Acidic Fibroblast Growth Factor Induces an Antifibrogenic Phenotype in Human Lung Fibroblasts

Carina Becerril, Annie Pardo, Martha Montaño, Carlos Ramos, Remedios Ramírez, and Moisés Selman

Instituto Nacional de Enfermedades Respiratorias; and Facultad de Ciencias, Universidad Nacional Autónoma de México, México DF, México

Acidic fibroblast growth factor (FGF-1), a prototype member of the heparin-binding growth factor family, influences proliferation, differentiation, and protein synthesis in different cell types. However, its possible role on lung extracellular matrix (ECM) metabolism has not been evaluated. In this study we examined the effects of FGF-1 and FGF-1 plus heparin on type I collagen, collagen-binding stress protein HSP47, interstitial collagenase (matrix metalloproteinase [MMP]-1), gelatinase A, and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 expression by normal human lung fibroblasts. Heparin was used because it enhances the biologic activities of FGF-1. Fibroblasts were exposed either to 20 ng/ml FGF-1 plus 100 μg/ml heparin for 48 h or to FGF-1 or heparin alone. Messenger RNA (mRNA) expression was analyzed by Northern blot. Collagen synthesis was evaluated by digestion of [^{3}H]collagen with bacterial collagenase, MMP-1 by Western blot, and gelatinolytic activities by zymography. Our results show that FGF-1 induced collagenase mRNA expression, which was strongly enhanced when FGF-1 was used with heparin. Likewise, both FGF-1 and FGF-1 plus heparin reduced by 70 to 80% the expression of type I collagen transcript, in part through effect on pro-α1(I) collagen mRNA stability. A downregulation of HSP47 gene expression was also observed. Synthesis of collagen and collagenase proteins paralleled gene expression results. FGF-1 activities were abolished with genistein, a tyrosine kinase inhibitor. Neither FGF-1 nor FGF-1 plus heparin affected the expression of TIMP-1, TIMP-2, and gelatinase A. These findings demonstrate that FGF-1, mostly in the presence of heparin, upregulates collagenase and downregulates type I collagen expression that might have a protective role in avoiding collagen accumulation during lung ECM remodeling. **Becerril, C., A. Pardo, M. Montaño, C. Ramos, R. Ramírez, and M. Selman. 1999. Acidic fibroblast growth factor induces an antifibrogenic phenotype in human lung fibroblasts. Am. J. Respir. Cell Mol. Biol. 20:1020–1027.**

Fibroblast growth factors (FGFs) are a class of heparin-binding proteins that constitute a multigene family of nine structurally related polypeptides. They have a broad spectrum of functions on various cell types, mediating their effects by binding to a family of high-affinity cell surface receptors with tyrosine kinase activity (1, 2).

Acidic FGF (FGF-1) has been extensively characterized at the molecular and biologic levels; FGF-1 and basic FGF (FGF-2) are regarded as prototypes of the FGF family. FGF-1 is a single-chain polypeptide of 155 amino acids with a molecular mass of $\sim$ 17 kD encoded by a single copy gene located on chromosome 3 (3). FGF-1 is a potent angiogenic factor and influences proliferation, differentiation, and other cellular activities in a variety of cell types (2, 4). Because FGF-1 is present in the extracellular matrix (ECM) in association with heparin sulfate proteoglycans, it has been suggested that FGF-1 may act as a local regulator for cell growth and differentiation during a number of physiologic processes, such as embryogenesis, angiogenesis, and tissue repair (2–5). However, although this growth factor is capable of inducing several mesenchymal cell functions (6, 7), its possible role on lung ECM metabolism has not been evaluated.

We have recently found that both FGF-1 and its receptor are upregulated during the development of experimental diffuse lung fibrosis (8). Macrophages and epithelial cells from rats exposed to paraquat plus hyperoxia ex-

pressed FGF-1 from the first week on, showing an increased upregulation after several weeks of injury. Interestingly, we occasionally observed that in the same areas where fibrotic lesions were present, macrophage expression of FGF-1 was mainly localized in foci where normal architecture was still present, suggesting that FGF-1 might be playing a protective role.

Supporting this point of view, FGF-1 appears to downregulate collagen gene expression in keloid fibroblasts (7, 9), and increased expression of this factor has been observed within atherosclerotic lesions, concomitant with decreased expression of $\alpha 1(I)$, $\alpha 2(I)$, and $\alpha 1(III)$ collagen messenger RNA (mRNA) levels (10). Therefore, we hypothesized that FGF-1 could have antifibrogenic properties.

To examine this hypothesis, we evaluated the possible roles of FGF-1 and of FGF-1 plus heparin on pro-$\alpha 1$ type I collagen; HSP47, a collagen-specific molecular chaperone binding stress protein; interstitial collagenase (matrix metalloproteinase [MMP]-1); gelatinase A (MMP-2); tissue inhibitor of metalloproteinase (TIMP)-1; and TIMP-2 expression by normal human lung fibroblasts.

Heparin was used in this study because it has been shown to increase the effect of FGF-1 greatly, probably changing the conformation of this growth factor and thus increasing its association contact for its receptor (11). Additionally, heparin stabilizes FGF-1, a function especially relevant for human FGF-1, which exhibits an unfolding transition at physiologic temperatures in the absence of polyanions (12).

Materials and Methods

Fibroblast Culture

Human lung fibroblast-like cells were obtained in our laboratory as previously described (13). Briefly, cells were derived from individuals having lobectomy or total lung resection for removal of a primary lung tumor; no morphologic evidence of disease was found in the tissue samples used for fibroblast isolation. Lung fibroblasts were isolated by trypsin dispersion, and cells were grown in F-12 (Ham's) medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum. The cells were cultured at 37°C in 5% CO_2/95% air in T-25 cm^2 Falcon flasks, using F-12K medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin. When fibroblasts reached early confluence, the medium was replaced with serum-free F-12K medium (SFM) containing either 20 ng/ml human recombinant FGF-1 (Sigma, St. Louis, MO), or 20 ng/ml FGF-1 plus 100 μg/ml heparin (Sigma), and the cells were incubated for 48 h. Serum-free cultures or 100 μg/ml heparin–treated fibroblasts were used as controls. Fibroblasts were obtained for RNA isolation and Northern blot analysis, and conditioned media were collected and stored at -20°C until assayed for Western blot analysis.

In parallel experiments, the time course of the effects of FGF-1 plus heparin on type I collagen and collagenase expression was also analyzed. For this purpose, stimulated cells were incubated for 1, 6, 12, 24, and 48 h and then collected for Northern blot analysis.

To analyze signal transduction pathways, fibroblasts were cultured for 1 h with either 25 μg/ml genistein (Sigma), a tyrosin kinase inhibitor, or 5 μm/liter bisindolylmaleimide (Boehringer Mannheim, Mannheim, Germany), a protein kinase C (PKC) inhibitor. Afterwards, cells were cultured in the presence of 20 ng/ml FGF-1 plus 100 μg/ml heparin for 48 h.

Complementary DNA Probes

Complementary DNA (cDNA) clones for human collagenase (MMP-1), TIMP-1, $\alpha 1(I)$ collagen, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the American Type Culture Collection (Rockville, MD). Human cDNA for collagenase type IV 72K (3,000 base pairs [bp]) cloned in a pBluescript vector was kindly provided by G. I. Goldberg (Washington University, St. Louis, MO). A 1.5-kb *Hind*III-*Eco*RI fragment of mouse HSP47 cDNA was kindly provided by Nobuko Hosokawa (Kyoto University, Kyoto, Japan). Human TIMP-2 (690 bp) cloned in pBluescript was kindly provided by Dylan Edwards (University of Calgary, Calgary, AB, Canada).

RNA Isolation and Northern Blot Analysis

Total cellular RNA from lung fibroblasts was isolated by the acid guanidinium thiocyanate/phenol chloroform extraction method (14). Total RNA (15 μg/lane) was fractionated on a 1% agarose gel containing 0.66 M formaldehyde. Ribosomal RNA (rRNA) was visualized with ethidium bromide, and the fractionated RNA was transferred onto a Nytran transfer membrane by capillary blotting overnight. RNA was immobilized by baking at 80°C for 2 h, and then prehybridized at 42°C for 18 h in 5× saline sodium citrate (SSC), 50% formamide, 5× Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS), containing 100 μg/ml of denatured salmon-sperm DNA. Hybridization was carried out at 42°C for 18 h in hybridization buffer containing 50% dextran sulfate plus heat-denatured [^{32}P]-labeled cDNA probes. Membranes were washed in 2× SSC/0.1% SDS at 42°C for 25 min, followed by 0.25× SSC/0.1% SDS at 55°C twice for 15 min, and 0.1× SSC/0.1% SDS at 65°C twice for 15 min. After drying, membranes were exposed to Kodak BIOMAX MS film (Rochester, NY) at -70°C with an intensifying screen. Equal loading of RNA samples was monitored by assessing the mRNA level of GAPDH. The cDNA probes were radiolabeled with a [^{32}P]deoxycytidine triphosphate to specific activity of 200×10^6 disintegrations per minute (dpm)/μg using a multiprime DNA labeling kit (Dupont NEP-103, Wilmington, DE).

RNA Stability Experiments

In certain experiments, actinomycin D (final concentration of 12.5 μg/ml) was added to serum-free subconfluent cultures to stop gene transcription. Fibroblasts were removed before actinomycin addition as a zero-time control. Actinomycin-treated cells, with or without exposure to 20 ng/ml FGF-1 plus 100 μg/ml heparin, were removed for total RNA isolation at 6, 12, 24, and 48 h after beginning of treatment. In another set of experiments, total RNA from cells exposed to FGF-1 plus heparin without actinomycin was isolated at the same time points. Northern hybridiza-

tion analysis was performed using $\alpha 1$-type I collagen cDNA. Differences in RNA loading were normalized using a cDNA probe for 18S rRNA. Collagen signal quantitated by densitometry was divided by the 18S rRNA signal and was expressed as the percentage of the zero-hour control.

Western Blot Analysis

Serum-free conditioned media were centrifuged at $300 \times g$ at 4°C for 30 min to remove cell debris, and were concentrated by lyophilization. Samples were solubilized in 500 μl of distilled water, and 10 μl were mixed with the same volume of Laemmli sample buffer and electrophoresed on 10% SDS–polyacrylamide gel electrophoresis (PAGE) (15). Western transfer to nitrocellulose filter was performed at 70 V for 3 h. After the nonspecific sites were blocked overnight with 4% (wt/vol) nonfat dried milk in phosphate-buffered saline (PBS), the membrane was incubated with antihuman interstitial collagenase rabbit immunoglobulin G (1:250 dilution in PBS/1% bovine serum albumin [BSA]) for 2 h at room temperature. Anticollagenase antibody was kindly provided by Eugene Bauer (Stanford University, CA) (16). Reaction with secondary antibody conjugated to peroxidase was for 1 h at room temperature. Finally, the filter was incubated for 15 to 20 min at room temperature in PBS containing 0.15% H_2O_2, 15% (vol/vol) methanol, and 4-chloro-naphthol at 6 mg/ml until the color developed.

SDS–PAGE Zymography

SDS polyacrylamide gels containing gelatin (1 mg/ml) were used to identify proteins with gelatinolytic activity from fibroblast-conditioned media. After electrophoresis, gels were placed in a solution of 2.5% Triton X-100 (2 $\times$ 15 min), washed extensively with water, and incubated overnight at 37°C in glycine (100 mM, pH 7.6) containing 10 mM $CaCl_2$ and 50 nM $ZnCl_2$. Identical gels were incubated in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA). Gels were stained with Coomasie Brilliant Blue R250 and destained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzymatic activity appeared as clear bands against a blue blackground. Molecular weights of the gelatinolytic bands were estimated by using prestained molecular-weight marker (Bio-Rad, Melville, NY).

Collagen Synthesis Assay

Collagen synthesis was evaluated according to Peterkofsky and Diegelmann (17). Cells were cultured in six-well tissue-culture plates with either FGF-1, heparin, or FGF-1 plus heparin in serum-free medium as described above. After 40 h, stimulation medium was replaced by fresh SFM containing 15 μCi/ml of [^{3}H]proline (New England Nuclear, Boston, MA), 50 μg/ml ascorbic acid, and 50 μg/ml β-aminopropionitrile. At the end of 8 h labeling, supernatants were collected on ice and dialyzed against distilled water containing protease inhibitors (25 mM N-ethylmaleimide, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA) and 0.02% sodium azide. To eliminate protease inhibitors, samples were dialyzed against deionized water,

lyophilized, and resuspended in Tris-HCl buffer, pH 7.6 (50 mM Tris-HCl and 10 mM $CaCl_2$). Aliquots were incubated with or without 36 μg/ml ultrapure bacterial collagenase (Sigma type VII) for 3 h at 37°C, and then were precipitated with cold 10% trichloracetic acid (TCA) and 0.5% tannic acid in the presence of 100 μl BSA, and washed twice in 5% TCA/0.25% tannic acid at 4°C. Supernatants were resuspended in 10 ml of scintillation counting fluid (Aquasol; New England Nuclear) to evaluate collagenous protein synthesis. The precipitates were hydrolyzed in 1 ml HCl, 6 N, for 24 h at 70°C, and also resuspended in 10 ml scintillation counting fluid for measurement of noncollagenous protein synthesis. Samples were counted in a scintillation counter (Beckman LS6000 SE, Palo Alto, CA). Collagen synthesis percent was calculated according to the following formula: % collagen synthesis = (dpm collagen $\times$ 100)/(dpm noncollagen protein $\times$ 5.4) + (dpm collagen).

In addition, hydroxyproline colorimetric analysis was performed in three independent experiments. For this purpose, conditioned media derived from four T-25 flasks of control and FGF-1 plus heparin–treated fibroblasts were pooled, dialyzed against distilled water, lyophilized, and resuspended in 1 ml distilled water. After acid hydrolysis, hydroxyproline was measured as described by Woessner (18), and results were expressed as micrograms of OH-proline per 10^6 cells per 48 h.

Results

Effect of FGF-1 plus Heparin on $\alpha 1$(I) Collagen, Interstitial Collagenase, and Stress Protein HSP47 Gene Expression

The effect of FGF-1 on pro-$\alpha 1$(I) collagen and HSP47 gene expression was examined by Northern blot analysis (Figure 1). Human lung fibroblasts grown at early confluence were incubated for 48 h in serum-free medium containing either 20 ng/ml FGF-1 plus heparin, 20 ng/ml FGF-1, or 100 μg/ml heparin. As compared with control SFM cultures, incubation of the cells with both FGF-1 alone and FGF-1 plus heparin resulted in a marked downregulation of pro-$\alpha 1$(I) collagen transcript levels. Heparin alone did not show any effect. When the signal of pro-$\alpha 1$(I) collagen mRNA was normalized by the level of GAPDH mRNA and quantified by densitometry from three different experiments, a reduction of 70 to 80% was noticed. HSP47 mRNA expression also revealed a significant decrease when fibroblasts were exposed to FGF-1 with and without heparin, thus paralleling pro-$\alpha 1$(I) collagen gene expression. The HSP47/GAPDH ratio for control, untreated fibroblasts decreased approximately 50% in the presence of FGF-1 and FGF-1 plus heparin.

By Northern hybridization, no MMP-1 transcript was detected in untreated fibroblasts cultured in SFM. Treatment with FGF-1 plus heparin strongly induced collagenase mRNA expression in all the tested fibroblast cell lines (Figure 1). Incubation with FGF-1 alone slightly induced MMP-1 mRNA expression. By contrast, exposure of fibroblasts to heparin did not induce expression of MMP-1.
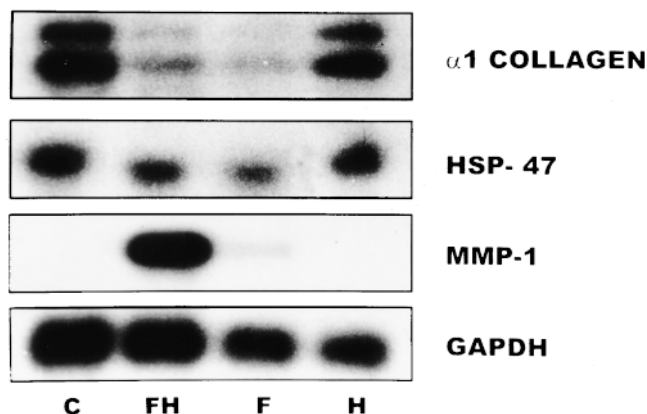
Figure 1. Effects of FGF-1, heparin, and FGF-1 plus heparin on α-1 type I collagen, HSP47, and MMP-1 gene expression. Representative Northern blot of 15 μg total cellular RNA per lane extracted from control (C) and stimulated fibroblasts. At early confluence, fibroblasts were cultured in serum-free medium and treated with 20 ng/ml FGF-1 plus 100 μg/ml heparin (FH), 20 ng/ml FGF-1 (F), or 100 μg/ml heparin (H) for 48 h. Both FGF-1 plus heparin and FGF-1 alone induced a downregulation in the expression of α-1(I) collagen and HSP47. Collagenase (MMP-1) was strongly upregulated by FGF-1 plus heparin.

Effect of FGF-1 plus Heparin on Collagen Biosynthesis and MMP-1 at the Protein Level

To determine whether the effect of FGF-1 plus heparin on α1(I) procollagen mRNA was reflected on procollagen biosynthesis, [³H]-labeled collagen sensitive to purified bacterial collagenase was measured. A significant reduction of collagen synthesis was observed after exposure to FGF-1, FGF-1 plus heparin, and even heparin alone. Al-
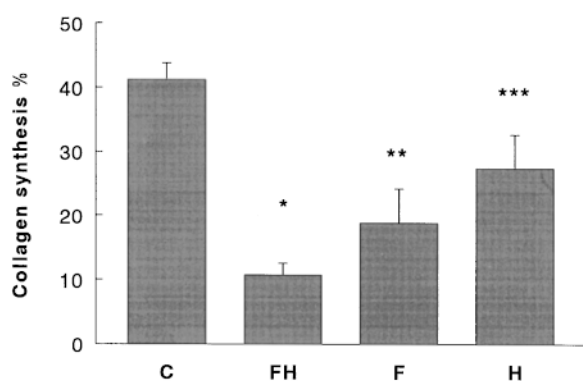

Figure 2. Effects of FGF-1 (F), heparin (H), and FGF-1 plus heparin (FH) on collagen biosynthesis. After 40 h stimulation in serum-free medium, triplicate wells of subconfluent cultures were incubated for an additional 8 h in fresh serum-free medium containing 15 μCi/ml of [³H]proline. Supernatants were collected, dialyzed against distilled water, and precipitated with cold 10% TCA/0.5% tannic acid. The precipitates were hydrolyzed in 1 ml HCl, 6 N, for 24 h at 70°C. Percent of collagen synthesis was calculated according to Peterkofsky and Diegelmann (17), as described in MATERIALS AND METHODS. *$P = 0.00025$; **$P = 0.003$; ***$P = 0.007$.

though basal levels of collagen production (expressed as percentages of protein synthesis) varied in the different cell lines, the magnitude of the response to the different stimuli was similar. Figure 2 illustrates the results obtained with one fibroblast cell line. In addition, total nonradiolabeled hydroxyproline content, measured in three different pools of conditioned media, showed a reduction of ∼ 25% in those samples treated with FGF-1 plus heparin as compared with controls (0.77 ± 0.06 versus 0.58 ± 0.05 μg OH-proline/10^6 fibroblasts, $P < 0.01$).

To examine the effect on MMP-1 at the protein level, immunoreactive collagenase was analyzed by Western blot in the conditioned media of cell lines under basal conditions and after stimulation with FGF-1, heparin, and FGF-1 plus heparin (Figure 3). A barely detectable level of MMP-1 was present in basal conditions; however, treatment with FGF-1 plus heparin induced a marked increase of interstitial collagenase in the medium. FGF-1 alone also induced an increase of MMP-1, whereas heparin exhibited no effect.

Effect of FGF-1 plus Heparin on Gelatinase A

To explore whether FGF-1 plus heparin provoked a change in MMP-2 gene expression, or gelatinase A activity, Northern blot analysis and gelatin zymography were performed. Under basal conditions, human lung fibroblasts expressed MMP-2 (Figure 4A). Exposure to FGF-1 alone or in combination with heparin had no detectable effect on gelatinase A mRNA gene expression as compared with GAPDH transcript levels.

Proteins secreted into the medium were also analyzed by gelatin zymography (Figure 4B). As seen with gelatinase A expression, no effect of FGF-1 alone or with heparin was observed on the 72-kD activated latent form of gelatinase A. Gelatinase activity was also observed at ∼ 52 kD, mainly in FGF plus heparin–stimulated fibroblasts (Figure 4B, *lane 2*). This gelatinolytic band was not attributable to collagenase-3 (MMP-13) because Northern blot analysis failed to reveal the mRNA expression. Therefore, this 52-kD gelatinolytic activity presumably represents the activated latent form of MMP-1. EDTA inhibited all gelatinolytic bands (not shown).

Effect of FGF-1 plus Heparin on TIMP-1 and TIMP-2 Gene Expression

Under basal conditions, human lung fibroblasts expressed a 0.9-kb TIMP-1 transcript. TIMP-2 was also expressed in
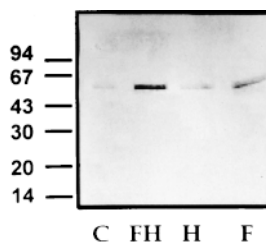

Figure 3. Effects of FGF-1, heparin, and FGF-1 plus heparin on MMP-1 at the protein level. Immunoblotting of conditioned media obtained from the same cell line. Media from control (C) and treated cultures were subjected to Western blot analysis, and interstitial collagenase (MMP-1) was detected using an antihuman collagenase antibody and a peroxidase detection system.
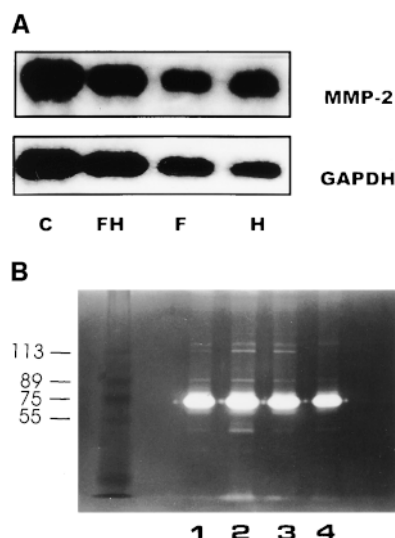
Figure 4. Effects of FGF-1, heparin, and FGF-1 plus heparin on MMP-2. (*A*) Northern blot analysis of 15 μg total cellular RNA per lane. At early confluence, fibroblasts were cultured in serum-free medium and treated with 20 ng/ml FGF-1 plus 100 μg/ml heparin (FH), 20 ng/ml FGF-1 (F), or 100 μg/ml heparin (H) for 48 h. (*B*) Gelatin zymography. Aliquots of conditioned medium were subjected to 8% SDS–PAGE containing 0.1% gelatin under nonreducing conditions. Following electrophoresis, gels were incubated overnight at 37°C in 100 mM glycine, pH 7.6, containing 10 mM $CaCl_2$ and 50 nM $ZnCl_2$. Gels were stained with Coomasie Brilliant Blue. *Lane 1*, control sample; *lane 2*, FGF-1 plus heparin; *lane 3*, FGF-1; *lane 4*, heparin. Sizes of prestained molecular-weight markers are indicated on the *left*.

basal conditions showing a 3.5-kb transcript and a slightly detectable 1.0-kb transcript. None of the treatments revealed any apparent effect on TIMP-1 and TIMP-2 mRNA gene expression when normalized to GAPDH levels (Figure 5).

Time-Course Expression of MMP-1 and Type I Collagen

To compare the kinetics of FGF-1 plus heparin effects on α1(I) collagen and interstitial collagenase, the time-course gene expression was determined by Northern blot analysis. Fibroblasts were stimulated with 20 ng/ml FGF-1 plus 100 μg/ml heparin, and specific mRNA levels were assessed from 0 to 48 h. The upregulation of collagenase

Figure 5. Effects of FGF-1 (F), heparin (H), and FGF-1 plus heparin (FH) on TIMP-1 and TIMP-2 expression. Total RNA was isolated from subconfluent cultures of human lung fibroblasts and hybridized with [^{32}P]-labeled complementary DNA probes of TIMP-1 and TIMP-2. C: control cells. Hybridization to GAPDH mRNA was used as a loading control.
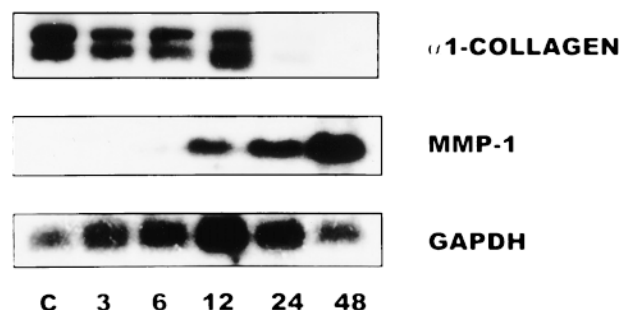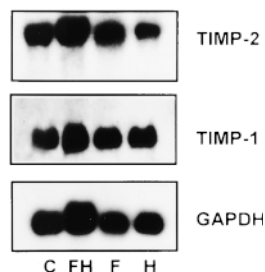

Figure 6. Time course of MMP-1 and type I collagen mRNA expression in FGF-1 plus heparin–treated cells. Fibroblasts were stimulated for the indicated hours and the samples were further processed for Northern blot analysis as described in Figure 5.

mRNA started as early as 6 h exposure, when a barely detectable transcript was observed. At 12 h there was a several-fold increase, and the highest transcript expression was observed at 48 h (Figure 6). Regarding pro-α1(I) collagen, mRNA expression decreased 50% at 12 h (as shown in a typical Northern blot [Figure 6]) or at 11 h (as analyzed from densitometric analysis of blots from three different experiments [Figure 7]).

To determine whether FGF-1 could influence mRNA stability, we performed mRNA analysis after treatment with actinomycin D in lung fibroblasts treated with FGF-1 plus heparin or in serum-free control cells. As shown in Figure 7, after actinomycin D the half-life of pro-α1(I) collagen mRNA in control cells was ∼ 18 h; whereas in fibroblasts incubated in the presence of FGF-1 plus heparin, half-life was further reduced to ∼ 5 h.

Effect of Tyrosine Kinases and PKC Inhibitors on MMP-1 Induction by FGF-1 plus Heparin

To examine the involvement of tyrosine kinases and PKC in the FGF-1–plus–heparin stimulation on MMP-1 expression, fibroblasts were cultured for 1 h with either 25 μg/ml genistein or 5 μM bisindolylmaleimide before exposure to FGF-1 plus heparin. Genistein, which inhibits tyrosine kinase activity by competing with adenosine triphosphate binding (19), blocked FGF-1–plus–heparin induction of collagenase (Figure 8, *lane 4*). On the other hand, the PKC inhibitor bisindolylmaleimide diminished MMP-1 expression ∼ 60% (Figure 8, *lane 3*).

Discussion

Low levels of FGF-1 are present in normal lungs, suggesting that it might influence or modulate cell growth, differentiation, and repair (20). Recently, we have demonstrated that FGF-1 and its receptor are upregulated during the development of an experimental model of pulmonary fibrosis (8). In this model, FGF-1 was expressed first by free alveolar macrophages and in more advanced stages by alveolar epithelial cells and fibroblasts. An unexpected observation was that the strongest expression of this growth factor was noticed in the neighborhood of areas where the lung architecture was still conserved. However, because this study was mostly descriptive it was unclear whether
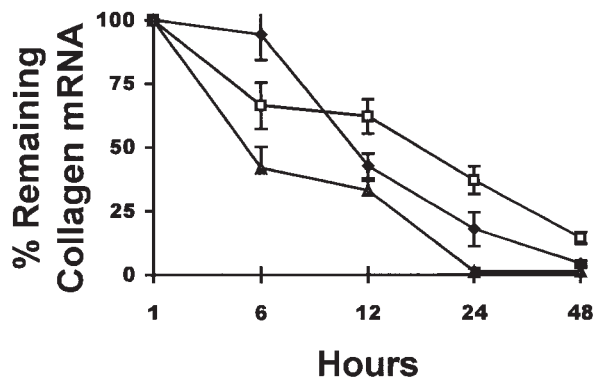
Figure 7. Effects of FGF-1 plus heparin on pro-α1(I) collagen mRNA stability. Subconfluent cultures of lung fibroblasts were maintained in serum-free medium, and treated with actinomycin D (*open squares*), FGF-1 and heparin plus actinomycin (*filled triangles*), and FGF-1 plus heparin in the absence of actinomycin (*filled diamonds*). Results are shown as percent of remaining pro-α1(I) collagen mRNA versus time and were derived from densitometric analysis of blots subsequently exposed to films. The α1-collagen/18S ratio in cells harvested before actinomycin D exposure (zero time) is expressed as 100%, and subsequent time points after exposure are expressed as percentages of that value. Each point represents the mean ± standard deviation of three different experiments.

FGF-1 was playing a protective role or whether its presence preceded the initiation of the fibrotic lesion.

Connective tissue accumulation, mainly fibrillar collagens, represents a crucial event in fibrosis, and it is probably the final result of a complex network of profibrogenic and antifibrogenic cytokines (21). Among them, transforming growth factor-β (TGF-β) is considered a prototype of a fibrogenic cytokine that maximizes collagen accumulation through several pathways implicated in collagen synthesis and degradation. Thus, it stimulates procollagen gene transcription, increases α-chain mRNA stability, inhibits MMP-1 expression, and stimulates TIMP-1 production (22–24).

According to this concept, and at least hypothetically, an antifibrogenic cytokine should be able to perform the opposite regulation, decreasing collagen synthesis and increasing interstitial collagenase expression.

In this context, in the present study we addressed this question by exploring the effect of FGF-1 on type I collagen and its molecular chaperone HSP47, interstitial collagenase MMP-1, gelatinase A, TIMP-1, and TIMP-2 expression by normal human lung fibroblasts.

Our results show that FGF-1, mainly in the presence of heparin, strongly upregulates collagenase expression and downregulates pro-α1(I) collagen and HSP47 gene expression. Heparin was used because this molecule has a pivotal role in the biologic effects of FGFs. Both heparin and heparin sulfate proteoglycans assist as low-affinity receptors, and as places for safekeeping of stored growth factor protecting for proteolysis. Additionally, it has been suggested that these molecules induce a conformational change promoting high-affinity receptor binding (11, 25, 26). A number of *in vitro* assays have clearly shown that binding to
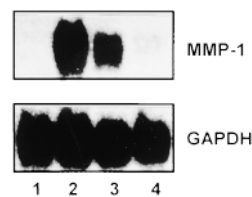

Figure 8. Effect of tyrosine kinase and PKC inhibitors on FGF-1 plus heparin–mediated MMP-1 induction. Fibroblast cells were pretreated with 5 μm bisindolylmaleimide (*lane 3*) or 25 μg/ml genistein (*lane 4*) for 1 h and stimulated with FGF-1 plus heparin for 48 h. *Lane 1*, nonstimulated cells; *lane 2*, fibroblast treated with FGF-1 plus heparin. Northern blot analysis was performed on MMP-1 expression. Levels of GAPDH mRNAs are shown as loading controls.

heparin-like glycosaminoglycans is required to elicit the FGF-1 activities (6, 8, 27), and the requirement for heparin is especially compelling in the case of human FGF-1 (28). Our results are in agreement with these observations because heparin strongly enhanced the effects of FGF-1, mainly on interstitial collagenase expression and synthesis.

Concerning collagen, our findings revealed that FGF-1 and FGF-1 plus heparin produced a considerable downregulation in the steady state of mRNA of α1(I) collagen, as well as in collagen biosynthesis. At least two potential steps are involved in post-transcriptional regulation. One is the RNA processing step, in which heterogeneous nuclear RNAs are spliced and polyadenylated, and the other comprises turnover of mRNA in the cytoplasm that affects the steady-state level of the message. We used actinomycin D to inhibit new transcription in lung fibroblasts and then to examine the stability of the existing pro-α1(I) collagen message in the cytoplasm. Treatment with FGF-1 plus heparin revealed a reduction in the half-life of pro-α1(I) collagen transcript. It is likely that FGF-1 regulates collagen message at the level of RNA export, splicing, or mRNA turnover.

Interestingly, the effect on collagen was accompanied by a decrease of HSP47 mRNA. HSP47 was included in this study because it is a collagen-binding stress protein that appears to act as a collagen-specific molecular chaperone during the biosynthesis, processing, and secretion of procollagen (29). HSP47 can specifically bind to newly synthesized procollagens types I through V, but does not bind to other ECM proteins (30). Furthermore, it has been demonstrated that HSP47 is induced in parallel with interstitial collagens during the development of hepatic fibrosis, supporting a direct role for this stress protein in the abnormal collagen deposit (31).

In general, coregulation of HSP47 and collagen has been well documented in situations in which collagen expression is increased. However, studies analyzing the correlation of HSP47 with collagen synthesis in an opposite situation, mainly in normal cells under physiologic stimuli, are scant. Our results clearly show that in normal human lung fibroblasts, FGF-1 induces a marked downregulation of HSP47 stress protein mRNA in parallel with a reduction α1(I) collagen mRNA, supporting the hypothesis that the expression of both molecules is closely correlated.

The accurate regulation of MMP expression is crucial for normal repair after lung injury. Concerning the degradation of fibrillar collagens, three human interstitial collagenases have been reported to date. Previous reports from

our laboratory have shown that expression of collagenase-1 by lung fibroblasts *in vitro* varies in different subpopulations of fibroblast-like cells (13). In the present study we found that FGF-1 plus heparin induced a marked upregulation of collagenase-1 in fibroblasts that did not express the enzyme at the basal level, as well as in producing MMP-1 fibroblasts (not shown). Collagenase-2 (MMP-8) seems to be circumscribed to neutrophils and chondrocytes (32, 33), and collagenase-3 (MMP-13) initially cloned from breast carcinoma (34) failed to be expressed in lung fibroblasts at the basal level or under FGF-1 stimulation (data not shown).

The constitutive production of tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 by human lung fibroblasts *in vitro* has been well documented. They bind in a 1:1 ratio to the active sites of all MMPs and with the latent forms of gelatinases. Northern blot analysis failed to demonstrate any changes in TIMP-1 and TIMP-2 mRNA levels from lung fibroblasts exposed to FGF-1 or FGF-1 plus heparin. Our results on TIMP expression differ from those produced in human vascular smooth-muscle cells by FGF-2, a closely related FGF. FGF-2 induced an $\sim$ 3-fold increase of TIMP-1 with a concentration-dependent decrease in TIMP-2 mRNA expression (35).

FGFs bind to two receptor classes with high and low affinity, respectively, at the cell surface. The high-affinity receptors are members of the family of transmembrane receptor tyrosine kinases (3). These receptors mediated the observed biologic activities of FGF-1, because the effect on interstitial collagenase was completely abolished by genistein, a tyrosine kinase inhibitor. There is also evidence that externally added FGF-1, after binding to its surface receptors, enters the cells and apparently the nucleus. It has been suggested that the effect of genistein on FGF-1 transport to the nuclear location is probably due not to inhibition of the tyrosine kinase of the receptor but to inhibition of some other kinase required for FGF-1 translocation (36). A current consensus in signal transduction is that signaling through growth-factor receptors with intrinsic tyrosine kinase activity integrates very rapidly downstream with serine/threonine kinases (37). The best-understood mechanism by which tyrosine kinases couple to serine/threonine phosphorylation is by stimulation of isoforms of the PKC family of serine/threonine kinases (38). In this context we explored the possible role of the PKC-dependent pathway in FGF-1–induced gene expression, and we demonstrated that it was also required for FGF-1 induction of fibroblast collagenase, as shown by bisindolylmaleimide effect.

In summary, our results show that FGF-1 induces a strong upregulation of interstitial collagenase with a downregulation of type I collagen and HSP47, without change in TIMP-1 expression. This divergent regulation suggests that the presence of FGF-1 shifts fibroblasts toward a matrix fiber–degradation phenotype, supporting the notion that it could be considered an antifibrogenic cytokine. Whether these properties might be useful *in vivo* is presently unknown.

Other molecules with similar biologic effects on fibroblasts have been shown to reduce lung fibrosis *in vivo*. For example: relaxin, which inhibits the TGF-β–mediated overexpression of interstitial collagens and stimulates MMP-1 expression *in vitro*, is also able to restore bleomycin-induced lung fibrosis (39). In other words, the induction of an ECM-degrading phenotype in lung fibroblasts might be associated with the inhibition of lung fibrosis *in vivo*. Additionally, several studies in human diseases have suggested that decreased collagenase activity plays a role in the development of pulmonary fibrosis. Patients with idiopathic pulmonary fibrosis usually exhibit a remarkable decrease in lung-collagen degradation (40). Furthermore, in chronic hypersensitivity pneumonitis, a disease in which most patients improve or heal but some evolve to diffuse lung fibrosis, we have demonstrated that healing appears to be associated with higher levels of lung collagenolytic activity, whereas progression to fibrosis seems to be related to a significantly lower collagenase activity together with high collagen synthesis (41).

Therefore, and at least theoretically, a cytokine capable of both increasing collagenase expression and synthesis and decreasing collagen expression and synthesis could have a role in inducing an antifibrogenic response.

In this sense, FGF-1 may provide a means of avoiding excess collagen accumulation in lung diseases that evolve to diffuse fibrosis. It has to be elucidated, however, whether the decreased expression of HSP47 with type I collagen and the upregulation of interstitial collagenase caused by FGF-1 *in vitro* may also induce inhibition of lung fibrosis *in vivo*.

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