Smoke extract stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activities

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1The First Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto 390-8621; 2Kyoto University Chest Disease Research Institute, Kyoto 606-01, Japan; and 3Overton Brooks Veterans Affairs and Louisiana State University Medical Centers, Shreveport, Louisiana 71101

Sato, Etsuro, Sekiya Koyama, Akemi Takamizawa, Takeshi Masubuchi, Keishi Kubo, Richard A. Robbins, Sonoko Nagai, and Takateru Izumi. Smoke extract stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activities. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1149–L1157, 1999.—Accumulation of monocytes and neutrophils and fibrous distortion of the airway are characteristics of airway disease secondary to smoking. The presence of inflammatory cells and fibrosis correlate, and, therefore, we postulated that lung fibroblasts might release chemotactic activity for neutrophils and monocytes in response to smoke extract. To test this hypothesis, human fetal lung (HFL 1) fibroblasts were cultured, and the supernatant fluid was evaluated for neutrophil (NCA) and monocyte (MCA) chemotactic activities with a blind well chamber technique. HFL 1 fibroblasts released chemotactic activity in response to smoke extract in a dose- and time-dependent manner (P < 0.05). Checkerboard analysis showed that the activity was predominantly chemotactic. Partial characterization of the released chemotactic activity revealed that the activity was partly heat labile, trypsin sensitive, and ethyl acetate extractable. Lipoxigenase inhibitors and cycloheximide inhibited the release of both NCA and MCA. Molecular sieve chromatography revealed that NCA and MCA were heterogeneous. NCA was inhibited by anti-human interleukin (IL)-8 and anti-granulocyte colony-stimulating factor antibodies and a leukotriene (LT) B4-receptor antagonist. Anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) and anti-monocyte chemoattractant protein (MCP)-1 antibodies and an LT B4-receptor antagonist inhibited MCA. Immunoreactive IL-8, granulocyte colony-stimulating factor, GM-CSF, and MCP-1 significantly increased in culture supernatant fluid in response to smoke extract. Finally, smoke extract augmented the expression of mRNAs of IL-8, GM-CSF, and MCP-1. These data demonstrate that lung fibroblasts release NCA and MCA in response to smoke extract and suggest that lung fibroblasts may modulate the inflammatory cell recruitment into the lung.

interleukin-8; granulocyte colony-stimulating factor; monocyte chemoattractant protein-1; granulocyte-macrophage colony-stimulating factor; fibrosis; chemotaxis; smoking

theory in the pathogenesis of emphysema is an abnor-
mal balance between proteases and antiproteases and/or oxidants and antioxidants in the lung (3, 15, 30). An increased number of neutrophils and monocytes activated by cigarette smoke produces large amounts of proteases and oxidants responsible for lung destruction (12, 33, 34). Senior et al. (28) reported that experimental emphysema was induced in animals by intratra-
cheal instillation of purified human neutrophil elastase. Monocytes also contain an elastase (27), and this protease, although differing from the neutrophil enzyme in amount and subcellular localization, is antigeni-
cally and biochemically closely related to the elastase of neutrophils. It is reported that macrophage elastase is sufficient for the development of emphysema that results from chronic inhalation of cigarette smoke in mice because macrophage elastase-deficient mice did not develop emphysema (11).

In a clinical study (13), increased numbers of macro-
phages, neutrophils, and eosinophils have been demonstrated in bronchoalveolar lavage fluid from smokers. Pulmonary tissue injury and dysfunction in cigarette smokers have been correlated with the number and activity of inflammatory cells (24). Although it has been proposed that an influx of inflammatory cells may occur because of chemotactic factors generated in the lung in response to smoke, including complement activation and macrophage- and airway epithelial cell-derived chemotactic factors, the precise mechanism remains to be elucidated.

Fibroblasts are the major cells responsible for the synthesis of matrix elements in soft connective tissue and constitute 35–40% of the cell in the interstitium of the lung. Although fibroblasts remain largely quiescent under normal conditions, they are activated to proliferate and synthesize various cytokines during inflammation, suggesting that they may contribute to the pathophysiology of certain lung diseases (8, 22). Consistent with this concept, peribronchial fibrous tissue in cigarette smoke-induced bronchiolitis correlates with the presence of inflammatory cells (21). Although chemotactic activity derived from macrophages and airway epithelial cells may recruit inflammatory cells from the interstitium to bronchial lumens or alveolar spaces, the regulation of inflammatory cell traffic from the vascula-
ture to the interstitium is uncertain.

Based on the above, we hypothesized that smoke extract might stimulate lung fibroblasts to release neutrophil (NCA) and monocyte (MCA) chemotactic activities. The results demonstrate that human fetal
lungs (HFL1) fibroblasts released NCA and MCA, which were heterogeneous and at least partially attributable to interleukin (IL)-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein (MCP)-1, in response to smoke extract. These data suggest that fibroblasts may regulate inflammatory cell traffic from the vasculature to the interstitium.

**MATERIALS AND METHODS**

HFL1 fibroblast cultures. HFL1 fibroblasts (diploid, passage 27) were purchased from a commercial source (American Type Culture Collection, Manassas, VA). The cells were suspended at 1 x 10^6 cells/ml in Ham's F-12 medium supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml), Fungizone (2 µg/ml), and 10% fetal calf serum (all from Gibco BRL, Life Technologies, Grand Island, NY). HFL1 fibroblast suspensions were added to 30-mm-diameter tissue culture dishes (Corning, Corning, NY) and cultured at 37°C in a 5% CO₂ atmosphere. After 4–6 days, the cells had reached confluence, and the culture medium was replaced with 2 ml of medium supplemented as above and cultured for an additional day.

Preparation of cigarette smoke extract. Smoke extract was prepared with a modification of the method of Carp and J anoff (6) and J anoff (14). Briefly, two cigarettes without filters were combusted with a modified syringe-driven apparatus. The smoke was bubbled through 50 ml of Hanks' balanced salt solution (HBSS; Gibco BRL). The resulting suspension was adjusted to pH 7.4 with concentrated NaOH and filtered through a 0.2-µm-pore filter to remove bacteria and large particles. The smoke extract was then applied to HFL1 fibroblast cultures within 30 min of preparation.

Exposure of HFL1 fibroblasts to smoke extract. Culture medium was removed from the cells by washing twice with serum-free Ham's F-12 medium, and the cells were incubated in the presence and absence of smoke extract (0, 0.5, 1, 5, and 10%) for 12, 24, 48, and 72 h at 37°C in a humidified 5% CO₂ atmosphere. Smoke extract was not cytotoxic as assessed by morphology and esterase staining (Sigma), with >99% viable cells. The cells were suspended in Gey's balanced salt solution (Gibco BRL) containing 2% bovine serum albumin (BSA; Sigma) at pH 7.2 to a final concentration of 3 x 10^6 cells/ml.

Mononuclear cells for the chemotaxis assay were obtained from normal human volunteers by Ficol-Hypaque density centrifugation to separate the red blood cells and neutrophils from the mononuclear cells. The mononuclear cells were harvested at the interface and centrifuged at 400 g for 5 min and washed three times with HBSS. The resulting cell pellet routinely consisted of ~30% monocytes and 70% lymphocytes by morphology and esterase staining (Sigma), with >98% viable cells. The cells were suspended in Gey's balanced salt solution (Gibco BRL) containing 2% BSA (Sigma) at pH 7.2 to a final concentration of 5 x 10^9 cells/ml.

Chemotaxis was performed in 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD) as previously described (10). The bottom wells of the chamber were filled with 25 µl of the chemotactic stimulus or medium in duplicate. A 10-µm-thick polyvinylpyrrolidone-free polycarbonate filter with a pore size of 3 µm for neutrophil chemotaxis and 5 µm for monocytes was placed over the samples. The silicon gasket and the upper pieces of the chamber were applied, and 50 µl of the cell suspension were placed into the upper wells. The chambers were incubated in 5% CO₂ humidified air at 37°C for 30 min for neutrophil chemotaxis and 90 min for monocyte chemotaxis. Nonmigrated cells were wiped away from the filter. The filter was immersed in methanol for 5 min, stained with a modified Wright's stain, and mounted on a glass slide. Cells that had completely migrated through the filter were counted with light microscopy, and 10 random high-power fields (HPF; x1,000) were counted.

**Fig. 1.** Dose-dependent release of neutrophil (A) and monocyte (B) chemotactic activities in response to smoke extract (n = 8 monolayers). *P < 0.05 compared with control culture supernatant fluid.
To ensure that monocytes and not lymphocytes were the primary cells that migrated in the monocyte chemotaxis assays, some membranes were stained with α-naphthyl acetate esterase according to the manufacturer’s directions (Sigma).

To determine whether the migration was due to movement along a concentration gradient (chemotaxis) or to stimulation of random migration (chemokinesis), a checkerboard analysis was performed with HFL1 fibroblast culture supernatant fluid obtained from cells cultured for 72 h in the presence of 5% smoke extract (38). This was done by placing various dilutions (1:1, 1:4, 1:16, 1:64, and 1:256) of the culture supernatant fluid with target cells below and above the membrane.

Partial characterization of NCA and MCA. Partial characterization of NCA and MCA released from the HFL1 fibroblast cultures was performed with culture supernatant fluid harvested after 72 h of incubation with 5% smoke extract. Sensitivity to proteases was tested by incubating the culture supernatant fluid with trypsin (100 µg/ml; Sigma) for 30 min at 37°C followed by addition of a 1.5 M excess of soybean trypsin inhibitor to terminate the proteolytic activity. Lipid solubility was evaluated by ethyl acetate extraction. Both the extracted and extractant fluids were evaluated for chemotactic activity. Heat sensitivity was determined by heating the supernatant fluid to 98°C for 15 min.

Molecular-sieve column chromatography. To determine the approximate molecular mass of the released chemotactic activity, molecular-sieve column chromatography was performed with a Sepadex G-200 column (Pharmacia, Piscataway, NJ) at a flow rate of 6 ml/h on 5% smoke extract-stimulated culture supernatant fluid harvested after 72 h of incubation. The culture supernatant fluid was eluted from the column with PBS, and the fractions were evaluated for NCA and MCA in duplicate.

Metabolic inhibitors of chemotactic activity. The effects of the nonspecific lipooxygenase inhibitors nordihydroguaiaretic acid (100 µM; Sigma) and diethylcarbamazine (1 mM; Sigma) and the 5-lipoxygenase inhibitor AA-861 (100 µM; Takeda Pharmaceutical, Tokyo, J apan) were used to assess the involvement of LTB₄ and PAF in NCA and MCA.

Measurement of LTB₄ and PAF. The concentration of LTB₄ in the culture supernatant fluid was measured by RIA as previously described (17). PAF was measured with a scintillation proximity assay (32). Briefly, this assay system combined the use of a high specific activity tritiated PAF tracer with an antibody specific for PAF and a PAF standard similar to the method used for the measurement of LTB₄.

Measurement of IL-8, G-CSF, MCP-1, GM-CSF, regulated on activation normal T cells expressed and secreted, and transforming growth factor-β. The concentrations of IL-8, G-CSF, and MCP-1 were measured in culture supernatant fluid harvested from cells cultured with 5% smoke extract after 72 h of incubation with an ELISA (R&D Systems, Minneapolis, MN). The minimum concentrations detected by these ELISAs were 10 pg/ml for IL-8, 31.3 pg/ml for MCP-1, and 0.31 ng/ml for transforming growth factor (TGF)-β. GM-CSF and regulated on activation normal T cells expressed and secreted (RANTES) kits were purchased from Amersham. The minimum concentrations detected with these methods were 2.0 pg/ml for GM-CSF and 15.6 pg/ml for RANTES. G-CSF was assayed with a commercial kit (Chugai, J apan).

Table 1. Checkerboard analysis of HFL1 fibroblast supernatant fluid

<table>
<thead>
<tr>
<th>Lower Well</th>
<th>RPMI</th>
<th>1:256</th>
<th>1:64</th>
<th>1:16</th>
<th>1:4</th>
<th>1:1</th>
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<tr>
<td><strong>Neutrophils</strong></td>
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<tr>
<td>RPMI</td>
<td>3 ± 2</td>
<td>3 ± 2</td>
<td>3 ± 1</td>
<td>6 ± 1</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>1:256</td>
<td>6 ± 1</td>
<td>4 ± 1</td>
<td>2 ± 1</td>
<td>7 ± 3</td>
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<td>4 ± 1</td>
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<tr>
<td>1:4</td>
<td>16 ± 2</td>
<td>17 ± 2</td>
<td>14 ± 3</td>
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<tr>
<td>1:1</td>
<td>22 ± 3</td>
<td>49 ± 3</td>
<td>24 ± 5</td>
<td>13 ± 2</td>
<td>7 ± 2</td>
<td>5 ± 1</td>
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</table>

| **Monocytes** |      |       |      |      |     |     |
| RPMI         | 4 ± 2 | 5 ± 1 | 6 ± 1 | 3 ± 2 | 4 ± 2 | 3 ± 1 |
| 1:256        | 3 ± 2 | 6 ± 2 | 9 ± 2 | 3 ± 1 | 3 ± 1 | 3 ± 1 |
| 1:64         | 3 ± 2 | 8 ± 2 | 10 ± 3 | 5 ± 2 | 5 ± 1 | 3 ± 1 |
| 1:16         | 9 ± 1 | 14 ± 3 | 14 ± 3 | 10 ± 2 | 5 ± 1 |       |
| 1:4          | 13 ± 2 | 14 ± 3 | 13 ± 1 | 20 ± 2 | 11 ± 3 | 7 ± 3 |
| 1:1          | 44 ± 3 | 43 ± 5 | 14 ± 1 | 13 ± 2 | 11 ± 4 | 14 ± 3 |

Values are means ± SE in cells/high-power field. HFL1, human fetal lung; RPMI, RPMI 1640 medium. Fluids were harvested after 72 h of incubation with 5% smoke extract. Lower wells contained dilutions of HFL1 fibroblast supernatant fluid. Upper wells contained dilutions of HFL1 fibroblast supernatant fluid with smoke extract.
Pharmaceutical, Tokyo, Japan), with a minimum detection limit of 1.0 pg/ml.

Evaluation of IL-8, G-CSF, MCP-1, and GM-CSF mRNA expression. The protein secretions of IL-8, MCP-1, and GM-CSF were augmented by smoke extract. RT-PCR was used to evaluate mRNA expression of IL-8, MCP-1, and GM-CSF in HFL1 fibroblast cultures in response to 5% smoke extract after 72 h of incubation. Total RNA was extracted from HFL1 fibroblast cultures as previously described (7). One microgram of total RNA was reverse transcribed with a commercially available kit (Promega, Madison, WI) and then amplified for 32 cycles in a Perkin-Elmer model 480 thermal cycler (denaturation at 94°C for 45 s, primer annealing at 72°C for 45 s, and primer extension at 72°C for 7 min). The sequences used in the present study were GM-CSF forward, 5’-GGA-GCATGTAATTGGC-3’, and reverse, 5’-ATCTGGTTGGAATTGGG-3’; IL-8 forward, 5’-TACTCCTCCTGGAATTGGG-3’, and reverse, 5’-ATCTGGTTGGAATTGGG-3’; MCP-1 forward, 5’-CAGCCAGATGCAATCAATGC-3’, and reverse, 5’-ATCTGGTTGGAATTGGG-3’; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5’-ACCACAGTCCATGCCATCA-3’, and reverse, 5’-TCCACCACCTGCTGCAAATGC-3’. Preliminary studies indicated that >32 cycles were subsaturating for the mRNA tested and thus were appropriate for a comparison of the relative levels of mRNA between groups. PCR products were separated by electrophoresis on a 3% agarose gel and visualized by 32P exposure. PCR band densities were determined by a NIH Image analytic program (National Institutes of Health, Bethesda, MD) on unaltered, computer-scanned images. GAPDH was used as a housekeeping gene for PCR, which was measured in both normal and stimulated RNA samples at each point. Integrated optical density measurement of 10 separate GAPDH samples did not vary >10% from the mean integrated optical density, which is an indication of the expected variation resulting from the experimental technique.

Statistics. In experiments where multiple experiments were performed, differences between groups were tested for significance with a one-way analysis of variance, with Duncan’s multiple range test applied to data at specific time and dose points. In experiments where single measurements were made, the differences between groups were tested with Student’s paired t-test. In all cases, a P value of <0.05 was considered significant. Data are expressed as means ± SE.

RESULTS

Release of NCA and MCA from HFL1 fibroblasts. HFL1 fibroblasts released NCA and MCA in a dose-dependent manner above a 1% concentration (P < 0.05; Fig. 1). HFL1 fibroblasts also released NCA and MCA in a time-dependent manner after 48 h (Fig. 2). Smoke extract itself was not chemotactic for neutrophils or monocytes (data not shown).
The chemotactic response to LTB₄ at a concentration of 10⁻⁷ M was 1,020 ± 674 cells/10 HPF for neutrophils and 756 ± 34 cells/10 HPF for monocytes.

Checkerboard analysis revealed that the HFL1 fibroblast culture supernatant fluid stimulated with smoke extract induced neutrophil and monocyte migration with increasing concentrations in the presence of a gradient (Table 1). Thus the migration of neutrophils and monocytes was predominantly consistent with chemotactic activity rather than chemokinetic activity.

Confirmation that the migrated cells were monocytes in the monocyte chemotaxis assay was shown by several experiments. Greater than 90% of the migrated cells were morphologically monocytes. Greater than 90% of the migrated cells were esterase positive. Lymphocytes purified by allowing the monocytes to attach to plastic and tested in the chemotaxis assay yielded 0–20% of the chemotactic activity of the monocyte preparation.

Partial characterization of NCA and MCA. Both NCA and MCA were heterogeneous in character. NCA and MCA both were partially sensitive to heat, ethyl acetate extractable, and partially digested by trypsin (Fig. 3).

Molecular-sieve column chromatography. The released chemotactic activity obtained from HFL1 fibroblast culture supernatant fluid incubated with 5% smoke extract for 72 h was evaluated by molecular-sieve column chromatography with a Sephadex G-200 column. These experiments revealed that the released NCA obtained from unstimulated cells was heterogeneous in size (Fig. 4A). At least four peaks of NCA that were just before BSA (molecular mass 66 kDa), before cytochrome c (molecular mass 12.3 kDa), just after cytochrome c, and near quinacrine (molecular mass 0.45 kDa) were separated by column chromatography. Stimulation with smoke extract resulted in each of these peaks becoming more prominent.

The released MCA from unstimulated and smoke extract-stimulated HFL1 fibroblast culture supernatant fluid was also heterogeneous and similar to the released NCA (Fig. 4B). At least four peaks of monocyte chemotactic activity that were just before BSA, before cytochrome c, just after cytochrome c, and near quinacrine were separated by column chromatography. Stimulation with smoke extract resulted in each of these peaks becoming more prominent.
Effects of metabolic inhibitors. The culture supernatant fluid incubated with 5% smoke extract for 72 h and with nordihydroguaiaretic acid, diethylcarbamazine, and AA-861 showed a significant decrease in NCA and MCA (Fig. 5). Cycloheximide also significantly inhibited the release of NCA and MCA (P < 0.05; Fig. 5).

Effects of LTB4- and PAF-receptor antagonists. Both total NCA and MCA and the lowest-molecular-mass NCA and MCA separated by column chromatography were significantly inhibited by addition of the LTB4-receptor antagonist ONO-4057 (Fig. 6). In contrast, the effects of the PAF-receptor antagonist TCV-309 were not significant for NCA and MCA. Each receptor antagonist at 10^{-5} M completely inhibited the neutrophil migration to 10^{-7} M concentrations of LTB4 and PAF, but the antagonists showed no inhibitory effects on complement-activated serum-induced NCA or MCA (data not shown).

Effects of smoke extract on the release of LTB4. The measurement of LTB4 by RIA revealed that HFL1 fibroblasts released LTB4 under basal unstimulated conditions (278 ± 34 pg/ml). There was no significant increase in LTB4 release from HFL1 fibroblasts cultured with 5% smoke extract for 72 h (289 ± 29 pg/ml). PAF was not detected in the unstimulated or smoke extract-stimulated culture supernatant fluid after 72 h of incubation.

Effects of anti-IL-8, anti-G-CSF, anti-GM-CSF, anti-MCP-1, anti-RANTES, and anti-TGF-β antibodies. Polyclonal blocking antibodies were evaluated for their capacity to reduce NCA and MCA. Anti-IL-8 and anti-G-CSF antibodies significantly reduced NCA. Anti-MCP-1 and anti-GM-CSF antibodies significantly reduced MCA (Fig. 7). The effects of anti-IL-8, anti-G-CSF, anti-GM-CSF, and anti-MCP-1 antibodies were also evaluated on the column chromatography fractions. These antibodies reduced the chemotactic activity at the corresponding molecular-mass peak (data not shown). In contrast, anti-RANTES and anti-TGF-β antibodies did not block MCA.

Effects of smoke extract on the release IL-8, G-CSF, GM-CSF, MCP-1, RANTES, and TGF-β. The concentrations of IL-8 and G-CSF significantly increased in the culture supernatant fluid in response to smoke extract (Fig. 8). The concentrations of GM-CSF and MCP-1 were also significantly increased (Fig. 9). HFL1 fibroblasts constitutively released TGF-β, but the concentration was not increased by smoke extract stimulation. RANTES was undetectable in both unstimulated and smoke-stimulated culture supernatant fluids.

Augmentation of IL-8, GM-CSF, and MCP-1 mRNA expression by smoke extract. Smoke extract treatment of HFL1 for 6 h resulted in the augmented expression of IL-8, GM-CSF, and MCP-1 mRNAs (Fig. 10). Although
we evaluated the expression of G-CSF, we could not detect a clear band of G-CSF mRNA after 40 cycles of amplification in both stimulated and unstimulated samples (data not shown).

**DISCUSSION**

In the present study, HFL1 fibroblasts released NCA and MCA in response to smoke extract in a dose- and time-dependent manner. The released activity was heterogeneous and chemotactic. Anti-IL-8 and anti-G-CSF antibodies and an LTB₄-receptor antagonist inhibited NCA. Anti-MCP-1 and anti-GM-CSF antibodies and an LTB₄-receptor antagonist inhibited MCA. In response to smoke extract, IL-8, G-CSF, MCP-1, and GM-CSF were all increased in HFL1 fibroblast culture supernatant fluid. Finally, the mRNA expression of IL-8, GM-CSF, and MCP-1 was augmented by smoke extract in HFL1 fibroblast monolayers. These data suggest that fibroblasts may play a role in the pathogenesis of smoking-induced lung diseases by releasing NCA and MCA in response to cigarette smoke.

Inflammatory cells are present in increased numbers in the lungs of smokers (13). Both in vitro and in vivo studies support the potential for inflammatory cells to damage lung parenchyma. The alteration of lung matrix could occur by a variety of mechanisms including the release of oxygen radicals and proteases (14, 23). The present study suggests that increased numbers of neutrophils and macrophages in the lung secondary to smoking may be due, at least in part, to NCA and MCA released from fibroblasts in response to smoke. Consistent with this hypothesis, peribronchial fibrosis and inflammation secondary to smoking often coexist (20, 31).

The present study demonstrates that several chemotactic factors were released by fibroblasts that may contribute to inflammatory cell recruitment. Partial characterization revealed that the released NCA and MCA were partially ethyl acetate extractable. Molecular-sieve column chromatography showed a large chemotactic peak in the lowest molecular-mass range, and an LTB₄-receptor antagonist inhibited this chemotactic activity. Furthermore, the concentration of LTB₄ was high enough to produce chemotactic activity (17, 19). Although smoke extract did not increase the release of LTB₄ into HFL1 fibroblast culture supernatant fluid, LTB₄ appeared to be one of the predominant chemotactic factors released.

In contrast, the trypsin sensitivity of the chemotactic activity along with the inhibition of the release of chemotactic activity by cycloheximide suggests that chemotactic activity released from HFL1 fibroblasts is at least partially dependent on protein synthesis. Molecular-sieve column chromatography revealed increases in the higher-molecular-mass peaks of chemotactic activity in response to smoke extract. The antibodies to IL-8, G-CSF, MCP-1, and GM-CSF inhibited NCA and MCA. Consistent with the inhibition of
chemotactic activity by blocking antibodies, these cytokines were significantly increased in the culture supernatant fluid in response to smoke extract. The expression of these cytokine mRNAs was augmented by smoke extract. These data suggest that these cytokines may play important roles in the recruitment of inflammatory cells into the lungs of smokers.

Early descriptions of cytokines focused on their production by immune and inflammatory effector cells. However, it is apparent that structural cells are also capable of releasing many cytokines. Fibroblasts are known to produce a variety of cytokines including IL-8 (25), GM-CSF (25), TGF-β (16), and MCP-1 (26) in response to a variety of stimuli. However, the relationship between smoking and the release of these cytokines has not been established. The present study demonstrated that HFL1 fibroblasts released these cytokines as chemotactic factors in response to smoke extract and suggest that these cytokines may play a role in smoking-induced lung disease by recruiting inflammatory cells.

The release of NCA and MCA from human lung fibroblasts was a two- to threefold increase from the constitutive release of NCA and MCA, and the induction of fibroblast chemotactic activity by smoke extract was modest compared with fractions isolated from unstimulated fibroblasts. However, the released NCA and MCA from fibroblasts in response to smoke extract was more than those from 10^6 alveolar macrophages/ml stimulated with lipopolysaccharide (data not shown). Thus fibroblasts may play an important role in the recruitment of inflammatory cells in response to smoke extract.

Although TGF-β was detected in the cell culture supernatant fluid, anti-TGF-β antibodies did not attenuate MCA. TGF-β induces monocyte chemotaxis at concentrations of 0.1–10 pg/ml (35). At higher concentrations, the chemotactic response of monocytes declines. TGF-β is usually released by cells in an inactive form (9, 36). The biologically inactive form of TGF-β is unable to bind to its receptor, and the release of inactive TGF-β may account for the lack of inhibition of MCA in the HFL1 fibroblast culture supernatant fluid by anti-TGF-β antibodies.

G-CSF has been reported to induce neutrophil migration at concentrations > 10–100 U/ml (7–70 ng/ml) (37). The concentration of G-CSF in the culture supernatant fluid was relatively low in the present study. However, blocking anti-G-CSF antibodies reduced NCA by up to 60%. Recently, Koyama et al. (18) have found that 10–1,000 pg/ml of G-CSF-induced significant NCA. Although it is possible that G-CSF may be facilitating the chemotactic response of other peptides (4), the concentration of G-CSF in the culture supernatant fluid exceeded the lower chemotactic threshold observed in our laboratory.

In conclusion, HFL1 fibroblasts release NCA and MCA in response to smoke extract in vitro. The released activity was heterogeneous and could be attributed to several cytokines. These data suggest that lung fibroblasts may play an important role in inflammatory cell recruitment in disorders associated with cigarette smoking.

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