Alveolar type II-like cells release G-CSF as neutrophil chemotactic activity

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Koyama, Sekiya, Etsuro Sato, Takeshi Masubuchi, Akemi Takamizawa, Keishi Kubo, Sonoko Nagai, and Takateru Izumi. Alveolar type II-like cells release G-CSF as neutrophil chemotactic activity. Am. J. Physiol. Lung Cell. Mol. Physiol. 19: L687–L693, 1998.—We evaluated the potential of A549 cells, an alveolar type II epithelial cell line, to release granulocyte colony-stimulating factor (G-CSF), in addition to interleukin (IL)-8 and leukotriene B4, as neutrophil chemotactic activity (NCA). Human recombinant IL-1β stimulated A549 cells to release NCA in a time- and dose-dependent fashion. The released NCA was blocked by mouse anti-human G-CSF polyclonal antibody. Molecular-sieve column chromatography revealed that IL-1β induced the release of a 19- to 20-kDa chemotactic mass that was inhibited by anti-human G-CSF antibody. IL-1β stimulated the release of G-CSF in a dose-dependent fashion, but the time-dependent profile of G-CSF showed that the concentration of G-CSF declined after 48 h. Tumor necrosis factor (TNF)-α, Escherichia coli lipopolysaccharide (LPS), and bradykinin (BK) stimulated A549 cells to release NCA that was inhibited by anti-G-CSF antibody. The release of G-CSF in response to TNF-α, LPS, and BK was significantly increased. The similar concentrations of human recombinant G-CSF (10–1,000 pg/ml) as in the supernatant fluid induced neutrophil chemotaxis. G-CSF mRNA was expressed time and dose dependently at 4 h and declined after 4 h in response to IL-1β as evaluated by RT-PCR. The expression of G-CSF mRNA was also observed by TNF-α, LPS, and BK stimulation. These data suggest that type II alveolar epithelial cells may produce G-CSF as NCA and may participate in the regulation of leukocyte extravasation.

granulocyte colony-stimulating factor; type II pneumocyte; neutrophil chemotaxis

SEQUESTRATION of peripheral blood neutrophils within the lung is characteristic of a number of acute pulmonary infections and lung injury (10, 11, 29). The presence of neutrophils is determined by the expression of adhesion molecules and local generation of chemotactic agents, which direct neutrophil migration from the vascular compartment to the alveolar space along chemotactic gradients. Substantial investigations have focused on alveolar macrophages as a primary source of chemotactic factors and chemokines (9, 17, 20). However, neutrophil chemotactic activity (NCA) has been reported to be produced by endothelial cells (24), fibroblasts (25), and alveolar and airway epithelial cells (13, 19, 22).

Alveolar type II epithelial cells (ATII cells) have been shown to play a key role in the regulation of the alveolar space. ATII cells synthesize and secrete surfactant, control the volume and composition of the epithelial lining fluid, and proliferate and differentiate into type I alveolar epithelial cells after lung injury to maintain the integrity of the alveolar wall (16). ATII cells have a role in modulating immunologic activity in the alveolar space. Both in vivo and in vitro data suggest that ATII cells could participate in the intraalveolar cytokine network by secreting interleukin (IL)-8 (11, 22), IL-6 (6), monocyte chemoattractant protein (MCP)-1 (23), regulated on activation normal T cells expressed and secreted (RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF)-β, interferon-γ, and IL-1β (14, 15).

Granulocyte colony-stimulating factor (G-CSF) plays significant roles in neutrophil migration (27), activation (26), and survival (5). G-CSF has been reported to be produced from macrophages, lymphocytes (4, 18), fibroblasts (2), and endothelial cells (12) in response to certain stimuli. However, the potential of ATII cells to produce G-CSF is uncertain, and the regulation of G-CSF release as NCA from ATII cells is undetermined. In the present study, we demonstrated that A549 cells released G-CSF as NCA in response to IL-1β, TNF-α, lipopolysaccharide (LPS), and bradykinin (BK). The expression of G-CSF mRNA was augmented in response to each stimulus. These data suggest that ATII cells may produce G-CSF as NCA and may participate in the regulation of neutrophil extravasation into the lung.

METHODS

Culture and identification of ATII cells. Because of difficulty in obtaining primary human type II epithelial cells of sufficient purity, A549 cells (American Type Culture Collection, Rockville, MD), an alveolar type II cell line derived from an individual with alveolar carcinoma (26), were used. These cells retained many of the characteristics of normal type II cells, such as surfactant protein, cytoplasmic multilamellar inclusion bodies, and cuboidal appearance, and have been extensively used to assess type II pneumocyte effector cell function (6, 14, 15, 21–23). A549 cells were grown as a monolayer on 100-mm tissue culture dishes. A549 cells were incubated in 100% humidity and 5% CO2 at 37°C with Ham's F-12 medium supplemented with penicillin (50 U/ml; Gibco, Grand Island, NY), streptomycin (50 µg/ml; Gibco), fungitoxin (2 µg/ml; Gibco), and 10% heat-inactivated FCS (GIBCO). The cells from a monolayer were harvested with trypsin (0.25%) and EDTA (0.1%) in PBS, centrifuged at low speed (250 g for 5 min), and resuspended in fresh medium at 1.0 × 10⁶ cells/ml in 35-mm tissue culture dishes. The cells were grown to confluence in the dish for 5–7 days. After the cells reached confluence, they were used for experiments.

Exposure of A549 cells to a variety of stimuli. Serum-containing medium was removed from the cells by washing...
them twice with serum-free Ham's F-12 medium; and then the cells were incubated with Ham's F-12 medium without FCS in the absence and presence of human recombinant IL-1β (500, 50, 5, and 0.5 pg/ml; Genzyme, Cambridge, MA), human recombinant TNF-α (1,000 U/ml; Genzyme), human recombinant interferon IFN-γ (500 U/ml; Genzyme), Escherichia coli LPS (serotype 0127:B11; Difco,Detroit, MI), BK (100 µM; Sigma, St. Louis, MO), histamine (100 µM; Sigma), and serotonin (100 µM; Sigma) and cultured for 12, 24, 48, and 72 h; the supernatant fluids were evaluated for neutrophil chemotaxis and G-CSF concentration.

For G-CSF mRNA expression, A549 cells were treated with IL-1β (500 pg/ml) for 2, 4, 8, and 12 h, washed with Hanks' balanced salt solution (GIBCO), and evaluated by RT-PCR. Because G-CSF mRNA was expressed most intensely after 4 h of exposure to IL-1β, dose-dependent expression was evaluated at IL-1β concentrations of 500, 50, and 5 pg/ml. For TNF-α (1,000 U/ml), LPS (10 µg/ml), and BK (100 µM) stimulation, A549 cells were treated for 4 h, and then mRNA evaluation was performed.

These stimuli did not cause cytotoxicity to A549 cells (≥95% of the cells were viable by trypan blue exclusion) after 72 h of incubation at the maximal doses.

A monoclonal antibody to G-CSF was generated by recombinant human growth-related protein-α (GCSF, IL-8, and LTB4) and incubated for 30 min in 37°C. The antibodies were then added to A549 cell supernatant fluids to inhibit the effects of G-CSF, IL-8, and LTB4. These samples were used for chemotactic assay. These antibodies and antagonist did not influence the neutrophil chemotactic response to endotoxin-activated serum and fMLP (data not shown).

Partial purification of G-CSF by molecular-sieve column chromatography. To determine the molecular weight of G-CSF in the A549 cell supernatant fluids, which were harvested after 48 h of incubation in response to IL-1β, TNF-α, LPS, and BK, molecular-sieve column chromatography was performed by using Sephadex G-100 (25 × 125 cm; Pharmacia, Piscataway, NJ) at a flow rate of 6 ml/h. The A549 cell supernatant fluid was eluted with PBS, and every fraction after the void volume was evaluated for G-CSF in duplicate. The neutrophil chemotactic peak at a molecular mass of 19–20 kDa was also demonstrated in this study.

Measurement of G-CSF and IL-8 in the supernatant fluids. The concentrations of G-CSF and IL-8 in the A549 cell supernatant fluids stimulated by a variety of stimuli were measured by ELISA according to the manufacturer's directions. G-CSF ELISA kit was purchased from Amersham, and the minimum concentration of G-CSF detected by this method was 31.9 pg/ml. IL-8 kit was obtained from R&D Systems (Minneapolis, MN), and the minimum concentration of detectable IL-8 was 31.3 pg/ml.

RT-PCR. RT-PCR was used to detect mRNA for G-CSF synthesis by A549 cells. Total RNA was extracted from A549 cells as previously described (3). One microgram of total RNA was reverse transcribed into cDNA with a cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany) and then amplified with Taq DNA polymerase (Boehringer Mannheim) for 27 cycles in a Perkin-Elmer Gene Amp PCR System 9600 (denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 30 s). The G-CSF sense, antisense, and probe used in the present study were 5'-GGTTAGAAGCAGGTAGGAAAG-3', 5'-AGGTTGCGTGAGAAGGGCTTA-3', and 5'-ACCCAGGTCATGCAGGCCTTC-GGCCTCT-3', respectively. Preliminary studies indicated that 27 cycles were subsaturating for mRNA tested and were thus appropriate for comparison of relative levels of mRNA between groups. PCR products were separated by electrophore
sis on a 3% agarose gel and were visualized by labeled 32P exposure. PCR band densities were determined by the NIH Image analytic program (National Institutes of Health, Bethesda, MD) on unaltered, computer-scanned images. β-Ac-
tin mRNA, which has been shown not to change by stimula-
tion, was measured in both normal and stimulated RNA samples at each point with the same cDNA that was analyzed for cytokines (data not shown). Integrated optical density measurements of 10 separate β-actin samples did not vary by >33% from the mean integrated, optical density, which is an indication of the expected variation resulting from the experimental technique.

Statistics. In experiments where a single measurement was made, the differences between groups were tested for significance with Student’s paired t-test. In all cases, a P value <0.05 was considered significant. Data are expressed as means ± SE.

RESULTS

Release of NCA from A549 cell monolayers. A549 cells released NCA in response to IL-1β in a dose-related fashion (P < 0.001; Fig. 1). The lowest dose of IL-1β to stimulate A549 cells was 5.0 pg/ml. Increasing concentrations of IL-1β up to 500 pg/ml progressively increased the release of NCA. The release of NCA began 12 h of exposure to IL-1β, and the released activity reached the plateau at 48 h (Fig. 2). TNF-α, LPS, BK, and IFN-γ also induced the release of NCA from A549 cells in a time- and dose-dependent fashion (data not shown). However, the releasing potential of NCA by histamine and serotonin was weak and not significant compared with control values. The chemotactic activities in response to fMLP and activated serum were 70.4 ± 8.7 and 85.6 ± 15.3 neutrophils/HPF, respectively. IL-1β by itself in the culture medium without cells and incubated identically did not show significant chemotactic activity for neutrophils (data not shown).

Inhibition of NCA by polyclonal antibodies to G-CSF and IL-8 and by LTB4-receptor antagonist. The neutral-
izing antibodies to G-CSF and IL-8 added to the A549 cell supernatant fluids, which were harvested after 48 h of incubation in response to IL-1β, at the suggested concentrations inhibited NCA (30 and 35%, respect-
ively). The LTB4-receptor antagonist also inhibited NCA by 40% (Fig. 3). The treatment with G-CSF antibody significantly inhibited NCA induced by TNF-α, LPS, and BK stimulation (Table 1). The inhibition was ≥40–50%. However, NCA induced by IFN-γ was not inhibited by G-CSF antibody. The combined use of IL-8 and G-CSF antibodies and the LTB4-receptor antago-

![Fig. 1. Dose-dependent release of neutrophil chemotactic activity in response to interleukin (IL)-1β from A549 cell monolayers after 72 h of incubation. Values are expressed as means ± SE; n = 8 monolayers. *P < 0.01 compared with medium alone.](image1)

![Fig. 2. Time-related release of neutrophil chemotactic activity in response to IL-1β (500 pg/ml) from A549 cell monolayers. Values are expressed as means ± SE; n = 8 monolayers. *P < 0.01 compared with medium alone.](image2)

![Fig. 3. Effects of anti-granulocyte colony-stimulating factor (G-CSF) antibody on released neutrophil chemotactic activity from A549 cell monolayers. Supernatant fluids from A549 cell monolayers. Superna-
tant fluids from A549 cell monolayers were harvested after 48 h of incubation in response to 500 pg/ml of IL-1β. LTB4, leukotriene B4; F-12, Ham’s F-12 medium. Values are expressed as means ± SE; n = 6 monolayers. *P < 0.01 compared with crude sample.](image3)
Table 1. Effect of G-CSF antibody on A549 cell supernatant fluid NCA induced by TNF-α, LPS, and BK

<table>
<thead>
<tr>
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<th>Supernatant</th>
<th>G-CSF antibody</th>
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<tbody>
<tr>
<td>TNF-α (1,000 U/ml)</td>
<td>62.7 ± 3.5</td>
<td>32.1 ± 3.1*</td>
</tr>
<tr>
<td>LPS (100 µg/ml)</td>
<td>54.5 ± 2.2</td>
<td>37.3 ± 2.5*</td>
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<tr>
<td>BK (100 µM)</td>
<td>51.9 ± 2.1</td>
<td>27.4 ± 3.1*</td>
</tr>
<tr>
<td>IFN-γ (500 U/ml)</td>
<td>45.3 ± 3.1</td>
<td>40.4 ± 5.1</td>
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<tr>
<td>Negative control</td>
<td>10.3 ± 1.2</td>
<td>12.4 ± 2.4</td>
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Values are means ± SE. G-CSF, granulocyte colony-stimulating factor; NCA, neutrophil chemoattractant activity; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; BK, bradykinin; HPF, high-power field; IFN-γ, interferon-γ. *P < 0.01 compared with crude supernatant fluid.

Partial purification of G-CSF by molecular-sieve column chromatography. Molecular-sieve column chromatography revealed that NCA was heterogeneous in its size. Three peaks of chemotactic activity were observed in IL-1β-stimulated A549 cell supernatant fluids (Fig. 4). The molecular-mass chemotactic peak at 19–20 kDa was inhibited by the addition of anti-G-CSF polyclonal antibody (28.5 ± 4.5 vs. 12.4 ± 2.5 cells/HPF; P < 0.01; n = 4 monolayers). The second molecular-mass peak was inhibited by IL-8 antibody (31.5 ± 3.1 vs. 14.3 ± 2.3 cells/HPF; P < 0.01; n = 4 monolayers), and the lowest-molecular-mass peak was inhibited by the LTB₄-receptor antagonist (34.3 ± 2.1 vs. 13.5 ± 1.8 cells/HPF; P < 0.01; n = 4 monolayers). TNF-α, LPS, and BK also induced three neutrophil chemotactic peaks at similar molecular masses. The molecular mass at 19–20 kDa in response to TNF-α, LPS, and BK was inhibited by anti-G-CSF antibody (Table 2).

Measurement of G-CSF and IL-8 in the supernatant fluids. The concentrations of G-CSF and IL-8 in A549 cell supernatant fluids in response to IL-1β were increased in a dose-dependent fashion (Fig. 5). The release of IL-8 was observed at the lower concentration of IL-1β. The concentration of IL-8 increased time dependently (Fig. 6B). However, the concentration of G-CSF declined after 48 h of incubation (Fig. 6A). TNF-α, LPS, BK, IFN-γ, histamine, and serotonin induced the release of IL-8 from A549 cells (Table 3). However, IFN-γ, histamine, and serotonin did not stimulate A549 cells to release detectable amounts of G-CSF.

Neutrophil migration induced by human recombinant G-CSF. Neutrophil chemotaxis assay to human recombinant G-CSF (Kirin Pharmaceutical, Tokyo, Japan) was performed at concentrations ranging from 1 pg/ml to 10 ng/ml. Human recombinant G-CSF at 10–1,000 pg/ml induced neutrophil migration in a dose-dependent manner and declined thereafter. The concentration of G-CSF that induced maximum neutrophil chemotaxis was 100 pg/ml (Fig. 7).

Induction of G-CSF mRNA by IL-1β, TNF-α, BK, and LPS. IL-1β at a concentration of 500 pg/ml induced the gene expression of G-CSF in a time-dependent manner (Fig. 8). The maximum expression of G-CSF mRNA was after 4 h of incubation, and then it declined. IL-1β induced G-CSF mRNA expression in a dose-dependent manner at 4 h (Fig. 9). TNF-α, LPS, and BK slightly induced G-CSF mRNA expression after 4 h of incubation (Fig. 9).

DISCUSSION

In the present investigation, we evaluated the potential of IL-1β to induce the release of G-CSF as NCA from A549 cells. IL-1β significantly stimulated A549 cells to release G-CSF in a dose- and time-dependent manner. Molecular-sieve chromatography showed that IL-1β induced three molecular masses (19–20 kDa, 8 kDa, and 400 Da) for NCA. Polyclonal blocking antibody to G-CSF significantly inhibited the chemotactic response in both crude and column-fractionated supernatants. The release of G-CSF was significantly augmented in response to IL-1β. TNF-α, LPS, and BK also induced the release of G-CSF as NCA. The G-CSF mRNA evaluated by RT-PCR showed a dose-dependent increase in the expression of G-CSF mRNA.

Table 2. Effects of G-CSF antibody on column chromatography-separated 19-kDa molecular-mass NCA induced by TNF-α, LPS, and BK

<table>
<thead>
<tr>
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<th>Fractionated</th>
<th>G-CSF antibody</th>
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<tbody>
<tr>
<td>TNF-α (1,000 U/ml)</td>
<td>29.3 ± 3.5</td>
<td>11.5 ± 2.8*</td>
</tr>
<tr>
<td>LPS (100 µg/ml)</td>
<td>20.4 ± 3.8</td>
<td>10.3 ± 2.3*</td>
</tr>
<tr>
<td>BK (100 µM)</td>
<td>29.4 ± 2.4</td>
<td>12.4 ± 2.2*</td>
</tr>
<tr>
<td>Negative control</td>
<td>12.1 ± 1.1</td>
<td>12.8 ± 2.9</td>
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Values are means ± SE. *P < 0.01 compared with crude fraction.

Fig. 4. Molecular-sieve column chromatographic finding of neutrophil chemotactic activity released from A549 cell monolayers in response to 500 pg/ml of IL-1β for 48 h of incubation. Values are representative data from 4 different supernatant fluids. Nos. on top and arrows, molecular-weight position; chemotactic peaks were inhibited by (left to right) anti-G-CSF polyclonal antibody, IL-8 polyclonal antibody, and LTB₄-receptor antagonist.
dency at 4 h and declined after 4 h in response to IL-1β. The expression of G-CSF mRNA was also observed with TNF-α, LPS, and BK stimulation. These data suggest that ATII cells may produce G-CSF as NCA in response to IL-1β, TNF-α, LPS, and BK and may participate in the regulation of leukocyte extravasation.

The characterization of released NCA in response to IL-1β is not complete in the present study because the blocking antibody to G-CSF attenuated the chemotactic activity up to 30% in response to IL-1β. Anti-G-CSF antibody inhibited NCA up to 40–50% in response to TNF-α, LPS, and BK. The involvement of IL-8 and LTB₄ as NCA was also significant. But the combined use of G-CSF and IL-8 antibodies and the LTB₄ receptor antagonist did not completely inhibit NCA. Because MIP-1α antibody did not influence NCA and MIP-1α was not in the supernatant fluids (data not shown), the involvement of MIP-1α was small. The possible candidates for NCA may involve complements, 12-hydroxyeicosatetraenoic acid (12-HETE), and 15-HETE. Although complements and 12- and 15-HETE may be involved in NCA released from A549 cells, IL-8, G-CSF, and LTB₄ explained 80–90% of NCA released from A549 cells in response to IL-1β and specific stimuli. The release of IL-8 was observed in response to a variety of stimuli, including IFN-γ, histamine, and serotonin. In contrast, the release of G-CSF needed a higher concentration of IL-1β and specific stimuli. The release of LTB₄ in response to IL-1β was not different from that of the unstimulated A549 cells (data not shown). The releasing pattern of LTB₄ was predominant at 24 h and then gradually increased. Thus the relevant role of these chemotactic factors may be dependent on the concentration of IL-1β or stimuli, i.e., at the lower concentration of IL-1β, the predominant NCA may be IL-8 and LTB₄ rather than G-CSF. However, the concentration of IL-1β in the bronchoalveolar lavage fluid was fairly high. Thus G-CSF may play an important role in neutrophil recruitment in lung inflammation.

Both in vivo and in vitro data suggest that ATII cells could participate in the intra-alveolar cytokine network by secreting IL-8 (21, 22), IL-6 (6), MCP-1 (23), RANTES, GM-CSF, and TGF-β (14, 15) constitutively and in response to TNF-α and IL-1β. In the present study, we illustrated that A549 cells produced G-CSF. Because G-CSF plays significant roles in neutrophil migration (27), activation (26), and survival (5), the production of G-CSF from A549 cells may suggest the
amplification of the inflammatory responses of the lung by type II epithelial cells in addition to the modulation of immunologic activity in the alveolar space.

Locally produced chemoattractants are likely to play an important role in the regulation of neutrophil extravasation and localization. CSFs, including G-CSF, GM-CSF, and M-CSF, can influence the migratory capacity of leukocytes, although somewhat conflicting results have been obtained (7, 28, 30). GM-CSF has been reported to inhibit the migration of leukocytes in an agarose assay (7, 30). GM-CSF and M-CSF, on the other hand, act as relatively potent chemoattractants for neutrophils and monocytes in Boyden chambers (28). The apparent conflict between reports on the influence of GM-CSF on chemotaxis might be explained by the different exposure conditions. Wang et al. (27) reported that G-CSF induced migration of neutrophils across polycarbonate or nitrocellulose filters and that this response involved chemotaxis. The concentration of G-CSF required for neutrophil migration by Wang et al. was >10–100 U/ml (7–70 ng/ml). The discrepancy of G-CSF concentration for neutrophil migration compared with the present study might be due to the differences in neutrophil separation and solutions used for neutrophil suspension because human recombinant G-CSF induced neutrophil migration at 10–1,000 pg/ml in the present study. The concentrations of G-CSF in the A549 cell supernatant fluid in response to a variety of stimuli reached this chemotactic concentration.

G-CSF activity is produced by various cell types including stimulated lymphocytes, activated macrophages (4, 18), fibroblasts (2), and endothelial cells (12) exposed to mononuclear phagocyte products. Therefore, it is conceivable that G-CSF production, triggered in these cell types directly by exogenous materials (endotoxin or antigens) or indirectly via monokine release, might serve to rapidly recruit neutrophils from the blood compartment to local inflammatory sites.

In conclusion, A549 cells released G-CSF as NCA in response to IL-1β, TNF-α, LPS, and BK. The expression of G-CSF mRNA was augmented in response to each stimulus. These data suggest that ATII cells may produce G-CSF as NCA and may participate in the...
regulation of neutrophil extravasation and lung inflammation.

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