Platelet-activating Factor Exerts Mitogenic Activity and Stimulates Expression of Interleukin 6 and Interleukin 8 in Human Lung Fibroblasts via Binding to its Functional Receptor

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Summary

Platelet-activating factor (PAF) is a potent proinflammatory phospholipid mediator of the lung. In this study, we demonstrate that PAF receptor mRNA and protein is expressed by human lung fibroblasts. Interaction of PAF with its specific receptor resulted in increases of tyrosine phosphorylation of several intracellular proteins, indicating that the PAF-receptor might be functionally active. PAF-induced transcription of protooncogenes c-fos and c-jun as well as of interleukin (IL)-6 and IL-8 genes in human fibroblasts. Transcription of the interleukins was followed by secretion of the respective proteins. Moreover, PAF enhanced proliferation of fibroblasts in a concentration-dependent manner. Using signaling inhibitors, we demonstrate that PAF-induced transcription of the c-fos, IL-6, and IL-8 genes, as well as proliferation, require activation of pertussis toxin–sensitive G proteins, tyrosine kinases, and protein kinase C (PKC). In contrast, transcription of c-jun was blocked by pertussis toxin, but not by inhibitors for tyrosine kinases or PKC. These data suggest that PAF stimulates distinct signaling pathways in human lung fibroblasts. In addition, the activation of human fibroblasts by PAF leads to enhanced proliferation and to the expression of proinflammatory cytokines, which may contribute to the pathophysiological changes in pulmonary inflammation.

Platelet-activating factor (PAF)[1] is a potent ether-linked phospholipid mediator that modulates vascular, muscular, and immune function in the human lung (1–5). Numerous studies established an important role of PAF in airway inflammation. Inhalation of PAF provokes asthma-like airway responses such as bronchoconstriction, bronchial hyperresponsiveness (1–3), increased vascular permeability (4, 5), and mucosal edema (4, 5). Recently, it has been shown that circulating PAF is markedly increased during acute asthmatic attacks (3, 6, 7).

PAF acts via binding to a specific receptor (8–11). The gene coding for the human PAF receptor has been recently cloned from leukocytes (8), and has been identified as a single copy gene on human chromosome 1 (12). Cloning studies of the PAF receptor revealed that the receptor shows homology to G protein–coupled receptors (8, 12–14). Coupling of the PAF receptor to pertussis toxin and cholera toxin–sensitive G proteins has been reported in human polymorphonuclear leukocytes, B lymphocytes, and platelets (15, 16). PAF activates tyrosine kinases, as indicated by increases in tyrosine phosphorylation of several intracellular proteins in the same cell types (17, 18). Furthermore, PAF induces activation of phospholipase C (PLC) (18); it mobilizes cellular calcium from intracellular stores (11, 19), and it increases the activity of protein kinase C (PKC) (15, 20–22). These PAF-mediated early biochemical events are followed by specific gene expression. For example, it has been demonstrated that PAF, via binding to the PAF receptor, promotes rapid induction of c-fos mRNA on human B-lymphoblastoid cells (19). In addition, PAF induces c-fos mRNA expression due to activation of tyrosine kinases and PKC activation in a human carcinoma cell line (22).

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[1]Abbreviations used in this paper: PAF, platelet-activating factor; PKC, protein kinase C; PLC, phospholipase C; ptyr, phosphotyrosine.

Increasing evidence suggests that PAF is capable of modulating inflammatory responses by enhancing the synthesis of certain IL (23–26) and adhesion molecules (27). Possible effects of PAF on lung fibroblasts are of particular interest, since fibroblasts are implicated in the pathophysiological changes of the lung in response to various stimuli (28, 29). However, modulatory effects of PAF on gene activation in human fibroblasts are still unknown. In this article, we demonstrate that functional PAF receptors are expressed on human lung fibroblasts. The receptor is coupled to G proteins, tyrosine kinases, and PKC signaling pathways. Moreover, activation of these cells by PAF is followed by c-fos, c-jun, IL-6, and IL-8 gene expression and proliferation. Thus, PAF is shown to have direct and profound biological effects on human lung fibroblasts, which could explain some clinical phenomena in asthmatics, such as leukocyte infiltration of the peribronchial tissue and subepithelial fibrosis (30).

Materials and Methods

Generation and Characterization of Primary Human Lung Fibroblasts. Primary lung fibroblasts cell lines were generated from small pieces (10 mm^2) of sterile lung biopsies (31) obtained from patients ($n = 10$) undergoing lung surgery for different reasons. Biopsies were taken from parts of the explants that appeared macroscopically normal. Cells were cultivated in RPMI 1640 supplemented with L-glutamine (4 mM; Seromed/Fakola, Basel, Switzerland) and 10% FCS (Gibco AG, Basel, Switzerland).

Immunostaining was performed to define the phenotype of the fibroblasts and to exclude any contamination with endothelial, epithelial, or smooth muscle cells. Briefly, cells were grown in Lab-Tek tissue culture chamber/slides (Miles, Scientific Division, Naperville, IL) containing eight chambers (0.9 × 0.9 cm) and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.01% (wt/vol) Triton X-100 for 10 min (32). To block nonspecific protein binding, cells were incubated in PBS containing 0.5% BSA (wt/vol: blocking buffer) for 20 min. Cells were incubated for 30 min with mAb specific to either smooth muscle cell actin, keratin, Factor VIII, fibronectin, laminin, or von Willebrand factor (Boehringer Mannheim, Mannheim, Germany). After three washes with blocking buffer, slides were further incubated for 30 min with either FITC-coupled anti–rabbit IgG or FITC-coupled anti–mouse IgG (Boehringer Mannheim). After washing, the preparations were mounted with Fluorosave reagent (Calbiochem-Novabiochem, Lucerne, Switzerland) and observed on a microscope equipped with epilumination and specific filters (560 nm) (Axiophot; Carl Zeiss Inc., Oberkochen, Germany), as described earlier (32).

Identification of PAF Receptors in Human Lung Fibroblasts. PAF receptor mRNA was identified by a PCR technique. Poly(A)$^+$ RNA was isolated from 10^7 cultured human lung fibroblasts or purified PBMC using an mRNA purification kit (Pharmacia, Dubendorf, Switzerland). Monocytes were purified by negative selection (11). First-strand synthesis and PCR were carried out as previously described (33, 34). Briefly, fibroblast and monocyte mRNA, random hexadeoxynucleotides at 0.2 mg/ml as primers, and Moloney murine leukemia virus reverse transcriptase (Pharmacia) were used for cDNA synthesis. PCR amplification of the cDNA encoding the PAF receptor was performed using two 27-mer oligonucleotide primers corresponding to sequences from

the initial 18 translated nucleotides (5' primer: 5' GTG GGA TCC ATG GAG CCA CAT GAC TCC 3) and 18 nucleotides from the 3' nontranslated region that occur 50 bp after the translation stop codon (3 primer: 5' GTG GAA TCC ATC CCT TCT TCC CCC AGC 3'), respectively (13). β-Actin oligonucleotides (Clontech-LucernaChem, Lucerne, Switzerland) were used to control cDNA synthesis and amplification conditions. For amplification, 1 μl cDNA was mixed with 2 μl of each primer (at final concentration 1 μM), all four deoxynucleotide triphosphates, and Taq polymerase (Perkin-Elmer Cetus, Rotkreuz, Switzerland) in a reaction buffer containing 1.5 mM Mg^{2+}. The final mixture was incubated for 30 cycles, each cycle consisting of 1 min denaturation at 94°C, 1 min annealing at 45°C, and 1 min extension at 72°C. After amplification, 8 μl of PCR product was run on a 1% agarose gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The radioactive probe was made by random priming, and the membrane was hybridized with a ^{32}P-labeled probe specific for human PAF receptor (kind gift from Dr. R.D. Ye, The Scripps Research Institute, La Jolla, CA). Membranes were then washed and autographed with intensifying screens at −80°C.

To determine PAF receptor surface expression on human lung fibroblasts, cells were harvested by using a cell scraper (Falcon-Becton Dickinson, Zurich, Switzerland) and by pipetting forcefully. After washing with staining solution (PBS/0.2% heat-inactivated FCS) cells (10^5) were incubated with polyclonal anti–human PAF receptor (11) or control Ab for 60 min at 4°C. Cells were then washed twice and incubated with FITC-conjugated, purified anti–rabbit IgG Ab (Inotech, Dottikon, Switzerland) for 30 min at 4°C. After washing, cells were analyzed by flow cytometry (EPICS-XL; Coulter, Hialeah, FL).

Detection of Tyrosine Phosphorylation by Immunoblotting. To demonstrate that the receptor for PAF is coupled to signaling pathways in fibroblasts, we investigated the level of tyrosine phosphorylation of intracellular proteins. Confluent cultures of cells (2 × 10^6) were stimulated with PAF (10^{-8} M) for 1, 3, 5, 10, and 30 min at 37°C. Further steps were performed as previously described (35). Briefly, cell pellets were lysed in SDS-PAGE sample buffer (10% SDS, 100 mM Tris [pH 6.8], 1% glycerol, 125 mg/ml bromophenol blue), and then sonicated, 1 mM dithiothreitol was added, and samples were boiled for 10 min. Protein concentrations were determined by the Coomassie blue assay using BSA as standard. Soluble proteins (100 μg/sample) were separated by SDS-PAGE (8%) and transferred onto polyvinylidene difluoride membranes (Millipore, Zürich, Switzerland). Filters were blocked in prewarmed blocking buffer (1% BSA, 0.01 M Tris [pH 7.5], 0.1 M NaCl, 0.1% Tween 20). Immunoblots were carried out by using an antiphosphotyrosine (ptyr) antibody, RC20, which was peroxidase conjugated (Affiniti, Nottingham, U.K.) at a 1:2500 dilution with prewarmed blocking buffer for 20 min at 37°C, and blots were washed extensively in blocking buffer without BSA and developed by an enhanced chemiluminescence technique (ECL kit; Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Detection of mRNA for Protooncogenes and ILs. Expression of transcripts for c-fos, c-jun, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-9, and IL-10 was determined using Northern blot analysis, as described earlier (36). Subconfluent fibroblast cultures were serum-deprived for 48 h before being stimulated with PAF (10^{-7} to 10^{-10} M) for various time periods (0, 0.5, 1, 2, 4, 6, 8, and 12 h). In further experiments, various drugs were used to antagonize PAF receptor binding or to inhibit signal transduction pathways before PAF stimulation (see below). Total RNA was extracted

(37), and 10 μg of total RNA were size fractionated in 1% agarose gels containing 2% formaldehyde. RNAs were transferred onto Hybond nylon membranes (Amersham) by capillary blotting overnight (10× SSC: 1.5 M NaCl, 0.15 M Na$_3$ citrate, pH 7.0) and cross-linked by UV irradiation. Membranes were prehybridized for 1 h at 45°C with a buffer containing 50% formamide, 1 M NaCl, 0.1 SDS, 10% dextran sulfate, 7.5% polyethylene glycol 6,000, and 1 mg/ml denatured salmon sperm DNA. Blots were hybridized overnight with 20 ng of 3′ end–labeled ([α-^{32}P]dATP) oligonucleotide probes (British Biotechnology, Cowley, U.K.) as described (31, 36). Blots were washed twice at room temperature with 5× SSC (15 min each) and a final wash with 1× SSC at 55°C. A random prime–labeled HLA-β cDNA probe (ATCC 57474; American Type Culture Collection, Rockville, MD) was used as constitutive control (31, 36). Blots were exposed to Kodak X-Omat films overnight or up to 48 h at −80°C. Hybridization signals were densitometrically analyzed using an automated scanner system and the NIH image program from Macintosh. Cells were preincubated for 30 min with 10 μM of actinomycin D to assess whether elevated levels of mRNAs are caused by increased transcription.

Expression of IL-6 and IL-8 Proteins. To confirm the data obtained at the mRNA level, we performed protein expression studies for ILs using ELISA techniques. Cells were seeded into 24-well cell culture plates (Falcon), cultivated until 80% confluence, and brought to quietitude by incubating cells for 48 h in low serum medium (0.1% FCS). Medium was replaced every 12 h. In some experiments, cells were preincubated with inhibitors (see below) before PAF stimulation (10^{-9} M). Aliquots of 100 μl cell supernatant were collected at 0, 6, 12, and 18 h after the addition of PAF. IL-6 and IL-8 were determined by ELISA (Advanced Magnetics, Cambridge, MA) as described (37).

Inhibition of PAF Receptor Binding and Signal Transduction Pathways. To demonstrate that the PAF-dependent activation of fibroblasts is mediated through interaction with its corresponding receptor (10), fibroblasts were preincubated in some experiments for 30 min with 10 μg/ml to 100 μg/ml of WEB 2170 (2 × 10^{-5} to 2 × 10^{-4} M), a specific PAF-receptor antagonist (kindly provided by Boehringer Ingelheim, Ingelheim, Germany).

To study the PAF receptor–coupled signaling pathways involved in the transcription of c-fos and c-jun, and the expression of IL-6 and IL-8, subconfluent layers of quiescent fibroblasts were stimulated for various time periods (0, 0.5, 1, 2, 4, 6, 8, and 12 h) with different PAF concentrations (10^{-7} to 10^{-10} M) in the presence or absence of specific inhibitors for G proteins, tyrosine kinase, or PKC. Involvement of pertussis toxin–sensitive G proteins (15, 16) in PAF-dependent cell activation was investigated by preincubating fibroblasts in the presence of 100 ng/ml pertussis toxin (List Biological Laboratories, Campbell, CA) for 16 h, followed by stimulation with 10^{-8} M PAF. Genistein (100 μg/ml; Sigma Chemical Co., St. Louis, MO) was incubated for 4 h to inhibit tyrosine kinases (38) before stimulation with 10^{-8} M PAF. To assess whether PKC activation (9, 10, 20, 21) is required in PAF-induced gene expression, fibroblasts were preincubated for 30 min in the presence of 10 mM polymyxin B or 10 mM staurosporine (Sigma) (10). The same preincubation steps were performed to analyze the effect of these inhibitors on PAF-mediated IL-6 and IL-8 protein expression.

PAF-stimulated Proliferation. The mitogenic activity of PAF and the effect of signal transduction inhibitors were assessed by incorporation of [^{3}H]thymidine (Amersham). Briefly, fibroblasts were seeded into 48-well cell culture plates (Falcon) and grown until 80% confluency. Fibroblast cultures were serum-deprived

for 48 h before being stimulated with 10^{-10} to 10^{-7} M PAF. Serum-deprived cells were pretreated with signal transduction inhibitors as described above before being stimulated with PAF. To exclude the possibility that other growth factors such as IL-6 or PDGF-BB are responsible for PAF-induced cell proliferation, we performed experiments using neutralizing monoclonal anti–PDGF-BB or anti–IL-6 antibodies (10 μg/ml, both from R & D). 18 h after stimulation, PAF-induced DNA de novo synthesis was assessed by addition of [^{3}H]thymidine (0.5 μCi/ml). 6 h later, [^{3}H]thymidine incorporation was stopped by washing the cells once with ice-cold PBS and fixation in methanol (twice, 5 min). After three washes with PBS, acid-soluble nucleotides were removed by a single wash with 0.1 M trichloric acid and three washes with H$_2$O. Subsequently, DNA was resolved in 0.3 M NaOH (15 min), and incorporated [^{3}H]thymidine was determined by liquid scintillation counting (39).

Statistical Analysis. Means ± SE for IL proteins were calculated on the basis of five different fibroblast cell lines, and each data point was determined in duplicate in each cell line. Statistical analysis of [^{3}H]thymidine incorporation was performed using Student's paired t test.

Results

Characterization of Human Lung Fibroblasts. Cultures of human lung fibroblasts were established from small lung biopsies. First outgrown cells could be observed between days 2–6 after setting up the cultures surrounding a piece of solid lung tissue. After the first passage of the primary cell cultures using trypsin-EDTA solution (0.05:0.02 wt/vol), morphological analysis by light microscopy revealed exclusively fibroblasts displaying a typical spindle shape morphology. Fig. 1 presents immunocytochemical characteristics of the cultured human lung fibroblast after three passages. In our cell preparations, all spindle-shaped cells stained positive for laminin and fibronectin, but were negative for immunostaining with mAbs against smooth muscle cell actin, keratin, Factor VIII, or von Willebrand factor. Therefore, no contaminations with cells such as smooth muscle cells, epithelial cells, or endothelial cells have been detected in any of the cell cultures used for the experiments.

PAF Receptor mRNA and Protein Expression by Human Lung Fibroblasts. As shown in Fig. 2 *a*, PAF receptor mRNA is expressed by human lung fibroblasts, as determined by a reversed transcriptase PCR (RT-PCR). Using specific primers corresponding to the sequence of the PAF receptor cDNA, a single PCR product with the expected size of 1,115 bp was amplified. Hybridization to a cDNA encoding for the human PAF receptor confirmed the identity of the PCR-amplified fragment (Fig. 2 *a*). Furthermore, the identity of the PCR fragment to the human PAF receptor was demonstrated by sequencing (data not shown).

To determine whether PAF receptor mRNA is also translated into protein, we applied a staining technique using anti–human PAF receptor antibody, followed by flowcytometric analysis. Human lung fibroblasts were positively stained with anti-PAF receptor antibody, as indicated by an
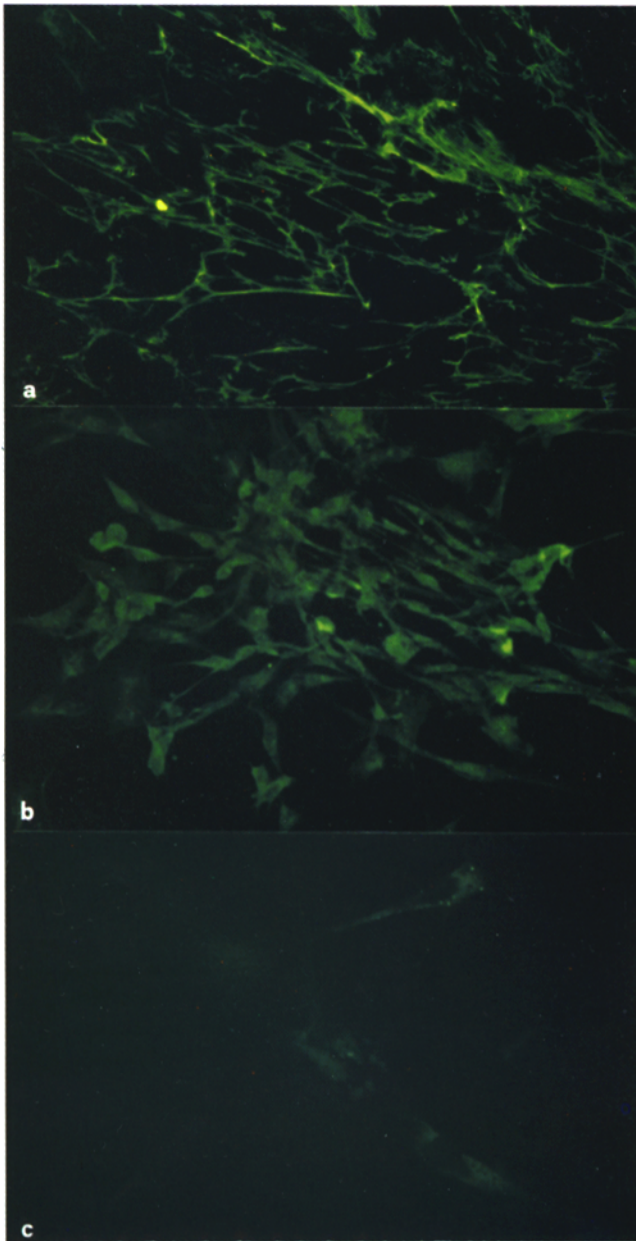
Figure 1. Immunohistological cell characterization. Immunohistological analysis of cultivated human lung fibroblasts by immunofluorescence. Cells were characterized using mAbs specific to (a) fibronectin, (b) laminin, or (c) von Willebrand factor, followed by FITC-conjugated second antibodies, as described in Materials and Methods.

increase of immunofluorescence compared to the control antibody (Fig. 2 b).

PAF Induces Increases in Tyrosine Phosphorylation in Human Lung Fibroblasts. Increases in tyrosine phosphorylation after PAF stimulation were previously observed in several cell types (17, 18, 40). Therefore, the expression of functional PAF receptors was assessed by the ability of PAF to induce increases in tyrosine phosphorylation of intracellular proteins. The addition of PAF to human lung fibroblasts led to rapid increases in tyrosine phosphorylation of several

intracellular proteins (Fig. 2 c). Tyrosine phosphorylation increased within 3 min, peaked at ~5 min, and declined to baseline levels within 30 min (Fig. 2 c). Thus, human fibroblasts express functional PAF receptors, being concordant with the observed PAF receptor mRNA and protein expression data.

PAF-induced Gene Expression. We determined PAF-induced expression of genes encoding for c-fos, c-jun, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, and IL-10 in human lung fibroblasts. PAF did not affect transcription of genes encoding IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, and IL-10 in cultivated human lung fibroblasts. At concentrations ranging from 10^{-7} to 10^{-10} M, PAF was capable of inducing the transcription of the c-fos, c-jun, IL-6, and IL-8 genes (Fig. 3). The kinetics of PAF-induced transcriptional changes were different for each of the four genes. The signal for mRNA coding for c-fos started 10 min after addition of PAF, with a peak at 30 min, and declined thereafter. Maximal amounts of mRNA coding for c-jun were observed 50–60 min after the addition of the stimulus. The transcription of the IL-6 gene peaked 1–2 h after the addition of PAF and declined thereafter. Furthermore, 4–6 h after the addition of PAF, mRNA expression of IL-8 was observed. The expression of the constitutive control gene (HLA-β) was not altered by PAF. Lyso-PAF failed to induce gene activation (data not shown). Pretreatment of fibroblasts with 10 μM actinomycin D abolished transcription of all four genes, suggesting that the increase of mRNAs is caused by de novo synthesis and not by accumulation of the respective mRNAs (data not shown).

PAF-induced transcription of IL-6 and IL-8 genes was followed by enhanced secretion of the corresponding proteins into the culture medium (Fig. 4). The PAF-induced secretion of both, IL-6 and IL-8, was dose-dependent. Protein concentrations of both ILs in the cell culture medium were significantly enhanced by PAF at concentrations $>10^{-10}$ M (Fig. 4). The stimulatory effect of PAF was maximal at a concentration of 10^{-8} M. Similar to the mRNA studies, lyso-PAF did not induce secretion of IL-6 or IL-8 protein, and PAF-induced secretion of the two ILs was inhibited in the presence of actinomycin D.

PAF Induces Gene Expression through Binding to its Specific Receptor. In the presence of the PAF receptor antagonists WEB 2170 (2×10^{-5} M), PAF-dependent transcription of c-fos, c-jun, IL-6, and IL-8 was abolished (Fig. 5). These results suggest that PAF-induced gene activation is mediated through interaction with its specific receptor.

PAF-induced Gene Expression Involves at Least Two Signaling Pathways. Analysis of the PAF receptor by cloning (8, 13, 14) and functional studies (15, 16) revealed that the receptor shows homology to G protein–coupled receptors. Therefore, we determined the effect of pertussis toxin on PAF-induced gene expression. As shown in Fig. 5, the transcription of c-fos, c-jun, IL-6, and IL-8 was abolished in the presence of pertussis toxin.

As depicted in Fig. 2, we observed increases in tyrosine phosphorylation in PAF-stimulated human fibroblasts. To
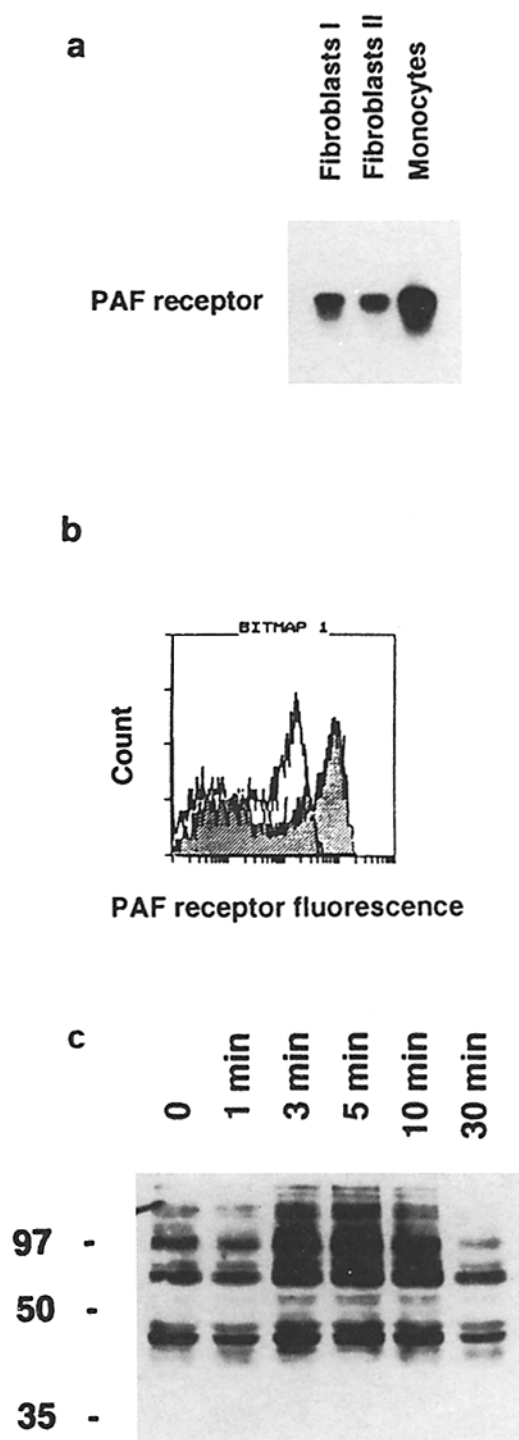
a

Fibroblasts I
Fibroblasts II
Monocytes

PAF receptor

b

BITMAP 1

Count

PAF receptor fluorescence

c

0   1 min   3 min   5 min   10 min   30 min

97 -

50 -

35 -

Figure 2. PAF receptor gene expression and activation of the PAF receptor induces tyrosine phosphorylation in cultivated human pulmonary fibroblasts. (a) PAF receptor mRNA is expressed in two preparations of human pulmonary fibroblasts. Purified human PBMC were used as positive control. Jurkat T cells were used as negative control (data not shown). mRNA expression was determined by combined PCR–Southern blotting technique, as described in Materials and Methods. Human β-actin amplification was equal (data not shown). (b) Surface PAF receptor protein expression on human pulmonary fibroblasts. Fibroblasts were stained with either specific polyclonal anti-PAF receptor antibody or nonspecific rabbit antibody, followed by second-step reagent (FITC-conjugated anti-

investigate a possible role for tyrosine kinases in PAF receptor–mediated gene activation, experiments with the tyrosine kinase inhibitor genistein were performed. Northern blot analysis revealed that PAF-stimulated transcription of c-fos, IL-6, and IL-8 genes was inhibited in the presence of genistein. In contrast, c-jun mRNA expression was not affected by this compound (Fig. 5). Similar results were obtained using polymyxin B to inhibit PKC activity (Fig. 5).

The data obtained with mRNAs encoding for IL-6 or IL-8 were confirmed at the protein level. As shown in Fig. 6, PAF-stimulated secretion of IL-6 and IL-8 was abolished in the presence of either one of the signal transduction inhibitors. In these experiments, we included a second PKC inhibitor, staurosporine (10 mM). Both PKC inhibitors, polymyxin B and staurosporine, showed similar inhibitory effects on the PAF-dependent secretion of IL-6 and IL-8 in human lung fibroblasts. Thus, tyrosine kinase and PKC, in addition to G protein activation, are essential for the expression of c-fos, IL-6, and IL-8 genes, but not that of c-jun.

PAF-induced Proliferation Depends on Tyrosine Kinase and PKC Activation. As demonstrated in Fig. 7 *a*, PAF induced a dose-dependent increase of de novo synthesis of DNA in cultivated human lung fibroblasts. Compared to unstimulated control cultures, the maximal mitogenic effect of PAF was observed at 10^{-7} to 10^{-8} M (+61%; Student's paired t test, $P <0.001$); lyso-PAF, at same concentrations, did not induce proliferation (Fig. 7 *a*). In the presence of 2–20 μM PAF receptor antagonist WEB 2170, PAF-mediated increase in proliferation was abolished (Student's paired t test, $P <0.005$), while WEB 2170 alone had only a minor effect on unstimulated control cells. These data further suggest that PAF interacts with its specific receptor in human fibroblasts. Moreover, incorporation of [^{3}H]thymidine was inhibited after pretreatment of fibroblasts with either genistein ($P <0.01$), pertussis toxin ($P <0.01$), or the PKC inhibitors staurosporine ($P <0.05$) or polymyxin B ($P <0.01$), while the compounds did not significantly alter spontaneous incorporation of [^{3}H]thymidine in unstimulated control cells (Fig. 7 *b*). Thus, similar to the c-fos, IL-6, and IL-8 gene expression data, tyrosine kinase and PKC activation is required, in addition to G protein activation, for PAF-induced proliferation.

To exclude the possiblity that the mitogenic effect of PAF is mediated by other growth factors such as PDGF (31) or IL-6 (37), we performed experiments in the presence of neutralizing monoclonal anti–PDGF-BB or anti–IL-6 antibodies. As depicted in Fig. 7 *c*, the neutralizing antibodies had no significant inhibitory effect in this system, suggesting that PAF-induced proliferation of human pulmonary

rabbit Ab). This figure is representative of two other experiments that yielded the same results. (c) PAF induces tyrosine phosphorylation of several cellular substrates in human pulmonary fibroblasts. Fibroblasts were stimulated with 10^{-7} M PAF for the indicated times. Tyrosine phosphorylation was determined by immunoblotting using an anti-ptyr antibody. Units are in kilodaltons.
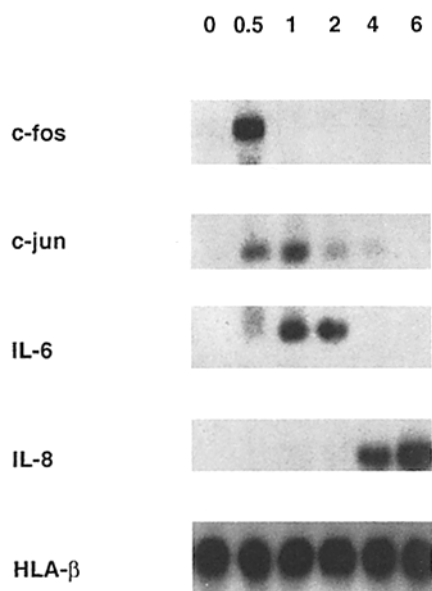
0 0.5 1 2 4 6

c-fos

c-jun

IL-6

IL-8

HLA-β

Figure 3. PAF induces gene transcription in fibroblasts. Kinetics of PAF-induced (10^{-8} M) gene transcription in cultivated human pulmonary fibroblasts by Northern blot analysis. The picture displays a characteristic time course in cell line L032; similar results were obtained with the nine other cell lines.

fibroblasts is not a consequence of PAF-induced growth factor mobilisation.

Discussion

PAF has been implicated in the pathogenesis of airway inflammation and mucus hypersecretion (1–3, 30, 41–44). Especially in pathophysiological events associated with asthma, PAF apparently plays a crucial role (42). For example, it has been demonstrated that circulating PAF levels are increased during acute asthmatic attacks (2, 6, 7). An enhanced release of PAF from granulocytes isolated from asthmatic patients has been reported (6, 7). In addition, platelets isolated from asthmatic individuals displayed an enhanced responsiveness to exogenous PAF (45–47). Furthermore, an increased expression of PAF receptor mRNA in asthmatic lung tissue has been observed (3, 9, 43, 48). Since fibroblasts represent a large portion of solid lung tissue and are situated adjacent to both airways and blood vessels, these cells are likely to participate in inflammatory processes and remodelling of the lung (23, 28, 29). However, direct stimulatory action of PAF on human lung fibroblasts has not yet been demonstrated. Therefore, we characterized PAF receptor gene expression, signal transduction pathways, the induction of protooncogenes and IL genes, as well as functional consequences of PAF receptor stimulation, in human lung fibroblasts.

Our results provide several lines of evidence for an involvement of fibroblasts in PAF-induced inflammatory responses of the lung. Using RT-PCR and flow cytometry, we have shown that cultivated lung fibroblasts express PAF receptor mRNA and protein for the recently cloned PAF

receptor. Furthermore, we have demonstrated the functional integrity of the PAF receptor and its coupling to pertussis toxin–sensitive G proteins, tyrosine kinases, and PKC in these cells. Moreover, PAF induced the transcription of genes encoding c-fos, c-jun, IL-6, and IL-8, and stimulated the proliferation of lung fibroblasts. The specificity of receptor–ligand interaction was confirmed using a PAF receptor antagonist, WEB 2170. In the presence of this compound, PAF receptor–mediated signaling, gene expression, and proliferation were inhibited. The specificity was further confirmed by the fact that lyso-PAF, a biological ineffective PAF analogue, did not exert any effect on human lung fibroblasts.

PAF stimulates tyrosine kinases as monitored by tyrosine phosphorylated proteins in human neutrophils (17), mono-
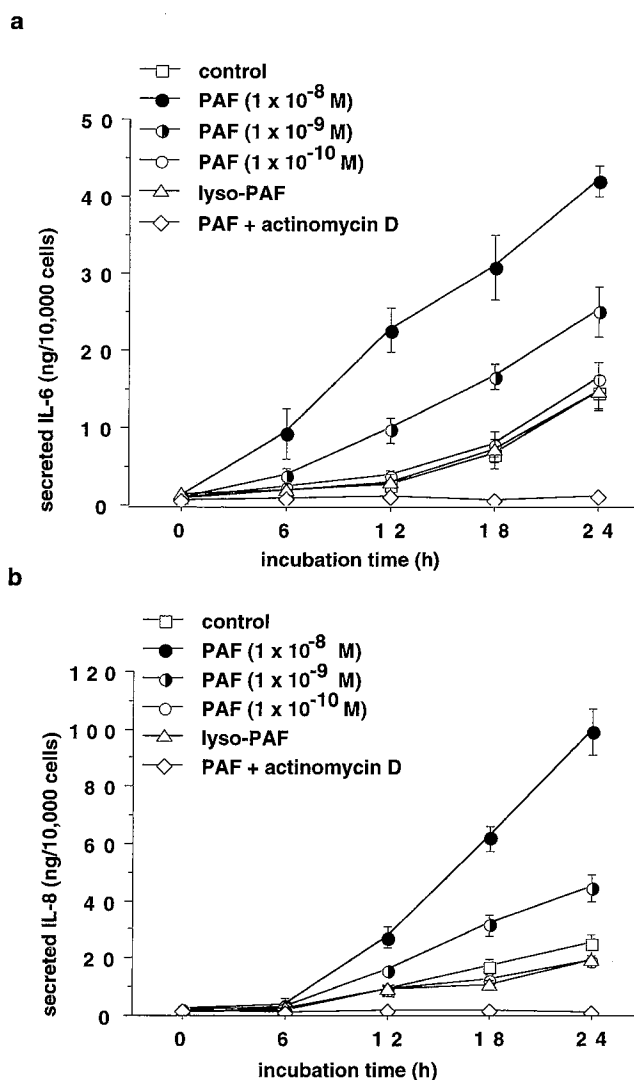

Figure 4. PAF induces secretion of IL-6 and IL-8 from fibroblasts. Kinetics of PAF-induced (10^{-8} M) secretion of IL-6 (a) and IL-8 (b) in cultivated human pulmonary fibroblasts. The amount of secreted interleukins was determined in 100 µl of culture medium obtained from subconfluent cultures of pulmonary fibroblasts (10^5 cell). Each data point represents mean ± SE of 30 independent experiments using 10 different cell lines.

cytes, B lymphoblastoid cells (18), and mouse fibroblasts (22). Therefore, we investigated whether stimulation of lung fibroblasts with PAF also results in tyrosine kinase activation. Indeed, PAF stimulated the tyrosine phosphorylation of several intracellular proteins. This suggests that the PAF receptor expressed on human lung fibroblasts is coupled to tyrosine kinases.

A central question is how the PAF receptor, which represents a member of the G protein–linked receptor superfamily (8, 9, 13, 15, 16, 40, 46), could activate one or more tyrosine kinases. We confirmed the linkage of the PAF receptor to G proteins since pertussis toxin blocked PAF-induced gene expression and proliferation. This indicates that direct activation of tyrosine kinases by the ligand-bound receptor and bypassing of G proteins may not occur in these cells. However, tyrosine kinases may be activated via a pertussis-sensitive G protein (40, 47). Our strategy to determine the relevance of tyrosine kinase activation in PAF-induced fibroblast activation was to use the tyrosine kinase inhibitor genistein in our systems. Genistein is known to specifically inhibit the activity of tyrosine kinases, whereas it has marginal effects on several serine/threonine kinases (38). The finding that genistein and PKC inhibitors blocked PAF-induced proliferation and expression of c-fos, IL-6, and IL-8, but not of c-jun, suggests a functional important signaling pathway involving the activation of G proteins, tyrosine kinases, and PKC. In contrast, PAF-induced transcription of c-jun is only sensitive to pertussis toxin, but

not to genistein or polymyxin B, indicating the existence of a PAF-inducible signaling pathway, which bypasses tyrosine kinases and PKC activation in human fibroblasts.

We have shown a rapid and transient transcriptional activation of c-fos and c-jun by PAF, as assessed by Northern blot analysis. The kinetics of the PAF-induced transcription of both transcription factors are similar to that observed with other mitogens such as angiotensin II (32) or PDGF isoforms (36). PAF-induced expression of both genes suggests that this phospholipid is a potent regulator of cell growth and differentiation. The protooncogenes c-fos and c-jun are members of the class of inducible genes termed cellular immediate early genes (48). Both protooncogenes encode proteins that are capable of forming a protein complex (AP-1) that binds specifically to transcriptional control elements (49). The fact that PAF is capable of upregulating transcription of c-fos and c-jun mRNA indicates a profound action of PAF on gene activation in fibroblasts. Our results are in agreement with recent studies demonstrating that PAF stimulates the expression of c-fos in human lymphoblastoid cells (19) and mouse A-431 cells (22).
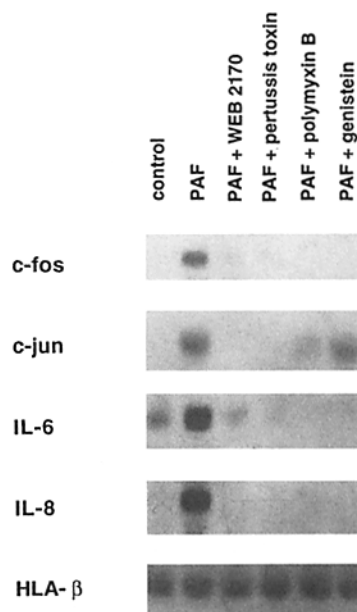

Figure 5. Intracellular signaling pathway in PAF-induced gene activation in fibroblasts. Effects of signal transduction inhibitors on PAF-induced gene transcription by Northern blot analysis. mRNAs were isolated at the time points of maximal transcription: c-fos, 30 min; c-jun, 60 min; IL-6 60 min; or IL-8, 4 h. The concentrations used were (a) PAF, 10^{-8} M; (b) WEB 2170 2 × 10^{-5} M; (c) pertussis toxin, 100 ng/ml; (d) genistein, 100 μg/ml; (e) polymyxin B, 10 mM. The picture displays a characteristic time course in cell line L035; similar results were obtained with the other nine cell lines.
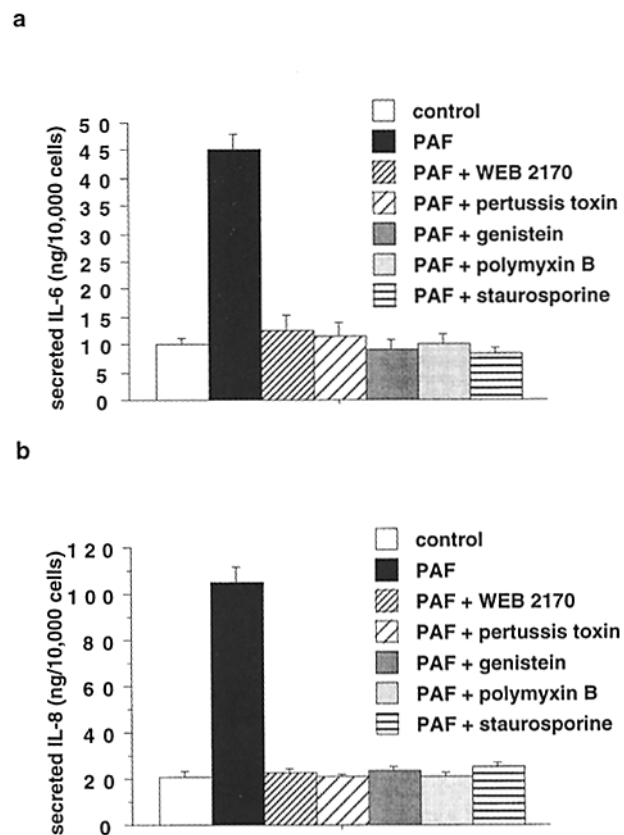

Figure 6. Intracellular signaling pathways involved in PAF receptor mediated interleukin secretion. Effects of signal transduction inhibitors on PAF-induced secretion of IL-6 or IL-8 in cultivated transduction inhibitors, as described in legend to Fig. 5. In addition, a second inhibitor of PKC, staurosporine (10 mM), was used. The amount of secreted interleukins was determined in 100 μl of culture medium that was obtained from subconfluent cultures of pulmonary fibroblasts (10^5 cell) 20 h after the addition of PAF. Each data point represents mean ± SE of 10 independent experiments.
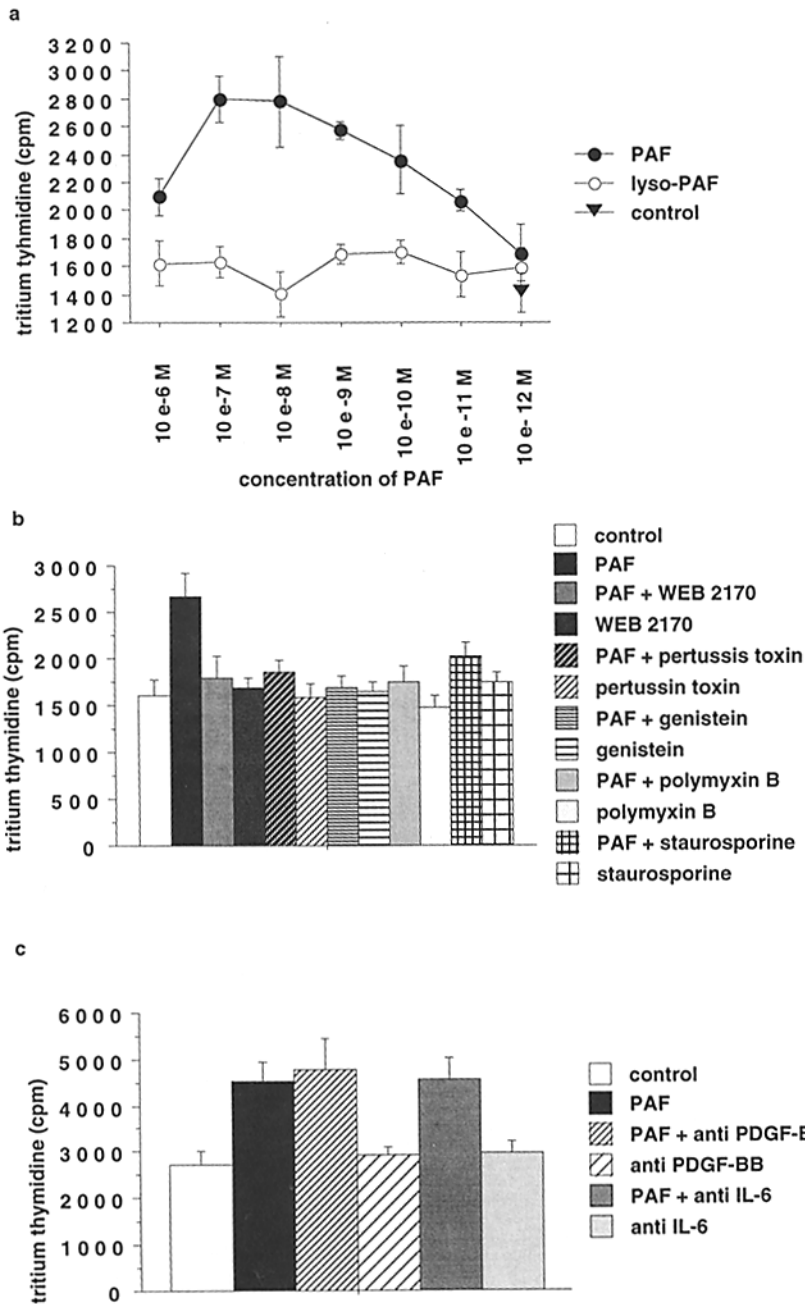
a

b

c

Figure 7. Mitogenic activity of PAF and its control. (a) Dose dependency of PAF-induced [³H]thymidine incorporation by human lung fibroblasts. (b) Inhibitory effects of various signal transduction inhibitors on PAF-induced de novo synthesis of DNA in cultivated human pulmonary fibroblasts. Fibroblasts were treated with the same concentrations of signal transduction inhibitors as described in legends to Figs. 5 and 6. (c) Effect of neutralizing anti–PDGF-BB and anti–IL-6 antibodies on PAF-dependent [³H]thymidine incorporation. The incorporated [³H]thymidine by pulmonary fibroblasts (10⁵ cell) was determined by liquid scintillation counting 24 h after the addition of PAF.

Connective tissue cells such as fibroblasts respond to cytokines and inflammatory mediators, including PAF (28, 29, 48). We have demonstrated earlier that human lung fibroblasts are capable of producing IL-6 after stimulation with the three PDGF isoforms (37). Comparing the potency of PDGF isoforms to equimolar concentrations of PAF, one has to note that PAF exerts a lower potency on stimulating the expression of the IL-6 gene in these cells. The observation that PAF stimulates the expression of IL-6 and IL-8 in cultivated human lung fibroblasts has some important implications for PAF-mediated inflammatory mechanisms in the human lung. IL-6 has been shown to induce a

variety of proinflammatory effects that are relevant to inflammation of the airways, including its ability to stimulate proliferation of thymocytes and T cells (49, 50), to stimulate cytotoxic T lymphocyte differentiation (51), to upregulate IL-4–dependent IgE production (52), and to mediate the terminal differentiation and Ig production of B cells (49). The proinflammatory effects of IL-8 are reflected by its ability to recruit neutrophils and eosinophils into inflammatory sites and to modulate the expression of various adhesion molecules in bronchial epithelial cells (53, 54) and lung macrophages (55). IL-8 also augments the production of proteases by neutrophils (56), generation of oxygen radi-

cals (57), and activity of 5-lipoxygenase in PMN cells (58). Our data obtained in vitro are consistent with the clinical observation of elevated levels of IL-6 and IL-8 in the bronchoalveolar lavages and biopsies of symptomatic asthmatic individuals (59, 60).

The finding that PAF stimulates the proliferation of cultivated human lung fibroblasts may reflect a key reaction of the cells to inflammatory mediators. It may contribute to the structural abnormalities found in asthmatic airways, namely to subepithelial fibrosis (30). Similar to the data obtained on PAF-induced expression of the IL-6 gene, the efficacy of PAF on cell proliferation is about 5 to 10 times lower compared to equimolar concentrations of PDGF-BB on the same cell type (31, 32, 36, 37). However, the PAF-induced proliferation of human lung fibroblasts does not involve the secreted IL-6 or other mitogens such as PDGF as we have demonstrated by the use of neutralizing anti–IL-6 or anti–PDGF-BB antibodies. Preliminary experiments using an antisense strategy to inhibit the expression of c-fos, NF-κB, or IL-6 revealed that the mitogenic potency of PAF is abolished in the presence of effective concentrations of an-tisense oligonucleotides directed against c-fos, NF-κB, or IL-6 mRNAs. Consistent with earlier studies on the mitogenic action of PDGF, our observation indicates that the three factors, c-fos, NF-κB, and IL-6, are essentially involved in the PAF-induced cell cycle progression (37; our unpublished observation).

In conclusion, PAF activation of human pulmonary fibroblasts, one of the most abundant cell types in the body, may amplify chronic inflammatory responses. We have demonstrated that human lung fibroblasts express functional PAF receptors that mediate PAF-induced transcription of c-fos, IL-6, and IL-8 genes upon binding of PAF. The signaling cascade resulting in transcription of the genes involves G proteins, tyrosine kinases, and PKC. In contrast, PAF-stimulated transcription of c-jun requires pertussis toxin–sensitive signaling pathways only. Thus, PAF plays a profound role in affecting human lung fibroblast physiology. Our data also support a model for a network of communication between structural tissue and inflammatory cells that collectively function to mediate pulmonary inflammation such as bronchial asthma.

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