Primary Human Alveolar Type II Epithelial Cell Chemokine Release
Effects of Cigarette Smoke and Neutrophil Elastase

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An early response to cigarette smoke is an influx of leukocytes into the lung. Alveolar epithelial type II (ATII) cells may contribute by releasing chemokines in response to cigarette smoke and neutrophil elastase (NE). Human ATII cells were purified from normal regions of lungs resected for carcinoma ($n = 14$). *In vitro*, these cells exhibited ATII cell characteristics: lamellar bodies, apical microvilli, tight junctions, and expressed surfactant apoprotein C. Basal ATII cell release of five chemokines ranked as follows: monocyte chemotactic protein (MCP)-1 > interleukin (IL)-8 > growth-related oncogene (GRO)-α > macrophage inflammatory protein (MIP)-1α > regulated on activation, normal T cell expressed and secreted (RANTES). MIP-1α and RANTES were often not detectable. After stimulation with a mixture of lipopolysaccharide/endotoxin (LPS), tumor necrosis factor-α, IL-1β, and IFN-γ, MCP-1 and IL-8 secretion rose 4–6-fold, whereas GRO-α rose 25-fold. NE stimulated IL-8 mRNA expression, and 10nM NE stimulated IL-8 secretion; however, 100nM NE caused a decrease in extracellular IL-8, MCP-1, and GRO-α, attributed to proteolysis. Cigarette smoke extract (CSE) inhibited IL-8 mRNA expression and release of all chemokines. Glutathione protected against the effects of CSE, suggesting oxidative mechanisms. GRO-α, important in growth and repair, was sensitive to both stimulation, by LPS:cytokines, and inhibition, by CSE. Thus, contrary to the original hypothesis, high concentrations of NE and CSE resulted in reduced extracellular chemokine levels. We hypothesize that reduced ATII cell–derived chemokine levels compromise alveolar repair, contributing to cigarette smoke–induced alveolar damage and emphysema.

Increased leukocyte migration into the lower respiratory tract is an early event in cigarette smokers (1), which persists in chronic smokers (2). Release of mediators by these cells is believed to perpetuate inflammation and precipitate lung injury leading to chronic obstructive pulmonary disease (COPD) (3, 4). The pattern of inflammation in COPD is complex. Neutrophils are markedly increased in conducting airway lumen, whereas the peripheral airways and lung parenchyma contain increased macrophages, which predominate, and T lymphocytes, especially CD8+, cytotoxic T cells, in the airway wall. Differences in the pattern of inflammation in different regions of the lung will reflect dose and site of delivery of cigarette smoke and consequent cellular responses to the cigarette smoke. Release of proinflammatory mediators, such as chemokines, by cigarette smoke–exposed resident phagocytes and pulmonary epithelial cells could sustain leukocyte recruitment. Chemokines are a family of cytokines with strong chemoattractant properties. Pulmonary secretions from smokers with bronchitis and COPD contain elevated levels of C-X-C and C-C chemokines, such as interleukin 8 (IL-8, CXCL8), monocyte chemotactic protein (MCP-1α, CCL2) and macrophage inflammatory protein (MIP-1α, CCL3) (5, 6). The source and relative contribution of these mediators to pulmonary inflammation is unclear. Human lung macrophages release IL-8, MCP-1, MIP-1α, and growth-related oncogene-α (GRO-α, CXCL1) (7, 8). Similarly, human bronchial airway cells release IL-8, MCP-1, MIP-1α, and GRO-α, as well as regulated on activation normal T cell expressed and secreted (RANTES, CCL5) (9–12). Chemokine production by large airway epithelial cells can be regulated by endotoxin and cytokines (8, 9, 13, 14), and cigarette smoke and neutrophil elastase (NE) upregulate IL-8 mRNA synthesis by primary human airway epithelial cells (11, 14). Thus, release of chemokines by both cell types could orchestrate leukocyte recruitment, whereas epithelial cells could specifically perpetuate neutrophil influx into the conducting airways of smokers and those with COPD via IL-8 release following stimulation by cigarette smoke and neutrophil-derived enzymes.

Similar processes may occur in the respiratory units. The alveolar epithelium is important in alveolar homeostasis, synthesizing surfactant, which maintains low surface tension and has other immunomodulatory functions. Alveolar type II (ATII) cells contain phase I and II biotransformation enzymes for xenobiotic metabolism and, following alveolar injury, ATII cells proliferate and differentiate to replace damaged alveolar type I epithelial cells. The alveolae are a major site of trafficking of inflammatory cells that can ultimately migrate through the airways and into parenchymal tissue. Furthermore, the respiratory unit is the site of the development of emphysema, a significant component of COPD, which is thought to be partly due to destructive properties of inflammatory cells. The predominance of monocytes in distal lung during COPD suggests a different pattern of mediator release in the respiratory units, compared with conducting airways, following cigarette smoke, possibly due to preferential release of monocyte chemoattractant chemokines by human alveolar epithelium. However, there are

TABLE 1

Subject age, sex, diagnosis, smoking history, and source of lung tissue used for alveolar type II epithelial cell isolation

Age	Sex	Smoke History	Lobe	Disease	LPS + CYTO	LPS + CYTO + CSE	NE	CSE + GSH	Subject No. and Symbol
77	M	Smoker, 10 cig/d; 40 yr	RU	Squamous cell carcinoma	+	+	+		1 ■
67	F	Smoker, 5 cig/d; 1 yr	LU	Lymphoma	+	+	+		2 ▲
55	F	Smoker, 40 cig/d; 30 yr	RU	Squamous cell carcinoma	+	+	+		3 ▼
58	F	Smoker, 20 cig/d; 20 yr	LU	Adenocarcinoma	+	+	+		4 ◆
66	F	Smoker, 20 cig/d; 30 yr	RU	Adenocarcinoma	+	+	+		5 ●
66	F	Smoker, 20 cig/d; 20 yr	LL	Adenocarcinoma	+	+	+		6 □
52	M	Nonsmoker	LL	Sarcoma	+		+		7 △
57	F	Smoker, 30 cig/d; 30 yr	L	Adenocarcinoma	+		+		8 ▽
73	F	Smoker, 20 cig/d; 40 yr	L	Squamous cell carcinoma	+	+		+	9 ◇
69	M	Smoker, 40 cig/d; 50 yr	LL	Squamous cell carcinoma	+	+		+	10 ○
73	M	Smoker, 20 cig/d; 35 yr	LU	Squamous cell carcinoma	+	+		+	11 ×
76	M	Smoker, 20 cig/d; 30 yr	RU	Large cell carcinoma	+	+		+	12 +
64	M	Smoker, 20 cig/d; 40 yr	RU	Carcinoid tumour	+	+		+	13 *
73	M	Smoker, 20 cig/d; 35 yr	LU	Squamous cell carcinoma	+	+		+	14 ⊥

Definition of abbreviations: L, left lobe; LL, left lingula lobe; LU, left upper lobe; RU, right upper lobe.

Smoking history is recorded as the number of cigarettes smoked per day (cig/day) followed by the number of years the subject smoked. The symbols denote the subject data described in Figures 3 and 4. Experimental conditions were as follows: LPS + CYTO = treated with LPS + cytokines; LPS + CYTO + CSE = treated with LPS + cytokines then exposed to CSE; NE = treated with NE; GSH = treated with glutathione; equivalent control groups were performed for each condition.

few studies of primary human alveolar epithelium. Human alveolar type I cells produce MCP-1 and RANTES (15), whereas primary human ATII cells produce MCP-1 (16); both chemokines are upregulated by tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (15, 16). This study aimed to examine chemokine synthesis by primary human ATII cells following stimulation with known mediators of inflammation (e.g., LPS [endotoxin] and cytokines). We hypothesized that cigarette smoke components and NE would upregulate ATII cell chemokine secretion in favor of monocyte recruitment, indicating a role for ATII cells in controlling the degree and profile of leukocyte recruitment, and hence inflammation, in the respiratory units of smokers and patients with COPD.

Materials and Methods

Isolation and Characterization of Human ATII Cells

ATII cells were isolated as described previously (17) from lobectomy specimens removed from 14 patients for carcinoma of the lung (mean age 66 yr [range, 52–77 yr], 8 males), 1 nonsmoker, 8 ex-smokers, and 5 current smokers (Table 1). A further set of patient samples were processed for subsequent ATII cell analysis ($n = 8$; details not given). Lung tissue with grossly normal appearance was taken from the pleural border, distal to the tumor, with the approval of the Royal Brompton and Harefield Ethics Committee. The tissue was rinsed with 0.15 M NaCl to remove excess blood and debris. It was then perfused repeatedly with 0.15 M NaCl, using a syringe, until the perfusate was virtually free from leukocytes ($< 10^4$/ml perfusate). The tissue was then fully inflated with trypsin (0.25% in HBSS; Sigma, Poole, UK) and incubated at 37°C for 45 min. The tissue was minced finely in newborn calf serum (NCS; Invitrogen, Paisley, UK). The mince was added to DNase (DNase I, Sigma; 1:1; 250 µg/ml HBSS) and shaken by hand for 5 min. This suspension was filtered through 400- then 40-µm mesh. The filtrate, containing single cells, was centrifuged and the type II cell–enriched pellet suspended in low protein hybridoma media (LPHM; Invitrogen. If LPHM is not available,

DCCM [React Scientific, Troon, UK] can be substituted) containing 100 µg/ml DNase, then plated into a large flask to allow adhesion of contaminating leukocytes and other cells (1–2 h). The supernatant containing nonadherent, type II cell–enriched cells was centrifuged at $300 \times g$ for 10 min. The pellet was resuspended in LPHM, 10% NCS (+ 2 mM glutamine, 100 U/ml penicillin, and 100 µgm/ml streptomycin for complete medium) and plated into plastic tissue culture flasks for 1–2 h to allow fibroblastic cells to adhere. The nonadherent type II cell–enriched supernatant was removed, centrifuged at $300 \times g$ for 10 min, and the pellet resuspended in LPHM, 10% NCS, complete medium. The cells were counted using a hemocytometer (cytospin preparations of these cells are typically 60–80% alkaline positive, i.e., type II cells) (17). The cells were plated onto collagen type 1–coated 96-well plates (75 µl, 1% Vitrogen-100 [Imperial Laboratories, London, UK] in sterile deionized water; dried overnight). Cells were allowed to adhere for at least 36 h, when nonadherent cells were removed and media changed every 24 h until confluence (2–3 d).

Characterization of Human ATII Cells

Human ATII cell preparations were examined for typical ATII cell characteristics, using a number of criteria.

Light microscopy. ATII cell phenotype was confirmed by positive staining for alkaline phosphatase (17) and surfactant protein-C (SP-C). Freshly washed, confluent cells were incubated with alkaline phosphatase stain (10 mg naphthol-AS-bisphosphate [Sigma, N-5625] in 40 ml dimethylsuphoxide, made up to 10 ml with 0.625M MgCl and 0.125 M amino methyl propanol in distilled water and filtered immediately before use) for 20 min at 30°C and washed with HBSS. Intense, pink staining identifies ATII cells. SP-C immunostaining was also performed on confluent cells, fixed with 4% paraformaldehyde, and pretreated by heat to facilitate antigen retrieval (in 0.01 M sodium citrate buffer, pH 6.0). Polyclonal rabbit anti-human pro–SP-C antibody (1:1,000 dilution; Research Diagnostics Inc., Flanders, NJ: negative control, pre-immune rabbit immunoglobulin) was used to identify pro–SP-C, visualized using Envision+ (rabbit) peroxidase DAB detection system (DakoCytomation, Cambridge, UK) according to the manufacturer's instructions. Cells were counterstained with Mayers hematoxylin.

Electron microscopy. To visualize ATII cell structure and lamellar bodies, the monolayer was detached using type I collagenase (Sigma), fixed in glutaraldehyde and prepared for electron microscopy using standard techniques.

Surfactant apoprotein mRNA expression. SP-A and SP-C mRNA expression was investigated by harvesting RNA using RNeasy mini kits (Qiagen, Crawley, UK) (17). Five micrograms of total RNA in loading buffer was resolved by electrophoresis and hybridized with random ^{32}P-dCTP primer-labeled cDNA encoding human SP-C, or SP-A, or β-actin (17).

Preparation of Cigarette Smoke Extract

Smoke from one commercial filterless medium tar cigarette (15 mg tar; 1.1 mg nicotine) was drawn through 1 ml serum-free LPHM using continuous negative pressure for 7 min. The cigarette burns to within 3 mm of the cigarette holder. This 100% solution was freshly generated for each experiment and diluted with LPHM as required immediately before use.

Effect of LPS:Cytokine Stimulation, Cigarette Smoke Extract, and NE on Human ATII Cells

The experiments were performed when the ATII cells had reached confluence, Days 3 and 4 after plating. Wells were divided into two groups and media replaced by serum-free LPHM (control group) or serum-free LPHM containing LPS (10 μg/ml; *Escherichia coli* serotype 055:B5; Sigma), IFN-γ (100 U/ml; R&D Systems Europe, Abingdon, UK), IL-1β (10 ng/ml; R&D Systems Europe), and TNF-α (10 ng/ml; R&D Systems Europe) (LPS:cytokine-stimulated group). After 24 h, media was removed and parallel groups of control and LPS:cytokine-stimulated cells were exposed to 0, 1, and 5% cigarette smoke extract (CSE) (*n* = 6 subject samples) and a control group exposed to 0, 1, 10, and 100 nM active human NE (Elastin Products, Owensville, MO; activity assessed as described previously) (18) in serum-free LPHM for 24 h (*n* = 8 subjects). Another six subject samples were exposed to CSE, in the absence and presence of 500 mM glutathione (GSH), to examine any protective effect of GSH. Each experimental condition used eight replicate wells. Media was harvested and stored at −20°C. Cellular mRNA was prepared as described above. Table 1 shows which experiments were performed on which patient ATII cell sample, the extent of which depended on ATII cell recovery. Thus, eight subject samples (subjects 1–8) were used to assess both stimulation with LPS:cytokine and the effects of NE on chemokine synthesis (Table 1). Six of these (Subjects 1–6) were also used to assess the effects of cigarette smoke. Samples from Subjects 1–6 were analyzed for all five chemokines; 7 and 8 were analyzed for IL-8 only. Another six samples (Subjects 9–14) were used to evaluate the ability of glutathione to prevent the effects of cigarette smoke on IL-8 synthesis. Thus, altogether, twelve of the fourteen subject samples were used to study the effects of cigarette smoke extract on IL-8 synthesis (Table 1).

Chemokine Enzyme-Linked Immunosorbent Assay

Chemokine release was measured in ATII cell–conditioned medium using enzyme-linked immunosorbent assay (ELISA). Matched pair antibodies were used to perform a standard sandwich ELISA to measure IL-8. Ninety-six-well plates were coated overnight with monoclonal anti–IL-8 (Genzyme, West Malling, UK). Nonspecific binding was blocked with 4% skimmed milk powder. Recombinant human IL-8 standards (Genzyme) or conditioned media were added and incubated for 2 h. Wells were incubated with biotinylated poly-

clonal rabbit anti–IL-8 antibody (Genzyme) for 1 h. Bound antibody was detected using streptavidin-conjugated horseradish peroxidase for 0.5 h, and visualized with diaminobenzidine substrate. MCP-1, MIP-1 α, GRO-α, and RANTES were determined using commercial ELISA kits (R&D Systems Europe), following the manufacturer's instructions. Replicate wells from all experiments were assayed. Unconditioned media was used as reagent blank. The lower limit of detection was 10 pg/ml in each case.

Immunoblotting for IL-8 of Conditioned Media from NE-Treated ATII Cells and Media Spiked with IL-8 and NE

To determine whether NE caused degradation of IL-8 released by NE-treated ATII cells, a further series of ATII cell preparations were generated and grown to confluence and serum depleted overnight. These cells were then exposed to NE (100 and 200 nM) for 24 h. In separate experiments, rhIL-8 (250 ng/ml; R&D Systems Europe) and NE (final concentration 50 and 25 nM) were incubated at 37°C in LPHM tissue culture media for up to 4 h. Both the ATII cell conditioned media and the IL-8–spiked media were processed for IL-8 immunoblot analysis. SDS-PAGE (15%) was performed on media samples (15 μl in running buffer) under reducing conditions. Immunoblot analysis was performed on Hybond-ECL nitrocellulose membranes (Amersham Biociences, Amersham, UK) following electroblotting (Biorad miniblot system) for 1 h at 100 V constant voltage. Proteins were visualized using polyclonal IL-8 antibody (Abcam, Cambridge, UK) chemiluminescence detection (ECL fluid; Amersham Biosciences). In addition, spiked media were examined following SDS-PAGE (15%) gel electrophoresis and silver staining to determine whether low molecular weight proteins appeared following co-incubation of rhIL-8 and NE.

Effect of NE on ATII Cell IL-8 mRNA Expression

To examine whether the apparent reduction in NE-treated ATII cell IL-8 release was due to inhibition of IL-8 mRNA expression, RNA was processed for Northern blotting as described above. In addition, separate experiments (*n* = 6) were carried out in which serum-starved ATII cells were exposed to 10 pM, 100 pM, 10 nM, 100 nM, and 200 nM NE, and 100 nM heat-inactivated NE (30 min at 60°C) for 24 h. The cell monolayer (at least two replicates per condition) was harvested using the cell lysis diluent provided by the manufacturer or using Tri Reagent (Sigma). Total RNA was prepared by chloroform:isopropanol extraction under RNase-free conditions and suspended in RNase-free water then washed with 75% ethanol. Total RNA was assessed using a Nanodrop ND-100 spectrophotometer. 0.75 μg of each RNA sample was used to quantify IL-8 mRNA using a Quantikine IL-8 mRNA detection kit (R&D Systems Europe).

Statistical Analysis

The data are expressed as median values per group and as individual data points to highlight between-subject variation. Statistical analysis was performed using Wilcoxon Signed Rank test for nonparametric paired data. Where analysis from a series of identical experiments were pooled and analyzed (IL-8 mRNA quantification), a paired *t* test was used. A *P* value of < 0.05 was regarded as significant.

Results

ATII Cell Purity

ATII cells reached confluence between 2 and 3 d after plating. Using this method, confluent cells are positive for alka-
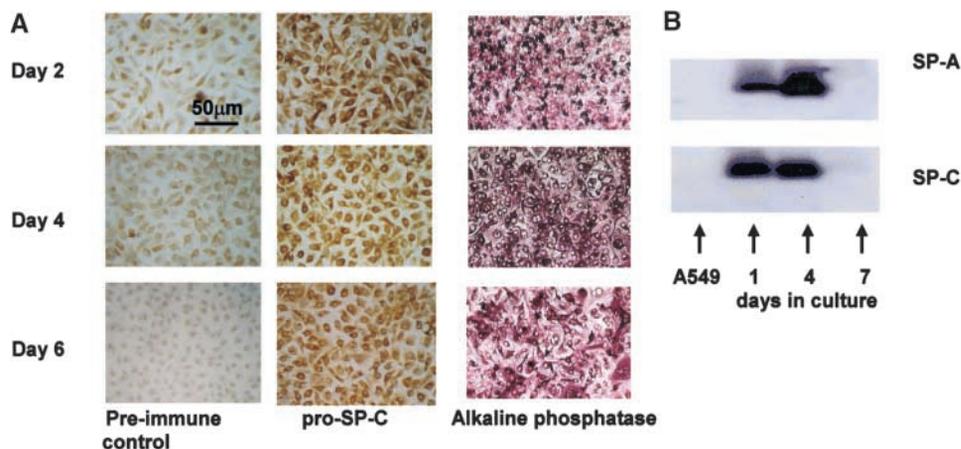
Figure 1. (*A*) Positive pro–SP-C and alkaline phosphatase staining of human ATII cell monolayers, Days 2, 4, and 6. (*B*) Northern blots of mRNA expression of SP-A and SP-C mRNA from ATII cells over 7 d of *in vitro* culture showing strong expression up to 4 d, and little expression by 7 d. A549 cells did not express either SP-A or SP-C mRNA.

line phosphatase and pro–SP-C (Figure 1) over 6 d in submerged tissue culture. We have found that removal of monocytes and other cells, initially by thorough saline perfusion and then by differential adhesion to tissue culture plastic before plating the remaining cells onto Vitrogen 100, consistently results in pure ATII cell monolayer preparations, as described here. Nevertheless, cytospins of the initial preparation, immediately before plating, are only 60–80% positive for alkaline phosphatase (17), but alkaline phosphatase–negative cells do not adhere and are removed during the medium change at 36 h. Thus, the purity of the final ATII cell monolayer preparation is not entirely dependent on the purity of the initial isolated cell preparation. Use of type I collagenase enabled the cells to be stripped off the Vitrogen substrate in sheets and preserved the cell–cell structure. Following transmission EM, intercellular tight junctions and apical microvilli were visible (Figures 2A and

2B) and in excess of 95% of the cells contained lamellar bodies. Fresh (Day 1) human ATII cell preparations expressed high levels of SP-A and SP-C mRNA (Figure 1B), which remained high during the first 4 d *in vitro* but had decreased by Day 7. In contrast, an A549 adenocarcinoma cell line (negative control) did not express surfactant apoprotein mRNA (Figure 1B).

Basal ATII Cell Chemokine Release

There was considerable heterogeneity in chemokine release between subject samples. For example, ATII cells from Subjects 9 and 11 released over twice as much IL-8 as the other ATII cell preparations (Figure 3A). Basal ATII cell IL-8 release was measured in all experiments, a total of 14 subject samples, with a median value of 1,560 pg/ml (range, 240–16,540; Figure 3A). The median value remained similar when the patient samples were analyzed as separate experiments. Thus, for the NE study (Subjects 1–8), the IL-8 median value was 1,030 pg/ml (range, 240–4,670; Figure 3B). In the assessment of five chemokines (Subjects 1–6), the IL-8 median value remained at 1,030 pg/ml (range 240–2,600). There were significant differences in the production of basal IL-8, MCP-1, RANTES, MIP-1α, and GRO-α. In the six samples where all five chemokines were measured, the median levels rank as follows: MCP-1 > IL-8 > GRO-α > MIP-1α > RANTES (median and range, pg/ml – MCP-1, 2,850 [790–4,710]; IL-8, 1,030 [240–2,600]; GRO-α, 180 [10–320]; MIP-1α, 0 [0 -210]; RANTES, 0 [0–25]). All six ATII cell preparations produced basal MCP-1, IL-8, and GRO-α (Figure 3A), whereas 5/6 and only 2/6 preparations produced measurable basal levels of MIP-1α and RANTES, respectively (the highest value for RANTES was 25 pg/ml). For these six subjects, MCP-1 and IL-8 levels were in the same order of magnitude and not significantly different, whereas basal release of GRO-α was significantly lower.

Effect of LPS:Cytokine Stimulation on ATII Cell Chemokine Release

Following stimulation with LPS:cytokines, release of all chemokines was significantly increased (Figure 3A). Where basal levels of chemokines had been undetectable (i.e., MIP-1α and RANTES), their release increased sufficiently
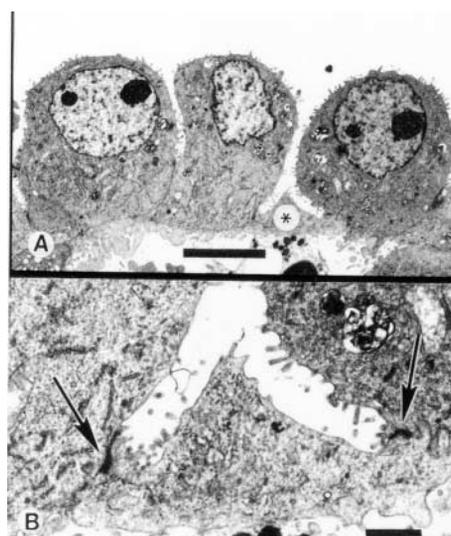

Figure 2. Electron microscopy of human ATII cells at Day 4 *in vitro.* (*A*) A string of cuboidal ATII cells, showing apical microvilli, tight junctions and lamellar bodies. *Bar:* 5 μm. (*B*) Enlarged view of human ATII cells (*asterisk* in *A*), highlighting tight junctions (*arrows*), microvilli, and lamellar bodies. *Bar:* 1 μm.
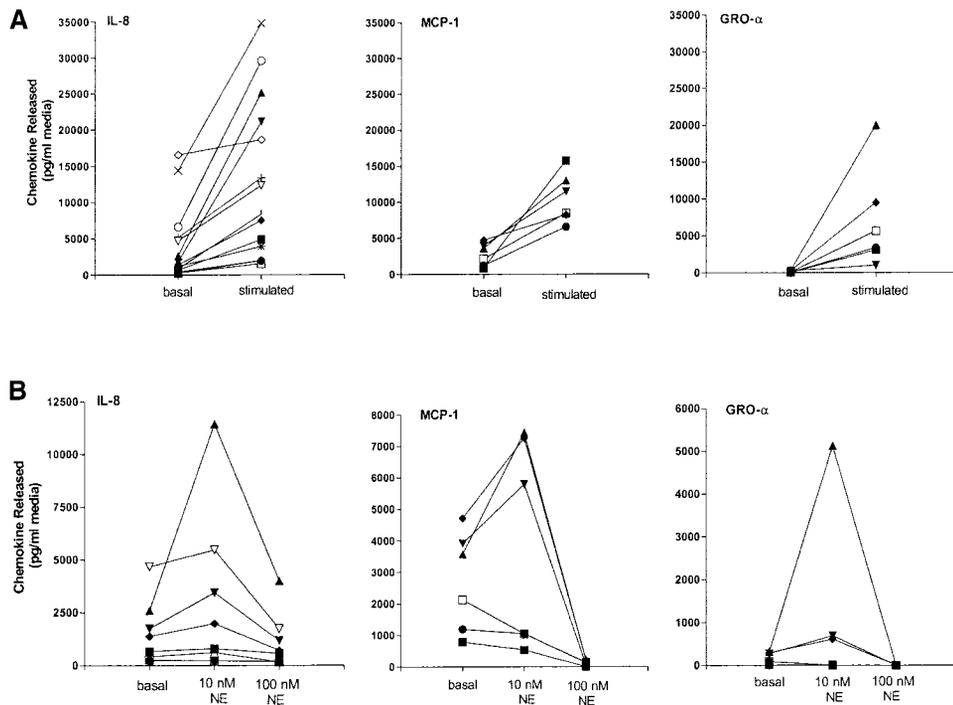
Figure 3. Chemokine release by LPS: cytokine-stimulated and NE-treated human ATII cells. Data are shown as individual ATII cell preparations. *Symbols* denote individual subject samples, as described in Table 1. (*A*) Effect of 24 h exposure to LPS:cytokine mixture on basal release of IL-8, MCP-1, and GRO-α. LPS stimulated release of all three chemokines. (*B*) Effect of NE on basal release of IL-8, MCP-1, and GRO-α. NE (10 nM) stimulated IL-8 release; 100 nM NE caused a decrease in extracellular levels of all three chemokines.

enough to be measurable. The rank order of chemokine release remained the same as that observed under basal conditions. Thus, for MCP-1 the median rose from 2,850 pg/ml (median and range, 790–4,710; $n = 6$) to 9,940 pg/ml (range, 6,530–15,750). For IL-8, release rose from a median of 1,560 pg/ml (range, 240–16,540; $n = 14$) to a median of 10,330 pg/ml (range, 1,520–34,770). GRO-α rose from 180 pg/ml (range, 10–320; $n = 6$) to 4,490 pg/ml (range, 1,000–20,000). Whereas the degree of increase in IL-8 and MCP-1 was of the same order, 4- to 6-fold, the increase in GRO-α was $\sim$ 25-fold and brought the levels of GRO-α into the same range as those for IL-8 and MCP-1. Stimulated RANTES release reached a median of only 30 pg/ml (median and range, 10–610) and MIP-1α reached 270 pg/ml (range, 40–14,610). Only one ATII cell sample, from Subject 2, had a value of MIP-1α above 1,000 pg/ml.

Effect of NE on ATII Cell Basal Chemokine Production

Analysis of conditioned media showed that there was a trend toward increased IL-8 release following exposure of ATII cells to 1nM NE (median and range, pg/ml, 1,190 [190–12,110] data not shown) which became significant following 10 nM neutrophil elastase ($P < 0.05$; median and range, pg/ml, 1,390 [220–11,440]; Figure 3B). However, there was no significant difference in the extracellular levels of other chemokines following treatment with 10nM NE. Although release of both MCP-1 and GRO-α increased in Subjects 2, 3, and 4, it decreased in Subjects 1, 5, and 6 (Figure 3B); of note is that the increase was observed in ATII cells that had had the highest basal levels of these chemokines. In contrast, 100 nM NE caused a significant decrease in detectable, extracellular, MCP-1α and GRO-α ($P < 0.05$) in conditioned media (Figure 3B). Compared

with basal release, the fall in IL-8 release following 100 nM NE fell in seven out of eight of the subjects studied, but was not significant. However, when IL-8 release was compared between treatment with 10 nM and 100 nM NE, it was lower in all samples treated with 100 nM NE (median and range, pg/ml, 630 [160–3,990]) compared with 10 nM NE ($P < 0.05$; median and range, pg/ml, 1,390 [220–11,440]). NE had no significant effect on the very low basal release of RANTES or MIP-1α (data not shown).

Effect of CSE on ATII Cell Chemokine Release

When ATII cells were exposed to 5% smoke extract, there was a significant fall in release of all chemokines, by both stimulated and unstimulated cells ($P < 0.01$; Figures 4A and 4B). However, the effect of 1% smoke extract was not significant. Thus, following exposure to 1% smoke extract, although there was a significant fall in GRO-α release ($P < 0.01$), IL-8 secretion was enhanced in half the subject samples, and decreased in the others (Figure 4). MCP-1α release increased in one subject sample and remained the same or fell in the other five subject samples (Figure 4). The cells were not necrotic and continued to exclude trypan blue and remained adherent (as assessed by uptake of 0.4% sulphorhodamine B stain, solubilized in 10 mM Tris and absorbance read at 510 nm) with unchanged protein and RNA levels, even when exposed to 5% smoke extract. Co-incubation of CSE-exposed ATII cells with 500 mM GSH significantly reversed the inhibitory effect of 5% CSE on ATII cell IL-8 release (Figure 4).

IL-8 Secretion and mRNA Expression

Because the initial studies suggested that NE inhibited ATII cell synthesis of IL-8, a further series of ATII cell samples
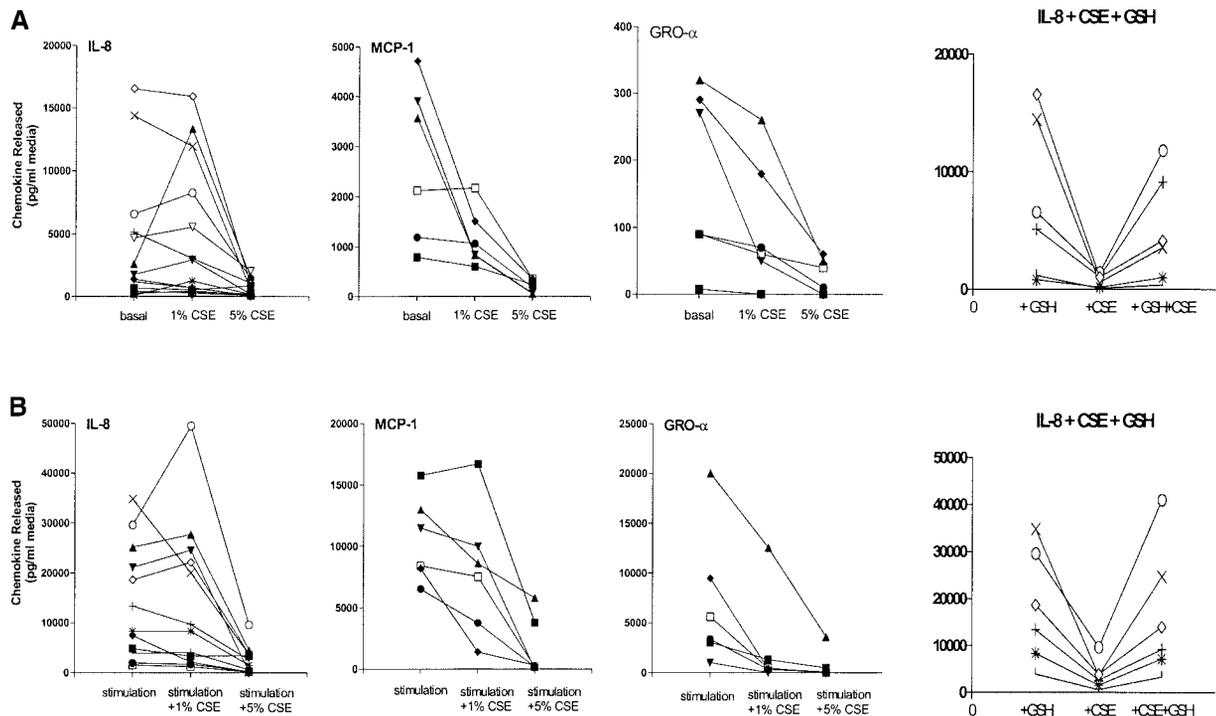
Figure 4. Effect of CSE on basal and LPS:cytokine-stimulated ATII cell release of IL-8, MCP-1, and GRO-α. Data are shown as individual ATII cell preparations. *Symbols* denote individual subject samples, as described in Table 1. (*A*) Effect of smoke extract on basal chemokine release. There was no clear-cut effect of 1% CSE on IL-8, and inhibition of MCP-1 and GRO-α; 5% CSE inhibited release of all three chemokines. This was prevented in the presence of 500 mM GSH. (*B*) Effect of CSE on LPS:cytokine-stimulated chemokine release. 5% smoke extract inhibited release of all three chemokines. This could be prevented by the presence of 500 mM GSH.

were investigated using IL-8 immunoblot analysis and Quantikine analysis of IL-8 mRNA. Immunoblotting of IL-8 secreted into media of NE-treated ATII cells confirmed a fall in IL-8 secretion at higher concentrations of NE (Figure 5A). Importantly, there were no IL-8–positive low molecular bands to suggest degradation of extracellular IL-8. Similarly, when IL-8 alone was incubated with NE, although the IL-8 band disappeared, there were no low molecular weight IL-8–positive bands to suggest degradation (Figure 5C). Nevertheless, SDS-PAGE of NE-exposed rhIL-8 shows reduced intact IL-8 and an increase in the appearance of low molecular weight bands, suggesting degradation of IL-8 (Figure 5B). mRNA expression was measured to determine whether reduced protein levels were due to transcriptional changes. Northern blotting showed that although CSE inhibited IL-8 mRNA, most markedly in LPS:cytokine-stimulated cells, both LPS:cytokine stimulation and NE upregulated IL-8 mRNA expression (Figure 6A). As this was in direct contrast to the reduction in extracellular IL-8 protein levels, observed following ATII cell treatment with NE, further experiments were performed. Quantification of IL-8 mRNA using Quantikine kits confirmed the semi-quantitative findings, that high concentrations of NE stimulate IL-8 mRNA levels (Figure 6B; $P < 0.05$).

Discussion

Using a straightforward method to isolate human alveolar ATII cells and study them in submerged culture at high purity we have shown that these cells are phenotypically characteristic of ATII cells *in vivo*, containing numerous lamellar bodies, with apical microvilli and tight junctions. They also express pro–SP-C protein and mRNA, a specific marker for ATII cells, as well as mRNA for another surfactant apoprotein, SP-A (19). ATII cells released basal levels of MCP-1, IL-8, and GRO-α; MIP-1α, and RANTES were released at very low levels, or were undetectable. When ATII cells were stimulated by exposure to a mixture of LPS and cytokines, there was an increase in release of all the chemokines, including RANTES, which was often not released basally. Although 10 nM NE stimulated IL-8 mRNA expression as well as protein release, extracellular chemokine levels decreased following 100 nM NE, probably due to proteolysis. CSE inhibited expression of IL-8 mRNA and chemokine protein secretion by ATII cells *in vitro*. This supports the concept that ATII cells can regulate the number and profile of resident leukocytes within the alveolar unit during health and disease, depending on endogenous and exogenous mediators, by chemokine release, and suggests that the ATII cell might be a useful therapeutic target for developing anti-inflammatory strategies.

ATII cell production of a panel of chemokines was assessed to determine the potential of the ATII cell to orchestrate leukocyte recruitment under basal and proinflammatory conditions. During an inflammatory event *in vivo*, ATII cells are likely to be exposed to more than one stimulant, possibly reflecting the presence of both exogenous and en-
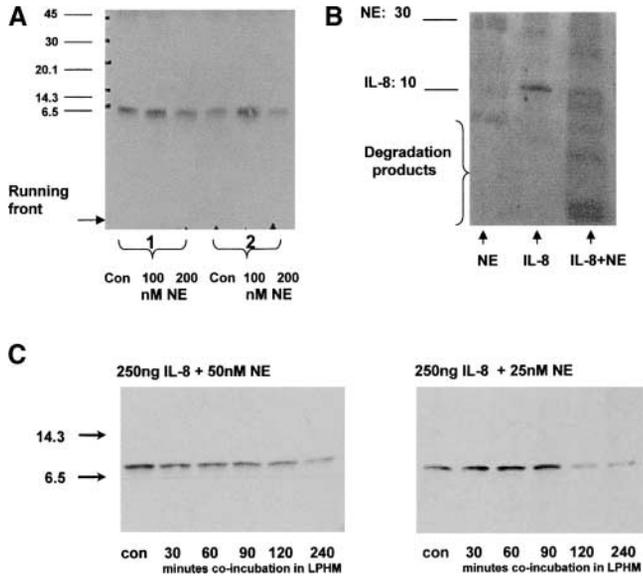
Figure 5. Effect of NE on extracellular IL-8 integrity. (*A*) Immunoblotting of IL-8 in conditioned media from ATII cells treated with 100 and 200 nM NE, showing a reduction of intact/immunoreactive IL-8 at high levels of NE; $n = 2$; con, control. (*B*) SDS-PAGE of NE, IL-8, and the same concentrations of NE incubated with IL-8 for 4 h at 37°C. Incubation of IL-8 with NE for 4 h resulted in disappearance of 30 kD neutrophil elastase, and appearance of low molecular weight degradation products. (*C*) Immunoblotting of IL-8 co-incubated with NE in tissue culture media for 4 h, showing disappearance of intact/immunoreactive IL-8.
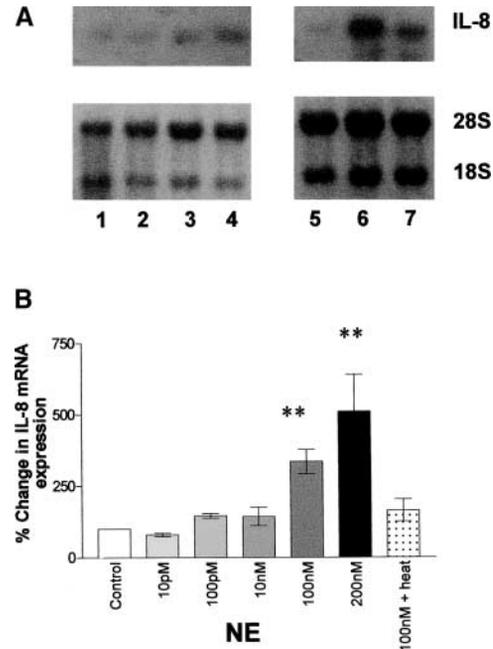

Figure 6. (*A*) Northern blot to show expression of IL-8 mRNA by human ATII cells following treatment with NE and CSE. *Lanes 1* and *5:* basal, control; *lane 2:* basal + 5% CSE; *lane 3:* basal + 10 nM NE; *lane 4:* basal + 100 nM NE; *lane 6:* LPS:cytokine-stimulated ATII cells; *lane 7:* LPS-cytokine-stimulated cells + 5% CSE. (*B*) Quantitative assessment of IL-8 mRNA expression by ATII cells following treatment with NE, showing increased expression at the highest concentrations of NE. Heat-treated NE had no effect. The data are expressed as percent change from untreated control, $n = 6$. **Significantly different from control, $P < 0.05$.

dogenous agents. We therefore used a mixture of stimulants containing bacterial endotoxin as well as TNF-α, IL1-β, and IFN-γ; these cytokines are released by activated human alveolar macrophages and lymphocytes, and are believed to mediate the inflammatory response in a number of pulmonary disease processes such as interstitial lung diseases (20), allergic lung disease (21), and COPD (22). Furthermore, *in vitro* studies of large airway epithelium and ATII cells indicate that simultaneous exposure to TNF-α and IFN-β has a synergistic effect on the release of MCP-1 (15, 23), IL-8, and RANTES (23). Thus, we reasoned that exposure to a mixture of stimulants would generate a maximal response by ATII cells.

To our knowledge, this is the first study to compare relative levels of release of a panel of CXC and CC chemokines by a series of primary human ATII cells. There was considerable between-subject variability in basal chemokine release, although it was invariability of the same order of magnitude. Basal release of IL-8 and MCP-1 by ATII cells was $\sim$ 20-fold greater than GRO-α, the next most abundant chemokine. Although there are no other such comparative studies of chemokine release, a previous study of primary human ATII cells (16) and another of human alveolar type I cells (i.e., differentiated ATII cells) (15) also demonstrated basal MCP-1 release *in vitro*. There are no similar human alveolar primary cell studies of IL-8 or GRO-α for comparison. MIP-1 α was released at extremely low levels and ATII cell release of RANTES was found to be poor, even when

stimulated. As Rosseau and coworkers (16) were only able to demonstrate low basal RANTES release by primary human alveolar type I cells, these combined findings suggest that ATII cell–derived MIP-1α and RANTES do not contribute to normal lung homeostasis. Thus, under resting conditions, ATII cells may modulate white blood cell migration into the alveoli, via MCP-1 and IL-8 release, ATII release of which is quantitatively similar. Although neutrophils normally form only a small proportion of peripheral airway lumen phagocytes, their tissue half-life is very short (1–2 d) and a continuous IL-8 gradient may ensure continuous replenishment. Macrophages are the major airway lumen phagocytes and peripheral blood monocyte precursors would be responsive to MCP-1.

MCP-1 is a chemoattractant for mononuclear phagocytes, CD45RO+ T lymphocytes, B cells, and NK cells. Increased MCP-1 and its receptor, CCR2, in the lung have been positively associated with leukocyte infiltration and lung disease (24, 25). Furthermore, *in vitro* studies with primary human alveolar type I and type II cells (15, 16) illustrate the role of alveolar epithelial cell–derived MCP-1 in monocyte migration across endothelial and epithelial monolayers. In their studies of human ATII cell MCP-1 secretion, and hence monocyte migration, Eghtesad and colleagues (16) showed MCP-1 stimulation by IL-1 and TNF-α, but

levels never quite reached the levels of LPS:cytokine stimulation shown in this study. TNF-α also stimulated primary human type I cell secretion of MCP-1 (and monocyte migration) (15), but, again, this never reached the levels described in the present investigation. Interestingly, this is in contrast to studies of the A549 adenocarcinoma cell line (26), where IL-1 and TNF-α induced higher levels (∼ 2.5-fold) of MCP-1 than those described here, for primary human ATII cells. In their studies of alveolar type I cells, Rosseau and associates (15) found that TNF-α–stimulated A549 cell MCP-1 secretion was low, suggesting inconsistencies between batches of A549 cells, possibly depending on culture conditions and passage number. The present, comparative, study, adds to previous studies of primary alveolar cells, showing that MCP-1 is a major chemokine produced by primary human ATII cells, contributing approximately half the total chemokines that were measured in the supernatants from unstimulated cells, and increasing nearly 4-fold following stimulation with known mediators of the inflammatory response. ATII cell–derived MCP-1 is therefore likely to be a crucial factor in controlling monocyte recruitment to the respiratory units in health and disease.

The marked increase in ATII cell IL-8 following LPS:cytokine exposure is of a similar magnitude to that seen in primary human airway epithelial cells following stimulation with a panel of cytokines (27). To our knowledge, there are no studies of primary human alveolar epithelial cell IL-8 or GRO-α release, but use of A549 cells as a surrogate suggest that these cells usually secrete comparatively lower levels of IL-8 (28). The concurrent dramatic increase in ATII cell GRO-α production following LPS:cytokine stimulation accounts for a significant proportion of the observed rise in CXC chemokines (∼ 30%) and suggests that it may be equally important during alveolar inflammation. Like IL-8, GRO-α attracts neutrophils, and an increase in ATII cell release of these CXC chemokines would therefore be likely to enhance neutrophil recruitment into the respiratory units following stimulation by proinflammatory mediators. However, the functional role of GRO-α is not entirely confined to attraction and activation of inflammatory cells; it has strong angiogenic and growth-promoting properties, which are important during wound repair (29, 30). Thus, within the challenged lung, in addition to its chemoattractant properties, GRO-α may be a paracrine and autocrine mediator of alveolar repair and maintain tissue homeostasis. During revision of the present manuscript, and in support of this suggestion, Vanderbilt and coworkers (31) reported an investigation of primary rat ATII cells, which illustrated increased expression of rat CXC chemokines GRO, CINC-2a, and MIP-2 (rat orthologs of human GRO-α, -β, and -γ) and their receptor, CXCR2, in response to lung injury.

Inhibition of chemokine release following exposure to 5% CSE did not fit with our original hypothesis: that peripheral inflammation in smokers' lungs is partly due to stimulated ATII cell chemokine release by cigarette smoke. Neither do these findings agree with previous experiments on primary human airway epithelium or the A549 adenocarcinoma cell line, where CSE was found to enhance IL-8 and MCP-1 expression and release (11, 28). This may reflect differential responses between alveolar epithelium and large airway epithelium (and carcinoma cells). Alternatively, the lack of agreement between studies may reflect the difference in concentration of CSE that was used. Previously, the smoke extract was approximately one tenth of the concentration of the 5% CSE used in the current study (11, 28). However, when we used 1% CSE, there were no significant differences in IL-8 release (Figure 4), and when we used concentrations of smoke extract that were the same as those used previously (11, 28), there was no change in IL-8 synthesis at all (data not shown). It is difficult to relate concentrations of CSE generated for experimental purposes with those that occur *in vivo*. Numerous studies have shown that the pattern and depth of inhalation vary markedly between individuals. Nevertheless, emphysema has been associated with high alveolar smoke exposure, suggesting that high smoke concentrations contribute to the pathology (32). In the present study, inhibition of IL-8 protein release by 5% CSE coincided with reduced IL-8 mRNA, whereas addition of glutathione completely removed the inhibitory effect of CSE on IL-8 release. Thus oxidative mechanisms are likely to be involved in the CSE-induced downregulation of chemokine transcription, and secretion, by ATII cells. However, this contrasts with a recent study (33) in which oxidative mechanisms and upregulation of NF-κB are thought to account for increased IL-8 release by human bronchial epithelial cells following exposure to particulate phase of cigarette smoke. Again, these differences may be due to fraction and concentration of the cigarette smoke preparation or to differential responses by cells obtained from different levels of the respiratory tract. There was no evidence of cell death, or loss of cells from the monolayer. Preliminary studies by us showed increased ATII cell DNA damage (using the TUNEL technique; Ref. 34 and data not shown) following CSE exposure, which was completely prevented by addition of glutathione. A similar effect of cigarette smoke–induced DNA damage has been shown using a human lung fibroblast cell line, which could be reversed by increased expression by transfected cells of glutathione S-transferase P1, i.e., increased generation of cellular antioxidant activity (35). Thus, this study of primary human ATII cells suggests that, unlike large airway epithelium, chemokine synthesis by alveolar epithelium is downregulated by cigarette smoke, via oxidative mechanisms, altering DNA transcription and causing inhibition of chemokine expression. Differential responses between proximal and distal airway epithelium to cigarette smoke and chemokine release may explain some of the differences in the inflammatory response and pathology of bronchitis and emphysema.

Lung tissue from heavy smokers with emphysema contains increased DNA fragmentation and apoptotic endothelial and epithelial cells in the peripheral lung, accompanied by reduced expression of vascular endothelial cell growth factor (VEGF) and its receptor, VEGF R2 (36). In addition, CSE downregulated VEGF and VEGFR2, and stimulated apoptosis, by endothelial cells *in vitro*, reflecting the role of VEGF as an endothelial cell growth factor. Our studies suggest that similar processes may be occurring with CSE-exposed ATII cells, leading to reduced chemokine release. In the present study, GRO-α was the most responsive to stimulation by proinflammatory mediators. Interestingly,

GRO-α was also the most susceptible to inhibition by CSE, falling significantly following exposure to both 1% and 5% CSE. This suggests that ATII cell GRO-α synthesis could be very sensitive to changes in the alveolar environment. GRO-α is a potent growth promoting chemokine involved, for example, in keratinocyte proliferation and angiogenesis (29). Studies of damaged human tissue, CXCR2-deficient (CXC chemokine receptor) rodents, and *in vitro* studies provide strong evidence that GRO-α, and perhaps IL-8, are important for normal wound healing to take place (29, 30). If CXC chemokines such as GRO-α and IL-8 play a role in growth and repair of the lung, exposure to cigarette smoke and downregulation of these growth-promoting chemokines might interfere with the repair process, possibly retarding both neovascularization and re-epithelialization. Such abnormal repair mechanisms could contribute to the pathologic processes that occur during the development of emphysema.

NE stimulates IL-8 mRNA expression by primary airway epithelial cells, A549 adenocarcinoma cells, and other airway cell lines (37). Thus, release of elastase by neutrophils may potentiate neutrophil inflammation by stimulating IL-8 release from epithelial cells. However, in the present study, although NE stimulated mRNA expression by human ATII cells, IL-8 protein release, which was stimulated at low concentrations of NE, decreased with increasing amounts of enzyme. This suggests that NE specifically upregulates ATII cell IL-8 messenger RNA, but ultimately inhibits protein synthesis and secretion. However, it is more likely that degradation of secreted IL-8 by NE at concentrations above 10 nM, as shown by us in this study, and previously by Leavell and coworkers (38), interfered with the immunoassay. Thus, NE was found to degrade IL-8 after 4 h at 37°C, i.e., within the 24-h time course of ATII cell NE exposure used in this study. Recently, Walsh and colleagues (39) showed that NE-stimulated release of IL-8 by a large airway epithelial cell line could be prevented by specific enzyme inhibition, suggesting that NE activation of IL-8 synthesis involves proteolytic activity, possibly via activation of surface receptors. *In situ*, the action of NE is controlled by inhibitors such as α1-proteinase inhibitor, secretory leukoprotease inhibitor, and possibly elafin. If these inhibitors are compromised leading to uncontrolled enzyme activity, as has been shown in lung secretions from subjects with COPD, low concentrations of active NE, below 10 nM, could stimulate epithelial cells to release elevated IL-8 and potentiate neutrophil inflammation. Our studies of NE activity in bronchoalveolar lavage from normal subjects show that, having accounted for dilution of epithelial secretions, active NE is present at ∼ 100 pM concentrations (40). Numerous studies, including our own (41), show that heavy smokers, and especially those with COPD, have elevated levels of NE. In addition, NE is most likely to exert its activity as concentrated enzyme in the "protected" pericellular region of the neutrophil or once adjacent to substrate, even in the presence of active inhibitors (37). This study demonstrates that higher concentrations of active, uninhibited NE would be likely to degrade any elevated, secreted IL-8, possibly as a feedback control mechanism. There is increasing evidence to suggest that the actions of extracellular chemokines are regulated by proteolytic enzymes, which may be activated or inactivated by cleavage of small, nondetectable fragments of the proteins. For example, cathepsin D specifically cleaves and inactivates MIP-1α and MIP-1β (42), MMPs 1, 3, 9, 13, and 14 cleave and inactivate the CXC chemokine stromal cell–derived factor-1 (43) and neutrophil gelatinase B (MMP9) potentiates IL-8 activity, degrades and inactivates GRO-α, and does not affect RANTES or MCP-2 (44). Unlike IL-8, extracellular GRO-α and MCP-1 increased in only three of the six ATII cell preparations following 10 nM NE. It is possible that, in the remaining cases, protease-stimulated chemokine release was insufficient to outweigh its proteolytic effects. This study suggests that there could be a fine balance in inflamed lungs, between the level of unopposed extracellular NE, chemokine synthesis and release, and proteolytic degradation of chemokines. Thus, although high concentrations of active elastase might prevent further inflammation by destroying chemokines, lower levels of elastase might potentiate inflammation, stimulating chemokine release without destroying subsequent chemokine activity.

Thus, this study of primary human lung ATII cells *in vitro* shows that chemokine production by these cells is highly responsive to known mediators of inflammation including LPS and cytokines, as well as cigarette smoke and NE. Release of chemokines by ATII cells within the alveolar region of the respiratory tract is likely to be significant in moderating the influx of inflammatory cells, under normal and challenging situations. As expected, a mixture of pro-inflammatory stimulants induced significant increases in all the chemokines. Neutrophil elastase also stimulated ATII cell IL-8 production; on the other hand, evidence also suggests that the concentration of active enzyme may be critical to the integrity and activity of any increased extracellular IL-8. In direct contrast, and against the working hypothesis, cigarette smoke inhibited ATII cell chemokine production. We hypothesize that this is a consequence of programmed, apoptotic, cell death. Lack of sufficient growth-promoting chemokines, such as GRO-α and IL-8, and consequent reduced or ineffective repair, may exacerbate such injury and contribute to cigarette smoke–associated emphysema.

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