Increased Expression of the Interleukin-8 Gene by Alveolar Macrophages in Idiopathic Pulmonary Fibrosis

A Potential Mechanism for the Recruitment and Activation of Neutrophils in Lung Fibrosis

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Abstract

Neutrophil migration into the airspaces of the lung is thought to contribute to the alveolar damage and subsequent fibrosis in idiopathic pulmonary fibrosis (IPF). Interleukin 8 (IL-8), a monocyte- and macrophage-derived cytokine, displays potent chemotactic and activating properties towards neutrophils and thus may contribute to the pathogenesis of IPF. The objective of this investigation was to quantify the spontaneous expression of IL-8 transcripts by alveolar macrophages from normal healthy volunteers and individuals with IPF. A quantitative assay employing reverse transcription of mRNA and the polymerase chain reaction was utilized. The level of IL-8 mRNA in alveolar macrophages was found to be significantly elevated in individuals with lone IPF or with lung fibrosis associated with connective tissue disorders compared to normal healthy controls. Moreover, the level of IL-8 mRNA in the 23 individuals with IPF correlated with the number of neutrophils per milliliter in their bronchoalveolar lavage (BAL) and with the degree of disease severity. In addition, the level of IL-8 protein in BAL was found to reflect the pattern of IL-8 mRNA expression by alveolar macrophages. These data suggest that IL-8 derived from alveolar macrophages may significantly contribute to neutrophil involvement in the pathogenesis of IPF. (*J. Clin. Invest.* 1991. 88:1802–1810.) Key words: interleukin 8 • interstitial lung disease • macrophages • polymerase chain reaction • pulmonary fibrosis

Introduction

Idiopathic pulmonary fibrosis (IPF)[1] is a chronic, usually fatal interstitial lung disease characterized by injury of alveolar parenchymal cells and by progressive fibrosis of the alveolar walls and interstitium (1, 2). The pathogenesis of IPF likely involves the chronic accummulation of inflammatory and immune effector cells in the lower respiratory tract. Studies of the bronchoalveolar lavage fluid (BALF) from patients with IPF demonstrate a severalfold increase in the total cell number characterized by increases in the number of macrophages and neutrophils (3–8). However, the processes controlling the influx and efflux of these inflammatory cells in the alveolar space are not well understood.

It has been hypothesized that sustained neutrophil accumulation in the alveolar space, persistent neutrophil-mediated injury, and destruction of alveolar architecture are the major factors that culminate in abnormal lung repair and interstitial fibrosis (9–11). The processes that sustain neutrophil influx into the alveolar space are unclear but appear to be mediated in part by neutrophil chemotactic factors released by macrophages (11–13). Macrophages from patients with IPF spontaneously release a chemotactic factor for neutrophils (11). This activity has been discriminated into a 400–600-D lipid-containing compound (11), and a 5–10-kD protein (14, 15). On the basis of similar biological activities and physicochemical properties, it has been suggested (16) that this latter protein may be interleukin 8 (IL-8), a recently purified (17), sequenced (18), and cloned (19) monocyte-derived neutrophil chemotactic and granule-releasing factor of molecular mass 8,400 D (16, 20).

While in vitro stimulation of human alveolar macrophages with tumor necrosis factor-α (TNFα), IL-1β, or lipopolysaccharide (LPS) has been shown to induce the expression (21, 22) and secretion (23) of IL-8, the role of this cytokine in the alveolar neutrophil infiltration seen in the lungs of patients with IPF remains to be determined. The present work was designed to address this question by quantifying the expression of the IL-8 mRNA by alveolar macrophages from normal individuals and patients with IPF by using a reverse transcription-polymerase chain reaction (RT-PCR) assay and by measuring the concentration of IL-8 protein in BALF using an ELISA. We found that patients with IPF, with or without an associated connective tissue disease, had an increased level of expression of the IL-8 gene by their alveolar macrophages and an elevation in the level of IL-8 protein in their BALF. Further, the level of expression of IL-8 mRNA correlated with the level of neutrophils found in BAL and the clinical severity of the disease. Thus, these findings support a role for IL-8 in the pathogenesis of IPF.

Methods

Study population

Patients with IPF. 14 patients with IPF were evaluated (Table I). IPF was diagnosed on the basis of a compatible history,

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Received for publication 27 December 1990 and in revised form 29 April 1991.

1. *Abbreviations used in this paper:* BALF, bronchoalveolar lavage fluid; CRP, clinical-radiographic-physiologic (score); CTD, connective tissue disease; IPF, idiopathic pulmonary fibrosis; RT-PCR, reverse transcription-polymerase chain reaction.

J. Clin. Invest.

0021-9738/91/12/1802/09 $2.00
Volume 88, December 1991, 1802–1810

physical examination, chest radiograph, and pulmonary physiologic evaluation. A confirmatory open lung biopsy was performed in 11 subjects. Five patients with IPF were receiving treatment at the time of lavage: prednisone alone, three; prednisone/cytoxan/colchicine, two. 13 patients were either never smokers or were ex-smokers (quit at least 6 mo before BAL), and there was one current smoker.

Patients with IPF associated with a connective tissue disease (IPF-CTD). Nine patients had IPF associated with a defined connective tissue disease. Six patients had progressive systemic sclerosis, one had dermatomyositis/polymyositis, one with systemic lupus erythematosis, and one had rheumatoid arthritis. Three patients with IPF-CTD were receiving treatment at the time of lavage: prednisone alone, two; cytoxan alone, one. All nine patients were either never smokers or were ex-smokers (quit at least 6 mo before BAL).

Normal control subjects. Analysis of cellular constituents of bronchoalveolar lavage fluid was performed in 10 healthy volunteers for comparison with the patients with IPF and IPF-CTD. Five were never smokers, four were current cigarette smokers, and one was an ex-smoker. These subjects had no respiratory symptoms, normal chest radiographs, and normal spirometric test results, lung volume, and diffusing capacity for carbon monoxide.

None of the subjects had evidence of hypersensitivity pneumonitis, left ventricular dysfunction, cardiac valvular disease, or significant occupational exposures. Informed consent was obtained from each patient and healthy volunteer, and the protocol was approved by the Institutional Human Subjects Review Committee.

Quantitation of clinical impairment

Before the BAL, a history, physical examination, chest radiograph, and physiologic evaluation were performed in all patients. The physiologic evaluation included spirometry, measurement of lung mechanics, diffusing capacity for carbon monoxide, and measurement of arterial blood gases at rest and during exercise. These procedures were performed as described elsewhere (24). Clinical impairment was quantitated by means of a clinical-radiographic-physiologic (CRP) score (24). The CRP score is derived from the assessment of eight variables: level of activity required to precipitate dyspnea; chest roentgenogram; forced vital capacity; forced expired volume in 1 s; thoracic gas volume of functional residual capacity; diffusing lung capacity for carbon monoxide corrected for alveolar volume; resting alveolar arterial oxygen pressure difference; and exercise-induced reduction in oxygen saturation indexed to the fraction of predicted maximal oxygen consumption achieved during exercise. In this scoring system, the higher the CRP score, the more severe the degree of impairment.

BAL

BAL was performed in all patients awake in an outpatient setting by previously reported methods (5). Sterile normal saline at room temperature was instilled through the bronchoscope in 60-ml aliquots to a total of 240 ml, with harvest of the fluid by immediate gentle hand suction applied to each instilling syringe. Differential counts on 400 cells were performed on Wright-Giemsa–stained cytocentrifuge preparations made from the pooled lavage fluid before centrifugation and resuspension. The cytocentrifuge preparations were made using a Cytospin-2 (Shandon Southern Instruments, Sewickly, PA), centrifuged at 400 *g* (2,000 rpm) for 15–20 s (Table I). The pooled lavage fluid was centrifuged at 800 *g* for 10 min, and the cell pellet was resuspended in serum-free RPMI 1640 culture medium without serum or antibiotics. Viability of the BAL cells was determined by trypan blue dye exclusion. Alveolar macrophages were purified by adherence to 100-mm diam plastic tissue culture dishes in serum-free RPMI 1640 at 37°C in an humidified atmosphere containing 5% CO_2. After 15 min of incubation the nonadherent cells were removed and the layer of adherent cells was washed twice with 10 ml of ice-cold sterile PBS. These conditions were selected to minimize the induction of IL-8 gene expression, which has been reported to be induced in monocytes incubated in serum containing medium for > 30 min (25). The purity of the macrophages was always greater than 98%.

Isolation of total cellular RNA

Total cellular RNA was extracted by lysis with 4 M guanidine isothiocyanate, and purified by centrifugation through 5.7 M cesium chloride at 100,000 *g* for 18 h (26). The RNA pellet was rinsed in 70% ethanol, resuspended in 300 μl of RNase-free water, ethanol-precipitated, dried, resuspended in 50 μl of RNAse-free water, and quantified by absorbance measure-

Table 1. Demographic, Clinical, and BAL Characteristics of the Study Population

	Healthy volunteers		Patients	
	Nonsmokers	Current smokers	IPF	IPF-CTD*
Age	30.2±1.9	31.4±1.8	62.6±3.7	49.5±3.5
Sex (female/male)	4/1	2/3	7/7	8/1
CRP score‡	ND	ND	45±4	31±6
White cells ($\times 10^6$)	9.29±0.91	33.70±9.92	28.15±6.17	48.19±12.80
Macrophages ($\times 10^6$)	7.68±0.77	31.28±9.63	19.59±4.97	35.99±12.16
Neutrophils ($\times 10^6$)	0.09±0.04	0.23±0.13	4.41±1.02	2.85±1.16
Eosinophils ($\times 10^6$)	0.02±0.02	0.01±0.01	1.96±0.86	1.78±0.91
Lymphocytes ($\times 10^6$)	1.51±0.38	2.18±0.67	2.17±0.40	7.56±2.78

Data are expressed as the mean±SE.

* IPF-CTD denotes idiopathic pulmonary fibrosis associated with a connective tissue disorder.

‡ The CRP score is a numerical scoring system which integrates clinical, radiological and physiological data (see text).

ments at 260 nm. The integrity of the purified RNA was determined by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of 2 μg of each RNA sample through a 1% agarose-formaldehyde ethidium bromide gel.

Reverse transcription of RNA

RNA from each patient was reverse transcribed as previously described (27). Each sample contained 1 μg of total cellular RNA, 50 mM Tris/HCl pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 1 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 20 U of placental ribonuclease inhibitor (RNAsin), 1 μg of oligo(dT) 15–18 primer, and 25 U of avian myeloblastosis virus reverse transcriptase in a final volume of 12.5 μl. After incubation at 42°C for 60 min, the samples were heated for 5 min at 94°C to terminate the reactions, and were stored at −20°C until used.

Preparation of oligonucleotide primers

Oligonucleotide primers were constructed from the published cDNA sequences of IL-8 and β-actin cDNA (19, 28). The primers were selected to: (1) span an intron-exon boundary in order to distinguish between amplification of mRNA and any contaminating genomic DNA (29, 30), and (2) as close as possible to the poly-A tail in order to increase the efficiency of the RT-PCR using oligo (dT) as the primer for reverse transcription. The primers were synthetized on a model 381A (Applied Biosystems, Inc., Foster City, CA) DNA synthesizer (using β-cyanomethylphos-phoramidate derivatives), purified by two extractions with phenol/chloroform (1:1 vol/vol), ethanol-precipitated, resuspended in RNase-free water, quantified by absorbance measurements at 260 nm, and stored at −20°C. The sequence of the IL-8 primers was (1) 5′ ATTTCTGCAGCTCTGTGTGAA 3′ (coding sense) corresponding to bases 144–164 of the cloned full-length sequence (2) 5′ TGAATTCTCAGCCCTCTTCAA 3′ (anticoding sense) which anneals to bases 378–398. The β-actin primers were (1) 5′ TCCTGTGGCATCCACGAAACT 3′ (coding sense) spanning bases 852–872 and (2) 5′ GAAGCATTTGCGGTGGACGAT 3′ (anticoding sense) spanning bases 1146–1166. The predicted sizes of the amplified IL-8 and β-actin DNA products were 255 and 314 bp, respectively.

Amplification of IL-8 and β-actin cDNAs

Each reverse transcription mixture was diluted 1:10 in RNase-free water and 3 μl (for IL-8) or 1 μl (for β-actin) were then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.7 μM of each primer) spanning the given sequence for amplification, 100 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.025% (vol/vol) NP40, 0.025% (vol/vol) Tween 20, 1.9 U of *Thermus aquaticus* DNA polymerase (Taq), and 1 μCi of [α-^{32}P]dCTP (sp act 3,000 Ci/mmol) in a final volume of 100 μl. As previously reported (31), inclusion of [α-^{32}P]dCTP in the PCR allows numerical quantification of the amplified products. The reaction mixture was then overlaid with 3 drops (~ 25 μl) of mineral oil and amplified for 20–34 cycles in a Perkin-Elmer/Cetus (Norwalk, CT) thermal cycler. The amplification profile consisted of denaturation at 94°C for 2 min, primer annealing at 50°C for 2 min, and extension at 72°C for 2 min. The reaction was terminated by chilling at 4°C.

Quantification of the amplified products

After completion of the PCR, the mineral oil was removed by extraction with 125 μl of chloroform. 60 μl of the upper aqueous phase was precipitated twice with 4 M ammonium acetate/100% ethanol (1:4 vol/vol) to remove the unincorporated [α-^{32}P]dCTP (32). Each amplified DNA was then vacuum-dried, resuspended in 25 μl of Tris-EDTA, pH 8, and electrophoresed through a 2% (wt/vol) agarose gel containing 0.5 μg/ml of ethidium bromide in 40 mM Tris-acetate buffer, pH 8.0, containing 1 mM EDTA. Gels were then photographed and dried and autoradiograms were prepared. Segments corresponding to the radioactive bands were excised from the gel and counted in a liquid scintillation counter.

Hybridization with end-labeled oligonucleotide probes

In experiments designed to validate the PCR products, a sense-on 19-mer oligonucleotide probe internal to the amplified fragment of IL-8 of sequence (5′)AAGACATACTCCAAACCTT(3′) corresponding to nucleotides 213–231 of the cDNA sequence was synthesized as described above. The oligonucleotide probe used to validate the β-actin PCR fragment was (5′)ATGAAGATCAAGATCATTG(3′). 100 ng of each probe were end-labeled with 200 μCi of [γ-^{32}P]ATP (sp act 3,000 Ci/mmol) using 8 U of T4 polynucleotide kinase in the presence of 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.5 μg of BSA in a final volume of 10 μl. Unlabeled PCR products were blotted onto nylon membranes, prehybridized in 10 ml of buffer composed of 5× sodium chloride (3M), monobasic sodium phosphate (0.2 M), EDTA (0.02 M), pH 7.4, 10× Denhardt's solution, 0.1% (wt/vol) SDS, and heat-denatured salmon sperm DNA (10 μg/ml). Hybridization was conducted in the presence of 10% (wt/vol) dextran sulfate and 5×10^6 dpm of the labeled oligonucleotide probe by incubation overnight at a temperature calculated to be 5°C below the melting point of the probe. Before autoradiography the blots were washed three times in 5× SSC for 20 min at room temperature, then for 1 min at melting point-5°C.

Quantification of IL-8 protein

IL-8 protein in BALF was determined using a sensitive ELISA kit obtained from R & D Systems, Minneapolis, MN. Briefly, 100-μl samples of BALF were dispensed into microtiter wells of the "Quantikine" kit and incubated at room temperature for 2 h. The plates were then rinsed four times with wash buffer and incubated with 200 μl of anti–human IL-8-horseradish peroxidase conjugate for 2 h. After further rinsing to remove excess conjugate, bound enzyme was detected by incubation with tetramethylbenzidine and hydrogen peroxide as substrate for 20 min before terminating the reaction with 50 μl of stop buffer and quantifying the absorbance at 450 nm. Values of IL-8 in BALF was determined by reference to an IL-8 standard curve constructed using 0–2,000 pg/ml of recombinant human IL-8.

Statistical analysis of data

One-way analysis of variance (ANOVA) or two sample *t*-tests were used to test for differences between group means depending on the number of groups involved. Sidak's multiple comparison procedure (33) was used when ANOVA indicated significant differences. Sidak's method adjusts the α level for individual comparisons in order to maintain an experiment-wise error rate of 0.05. Correlation coefficients and associated tests

of significance were computed using standard linear regression methods.

Results

Verification of PCR products as predicted segments of spliced IL-8 and β-actin mRNAs. To verify that the amplification products derived from the IL-8 and β-actin primers were authentic, we (1) determined the sizes of the amplified products and (2) prepared Southern blots of the amplified DNAs which we then probed with end-labeled oligonucleotides composed of sequences that were internal to the primers. The RNA was isolated from macrophages derived from an individual with IPF. As can be seen in Fig. 1, *A* and *B* (lane *1*) the amplification products of both IL-8 and β-actin were identical to the predicted sizes for the mRNA template of 255 and 314 bp, respectively. Bands of higher molecular weight were not detected indicating that genomic DNA was not being amplified. Fig. 1, *A* and *B* (lane *2*) illustrates that hybridization of Southern blots of the amplification products with end-labeled IL-8 and β-actin oligonucleotide probes whose sequences were internal to the amplified sequences, produced hybridization signals that corresponded in sizes to the ethidium bromide–stained gel, thus confirming that the amplified sequences consisted of authentic IL-8 and β-actin sequences. A very low hybridization signal was seen at ~ 506 bp in Southerns probed with the IL-8 oligonucleotide probe. The identity of this faint band is not known although could conceivably represent an amplification product derived from contaminating genomic DNA. This band is eliminated during excision and quantification of the IL-8 mRNA amplification product.

Kinetics and yield of amplification of IL-8 mRNA. Previous studies have shown that the amount of amplified fragments is proportional to the quantity of input mRNA during the exponential phase of the amplification process (34, 35). We have taken three approaches to ensure that the measurements taken in this study were during the exponential phase. First, we have varied the number of cycles of amplification to determine the points at which amplification is within the exponential phase. cDNAs were amplified from two normal subjects and two patients with IPF. As illustrated in Fig. 2, the amplification of IL-8 mRNA remained in the exponential phase at 28 and 31 cycles, while the plateau of amplification was reached at 34 cycles.

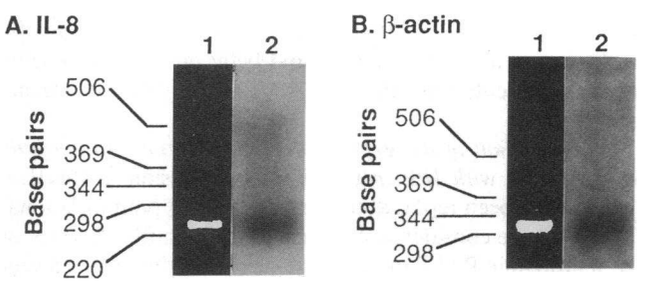

Figure 1. Amplification products obtained with IL-8 (*A*) and β-actin (*B*) specific primers and RNA derived from alveolar macrophages from a healthy volunteer. Lane *1* shows the ethidium bromide staining patterns and lane *2* shows the autoradiographic signals obtained following Southern transfer of the amplified products onto nylon membranes, and hybridization with end-labeled sense-on oligonucleotide probes whose sequences were internal to the pirmers. Amplification was for 35 cycles.

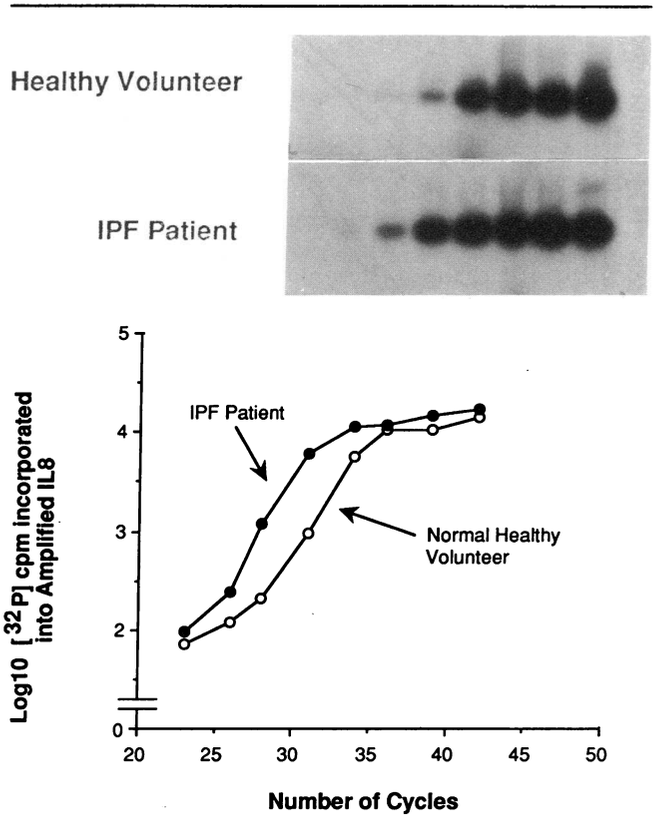

Figure 2. Amplification of IL-8 mRNA. *Top,* cDNA derived from alveolar macrophages from a normal subject or from a patient with idiopathic pulmonary fibrosis were amplified with IL-8 specific primers for the indicated number of cycles in the presence of [α-^{32}P]dCTP. After being dried, the gels containing the IL-8 cDNA fragments were exposed to X-ray film for 8 h at room temperature with an intensifying screen. The figure shows the corresponding autoradiograms. These data are representative of duplicates and of two separate experiments done on a total of two normal smokers and two patients with idiopathic pulmonary fibrosis. *Bottom,* semilogarithmic representation of the extent of IL-8 amplification, as measured by counting the amount of [^{32}P] incorporated into the fragments visualized in top panel.

Second, the efficiency of amplification is known to be constant for a given set of primers (34), and thus the number of cycles after which the amplification ceases to be exponential is dependent on the abundance of starting cDNA. To verify that the amplification remained in the exponential phase under varying conditions of input cDNA, we amplified the IL-8 cDNA derived from each RNA sample at 28, 31, and 34 cycles thereby allowing us to verify that the efficiency of the amplification process was similar in the different groups studied. A representative sample of two healthy individuals and two patients with IPF is shown in Fig. 3. It will be seen that the amplification of the IL-8 cDNA remained in the exponential phase for the two different patient groups.

Third, we also determined the relationship between input cDNA and the amount of amplified ^{32}P-labeled product. Increasing amounts of input RNA were reverse transcribed, am-

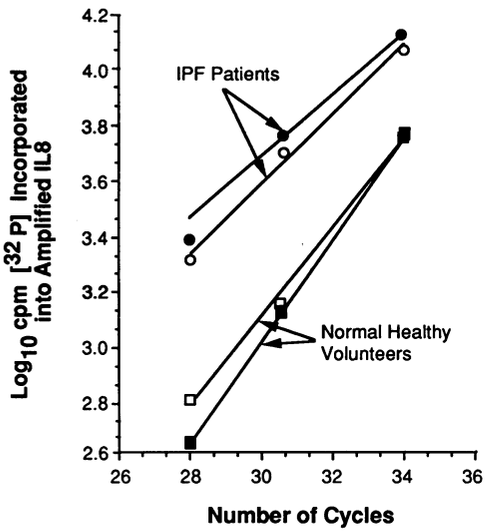

Figure 3. Analysis of the efficiency of IL-8 amplification according to the different groups of subjects. The logarithm of the radioactivity incorporated into the amplified fragments is plotted versus the number of cycles. Each point is the counts per minute obtained for normals nonsmokers (■), normals smokers (□), IPF patients (○), or patients with IPF-CTD (●).

plified for 31 cycles and the amount of labeled product quantified. As shown in Fig. 4, a linear relationship between input cDNA and output of [^{32}P]IL-8 cDNA was observed. Collectively, these data demonstrate that when the analysis is performed in the exponential rate, the quantitative PCR assay employed in this study can resolve less than twofold differences in input mRNA.

To determine that differences in the IL-8 mRNA measurements between individuals were not due to random variations in the input quantity of RNA or in the efficiency of the reverse transcription, we amplified a β-actin mRNA sequence at 20, 23, and 27 cycles for each individual and repeated these determinations two further times. We verified that the amplification

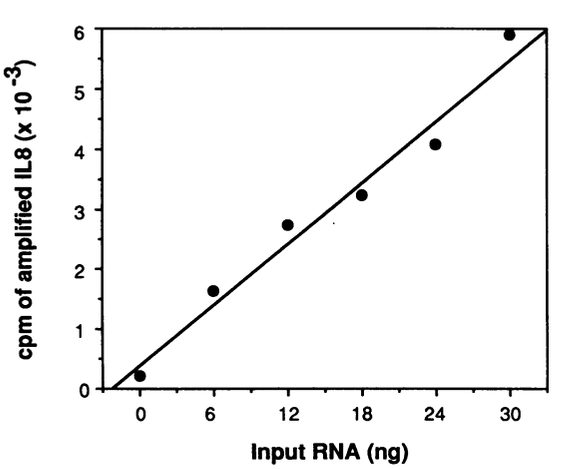

Figure 4. Quantitative analysis of the IL-8 mRNA level. A cDNA mixture from one individual was serially diluted, i.e., 30, 24, 18, 12, or 6 ng of RNA equivalent from a same individual were subjected to 31 cycles of amplification. The figure shows a linear relationship between the input quantity of cDNA and the radioactivity of the corresponding amplified bands.

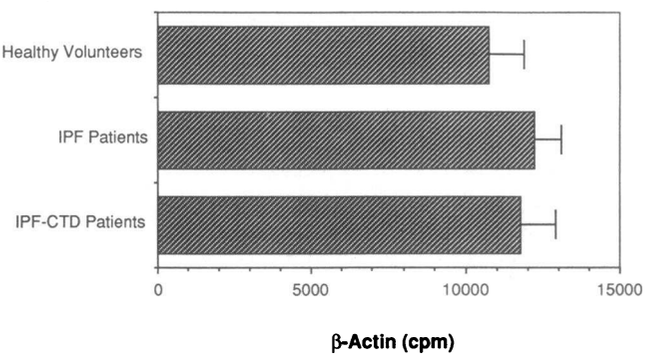

Figure 5. Amplification of β-actin specific mRNA in alveolar macrophages derived from healthy volunteers, IPF patients and patients with IPF-CTD. Amplification was for 23 cycles.

did not reach a plateau at 20 and 23 cycles and that the efficiency was similar in the four groups (data not shown). As shown in Fig. 5, there was no significant differences of β-actin expression at 23 cycles between healthy volunteers, or patients with IPF or IPF-CTD ($P = 0.1795$).

Comparison of the expression of IL-8 mRNA in normals and patients with IPF. Three determinations of IL-8 and β-actin were conducted at 31 and 23 cycles, respectively, for each patient in at least two separate experiments. Samples from normal individuals and patients were always run in parallel. A linear regression of IL-8 against β-actin revealed a significant correlation between levels of β-actin and IL-8 counts ($r = 0.53$, $P = 0.009$). However, a regression of the IL-8/β-actin ratio against β-actin revealed that these two parameters were not correlated ($r = 0.14$, $P = 0.513$), indicating that normalization of the IL-8 values to the internal standard allowed quantitative comparisons independent of input RNA.

The IL-8/β-actin ratios were quantified in 10 healthy volunteers and 23 patients with IPF. As shown in Fig. 6 *A*, the mean±SEM of the IL-8/β-actin ratio in alveolar macrophages from normal never-smokers was 0.16±0.01 and for current smokers it was 0.19±0.02 ($P = 0.27$). The IL-8/β-actin ratio from patients with IPF was 0.53±0.07 and for patients with IPF-CTD it was 0.38±0.04. There was no significant difference between the IPF and IPF-CTD patients ($P = 0.077$). Both patient groups had significantly higher IL-8/β-actin ratios compared to normals (normal never-smokers vs. IPF or IPF-CTD, $P = 0.0001$ and $P = 0.0006$, respectively; current smokers vs. IPF or IPF-CTD, $P = 0.0002$ and $P = 0.0012$, respectively). In addition, no differences were found between the IL-8/β-actin ratios in patients with IPF or IPF-CTD, with or without treatment.

Comparison of the expression of IL-8 protein in normals and patients with IPF. Although the expression of the IL-8 mRNA has been reported to be regulated at a pretranslational level (22), we considered it important to quantify the level of IL-8 protein in BALF in samples obtained at the time of alveolar macrophage isolation. The IL-8 protein concentration in BALF was determined using a sensitive ELISA. The mean±SEM of the IL-8 concentrations in BALF derived from healthy nonsmokers and healthy smokers were 11.0±3.6 and 22±9.4 pg/ml, respectively. These values were not significantly different ($P = 0.995$). By contrast, the concentration of IL-8 in BALF derived from patients with IPF was 58.5±11.2 and was significantly elevated ($P = 0.005$) compared to healthy individ-

uals (Fig. 6 *B*). The concentration of IL-8 in BALF in individuals with IPF-CTD was 32.7±7.4 and was not significantly different from healthy controls ($P = 0.447$) of from patients with IPF ($P = 0.121$).

Correlation of IL-8/β-actin ratio levels and disease severity. We have investigated possible relationships between the IL-8/β-actin ratio and several biological and clinical parameters. These parameters included number of white cells, macrophages, lymphocytes, neutrophils, or eosinophils in the lavage; age; smoking status, duration of illness, lung function parameters (total lung capacity, forced vital capacity, carbon monoxide diffusion capacity/alveolar ventilation, resting arterial oxygen pressure [Pao_2]); the CRP score, an index of disease severity. A linear regression model was fitted to each of the quantitative variables individually.

Three variables were found to have a significant correlation with the IL-8/β-actin ratio. As can be seen in Fig. 7 *A*, the number of neutrophils/ml of BAL fluid increased as the IL-8/β-actin levels increased ($r^2 = 0.63$; $P < 0.0001$). The Pao_2 (Fig. 7 *B*) decreased as the IL-8/β-actin ratio increased ($r^2 = 0.313$, $P = 0.005$), suggesting that the higher the IL-8/β-actin levels, the more severe the pulmonary dysfunction. The composite CRP score, a measure of the degree of clinical impairment, was consistent with this trend. That is (Fig. 7 *C*) patients with the more severe degrees of clinical impairment had a higher level of IL-8/β-actin compared to those less ill ($r^2 = 0.325$, $P = 0.007$). A multiple linear regression of all variables tested indicated that this relationship between neutrophils/ml and the IL-8/β-actin ratio was the most significant. The concentration of IL-8 protein in BALF was not found to be significantly correlated with any of the above measured parameters.

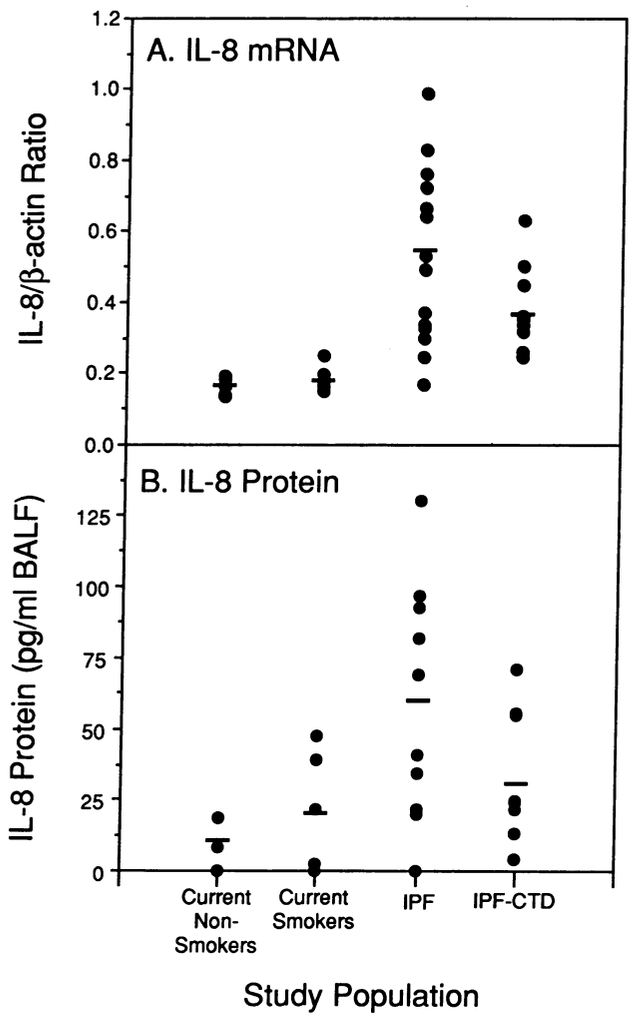

Figure 6. (*A*) Differences in the IL-8 gene expression by alveolar macrophages from normals individuals and patients with IPF and IPF-CTD. The quantitative results are expressed as ratios of the intensity of the IL-8 band at 31 cycles to the intensity of the β-actin band at 23 cycles used as standard. Each value is the mean of triplicate determinations for each patient in two or three separate experiments. (*B*) Concentrations of IL-8 protein in BALF from normal individuals, and patients with ILF and IPF-CTD. The results are expressed as pg of IL-8 per ml of BALF.

Discussion

The pathogenesis of IPF remains unknown. However, current concepts suggest that the chronic presence of neutrophils in the alveolar structures plays a prominent role in the parenchymal injury and destruction seen in this disease (9–13). A number of studies have suggested that the alveolar macrophage actively participates in the control of the neutrophil traffic in IPF by synthesizing and releasing neutrophil chemoattractant(s) (36). A number of alveolar macrophage inflammatory mediators, such as leukotnene B_4 (37), platelet-derived growth factor (38), and platelet-activating factor (39), have been reported to be potent neutrophil attractants in vitro. However, these factors do not demonstrate selectivity for a particular leukocyte (21). Further, these data are largely derived from in vitro studies and cannot be extrapolated to the profile of chemotactic factors that may be released by alveolar macrophages under pathological conditions in vivo. Recently, a novel polypeptide has been isolated, sequenced and cloned from human blood and lung mononuclear leukocytes and found to have potent and selective chemotactic activity for neutrophils. It has been proposed that this 8,400-D protein, IL-8 or neutrophil attractant/activation protein-1(NAP-1), could be a prime candidate in mediating the sustained accumulation of neutrophils in the lungs of patients with acute/chronic inflammatory lung diseases, such as IPF.

In this study, we developed a quantitative assay for IL-8 mRNA to enable us to examine the expression of this cytokine by alveolar macrophages immediately after sampling from the lung. This approach was adopted to provide information about one of the major cell types implicated in producing IL-8, without activating the cell in vitro. Hence the data obtained in this study would closely reflect the level of IL-8 expression by alveolar macrophages in vivo. IL-8 mRNA expression was quantified by reverse transcription combined with amplification by the PCR. Such an approach has been shown to be a useful method for detecting small amounts of transcripts when applied to the quantification of RNAs from small numbers of cells or to characterize low abundant transcripts (31, 40, 41). We demonstrate that the IL-8 gene is expressed in increased amounts by alveolar macrophages from patients with IPF, with or without an associated connective tissue disease, compared to those from normal individuals. Importantly, the level of alveolar macrophage IL-8 mRNA expression correlated positively with the number of neutrophils recovered by BAL. This ap-

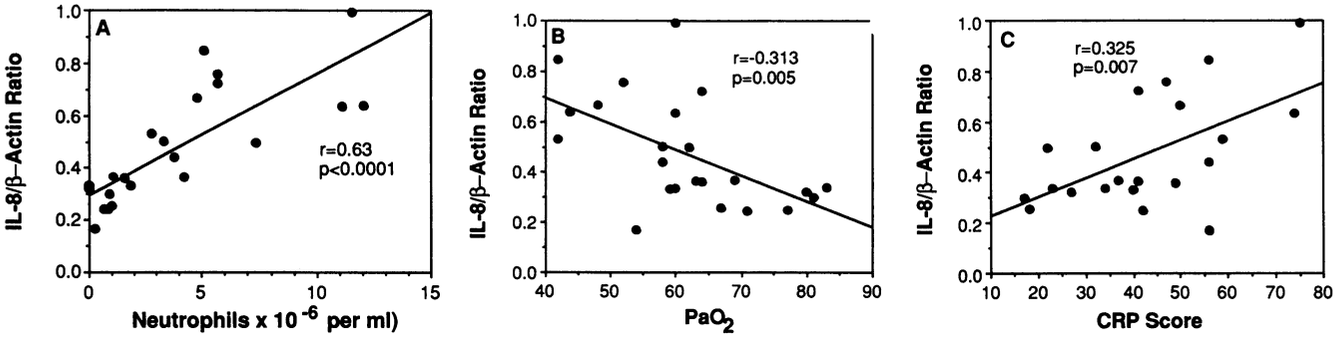

Figure 7. Correlation between the IL-8/β-actin ratio and some clinical or bronchoalveolar lavage parameters of the 23 patients with CFA. The ratio IL-8/actin was plotted as a function of (*A*) the number of neutrophils per milliliter of BAL, (*B*) the resting PaO_2, or (*C*) the CRP score. The R^2 values were for (*A*) 0.679 ($P = 0.0001$), (*B*) 0.3134 ($P = 0.005$), and (*C*) 0.325 ($P = 0.007$).

pears to be a selective effect since there was no correlation with other cell types recovered in BAL. In addition, the alveolar macrophage IL-8 gene expression correlated positively with parameters of disease activity. That is, those patients with more severe clinical impairment, as assessed by gas exchange or by a composite clinical-radiographic-physiologic scoring system (CRP score), had higher levels of IL-8 gene expression.

By employing a sensitive ELISA, we were also able to detect a similar pattern in the concentration of IL-8 protein in BALF thus indicating that antigenic protein was synthesized and secreted by the cells of the lung. Thus, these findings suggest that IL-8 may operate in vivo in the lung as a neutrophil chemotactic factor and thus is relevant to the pathogenesis and course of this disease. However, it should be emphasized that while detection of IL-8 protein provides an important link between the observed increase in the IL-8 mRNA expression by alveolar macrophages and the recruitment of neutrophils to the airspaces, such measurements fail to provide information about the cell types responsible for the production of IL-8. Furthermore, given the great stability of IL-8, quantification of IL-8 protein in BALF provides an indication of the net accumulation of this cytokine in excess of that required for receptor binding but fails to yield any information about the time or place of its accumulation.

Macrophages are found in increased numbers and in an activated state both within the alveolar space and within the alveolar interstitium in the lungs of patients with IPF. These cells appear to play a central role in the pathogenesis of IPF. Whereas resting alveolar macrophages demonstrate little effector cell function, stimulated macrophages produce a variety of enzymes, complement components, cytokines, and other mediators of inflammatory and fibroblast cell function that could initiate or maintain the inflammatory and immune processes that precede and result in the fibrotic stage of this disease. Sustained neutrophil accumulation in the alveolar space and persistent neutrophil mediated injury and destruction of alveolar architecture are believed to be major factors that culminate in abnormal lung repair and interstitial fibrosis (9–11). Since neutrophils are not found in the normal lung, processes that sustain neutrophil influx into the alveolar space have been sought. Activated macrophages from patients with IPF appear to amplify the alveolar inflammatory process by recruiting neutrophils into the alveolar space through the release of chemotactic factors (11–13). A specific factor for neutrophil recruitment and retention in the lung has not been identified.

Several lines of evidence support a role for the neutrophil in mediating the parenchymal injury found in patients with IPF (9, 10, 42–44). The number of neutrophils in BALF and lung tissue from patients with IPF is increased and these neutrophils are capable of injuring lung cells and degrading interstitial matrix components by secreting proteases and oxidants, many of which are found in the BALF of patients with IPF. The data in this study therefore are important since they delineate further a mechanism by which neutrophils may be attracted to the lungs of patients with IPF: alveolar macrophages of patients with lavage neutrophilia exhibit an overexpression of the IL-8 gene.

In the present study, the IL-8 gene expression by alveolar macrophages from the IPF and IPF-CTD patients studied appears to be specifically related to disease and not to some other exogenous stimulus. Endotoxin and LPS have been shown to stimulate the expression of IL-8 by monocytes (17). While we cannot be sure that endotoxin, possibly present in the respiratory tract and/or in the short term cultures, could have accounted for the differences noted in IL-8 expression, we did not detect any obvious overexpression of IL-1 expression in our patients compared to the normal controls (P.C. Carre and D. W. H. Riches, unpublished observations). Endotoxin and LPS, even at minute concentration is well known to induce macrophage IL-1 gene expression in alveolar macrophages (45, 46). Alternatively, it has been shown that particulates present in cigarette smoke can stimulate alveolar macrophages in vitro and in vivo to exhibit a neutrophil chemotactic activity (47). However, in the present study there was no difference in the extent of IL-8 expression between normal non-smokers and normal smokers. Furthermore, most of our patients were non-smokers or ex-smokers. Consequently, the overexpression of IL-8 by alveolar macrophages in this study cannot be attributed to the presence of cigarette smoke in the respiratory tract.

In conclusion, we have shown that the IL-8 gene is overexpressed by alveolar macrophages from patients with ILD. As the principal resident cell in the lung, the alveolar macrophage plays a central role in host defense and injury. The generation of IL-8 by these cells provides a mechanism for the initiation and amplification of inflammation in the lung. The mechanism(s) by which alveolar macrophages are activated to express the IL-8 gene is unknown. A recent study suggested that neutrophil accumulation and activation in the lungs after connective tissue injury is mediated, at least in part, by factors released from alveolar macrophages (48). Indeed, when stimulated in vitro by collagen peptides, macrophages released a neutrophil

chemotactic factor whose physicochemical characteristics resembled those of IL-8 (48). Since collagen injury is a usual feature in lung fibrosis, it is possible that in this situation, fragments from collagen breakdown are responsible for the activation of the expression of the IL-8 gene by alveolar macrophages. Although an increase in the synthesis and release of IL-8 in the BAL of patients with ILD remains to be determined, we believe that the quantification of the mRNA is a good basis for hypothesizing that IL-8 production by alveolar macrophages may be an important mechanism by which neutrophils are attracted to the lungs in pulmonary fibrosis. Indeed, in vitro studies have shown that IL-8 is regulated at the transcriptional level in alveolar macrophages and that induction of IL-8 expression is followed by synthesis and release of the protein (22). Whether manipulations of IL-8 production at the gene level or antagonism of the actions of IL-8 at the level of the neutrophil receptor could alter the influx of neutrophils into the lung and subsequently the fibrotic process remains to be established.

Acknowledgments

We are indebted to Dr. Ed Palmer for helping to establish the PCR protocol used in this study and to Dr. Brian Kotzin for productive discussions. We would also like to acknowledge Dr. David Ikle, William Kastner and Dr. Lynn Ackerson for the statistical analysis of the data, S. Arlene Niccoli, Trudy McDermott, Martin Wallace, and Alma Kervitsky for their contribution to the normal volunteer and patient studies, Joel Boymel for preparing of oligonucleotides, and Nadia De Stackleberg, Barry Silverstein, and Leigh Landskroner for preparing the figures and Georgia Wheeler for preparing the manuscript. We also wish to thank May Gillespie for conducting the IL-8 ELISA. Finally, we would like to thank Dr. Peter Henson for his enthusiastic input into all phases of this work.

This study was supported in part by grants CA-50107 and SCOR grant HL-27353 from National Institutes of Health.

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