, mice were given intratracheal (IT) injections of saline, an acidic solution, or an acidified solution containing sterile gastric particles. Animals were euthanized at 4, 6, and 24 h to examine lung injury. To determine the impact of CXCR2 on increasing levels of lung injury, the expression of the receptor was examined on peripheral blood neutrophils and lung tissue. In another set of experiments, mice were pretreated with CXCR2 blocking antibodies and euthanized at 6 h after IT injection to evaluate lung inflammation and injury. The role of CXCR2 was further investigated in CXCR2-deficient mice given either acid, acid solution containing gastric particles, or a non-acidified solution containing gastric particles.

Animals. Female, ICR mice (23–25 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). The mice were housed in a temperature-controlled room with a 12-h dark/light cycle and allowed food and water ad libitum. CXcr2−/− mice and appropriate wild-type controls (BALB/c) were obtained from Jackson Laboratories (Bar Harbor, ME). The University Committee on Use and Care of Animals approved all of the experiments.

Aspiration. Mice were anesthetized with isoflurane, and 80 µl of solution were delivered IT as previously described (17). The acidic solution consisted of saline titrated to a pH of 1.15 with hydrochloric acid. The solutions containing gastric particles were prepared as previously reported by Knight et al. (7, 9, 24). Briefly, the stomach contents of healthy mice were washed with saline, filtered through a 200-µm mesh, and autoclaved. The particles were resuspended (40 mg/ml) in saline or saline titrated to a pH of 1.15.

Death and sample harvest. The mice were anesthetized with intraperitoneal injections of 87 mg/kg ketamine (Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and 13 mg/kg xylazine (Rompun; Bayer, Shawnee Mission, KS). A 20-µl blood sample was collected in EDTA from the tail vein, and a complete blood count was performed using a Hemavet Mascot Hematology System Counter (CDC Technologies, Oxford, CT). The mice were exsanguinated by the retroorbital route and killed by cervical dislocation. A BAL was performed by injecting two separate 1-ml volumes of warm Hanks’ balanced salt solution (HBSS without CaCl2, MgSO4, or phenol red; Gibco, Grand Island, NY) into the trachea. After BAL, the right ventricle was perfused with 2 ml of saline. With the trachea cannulated, 10% buffered formalin was infused to a standardized pressure, and the lungs were placed in formalin for histology and immunohistochemistry.

BAL cell counts and differential. The 1-ml samples were centrifuged (600 g, 3 min), and the supernatant from the first sample retrieved from each mouse was stored at −20°C. The two cell pellets were pooled for total counts with a Coulter Counter model Z1 after RBC lysis with Zap-Oglobin II (Miami, FL). Slides were loaded with 1 × 105 cells, centrifuged (700 g, 3 min), and stained with Diff-Quick (Baxter, Detroit, MI). Differentials (300 cells) were counted with a light microscope.

Albumin ELISA. Albumin was measured in BAL fluid using a direct ELISA as previously described (16). A standard curve of mouse albumin (Sigma) and lavage samples (1:100) were diluted in borate buffer (120 mM NaCl, 50 mM H3BO3, 16 N NaOH). A polyclonal, rabbit anti-mouse albumin antibody (6.9 µg/ml) diluted in 1% BSA in PBS (pH 7.4) was used as the primary antibody. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). The color reagent was 3,3',5,5'-tetramethylbenzidine (TMB), and the reaction was stopped with 1.5 N sulfuric acid. The absorbance was read at 450 and 630 nm.

Histology and lung injury score. The lung samples were fixed in formalin, embedded, sectioned, and stained (hematoxyl and eosin). The sections were evaluated under light microscopy and scored with a modification of a previously described system (1, 20) by a blinded observer. Scores (0–4) were assigned for hemorrhage, infiltration of neutrophils, and alveolar wall thickness. Cumulative numbers for each lung were used to determine the mean scores for each group.

Esterase staining. For identification of neutrophils, histological slides were deparaffinized and rehydrated. The slides were stained for naphthol AS-D chloroacetate esterase as per the manufacturer’s instructions (Naphthol AS-D Chloroacetate Esterase Kit; Sigma-Aldrich, St. Louis, MO) and counterstained with hematoxylin. For each section from the wild-type and CXcr2−/− mice, three photomicrographs (Zeiss Axio microscope and digital camera, Carl Zeiss Microimaging, Germany) of cellular aggregates were obtained by an unbiased observer. Using imaging software (NIH ImageJ, version 1.60), areas were selected randomly within the aggregates. Neutrophils within these areas, identified by red cytoplasmic staining and typical polymorphonuclear morphology, were counted. The results from each count were expressed as neutrophil number/µm² and averaged for the section. These results were then averaged for each group of mice (n = 3/group).

Cytokine ELISA. Cytokines were measured in plasma (1:10 dilution) and lung lavage (1:2 dilution). Methylated pairs (biotinylated and non-biotinylated) of anti-murine antibodies against CXCL1/KC and CXCL2/MIP-2a along with their recombinant proteins (R&D Systems, Minneapolis, MN) were used in protocols for sandwich ELISAs previously described by this laboratory (19). Peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and the color reagent TMB were used as the detection system. The results were read at absorbances of 450 and 630 nm.

Flow cytometry. Peripheral blood samples were subjected to hypotonic lysis, washed, and resuspended in PBS with 0.1% sodium azide and 1.0% bovine calf serum (HyClone, Logan, UT) at 1.0 × 10^6 cells/ml. The suspensions were incubated for 5 min (4°C) with 0.5 µg Fc-yII/III reagent (BD Pharmingen) to block Fc receptors. The cells were incubated (30 min at 4°C) with 2.5 µg/ml each of fluorescently labeled anti-mCXCR2 (R&D Systems), anti-mLy-6G (BD Pharmingen), and anti-m-Gr-1 (Ly-6G and Ly-6C; BD Pharmingen) antibodies with recommended control antibodies (rat PE IgG1, FITC IgG2a, and APC IgG2b, respectively). The fluorescence was measured using a CytoFACS FC 500 Beckman Coulter, Fullerton, CA) flow cytometer using a 488-nm excitation laser (peak emission 515–545 nm). Compensation was performed utilizing WinList for 32 software (Verity Software House, Topsham, ME).

Immunohistochemistry. Lung sections were deparaffinized and rehydrated. Antigen retrieval was performed by microwave rapid boiling in Antigen Retrieval Citra Plus solution (BioGenex, San Ramon, CA). Sections were incubated with a peroxidase blocking solution (Super Sensitive Link-Label IHC Detection System; BioGenex) followed by a primary rat anti-mCXCR2 (5 µg/ml; R&D Systems) antibody. The specificity of this antibody has been demonstrated on lungs of mice deficient in CXCR2 (22). A biotinylated anti-IgG (MultiLink, BioGenex) was used as the secondary antibody. Negative controls were treated in the same manner but lacked primary antibody. The sections were incubated with peroxidase-conjugated streptavidin in PBS followed by a 3,3′-diaminobenzidine solution, counterstained with hematoxylin, and mounted for microscopy.

Quantitative real-time RT-PCR. Lung tissues were isolated from female ICR mice 6 h post-IT injection. RNA was purified from 100 mg of lung tissue homogenized in 1 ml of TRIzol (Invitrogen Life Technologies, Carlsbad, CA) using the guanidine hydrochloride/chloroform extraction method according to the manufacturer’s instructions. RNA was quantified by measuring the absorbance at 260 and 280 nm. The cDNA was generated with MuLV reverse transcriptase using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on cDNA using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and an iCyler real-time instrument (Bio-Rad). Primer sequences for CXCR2 were 5′-GGTCTGATCTGCGTATCCCTGCTCAG-3′ and 5′-TAGCCATGATCTGGAAGTCCATG-3′ (Sigma-Aldrich). The housekeeping gene used to normalize samples was β-actin.
with the primer set consisting of 5'-GTCGTACCACCTGCGATTGT-3' and 5'-CTCTCAGCTTGTTGGTGTAAA-3' (Invitrogen Life Technologies). PCR reactions were performed with a hot start at 95°C for 1 min followed by 40 cycles of 95°C for 20 s, annealing temperature of 60°C for 30 s, and extension at 72°C for 20 s. The threshold cycle was calculated automatically by iCycler using the maximum curvature approach with the data analysis window set at 95% of a cycle and centered at the end of the cycle. Amplification was followed by melting curve analysis. Results are presented as the ratio of CXCR2 to β-actin. The amplicons were loaded on a 1.5% agarose gel for electrophoresis, and band size consistent with CXCR2 was verified.

CXCR2 neutralization. Mice were treated with antiserum directed against mCXCR2 (a generous gift from Dr. Robert Strieter, Univ. of California, Los Angeles, CA). These antibodies neutralize the activity of CXCR2 in vivo without increasing peripheral blood neutrophil counts (14, 27). A 0.5-ml dose was given subcutaneously 15 h and again 2 h before IT injection. Animals were euthanized 6 h after the IT injection.

Statistical analysis. Summary data were expressed as means ± SE. The Student’s t-test and ANOVA with post hoc Tukey’s multiple comparison test were used to analyze differences among groups. These analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Lung inflammation and injury in response to aspiration. To verify the inflammation and lung injury in the aspiration model, mice were given IT injections and euthanized 4, 6, or 24 h later. There were significant increases (P < 0.05) in total cell counts in BAL fluid at 6 h (0.26 ± 0.18 vs. 0.92 ± 0.30 vs. 1.63 ± 0.57 × 10⁶ cells/mouse) and at 24 h (0.23 ± 0.03 vs. 1.40 ± 0.23 vs. 2.71 ± 0.15 × 10⁶ cells/mouse) after IT injections of saline, acid, or acid with particles, respectively. Differential counts demonstrated increases in macrophage numbers in the acid and acid + particles groups (0.31 ± 0.05 and 0.30 ± 0.04 × 10⁶/mouse, respectively) at 6 h compared with the saline group (0.19 ± 0.05 × 10⁶/mouse). These differences became significant with further increases of the macrophage counts in the acid and acid + particles groups (0.35 ± 0.03 and 0.38 ± 0.09 × 10⁶/mouse, respectively) compared with saline (0.17 ± 0.03 × 10⁶/mouse) at 24 h. There were also significant increases in the number of neutrophils in the BAL fluid within 4 h of IT injection in both the acid and acid + particles groups. At later time points, differences between the acid and acid + particles groups became more pronounced, and the increases paralleled the severity of the insult (Fig. 1). The degree of lung injury demonstrated by BAL fluid albumin levels and histology also increased with increasing severity of the insult. Albumin levels in BAL fluid, determined by ELISA, showed significant, incremental increases at 6 h after IT injection (Fig. 2A). The lung injury scores for tissues harvested from each group demonstrated a similar pattern that persisted for 24 h (Fig. 2B). In the acid group, histology revealed scattered neutrophils in the perivascular, peribronchiolar, and parenchymal tissues. In the acid + particles group, neutrophils were found in the same pattern as in the acid group with the additional finding of multiple, discrete foci of aggregated cells.

Aspiration increases CXC chemokines. To examine factors affecting early neutrophil recruitment, chemokine concentrations were evaluated. In plasma, CXCL1/KC and CXCL2/MIP-2α levels were below the limit of detection by the assay (40 and 20 pg/ml, respectively) for all the injury groups. In BAL fluid, CXCL1/KC and CXCL2/MIP-2α demonstrated early, significant increases that paralleled the severity of the lung inflammation associated with each type of injury (Fig. 3, A and B).

Systemic effects of aspiration. To examine the effects of aspiration on systemic neutrophils, complete blood counts were performed on animals 4 h after aspiration. Aspiration of acid was associated with a significantly increased (P < 0.05) percentage of neutrophils in the peripheral blood (45.9 ± 3.6%) compared with aspiration of saline (26.7 ± 4.6%). However, there was no significant difference between the acid and acid + particles (36.8 ± 3.2%) groups. Although aspiration of acid was associated with a trend toward a higher mean neutrophil count, there were no significant differences in absolute counts among the saline, acid, and acid + particles groups (1.5 ± 0.5, 3.0 ± 0.5, and 2.5 ± 0.3 × 10⁶/l, respectively). The surface expression of CXCR2 on systemic neutrophils was also examined in peripheral blood harvested 4 h after IT injections, just before peak neutrophil recruitment to the lung. During evaluation by flow cytometry, cells were gated based on Ly-6G expression (Fig. 4, A and B), which would discriminate granulocytes (neutrophils and eosinophils) from monocytes (Ly-6G-). The mean percentages of Ly-6G+ cells with detectable surface expression of CXCR2 (Fig. 4C) were not significantly different among the saline, acid, or acid + particles groups (94.9 ± 1.7, 91.4 ± 1.5, and 86.8 ± 5.1%, respectively). When absolute numbers of CXCR2-expressing granulocytes were calculated (Fig. 4D), the mean value for the acid group (2.7 ± 0.4 × 10³/µl) was higher than for the saline group (1.5 ± 0.4 × 10³/µl); however, this difference did not reach statistical significance. There was no difference in numbers expressing CXCR2 between the acid group and the acid + particles groups (2.3 ± 0.2 × 10³/µl). These numbers were primarily a reflection of neutrophil counts since eosinophils represented <2.0% of the total granulocyte counts (data not shown). Gr-1 expression (Ly-6G and Ly-6C) was used to distinguish monocytes (Gr-1+ , Ly-6G-) from granulocytes (Gr-1+ , Ly-6G+) expressing CXCR2. In the acid group, the
percentage and absolute number of CXCR2+ monocytes (26.2 ± 4.8% and 5.9 ± 0.9 × 10^6/μl, respectively) were significantly increased (P < 0.05) compared with the saline group (8.1 ± 1.7% and 1.7 ± 0.3 × 10^6/μl, respectively) but not the acid + particles group (13.9 ± 3.9% and 3.1 ± 0.9 × 10^6/μl, respectively).

Expression of CXCR2 in lung tissue increases after acid aspiration. The expression of chemokine receptors on lung tissue may influence neutrophil recruitment and vascular permeability. Therefore, the surface protein expression of CXCR2 was examined with immunohistochemistry on lung tissue harvested after BAL and vascular perfusion (n = 3/group). At 6 h after IT saline, immunohistochemistry revealed relatively little CXCR2 expression (Fig. 5A). Acid aspiration caused an increase in CXCR2 expression (Fig. 5C), which was evident on epithelial cells, endothelial cells, and macrophages (Fig. 5). Similar changes were noted after the aspiration of acid containing gastric particles (Fig. 5D). Although the expression appeared intensified around the aggregates of cells in the acid + particles group, further analysis did not suggest an overall increase in expression when particulates were added to the acid solution. Real-time RT-PCR of whole lung tissues was performed with primers for CXCR2 and β-actin. Although the lung insults resulted in increased expression compared with saline, there was no difference in expression between the acid and acid + particles groups (Fig. 6).

CXCR2 neutralization decreases BAL fluid neutrophil counts. Mice were treated with anti-CXCR2 antibodies or nonspecific control antibodies before induction of lung injury. Six hours after IT injections of the acidic solution, anti-CXCR2 antibody-treated mice showed a significant reduction of neutrophil recruitment in the BAL fluid (Fig. 7A). There was no

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significant difference in BAL fluid albumin levels between the animals treated with anti-CXCR2 antibodies (1.7 ± 0.2 μg/ml) and control mice (1.9 ± 0.2 μg/ml). The neutralization experiment was repeated in animals given acid with gastric particles. Although the identical treatment did reduce neutrophil recruitment (Fig. 7B), CXCR2 neutralizing antibodies had relatively less effect after aspiration of acidified gastric particles than after aspiration of acid alone. In addition, the animals given CXCR2 neutralizing or control antibodies before aspiration of particulates showed no differences in histological scores (2.5 ± 0.8 vs. 2.0 ± 0.5, respectively) or in BAL fluid albumin levels (3.3 ± 0.3 vs. 3.5 ± 0.3 μg/ml, respectively).

Pulmonary inflammation in CXCR2-deficient mice. Because antibodies could not provide a complete block of all receptors, the neutralization study findings were verified by giving IT injections to mice lacking CXCR2 and wild-type controls. It is well-established that CXCR2-deficient mice have higher peripheral neutrophil counts than wild-type animals (2). This difference between wild-type and Cer2−/− mice was not apparent 6 h after aspiration of acid (4.2 ± 0.5 and 4.0 ± 1.4 x 10^3/μL, respectively) but was pronounced at 24 h (1.1 ± 0.4 and 5.2 ± 1.6 x 10^3/μL, respectively). In response to the acid solution, the BAL fluid of wild-type mice revealed neutrophil counts that increased over 24 h, similar to the results of
previous experiments (Fig. 8A). However, BAL fluid neutrophil counts from Ccr2−/− mice did not show an increase in recruitment over 24 h and were significantly decreased in the Ccr2−/− mice compared with the wild-type mice at that time point (Fig. 8A). The BAL fluid macrophage counts were not significantly different between the wild-type and Ccr2−/− mice at either 6 h (0.21 ± 0.11 and 0.23 ± 0.03 × 10⁶/mouse, respectively) or 24 h (0.36 ± 0.11 and 0.37 ± 0.08 × 10⁶/mouse, respectively). The mean lung injury scores from wild-type mice increased by 50% from 6 to 24 h (P < 0.05), but no significant increase was noted in the Ccr2−/− mice over that time frame (Fig. 8B). The 24-h lung injury scores for the Ccr2−/− mice were significantly lower than for the wild-type mice. In response to acid solution containing gastric particles, peripheral neutrophil counts in wild-type animals were slightly lower than in the mice lacking CXCR2 (3.0 ± 0.3 and 4.3 ± 2.6 × 10³/µl, respectively) at 6 h, and, as with the acid aspiration groups, this difference was more pronounced at 24 h after aspiration (2.4 ± 0.1 and 11.2 ± 6.0 × 10³/µl, respectively). The BAL fluid neutrophil counts from wild-type mice increased significantly over 24 h (Fig. 8C). However, Ccr2−/− mice did not demonstrate an increase in BAL fluid neutrophils over time, and final counts at 24 h were 60% lower than those seen in wild-type mice (P < 0.01). The BAL fluid macrophage counts were not significantly different between the wild-type and Ccr2−/− mice at either 6 h (0.21 ± 0.06 and 0.22 ± 0.07 × 10⁶/mouse, respectively) or 24 h (0.26 ± 0.04 and 0.30 ± 0.06 × 10⁶/mouse, respectively). Interestingly, the lung injury scores for wild-type and Ccr2−/− mice were not significantly different at either 6 or 24 h after IT injection of the acid solution containing gastric particles (Fig. 8D). To better delineate the effects of CXCR2 on the different types of lung injury, a solution of gastric particulates suspended in non-acidified saline (particles) was given to wild-type and Ccr2−/− mice. The BAL fluid neutrophil counts from wild-type mice at either 6 h (0.21 ± 0.11 and 0.23 ± 0.03 × 10⁶/mouse, respectively) or 24 h (0.26 ± 0.04 and 0.30 ± 0.06 × 10⁶/mouse, respectively). Interestingly, the lung injury scores for wild-type and Ccr2−/− mice were not significantly different at either 6 or 24 h after IT injection of the acid solution containing gastric particles (Fig. 8D). To better delineate the effects of CXCR2 on the different types of lung injury, a solution of gastric particulates suspended in non-acidified saline (particles) was given to wild-type and Ccr2−/− mice. The BAL fluid neutrophil counts from wild-type mice at either 6 h (0.21 ± 0.11 and 0.23 ± 0.03 × 10⁶/mouse, respectively) or 24 h (0.26 ± 0.04 and 0.30 ± 0.06 × 10⁶/mouse, respectively). Interestingly, the lung injury scores for wild-type and Ccr2−/− mice were not significantly different at either 6 or 24 h after IT injection of the acid solution containing gastric particles (Fig. 8D). To better delineate the effects of CXCR2 on the different types of lung injury, a solution of gastric particulates suspended in non-acidified saline (particles) was given to wild-type and Ccr2−/− mice. The BAL fluid neutrophil counts from wild-type mice at either 6 h (0.21 ± 0.11 and 0.23 ± 0.03 × 10⁶/mouse, respectively) or 24 h (0.26 ± 0.04 and 0.30 ± 0.06 × 10⁶/mouse, respectively). Interestingly, the lung injury scores for wild-type and Ccr2−/− mice were not significantly different at either 6 or 24 h after IT injection of the acid solution containing gastric particles (Fig. 8D). To better delineate the effects of CXCR2 on the different types of lung injury, a solution of gastric particulates suspended in non-acidified saline (particles) was given to wild-type and Ccr2−/− mice. The BAL fluid neutrophil counts from wild-type mice at either 6 h (0.21 ± 0.11 and 0.23 ± 0.03 × 10⁶/mouse, respectively) or 24 h (0.26 ± 0.04 and 0.30 ± 0.06 × 10⁶/mouse, respectively). Interestingly, the lung injury scores for wild-type and Ccr2−/− mice were not significantly different at either 6 or 24 h after IT injection of the acid solution containing gastric particles (Fig. 8D).
mentioned. Interestingly, these discrete areas of inflammation were also evident in the CXCR2-deficient animals (Fig. 9, A and B). This ongoing inflammation may explain the lack of difference in the lung injury scores between the Ccr2−/− and wild-type mice, despite the significant difference in BAL fluid neutrophil counts. Esterase staining was used to distinguish neutrophils from the mononuclear cell components of the focal lesions seen in the CXCR2-deficient mice and wild-type mice (Fig. 9, C and D). Histomorphometric analysis of the focal lesions was performed by counting the neutrophils present in focal lesions. These results suggested no significant difference between the CXCR2-deficient mice and wild-type mice with regard to the number of neutrophils/unit area within the aggregates (0.015 ± 0.001 neutrophils/µm² vs. 0.015 ± 0.002 neutrophils/µm², respectively).

**DISCUSSION**

In this study, the severity of aspiration-induced pulmonary inflammation and lung injury was intensified as the complexity of the aspiration insult was increased. The pulmonary neutrophil recruitment seen after acid aspiration was significantly increased when sterile gastric particles were added to the acidic aspirate and was accompanied by relative increases in the BAL fluid concentrations of the CXC chemokines. These results are consistent with the findings in the original studies by Knight et al. (9, 11) and verify the replication of the model. In the original studies, inflammation and injury induced by an acid solution containing sterile gastric particles was significantly increased over the combined results from the two separate components, documenting a synergistic effect of the dual insult (9, 11). This dual insult was associated with BAL fluid concentrations of the CXC chemokines that were higher in magnitude and of longer duration compared with fluid from rats exposed to the individual insults (9). Similarly, BAL fluid levels of mCXCL1/KC were significantly increased in mice after the dual injury compared with either acid or particle injury (21). The results of those studies showed a strong association of certain ELR+ CXC chemokines (CXCL1/CINC-1 or KC and CXCL2/MIP-2α) with the progressive severity of acid aspiration lung injury and composition of the aspirated material. In addition, recent work suggested that the BAL fluid concentrations of chemokines, alone or in combination with other cytokines, may be used at distinct time points to discriminate between different aspirates. Consequently, they may be used to predict the risk of secondary complications such as pneumonia or acute respiratory distress (7). However, the relative importance of the CXC chemokines to lung inflammation and injury was not demonstrated definitively with neutralization studies in a model of particulate aspiration.

In this study, we expanded the mechanistic evaluation of aspiration lung injury and examined factors other than chemokine concentration that could alter neutrophil recruitment. The percentage of peripheral blood neutrophils increased after acid was...
delivered to the lung. Other studies have shown downregulation of surface CXCR2 on neutrophils in response to sepsis and trauma (23, 26); however, aspiration did not significantly alter the percentage of granulocytes with surface expression of the receptor CXCR2. Overall, the results found no significant differences in peripheral blood neutrophils among the groups that would adequately explain the differences in pulmonary recruitment seen between groups. Of particular note, there was no difference in surface CXCR2 expression between the acid and acid + particles groups, suggesting other factors impact differences in neutrophil recruitment between these groups. Since the in vivo findings of increasing lung injury could not be explained by the chemokine receptor on neutrophils, other aspects of CXCR2 expression were examined. Previous studies using chimeric mouse models demonstrated that expression of CXCR2 on epithelial and endothelial cells was an essential factor for LPS-induced neutrophil recruitment and lung injury (22). In addition, we have shown via immunohistochemistry that acid aspiration increases CXCR2 expression in lung tissue (18). Those results were confirmed in this current study in which increased expression was shown on epithelial cells, endothelial cells, and alveolar macrophages. These findings were further verified by demonstrating increased expression in whole lung tissue with real-time RT-PCR. Studies of LPS-induced pulmonary injury have shown that the amount of CXCR2 expression is a rate limiting factor for neutrophil recruitment when Ccr2−/− mice were compared with heterozygous mice (22). Interestingly, PCR of the whole lung did not demonstrate differences in CXCR2 expression between the acid and acid + particles groups. This would suggest that the expression of this receptor is not the rate limiting factor in acid aspiration complicated by particulate matter. Alternatively, the increased injury could be dependent on chemokine concentration or the influence of factors other than CXC chemokines.

To verify the role of CXCR2 and ultimately of the CXC chemokines, neutrophil recruitment was examined after neutralization of the receptor. Previous studies have examined the results of neutralizing specific chemokines (16, 25). In the current study, neutralization of CXCR2 blocked the combined effects of mCXCL1/KC and CXCL2/MIP-2α in addition to any other ELR+ CXC chemokines (i.e., CXCL5/LIX, CXCL5/ENA-78) that had not previously been evaluated in acid aspiration. Although antibody treatment would not result in complete receptor blockade, specific antibodies against CXCR2 caused an 80% decrease in BAL fluid neutrophil counts after
aspiration of acid. Identical treatment resulted in only a 33% reduction in recruitment after aspiration of acid with particles. The differences in results may have been due to competition with the greatly increased chemokine concentrations in the acid + particles group or the possibility that other chemokine/receptors play a more important role in complex injury. To examine these possibilities, CXCR2-deficient mice were used. In the absence of CXCR2, recruitment of neutrophils into the airways was significantly reduced after exposure to either acid or acid with gastric particles. These results were similar to those found in studies of LPS-induced lung injury. After LPS, the initial margination and tight adherence of neutrophils occurred in the pulmonary vasculature even in the absence of CXCR2 (22, 29). However, further transendothelial and transepithelial migration of neutrophils was fully dependent on CXCR2 (22). Those studies suggested that CXCR2 inhibitors would be beneficial in treating inflammatory lung injury. The BAL fluid neutrophil counts documented after aspiration of acid or acid with particles would also suggest that CXCR2 inhibitors would be beneficial.

With regard to lung injury scores, the effect of CXCR2 expression was more dependent on the type of injury. After exposure to an acid insult, there was a significant increase in lung injury scores over 24 h in wild-type mice. However, acid aspiration did not elicit an increase in lung injury in the Ccr2−/− mice, and scores were significantly lower compared with wild-type mice at 24 h. In contrast, the lung injury scores derived from lungs exposed to acid with particulate material showed no significant difference between the Ccr2−/− mice and their wild-type controls. Most notably, the distinct foci of cells observed after exposure to acid with particles were evident in the CXCR2-deficient mice as well as the wild-type animals. This ongoing inflammation contributed to the lung injury scores and may explain the lack of difference in the two groups. Further investigation of injury caused by particulate material without the acid component demonstrated some influence of CXCR2 on airway neutrophil recruitment at the early time point (6 h). However, the later time point was not influenced by the presence of CXCR2 and neither were the lung injury scores. Studies by Raghavendran et al. (21) first documented the presence of organizing granulomas in mice after aspiration of acidified gastric particles. These granulomas were characterized by mononuclear cell infiltrates and distinct areas of consolidated injury, documented within 24 h of particulate aspiration. In our study, esterase staining and typical polymorphonuclear appearance confirmed that neutrophils also accumulated in the consolidated areas within 6 h. The recruitment of neutrophils to these aggregates occurred in equal numbers in wild-type and CXCR2-deficient mice, apparently independent of CXCR2 expression. The granulomas described in previous studies were not present in CCL2/MCP-1 knockout mice that displayed a diffuse pneumonitis compared with wild-type mice (21). Ironically, the containment afforded by the granulomas appeared to promote a survival advantage in those studies (21). Therefore, the use of CXCR2 inhibitors may prove beneficial in cases of acid aspiration by eliminating diffuse neutrophil recruitment; however, it appears that focal recruitment of neutrophils in response to more complex injury is dependent on factors other than CXC ELR+ chemokine gradients.

CONCLUSIONS

Although chemokine concentration is an important regulator of aspiration lung injury, the results of our studies suggest that
additional factors impact neutrophil recruitment. Aspiration of acid caused an increase in the percentage of peripheral blood neutrophils, and these cells maintained their expression of CXCR2. CXCR2 expression on lung tissue increased after aspiration of acid also included gastric particulates, the aspiration of gastric contents during anesthesia reported to the Departmental Quality Assurance Committee. J Clin Anesth 18: 102–107, 2006.


