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Acidic fibroblast growth factor decreases α -smooth muscle actin expression and induces apoptosis in human normal lung fibroblasts

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Ramos, Carlos, Martha Montaña, Carina Becerril, José Cisneros-Lira, Lourdes Barrera, Víctor Ruíz, Annie Pardo, and Moisés Selman. Acidic fibroblast growth factor decreases α -smooth muscle actin expression and induces apoptosis in human normal lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 291: L871–L879, 2006. First published June 9, 2006; doi:10.1152/ajplung.00019.2006.—Fibroblast/myofibroblast expansion is critical in the pathogenesis of pulmonary fibrosis. To date, most research has focused on profibrotic mediators, whereas studies on antifibrotic factors are scanty. In this study, we explored the effects of acidic fibroblast growth factor (FGF-1) and FGF-1 plus heparin (FGF-1+H) on fibroblast growth rate, apoptosis, and myofibroblast differentiation. Heparin was used because it participates in FGF-1 signaling. Growth rate was evaluated by WST-1 colorimetric assay, DNA synthesis by [³H]thymidine incorporation, and apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and cleaved caspase 3. Expression of α -smooth muscle actin (α -SMA) was examined by immunocytochemistry, flow cytometry, real-time PCR, and immunoblotting. Despite the induction of DNA synthesis, FGF-1+H significantly reduced fibroblast growth rate. This correlated with a significant increase in apoptosis, evaluated by TUNEL ($41.6 \pm 1.4\%$ vs. $12.5 \pm 0.6\%$ from controls; $P < 0.01$) and cleaved caspase 3 (295 ± 32 vs. 200 ± 19 ng/10⁶ cells from controls; $P < 0.05$). Double immunostaining (α -SMA-TUNEL) revealed that the levels of induced apoptosis were similar in fibroblasts and myofibroblasts. FGF-1+H inhibited the effect of TGF- β 1 on myofibroblast differentiation. α -SMA-positive cells were reduced by immunocytochemistry from $44.5 \pm 6.5\%$ to $10.9 \pm 1.9\%$ and by flow cytometry from $30.6 \pm 2.5\%$ to $7.7 \pm 0.6\%$ ($P < 0.01$). Also, FGF-1+H significantly inhibited the TGF- β 1 induction of α -SMA quantified by real-time PCR and Western blot. This decrease was associated with a 35% reduction in TGF- β 1-induced collagen gel contraction. The effect of FGF-1+H was mediated by a significant decrease of TGF- β 1-induced Smad2 phosphorylation. FGF-1 alone exhibited similar but lower effects. These findings suggest that FGF-1 can have an antifibrogenic role, inducing apoptosis of fibroblasts and inhibiting myofibroblast differentiation.

lung fibrosis; myofibroblasts; transforming growth factor- β 1; fibroblast growth factor

HOST RESPONSE TO INJURY is characterized by the formation of granulation tissue, which develops from the connective tissue surrounding the damaged area and contains inflammatory cells, fibroblasts, and myofibroblasts. Disappearance of myofibroblasts has been postulated as a key step in the resolution of injury, and actually, in a completely healed wound, few if any myofibroblasts are detected (8). By contrast, excessive prolifer-

ation or persistent presence of these cells plays a relevant role in the fibrotic response (34, 36).

Pulmonary fibrosis is the final result of a variety of interstitial lung diseases and is characterized by fibroblast migration and proliferation, differentiation to myofibroblasts, and exaggerated extracellular matrix accumulation (22, 35). Myofibroblasts contribute to the scar formation, synthesizing extracellular matrix components and inducing tensile force through the neoformation of α -smooth muscle actin (α -SMA) containing cytoplasmic stress fibers (8, 24).

The mechanisms responsible for the expansion of the population of myofibroblasts are not accurately known (33, 34, 36). However, transforming growth factor β ₁ (TGF- β 1), a potent profibrogenic mediator, is a strong inducer of fibroblast differentiation to myofibroblasts (6, 24).

The mammalian fibroblast growth factors (FGFs) constitute a large family of at least 23 polypeptides, comprising structurally related ligands involved in a number of biological processes from embryogenesis to adult homeostasis (19). The effects of FGFs are mediated by binding to the FGF receptor (FGFR) family of receptor tyrosine kinases (19, 30, 44). Heparin and heparan sulfate glycosaminoglycans act as low-affinity receptors and are part of a dual-receptor system, which, along with a cell surface tyrosine kinase receptor, transduces ligand activation along appropriate cytosolic and nuclear pathways (20, 21, 26). FGF-1, the first described of this family, regulates cell growth and differentiation and participates in several physiological processes, including angiogenesis and tissue repair (19).

Our group (1) has previously found that FGF-1 and its receptors are upregulated during the development of experimental pulmonary fibrosis. Interestingly, our group (1) observed that the expression of this factor was abundant in macrophages located in zones with normal pulmonary architecture, suggesting that FGF-1 could play a protective role. Afterward, our group (2) demonstrated that, in human lung fibroblasts, FGF-1 induces a decrease in the expression of type-I collagen and Hsp⁴⁷ chaperonin, as well as an increase in the expression of matrix metalloproteinase (MMP)-1.

In this study, we explored the effects of FGF-1 on growth rate, apoptosis, and myofibroblast phenotype in human normal lung fibroblasts. Our results show that FGF-1, especially in combination with heparin, reduces fibroblast growth rate by inducing programmed cell death and inhibits the induction or reverts the myofibroblast phenotype induced by TGF- β 1, downregulating the expression of α -SMA.

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MATERIALS AND METHODS

Fibroblasts isolation and culture. Primary human lung fibroblasts were obtained as previously described (2). Fibroblasts (primary cell-line N12; passages 8–10) were cultured at 37°C in 5% CO₂-95% air in 25-cm² Falcon flasks containing Ham's F-12 medium supplemented with 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2.5 mg/ml of amphotericin B. Fibroblasts showed a doubling time of 21–24 h.

Cells reaching ~75% confluence were stimulated with FGF-1 (20 ng/ml), heparin (100 µg/ml), FGF-1 plus heparin (FGF-1+H), or TGF-β1 (5 ng/ml). Recombinant human FGF-1 (R&D Systems, Minneapolis, MN) had >97% purity and endotoxin level of < 1.0 endotoxin units per 1 µg of the cytokine. The doses of FGF-1 and heparin were selected according to previous studies (2). Heparin was used because it plays an essential role in FGF signaling by direct association with FGF and FGFR (20, 28). Fibroblasts without stimuli were used as controls. The protocol was accepted by the Ethics Committee of the National Institute of Respiratory Diseases.

Growth rate assay. Early confluent fibroblasts were trypsinized, harvested, resuspended in Ham's F-12 medium supplemented with 10% FBS. Cells were plated in 96-well culture plates at a density of 15 × 10⁴ cells/well and incubated at 37°C in 5% CO₂-95% air. After 24 h, culture medium was replaced by 1% FBS-supplemented medium alone or by 1% FBS with FGF-1, heparin, or FGF-1+H. Cell number was examined at 3 and 6 days by use of the cell proliferation reagent WST-1 (Roche Applies Science, Indianapolis, IN) as previously described (29, 40). Absorbance was analyzed on an ELISA plate reader at 450 nm, with a reference wavelength of 620 nm.

Thymidine uptake assay. Fibroblasts (4 × 10⁴) were grown to ~75% confluence in 24-well culture plates, and then growth was arrested by incubation with serum-free medium for 24 h. The cells were stimulated with FGF-1, heparin, or FGF-1+H for 18 h. Then, [³H]thymidine (2 µCi/ml) was added, and the cells were incubated for another 6 h. Fibroblasts were washed twice with PBS, 5 times with 5% TCA, and lysed in 0.1 N NaOH-0.1% SDS. The radiolabel incorporation was measured in 100-µl aliquots of each lysate using a scintillation counter. Results were expressed as percentage of stimulation over control.

Apoptosis assays. Apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and by measuring active (cleaved) caspase 3. DNA fragmentation examined by TUNEL was done with an in situ cell death detection commercial kit (Roche Mol Biochem, Mannheim, Germany) (29). Fibroblasts (1 × 10⁴ cells by cm²) were plated on coverslips and cultured for 24 h in the presence of FGF-1+H, FGF-1, or heparin alone. Control cells were incubated in serum-free medium; fibroblasts incubated with 2 U/ml RNase/DNase for 30 min at 37°C were used as positive controls. Cells were fixed in freshly prepared cold 4% paraformaldehyde for 15 min at room temperature, washed with PBS, and permeabilized with 2 ml of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Fifty microliters of TUNEL reaction mixture were added to the slides, and they were incubated in a humidified chamber at 37°C for 1 h. The slides were mounted with 50 µl of 50% glycerol in PBS and visualized with an Olympus microscope equipped with fluorescein filters. The images were captured by a color charge-coupled device camera (JVC), imported to Adobe Photoshop software, and printed on a standard ASA 100 color film (Kodak). Apoptotic cells showing brown nuclei were counted at different random fields until at least 500 cells were completed.

ELISA assay for cleaved caspase 3. Cleaved caspase 3 (Asp¹⁷⁵) was analyzed by Sandwich ELISA kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instructions. Fibroblasts were stimulated with FGF-1+H during 12 h, and the whole cell lysate was obtained in ice-cold 1 × cell lyses buffer plus 1 mM PMSF. The levels of cleaved caspase 3 were spectrophotometric determined by reading absorbance at 450 nm. Results were expressed as nano-

grams of cleaved caspase 3/10⁶ cells. In addition, cleaved caspase 3 was examined by Western blot (see *Western blot analysis*).

Myofibroblast phenotype. Fibroblasts were stimulated with TGF-β1 during 24 h before the use of FGF-1, FGF-1+H, or heparin, and the expression of α-SMA was evaluated by real-time RT-PCR, immunocytochemistry, flow cytometry, and Western blot. In parallel experiments, cells were stimulated simultaneously with TGF-β1 and FGF-1, FGF-1+H, or heparin, and α-SMA was evaluated by Western blot and real-time RT-PCR.

RT-PCR and quantitative real-time PCR amplification. One microgram of total RNA was reverse transcribed using Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). Quantitative real-time PCR amplification was performed with an i-Cycler iQ detection system (Bio-Rad, Hercules, CA). PCR was performed with a cDNA working mixture in a 20-µl reaction volume containing 3 µl of cDNA, 20 mM Tris·HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 200 µM dNTP, and 1.25 units of Taq Gold DNA polymerase (Roche, Branchburg, NJ). For α-SMA, 1 µl of 20× TaqMan MGB probe and FAM dye labeled (Assay on Demand Hs00166915_m1; Applied Biosystems, Foster City, CA) primers were also included. For r18S, the following sequences of the PCR primer pairs and probes were used: sense, TGCGAATGGCTCAT-TAAATCAGTT; antisense, CCGTCGGCATGTATTAGCTCTAG; and probe, TET-CCTTTGGTTCGCTCGCTCCTCTCCC-MGNFQ. Each of them was used in 1 µM concentration. A dynamic range was built with each product of PCR on copy number serial dilutions of 1 × 10¹⁰, 1 × 10⁸, 1 × 10⁶, 1 × 10⁴, and 1 × 10²; all PCRs were performed in triplicate. Results were expressed as the number of copies of the target gene normalized to 18S rRNA.

Immunocytochemistry. Fibroblasts (1 × 10⁴ cm²) were plated on coverslips and incubated with serum-free medium containing 5 ng/ml of TGF-β1 for 24 h. The medium was replaced by serum-free medium containing FGF-1, heparin, FGF-1+H, or IL-1β (10 ng/ml), and cells were incubated for other 24 h. Parallel controls were incubated in serum-free medium for 48 h. Fibroblasts were fixed with acetone-methanol (1:1) at -20°C for 2 min and incubated with the human monoclonal α-SMA antibody (Biocare Medical, Walnut Creek, CA) at 37°C for 30 min followed by biotinylated goat anti-mouse IgG for 20 min (Biogenex, San Ramon, CA). 3-Amino-9-ethyl-carbazole (BioGenex) in acetate buffer containing 0.05% H₂O₂ was used as substrate. Cell nuclei were counterstained with hematoxylin.

Double immunostaining for α-SMA and apoptosis. Cells were plated on coverslips, and myofibroblast phenotype was induced by incubation with TGF-β1 (5 ng/ml) in serum-free medium for 24 h. The cells were incubated for an additional 24 h with medium containing FGF-1+H. Cells were fixed, permeabilized, and incubated at 37°C for 30 min with anti-human α-SMA antibody (DAKO, Carpinteria, CA) diluted 1:20. Fifty microliters of TUNEL reaction mixture (Boehringer in situ cell death detection kit-fluorescein) were added to the slides, and they were incubated in a humidified chamber at 37°C for 1 h followed by anti-mouse-Texas Red-conjugated IgG (Jackson ImmunoResearch Laboratories) diluted 1:100 for 40 min. The slides were mounted with medium for fluorescence (Vector, Burlingame, CA).

Flow cytometry. Fibroblasts treated as described for immunocytochemistry were isolated from six-well culture plates, washed twice with staining buffer (BD Pharmingen), resuspended, and incubated in Cytofix/cytoperm solution (BD Pharmingen). Cells were washed twice with Perm/wash solution (BD Pharmingen), resuspended in the same buffer, and incubated with anti-human α-SMA-FITC conjugate (Sigma, St. Louis, MO) antibody diluted 1:400. Finally, cells were washed and resuspended in PBS containing DNase-free RNase and 5 µg/ml of propidium iodide. Anti-α-SMA fluorescence and DNA content data were acquired in log and linear scales, respectively, using a FACSaria (Becton Dickinson) flow cytometer (40).

Western blot analysis. The whole cell lysates were prepared in RIPA buffer (50 mM Tris·HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% Triton X-100 plus proteinase inhibitors; Sigma). Protein concentration was determined by Bradford assay, and samples

containing 30 μg were separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). After membranes were blocked in 5% nonfat milk in 20 mM Tris·HCl, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature, the membranes were incubated overnight at 4°C with different primary monoclonal antibodies: anti caspase 3 (1:1,500; Cell Signaling Technology), anti- α -SMA (1:300; Sigma), anti Smad2 (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-Smad2 (1:1,000; Calbiochem, San Diego, CA). Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Amersham Biosciences).

Collagen gel contraction assay. Gels were prepared mixing 250 μl of rat tail type I collagen from a collagen stock solution (4 mg/ml, BD Biosciences, Bedford, MA) with 200 μl of 2.5 \times Ham's nutrient medium (containing 0.25% FBS), 50 μl of HEPES buffer (0.2 M HEPES buffer, 0.26 M sodium bicarbonate, 0.05 M sodium hydroxide), and 5 $\times 10^5$ fibroblasts. Collagen and cell suspensions were incubated in 24-well plates (Corning New York) at 37°C for 1 h to polymerize the collagen, and the gels were gently cut away from the sides of the well and lifted off the bottom by adding serum-free medium. Cells were stimulated during 24 h with 5 ng/ml of TGF- β 1, and then the medium was replaced by fresh serum-free medium containing either TGF- β 1, FGF-1, heparin, FGF-1+H, and IL- β 1 at the concentrations mentioned above, and the cells were cultured for additional 24 h. Gels were photographed using a camera (Olympus SC-35) connected to inverted microscope and the diameter was measured at 24 h.

Statistical analysis. Results are presented as means \pm SD. Data were analyzed with ANOVA and Dunnett's multiple comparison test. A *P* value of <0.05 was considered statistically significant.

RESULTS

FGF-1 reduces growth rate of lung fibroblasts. Human lung fibroblasts were cultured in 1% FBS-supplemented medium and exposed to FGF-1+H and FGF-1 or heparin alone, and cell growth was evaluated by the WST-1 assay. We have previously demonstrated that direct cell counts confirm the WST-1 cell number assay (40). As shown in Fig. 1A, FGF-1+H induced from day 3 on a significant loss of cell density as measured by the WST-1 assay ($-14.6 \pm 0.01\%$ and $-36 \pm 2.1\%$ at 3 and 6 days; $P < 0.001$). FGF-1 alone also reduced growth rate, although to a lesser magnitude compared with FGF-1+H ($P < 0.01$).

FGF-1 induces fibroblast DNA synthesis. The mitogenic effect of FGF-1 on fibroblasts was studied by the incorporation of [^3H]thymidine. Treatment with FGF-1 alone and FGF-1+H provoked a significant increase of [^3H]thymidine incorporation ($49 \pm 2.2\%$ and $128 \pm 17\%$ over nonstimulated cells; $P < 0.05$ and $P < 0.01$, respectively; Fig. 1B). Heparin alone showed no effect.

FGF-1 induces apoptosis of lung fibroblasts and myofibroblasts. To determine whether apoptosis was playing a role in the reduction of the growth rate, we analyzed chromatin condensation by TUNEL staining. Treatment for 24 h with FGF-1+H and FGF-1 alone caused a significant increase of apoptotic fibroblasts ($41.6 \pm 1.4\%$ and $22.7 \pm 2.5\%$ vs. $12.5 \pm 0.6\%$ from controls; $P < 0.01$; Fig. 2A). Heparin alone showed no effect. Figure 2, B and C, illustrates the increase in the number of apoptotic nuclei when cells are incubated with FGF-1+H.

Because the percentage of myofibroblasts in these experiments was low (<8%), our results suggested that FGF-1+H was inducing cell death on fibroblasts. To determine whether

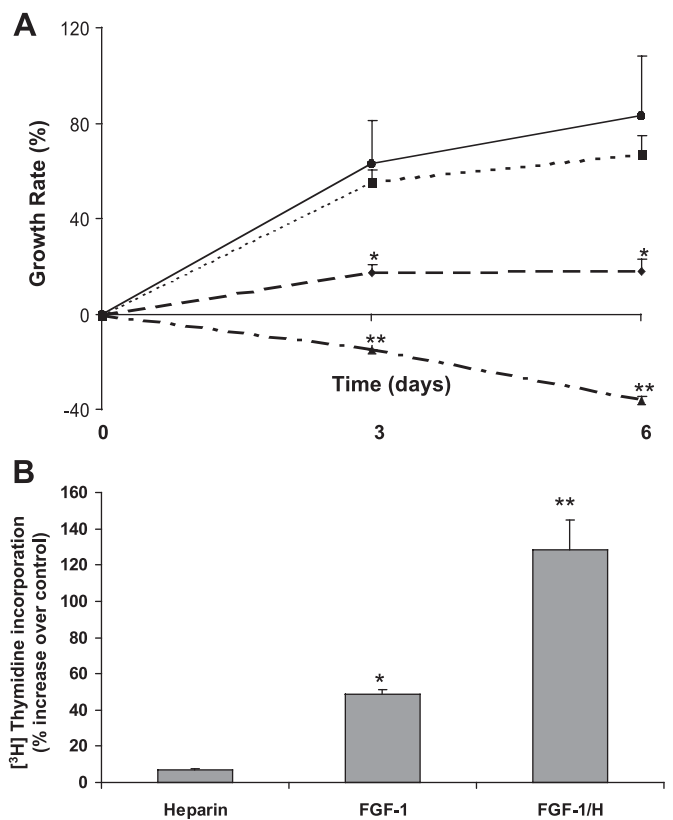


Fig. 1. A: effect of fibroblast growth factor (FGF-1) plus heparin (FGF-1+H) on fibroblast growth rate. Human lung fibroblasts (1×10^3 /well) were cultured in 96-well culture plates. At 3 and 6 days, cell number was estimated by WST-1 assay. Each point represents mean \pm SD of 3 independent experiments performed in triplicate. ●, 1% FBS medium (control); ■, 1% FBS medium plus 100 $\mu\text{g/ml}$ heparin; ◆, 1% FBS medium plus 20 ng/ml FGF-1; ▲, 1% FBS medium plus FGF-1+H. * $P < 0.01$ and ** $P < 0.001$ compared with untreated controls. B: effect of FGF-1+H on DNA synthesis. Subconfluent fibroblasts were incubated in serum-free medium for 24 h and then stimulated with heparin, FGF-1, or FGF-1+H for 18 h. Cells were incubated with [^3H]thymidine (2 $\mu\text{Ci/ml}$) for an additional 6 h. * $P < 0.05$ and ** $P < 0.01$.

FGF-1+H induced apoptosis also on myofibroblasts, a double immunostaining for α -SMA and TUNEL was performed. Fibroblasts were treated with TGF- β 1 for 24 h to induce myofibroblast transformation, and then with FGF-1+H for 24 additional hours. Figure 2D illustrates chromatin condensation by TUNEL in α -SMA-positive and -negative cells. Quantitation of apoptotic cells at different random fields showed a similar percentage of induced apoptosis in myofibroblasts and fibroblasts ($42.7 \pm 10.4\%$ and $43.3 \pm 3.6\%$, respectively).

Apoptosis was further confirmed by Western blot and ELISA for cleaved caspase 3. As illustrated in Fig. 3, fibroblasts incubated with FGF-1+H showed an increase of cleaved caspase 3 as evaluated by Western blot. Consistent with TUNEL and Western blot assays, we found that lung fibroblasts treated during 12 h with FGF-1+H showed a significant increase in cleaved caspase 3, as measured by ELISA (295 ± 32 vs. 200 ± 19 ng/ 10^6 fibroblasts from controls; $P < 0.05$, Fig. 3B).

FGF-1 reduces the percentage of myofibroblasts induced by previous treatment with TGF- β 1. The effect of FGF-1 on the expression of α -SMA, the hallmark for myofibroblasts, was examined by different assays. In these experiments, cells were

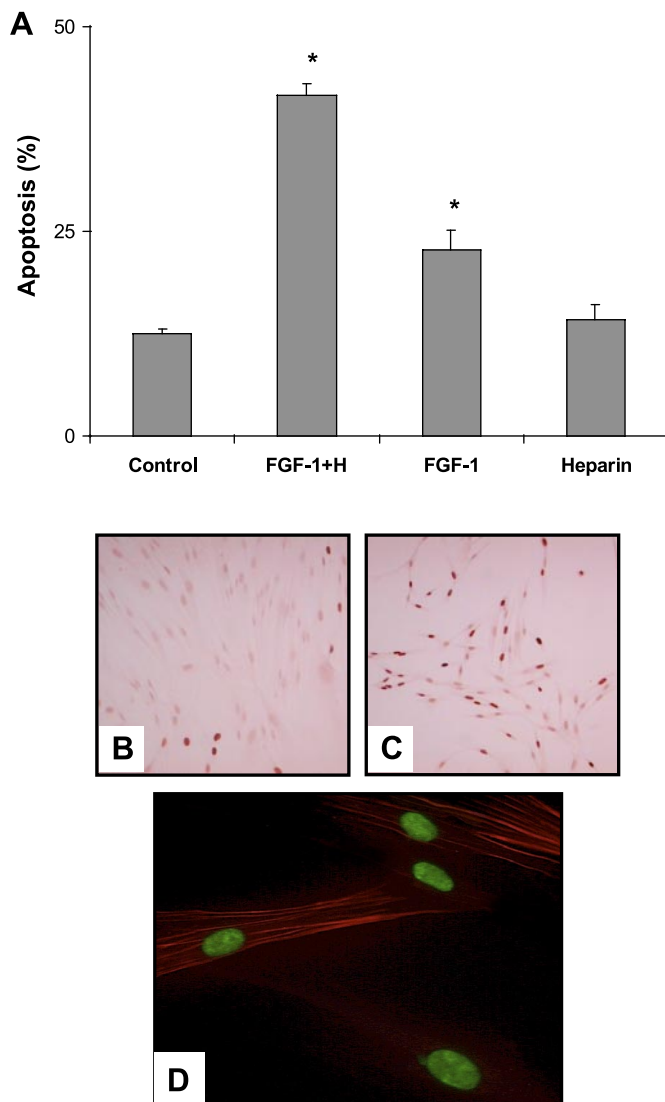


Fig. 2. FGF-1 induces fibroblast and myofibroblast apoptosis as detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction technique. *A*: percentage of TUNEL-positive cells. Bars represent mean \pm SD of 3 independent experiments. * $P < 0.01$ compared with untreated controls. *B*: nonstimulated control cells showed scarce positive apoptotic cells. *C*: numerous apoptotic cells (brown nuclei) were observed after incubation with FGF-1+H. *D*: double immunostaining with TUNEL and anti- α -smooth muscle actin (α -SMA). Two myofibroblasts and 2 fibroblasts (α -SMA-negative cells) showing fluorescent apoptotic nuclei can be observed.

previously stimulated with TGF- β 1 for 24 h. TGF- β 1 stimulation resulted in a significant increase in the number of myofibroblasts ($44.5 \pm 6.5\%$ vs. $3.3 \pm 0.7\%$ from controls; Fig. 4A) as shown by immunocytochemistry. When TGF- β 1-stimulated fibroblasts were incubated in the presence of FGF-1+H, a significant decrease in the percentage of myofibroblasts was observed ($10.9 \pm 1.9\%$ vs. $44.5 \pm 6.5\%$; $P < 0.01$). IL-1 β , a known suppressor of α -SMA gene expression (45), showed a similar effect ($12.5 \pm 0.75\%$; $P < 0.01$). A strong decrease was also observed when the myofibroblasts were incubated with FGF-1 alone ($17.2 \pm 3.3\%$ vs. $44.5 \pm 6.5\%$; $P < 0.01$). Heparin alone had no effect (not shown). Positive α -SMA cells displayed the typical red intracellular microfilaments, as illus-

trated in Fig. 4C. Most cells exposed to FGF-1+H exhibited an elongated, spindle-shaped appearance (Fig. 4D).

To corroborate the effect of FGF-1+H on TGF- β 1-induced myofibroblast differentiation, the proportion of α -SMA-positive fibroblasts was further analyzed by flow cytometry. Approximately 5% of nonstimulated human lung fibroblasts were positive for α -SMA. As exemplified in Fig. 5, treatment with TGF- β 1 resulted in an approximately sixfold increase in the proportion of myofibroblasts ($30.6\% \pm 2.5\%$ vs. $4.1 \pm 0.5\%$ from controls; $P < 0.01$). Consistent with the immunocytochemical results, treatment with FGF-1 alone and FGF-1+H caused a significant reduction in the proportion of myofibroblasts ($11.4 \pm 1.3\%$ and $7.7 \pm 0.6\%$, respectively; $P < 0.01$; Fig. 5C). Heparin alone showed no effect. The incubation of the fibroblasts with FGF-1+H without previous stimulus with TGF- β 1 did not show differences with control cells.

FGF-1 inhibits the expression of α -SMA used either simultaneously or after TGF- β 1 stimulation. The effect of FGF-1+H on α -SMA protein expression was further analyzed by real-time PCR and immunoblotting. In these experiments, human lung fibroblasts were incubated either simultaneously with TGF- β 1 and FGF-1+H or preincubated with TGF- β 1 for 24 h and then exposed to FGF-1+H for 24 h.

The expression of α -SMA mRNA as measured by quantitative real-time RT-PCR increased ~ 10 -fold when fibroblasts were treated with TGF- β 1. Coincubation of cells with FGF-1+H either simultaneously or 24 h after TGF- β 1 stimulation caused a significant reduction in the expression of α -SMA ($P < 0.01$; Fig. 6A).

These results were confirmed at the protein level. TGF- β 1 induced a significant increase in cellular levels of α -SMA, detected as a single 42-kDa band (Fig. 6B). Simultaneous coincubation of the fibroblasts with TGF- β 1 and FGF-1+H resulted in α -SMA expression similar to nonstimulated control cells. When the fibroblasts were first preincubated with TGF- β 1 and then treated with FGF-1+H, a substantial suppression of TGF- β 1-stimulated α -SMA expression was also observed. FGF-1+H alone had no effect of α -SMA protein expression (Fig. 6B).

FGF-1 reduces TGF- β 1-induced collagen gel contraction. Collagen gel tightening analysis was performed to examine the ability of human lung fibroblasts to reorganize and contract three-dimensional collagen gels (25). When fibroblasts were cultured in three-dimensional collagen gels in serum-free medium containing TGF- β 1 for 24 h, the diameter of the gel decreased $\sim 50\%$ of the original size (from 1.55 ± 0.04 to 0.85 ± 0.04 cm) (Fig. 7). The inclusion of FGF-1+H to these fibroblast-populated collagen lattices caused significant reduction of gel contraction (from 0.85 ± 0.04 to 1.15 ± 0.04 cm; $P < 0.01$). This decrease was similar to that observed with IL-1 β . FGF-1 alone also caused an $\sim 20\%$ decrease in the gel contraction induced by TGF- β 1 (1.04 ± 0.04 cm; $P < 0.01$). Heparin alone had no effect (not shown).

FGF-1 inhibits phosphorylation of Smad2. To elucidate the mechanism by which FGF-1 inhibits TGF- β 1-mediated myofibroblast differentiation, we examined the influence of FGF-1 on Smad signaling pathways. TGF- β 1 predominantly transmits the signals through serine and/or threonine receptor kinases and cytoplasmic proteins called Smads. The activated TGF- β type 1 receptor kinase phosphorylates Smad2 and Smad3, which then form heterodimers with the co-Smad, Smad4. The

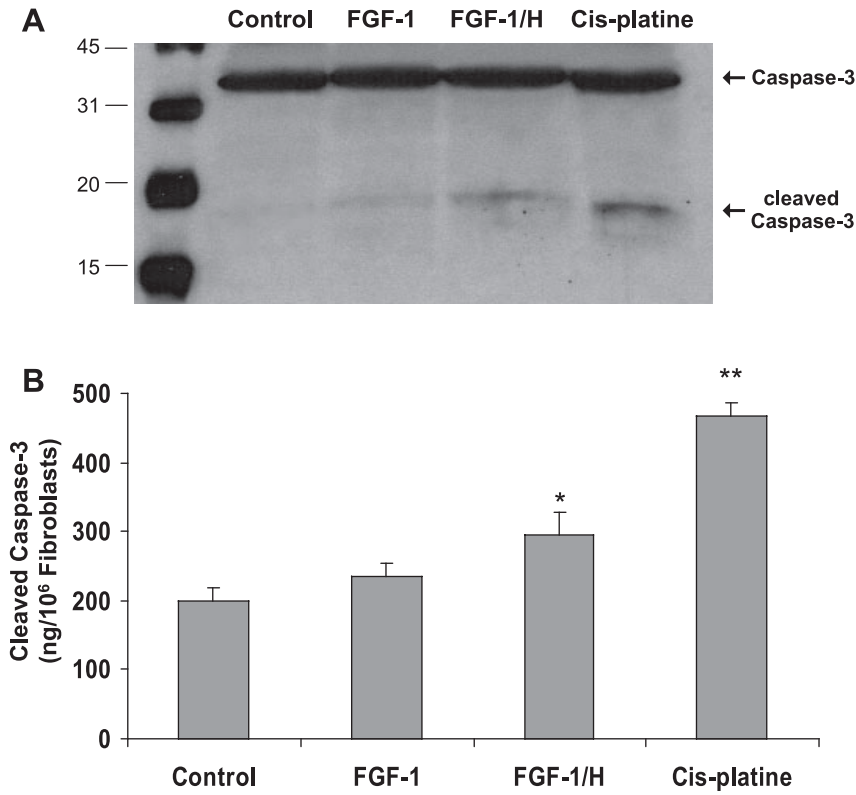


Fig. 3. FGF-1+H leads to increased caspase 3 activation. A: cell lysates were assayed for caspase 3 activation by Western blot analysis. Cisplatin was used as a positive control. Left numbers are in kDa. B: cleaved caspase 3 measured by ELISA. **P* < 0.05 and ***P* < 0.01 compared with control nonstimulated cells.

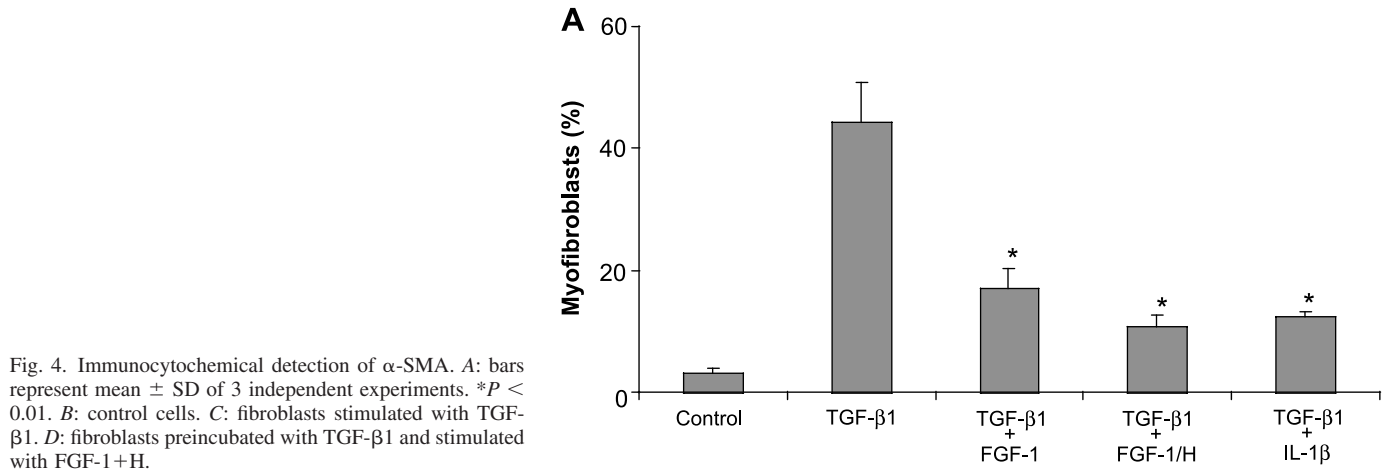


Fig. 4. Immunocytochemical detection of α -SMA. A: bars represent mean \pm SD of 3 independent experiments. **P* < 0.01. B: control cells. C: fibroblasts stimulated with TGF- β 1. D: fibroblasts preincubated with TGF- β 1 and stimulated with FGF-1+H.

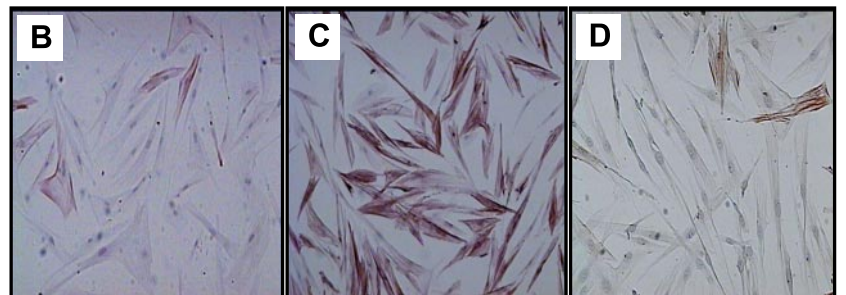
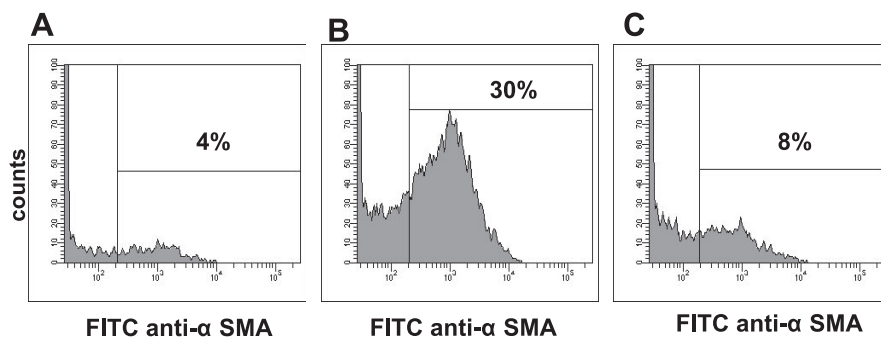


Fig. 5. A representative flow cytometry experiment showing α -SMA expression by human lung fibroblasts, cultured under control conditions (A), stimulated with TGF- β 1 (B), or preincubated with TGF- β 1 and then stimulated with FGF-1+H (C). The percentage of cells with positive α -SMA staining within the set markers is indicated.



complex translates to the nucleus and regulates gene transcription by binding directly to DNA via interactions with DNA-binding proteins and/or transcriptional coactivators and corepressors (27, 32). We first analyzed the kinetics of Smad2 phosphorylation triggered by TGF- β 1 in human lung fibroblasts by immunoblot analysis. As shown in Fig. 8A, Smad2 phosphorylation was detected as early as 15 min, reached the peak level at 30 min, remained elevated for 1 h, and then declined close to baseline level at 3 h.

We then evaluated whether FGF-1+H modulates Smad2 phosphorylation triggered by TGF- β 1. Fibroblasts incubated with TGF- β 1 and FGF-1+H for 30 min showed a significant decrease of TGF- β 1-induced Smad2 phosphorylation (Fig. 8B). On the other hand, FGF-1+H had no effect on the expression of the inhibitory Smad6 (data not shown).

DISCUSSION

Independent of etiology, pulmonary fibrosis is characterized by fibroblast migration/proliferation and differentiation to myofibroblasts. Subsequently, the exaggerated expansion of fibroblasts and myofibroblasts in the lung parenchyma results in tissue remodeling with the accumulation of extracellular matrix molecules (22, 34, 36). Myofibroblasts actively synthesize extracellular matrix components, are resistant to apoptosis, and have contractile properties, playing a critical role in the pathogenesis of pulmonary fibrosis (4, 11, 12, 22, 23, 34, 45). Also, myofibroblasts derived from fibrotic lungs synthesize and release angiotensinogen and induce apoptosis of alveolar epithelial cells contributing to the altered alveolar reepithelialization (38, 39, 42). In this context, the search of mediators capable of decreasing the number of myofibroblasts and reverting these processes is important, not only for understanding fibrogenic mechanisms but also to open new therapeutic approaches. Actually, in normal wound healing, as well as in self-limiting models of lung fibrosis, the number of myofibroblasts gradually decreases as the healing process or active

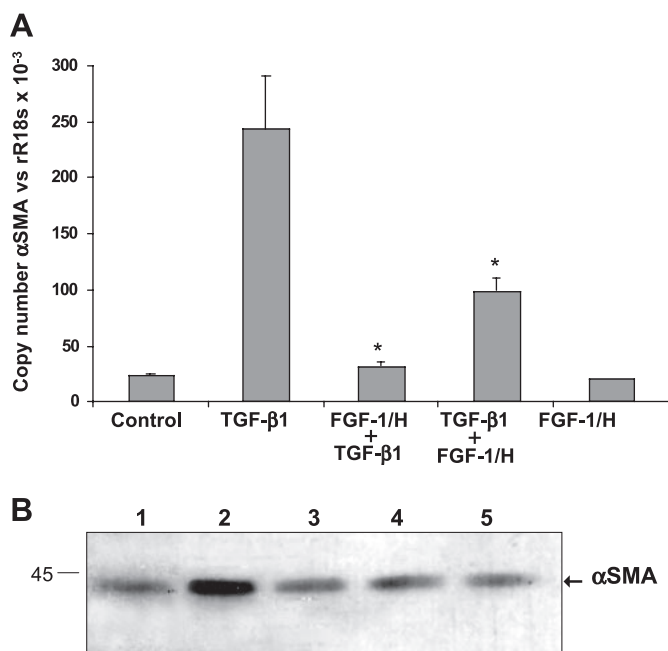


Fig. 6. Effect of FGF-1+H on TGF- β 1-induced α -SMA expression. A: cells were treated with FGF-1+H simultaneously (FGF-1+H + TGF- β 1) or 24 h after the use of TGF- β 1 (TGF- β 1 + FGF-1+H). α -SMA mRNA abundance was analyzed by real-time RT-PCR, and the 18S rRNA gene was used for normalization. * $P < 0.01$. B: Western blot analysis of α -SMA protein in human lung fibroblasts. Lysates from control cells (lane 1) and cells stimulated with TGF- β 1 (lane 2), TGF- β 1 and FGF-1+H used simultaneously (lane 3), or prestimulated with TGF- β 1 for 24 h and then with FGF-1+H (lane 4), and FGF-1+H alone (lane 5) were run on SDS-PAGE and immunoblotted for α -SMA. Left number is in kDa.

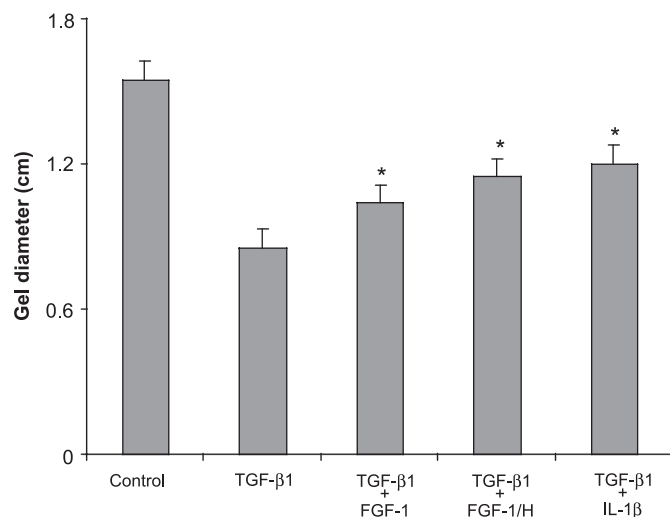


Fig. 7. Effect of the FGF-1 with heparin on 3-dimensional collagen gel dimension. Gel contraction was induced by incubation with TGF- β 1 in serum-free medium for 24 h. Subsequent addition of FGF-1 alone and plus heparin, as well as IL-1 β , reduced the contraction of the fibroblast-populated collagen gels. Gel areas were quantified by densitometry using a digital photographic system (DC120 digital Kodak) and Kodak Digitalis Science DC1 software. Results were calculated from duplicate wells of at least 3 different experiments and are expressed as means \pm SD. * $P < 0.01$ compared with cells exposed to TGF- β 1.

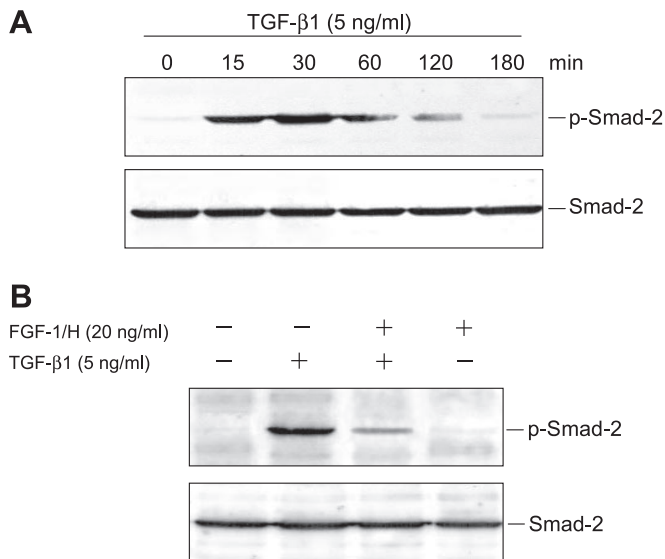


Fig. 8. FGF-1 inhibits TGF- β 1-mediated Smad signaling. **A:** kinetics of Smad2 phosphorylation caused by TGF- β 1 in human lung fibroblasts. Cells were incubated with TGF- β 1 for various periods of time as indicated. Of the whole cell lysates, 30 μ l of protein were probed with antibodies against phospho-Smad2 (p-Smad2) and total Smad2 by Western blot. **B:** effect of FGF-1+H on TGF- β 1-mediated Smad2 phosphorylation. Cells were treated with 5 ng/ml TGF- β 1 or 5 ng/ml TGF- β 1 plus 20 ng/ml FGF-1 plus 100 μ g/ml heparin simultaneously for 30 min.

fibrosis is successfully completed or terminated (23, 24, 45). By contrast, the persistence of fibroblasts/myofibroblasts in injured tissues is associated with progressive fibrotic disease in diverse tissues. Importantly, different studies strongly suggest that myofibroblast disappearance occurs via programmed cell death and support the notion that augmentation of myofibroblast apoptosis may inhibit the scar formation and even promote the resolution of fibrosis (3, 7, 18).

FGF signaling is required for formation of new alveoli, for protection of alveolar epithelial cells from injury, and for migration and proliferation of putative alveolar stem/progenitor cells during lung repair (43). During embryonic development, FGF-1 participates in the regulatory network involving epithelial-mesenchyma interactions, controlling proliferation, differentiation, and pattern formation (13, 43).

The physiological role of FGF-1 in adult lung is presently unclear. Selective inhibition of FGFR signaling in the postnatal mouse lung did not alter lung morphogenesis or function under physiological conditions but rendered male mice susceptible to oxygen-induced injury (9). Likewise, we have shown that FGF-1 and FGFR are increased during the development of experimental lung fibrosis (1). Interestingly enough, FGF-1 was mostly observed in areas where the pulmonary architecture was preserved, suggesting a protective effect. Supporting this hypothesis, severe inflammation and increased expression of proinflammatory cytokines were noted in the lungs of transgenic mice in which a soluble FGFR was conditionally expressed in respiratory epithelial cells to inhibit FGF activity (9).

In addition, we have previously demonstrated that FGF-1 decreases collagen and Hsp⁴⁷ expression and increases MMP-1 expression *in vitro*, suggesting an antifibrogenic role (2).

In the present work, we focused on other possible antifibrotic effects of FGF-1, primarily on cell growth and apoptosis

and its capacity to revert the differentiation to myofibroblasts induced by TGF- β 1. Our results showed that FGF-1 causes programmed cell death of fibroblasts, which was reflected in an important decrease of the rate of cell growth despite the induction of DNA synthesis measured by [³H]thymidine incorporation. Of interest is the finding that FGF-1 could stimulate DNA synthesis and apoptosis. This dual effect was probably related to the significant heterogeneity of the fibroblast population that includes among others the presence of fibroblasts, proto-myofibroblasts, and myofibroblasts (37). Likewise, our group (40) previously demonstrated that primary human lung fibroblasts in culture display important differences in cell size that show significant differences in cell proliferation and other activities. Additionally, a particular signal, such as a growth factor, can lead to proliferation, cell cycle arrest, or apoptosis in the same cell type, depending on the presence or absence of other factors, such as integrin ligation, cytoskeletal organization, or activation of parallel signaling pathways.

The apoptotic effect observed in this study seemed to be mediated by caspase 3 activity because the active form was observed by Western blot and ELISA. The effect of FGF-1 on programmed cell death was stronger when used in combination with heparin. This is probably because heparin (and heparan sulfate) plays an essential role in FGF signaling by direct association with FGF and FGFR in a ternary complex on the surface of the cell, inducing the more stable conformation and the higher affinity between FGF-1 and FGFR (20, 21).

Likewise, FGF-1+H strongly inhibited (when used simultaneously) and reverted (when used subsequently) the TGF- β 1-induced switching of the human lung fibroblasts to myofibroblasts that was accompanied by a change in cell morphology from a typical flattened, irregular shape characteristic of a myofibroblast-like phenotype to an elongated, spindle-shaped appearance (17, 26). The effect of FGF-1+H on myofibroblast phenotype was demonstrated by several complementary techniques, including flow cytometry, immunocytochemistry, and quantification of α -SMA by real-time RT-PCR and Western blot. Furthermore, FGF-1+H also decreased collagen gel contraction induced by TGF- β 1. It is known that a typical feature of myofibroblasts, their contractile activity, depends on α -SMA expression and organization. Thus the increased expression of α -SMA in TGF- β 1-treated cells results in an increase in the ability of the fibroblasts to contract collagen gels. In this context, we showed that the TGF- β 1-stimulated collagen gel contraction was strongly inhibited by FGF-1+H.

Myofibroblast phenotype reversal induced by FGF-2 plus heparin has been described in human synovial and human breast gland fibroblasts (17, 31). More recently, it was shown that FGF-2 enhances telomerase expression and significantly reduces α -SMA expression (14). Also, a similar effect has been previously found for FGF-1+H in rabbit corneal fibroblasts (16).

To elucidate the mechanisms likely implicated in the FGF-1+H reversion of myofibroblastic differentiation induced by TGF- β 1, we investigated its effect on the Smad signaling. It is well documented that TGF- β 1, on binding to its receptors, initiates Smad2 and -3 phosphorylation, which in turn bind to co-Smad4 and translocate into the nuclei, where they control the transcription of TGF- β 1-responsive genes (27, 32). Our findings provide evidence that FGF-1+H strongly inhibits the phosphorylation of Smad2 a major signal transducer of TGF-

β 1. This inhibition may subsequently disrupt the cascade of signal transduction processes blocking myofibroblast differentiation.

Together, our findings suggest a potential antifibrotic role of FGF-1. The induction of fibroblast apoptosis and the inhibition or reversion of myofibroblast phenotype should have a profound effect of lung fibrogenesis. In addition, our group (2) previously demonstrated that FGF-1 decreases type I collagen expression and increases MMP-1 expression sustaining the notion that this mediator may help to control the aberrant matrix remodeling.

Supporting a possible protective role of FGF-1 in lung disorders, it has been demonstrated that adenovirus-mediated FGF-1 overexpression in the lungs causes alveolar and bronchiolar epithelial cell proliferation and increases survival in hyperoxia-induced lung injury (15). Moreover, it has been demonstrated that early administration of heparin results in virtual abrogation of bleomycin-induced lung fibrosis (5). Although this attenuation is often attributed to its effect on modulating alveolar fibrin generation, it can be speculated that another heparin effect may be to augment endogenous FGF-1 signaling in the recovery process.

In summary, our findings indicate that FGF-1 provokes fibroblast apoptosis and counteracts with TGF- β 1 in the induction of myofibroblast differentiation through inhibition of Smad-2 phosphorylation, suggesting that it may have a beneficial effect during the development of pulmonary fibrosis. Future work will be required to elucidate whether lung fibroblasts from subjects with pulmonary fibrosis behaved similarly or differently from normal lung fibroblasts when exposed to FGF-1+H.

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