Matrix Metalloproteinase (MMP)-1 Induces Lung Alveolar Epithelial Cell Migration and Proliferation, Protects from Apoptosis, and Represses Mitochondrial Oxygen Consumption*

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Idiopathic pulmonary fibrosis is a devastating lung disorder of unknown etiology. Although its pathogenesis is unclear, considerable evidence supports an important role of aberrantly activated alveolar epithelial cells (AECs), which produce a large variety of mediators, including several matrix metalloproteases (MMPs), which participate in fibroblast activation and lung remodeling. MMP-1 has been shown to be highly expressed in AECs from idiopathic pulmonary fibrosis lungs although its role is unknown. In this study, we explored the role of MMP-1 in several AECs functions. Mouse lung epithelial cells (MLE12) transfected with human MMP-1 showed significantly increased cell growth and proliferation at 36 and 48 h of culture (p < 0.01). Also, MMP-1 promoted MLE12 cell migration through collagen 1, accelerated wound closing, and protected cells from staurosporine- and bleomycin-induced apoptosis compared with mock cells (p < 0.01). MLE12 cells expressing human MMP-1 showed a significant repression of oxygen consumption ratio compared with the cells with the empty vector. As under hypoxic conditions hypoxia-inducible factor-1α (HIF-1α) mediates a transition from oxidative to glycolytic metabolism, we analyzed activation of HIF-1α. Higher activation of this factor was detected in MMP-1-transfected cells under normoxia and hypoxia. Likewise, a significant decrease of both total and mitochondrial reactive oxygen species was observed in MMP-1-transfected cells. Paralleling these findings, attenuation of MMP-1 expression by shRNA in A549 (human) AECs markedly reduced proliferation and migration (p < 0.01) and increased the oxygen consumption ratio. These findings indicate that epithelial expression of MMP-1 inhibits mitochondrial function, increases HIF-1α expression, decreases reactive oxygen species production, and contributes to a proliferative, migratory, and anti-apoptotic phenotype.

Idiopathic pulmonary fibrosis (IPF)3 is a progressive and destructive lung disorder of unknown etiology. Although the natural history and the pathogenic mechanisms remain unknown, a growing body of evidence indicates that the disease is the result of an abnormal behavior of the alveolar epithelial cells (AECs) that induce the migration, proliferation, and activation of mesenchymal cells, with the formation of fibroblast and myofibroblasts foci (1–3).

Gene expression profiling of IPF lungs has revealed the increase of several matrix metalloproteases (MMPs) which appear to play an important role in the fibrotic response (4, 5). Intriguingly, one of the most up-regulated MMPs is MMP-1 (collagenase I) (4, 5), an enzyme able to cleave fibrillar collagens (types I and III), the extracellular matrix molecules that are exaggeratedly accumulated in IPF (4, 5). Moreover, MMP-1 is implicated in diseases that, in contrast to fibrosis, are characterized by excessive extracellular matrix degradation, such as rheumatoid arthritis and lung emphysema (6–10). Studies regarding the distribution of MMP-1 have partially elucidated this paradox. Thus, we have shown that MMP-1 is localized primarily in IPF lungs in the reactive alveolar epithelium and is virtually absent in the interstitial compartment where collagen is being accumulated, suggesting that the role of MMP-1 in IPF goes far beyond that of digesting fibrillar collagens (11).

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§ The abbreviations used are: IPF, idiopathic pulmonary fibrosis; AEC, alveolar epithelial cell; BAL, bronchoalveolar lavage; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; HIF, hypoxia-inducible factor; MMP, matrix metalloprotease; OCR, oxygen consumption ratio; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

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Actually, strong evidence has demonstrated that MMP-1 is a multifunctional protease that in addition to degrading collagen and noncollagenous components of the extracellular matrix, also processes cytokines such as interleukin (IL-1)-β and tumor necrosis factor (TNF)-α, insulin growth factor-binding proteins, and several chemokines (12–14). Furthermore, recent evidence suggests that MMP-1 may have intracellular functions (15).

In this context, the epithelial localization of MMP-1 in IPF lungs, as well as its non- extracellular matrix-related functions, suggests that MMP-1 may be a potential regulator of local epithelial/mesenchymal interaction or influence the epithelial behavior during the evolution of fibrosis. However, to our knowledge no studies on the biological role of MMP-1 in lung AECs have been reported to date.

In this study we aimed to explore the putative functions of the lung epithelial expression of MMP-1. We used mouse lung alveolar epithelial cells transfected with human MMP-1, and A549 human AECs silenced with specific MMP-1 shRNA. Our results showed that MMP-1 is implicated in AEC migration, proliferation, and resistance to apoptosis. We also documented that MMP-1 represses oxygen consumption ratio, up-regulates the expression of hypoxia-inducible factor (HIF-1α), and decrease reactive oxygen species (ROS) production.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The mouse lung epithelial cell line MLE12 and human lung epithelial cells A549 were purchased from ATCC (Manassas, VA). MLE12 cells were cultured in a CO2 incubator chamber (Billups-Rothenberg Inc.) at 37 °C in Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (DMEM/F-12; Invitrogen), supplemented with 2% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% l-glutamine, 1% HEPES, 1% insulin/transferin/sodium selenite, 0.01% β-estradiol, and 0.01% hydrocortisone. A549 cells were cultured in F12K medium with 10% FBS. For hypoxic conditions, cells reaching 75% confluence were harvested and total RNA was isolated with TRIzol reagent (Invitrogen). cDNA from 1 μg of total RNA was synthesized with the Ambion RETROscript® First Strand Synthesis reagent, 50 ng of each cDNA in 2X master mix (Thermo Fischer Scientific). The primers used were: sense, 5'-AGGTTATC-CAAAAATGATAG-3’ and antisense, 5’-TGCAAGTTGAAC-CAGCTATTA-3’. The amplification reactions were carried out for 35 cycles at 95 °C for 30 s, 55 °C for 20 s, and 72 °C for 20 s, followed by extension at 72 °C for 7 min. The amplification products were run on 1% agarose gels stained with 0.5 μg/ml ethidium bromide and visualized on a UV transilluminator.

**Gene Expression Assay**—Untransfected MLE12 cells (WT) and cells transfected with pQCXIP (mock) or with pQCXIP +MMP-1 plasmids were plated in 35-mm culture dishes. After reaching ~80% confluence, the cells were harvested and total RNA was synthesized with the Ambion RETROscript® First Strand Synthesis kit (Invitrogen). The amplification of mRNA encoding the human MMP-1 was performed by PCR with 50 ng of each cDNA in 2X master mix (Thermo Fisher Scientific). The primers used were: sense, 5’-GGTTCGCAAATGATAG-3’ and antisense, 5’-TGCAAGTTGAAC-CAGCTATTA-3’. The amplification reactions were carried out for 35 cycles at 95 °C for 30 s, 55 °C for 20 s, and 72 °C for 20 s, followed by extension at 72 °C for 7 min. The amplification products were run on 1% agarose gels stained with 0.5 μg/ml ethidium bromide and visualized on a UV transilluminator.

**Quantitative Real-time PCR**—The MMP-1 expression in A549 cells was determined by real-time PCR using the TaqMan Gene Expression Assay (Hs00899658_m1) and normalized with hypoxanthine-guanine phosphoribosyltransferase (Applied Biosystems). The amplification reactions were done in a Rotor Gene Q (Qiagen) with 60 ng of cDNA and Maxima Probe qPCR Master Mix (Thermo Fisher Scientific). The relative quantitation method was used to analyze the results of two independent experiments made in triplicate.

**Transfection of MMP-1**—Full-length human Mmp-1 cDNA cloned into the pSP64 vector was obtained from ATCC (57684). To confirm orientation, this fragment was excised with Aval and BglII restriction enzymes (New England Biolabs) and sequenced. Then, the fragment was cloned into the pQCXIP expression vector (Clontech) designed to express a target gene along with the puromycin selection marker. The pQCXIP +MMP-1 or pQCXIP (mock) plasmids were transfected into MLE12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol and maintained in medium plus puromycin (10 μg/ml; Sigma). Resistant cells were cultured in selection medium and expanded.

**growth rate assay and proliferation was determined by meas-
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uring bromodeoxyuridine (BrdU) incorporation with the Cell Proliferation ELISA kit (Roche Applied Science). After 12, 24, 36, and 48 h in DMEM/F-12 medium supplemented with 2% FBS, cells were incubated for 4 h with BrdU labeling solution. The assay was performed according to the manufacturer's instructions. Absorbance values were measured at 450 nm, and the experiments were done twice in triplicate.

Wound Healing Assay—Cells overexpressing MMP-1 and mock (1 × 10⁴) were plated in 35-mm cell culture dishes. After 24 h, an artificial wound was created by disrupting the monolayer with a sterile plastic pipette tip (200 µl). Cultures were maintained in DMEM/F-12 medium with 2% FBS and were photographed at 24, 36, and 48 h using an inverted phase contrast microscope (Olympus). The assay was performed twice.

Epithelial Cell Migration Assay—Cells reaching 80% confluence were harvested and resuspended in DMEM containing 5% BSA or harvested and pretreated for 2 h with 10 µg/ml mitomycin C (Sigma). The cells (3 × 10⁵) were added to the upper chamber of collagen-coated Boyden chambers (QCM™ Haptoptaxis Cell Migration Assay, Collagen I; EMD Millipore). Cells on BSA-coated chambers were used as blanks for each sample.

Fibroblast Migration—Co-cultures of epithelial cells and fibroblasts were performed as follows. MLE cells (MMP-1 or mock) and A549 cells (control or shMMP-1) pretreated 18 h with 10 ng/ml mitomycin C (Promocell; Heidelberg, Germany) were plated in Transwell inserts with a polycarbonate membrane collagen I-coated (CytoSelect 24 Wells Cell Haptoptaxis Assay; Cell Biolabs, San Diego, CA). The inserts were immediately placed into the wells where epithelial cells were seeded and migratory fibroblasts were stained and quantified 8 h later according to the manufacturer's instructions. As controls, inserts with fibroblasts were placed into the wells with medium alone. Two independent experiments were performed in duplicate.

Flow Cytometry—Cells were grown to ~75% confluence in 12-well plates and were induced to undergo programmed cell death by incubation for 3 h with staurosporine (1 µM) or for 48 h with bleomycin (10 milliunits) (both from Sigma-Aldrich). After stimulation, cells were harvested and double stained with annexin-V phycoerythrin-conjugated and 7AAD (BD Biosciences) following the manufacturer's instructions. Cells were acquired with the BD FACScalibur flow cytometer (BD Biosciences), and results were calculated by analysis of early apoptotic cells (annexin-V⁻, 7AAD⁻) with the flow cytometry analysis software FlowJo (Tree Star, Inc., Ashland, OR). Two independent experiments were performed.

Detection of ROS—Intracellular ROS and mitochondrial ROS generated in mock- and MMP-1-transfected cells was assessed using H₂DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) and MitoSOX reagents (Molecular Probes, Invitrogen). Briefly, the cells were plated on 6-well culture plates at a density of 3.5 × 10⁴ and exposed to normoxia or hypoxia for 24 h. After 45 min of incubation with a 5 µM concentration of each reagent, the cells were harvested, and stained cells were analyzed by flow cytometry.

Western Blotting—Cells expressing human MMP-1 and mock were plated on 100-mm culture dishes and grown until they reached 75% confluence. Then, cells were exposed to hypoxic or normoxic conditions. Cells were lysed with radiomune precipitation assay buffer (Sigma-Aldrich) to obtain total cell extracts, and nuclei lysates were obtained with a nuclear extraction kit (Epigentek). In parallel, serum-free conditioned media obtained from cells grown in normoxic conditions were concentrated by lyophilization and were reconstituted in water. Proteins from total extracts (30 µg), nuclei (20 µg), or conditioned media (50 µg) were mixed with Laemmli buffer (v/v) and separated on 10% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences), blocked with 5% (w/v) nonfat dried milk in PBS for 1 h, and incubated overnight at 4 °C with a goat anti-HIF-1α for lysates (1 µg/ml; Santa Cruz Biotechnology), or with a rabbit polyclonal anti-MMP-1-hemopexin domain (2 µg/ml; Abcam) for media. After washing with PBS-Tween 20 0.05%, membranes were incubated with the corresponding secondary antibody HRP-conjugated (1:10,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Bands were detected using a chemiluminescence detection system (Pierce). Detection of β-actin (1 µg/ml; Santa Cruz Biotechnology) was used to test equal loading for HIF-1α in total cell extracts, and anti-histone H3 (2 µg/ml; BioVision, Milpitas, CA) for nuclear extracts.

Oxygen Consumption Measurement—Oxygen consumption was studied with a Seahorse Biosciences instrument (model XF24) in 24-well plates. 40,000 cells were seeded per well in complete medium the day before the experiment was performed. Then, the cells were equilibrated at 37 °C for 1 h in an incubator lacking CO₂. Three measurements of O₂ concentration were developed in basal conditions and after the injection of oligomycin A (5 µM), FCCP (10 µM), and antimycin/rotenone (2 µM/2 µM) to analyze the metabolic mitochondrial profile of the cells. Measurements were normalized by the cell number in each well. To subtract the nonmitochondrial respiration background, oxygen consumption ratio (OCR) values after the antimycin/rotenone injection were subtracted from each data.

Immunohistochemistry and Immunofluorescence—Lung tissue sections were deparaffinized, rehydrated, and then incubated for 30 min with H₂O₂ (3%) followed by antigen retrieval with citrate buffer (10 mM, pH 6.0) for 5 min in a microwave. Tissue samples were incubated overnight at 4 °C with rabbit anti-MMP-1 (1:100 dilution, IM35; Calbiochem) or Pan Cytokeratin (Ab 961; Abcam). A secondary biotinylated anti-IgG followed by horseradish peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) was used according to the manufacturer’s instructions. 3-Amino-9-ethylcarbazole (BioGenex) in acetate buffer containing 0.05% H₂O₂ was used as a substrate. Tissue sections were counterstained with hematoxylin.

For immunofluorescence, cells transfected with MMP-1 and mock were plated on glass coverslips and exposed to normoxia and hypoxia for 24 h, followed by fixation with methanol for 10 min to ~20 °C. Cells were permeabilized with 0.01% Triton X-100 in PBS. Coverslips were incubated overnight at 4 °C with a rabbit polyclonal anti-MMP-1 (Epitomics 1:100) or rabbit polyclonal anti-HIF-1α (2 µg/ml; GeneTex, Irvine CA). After being washed, cells were incubated with Alexa Fluor 546-con-
jugated antibody for 1 h at room temperature in the dark. Nuclei were labeled with DAPI, and coverslips were mounted and visualized in a confocal laser-scanning microscope (Olympus FluoView™ FV1000).

Quantification of MMP-1 in Human Bronchoalveolar Lavage (BAL) Fluids—BAL was performed through flexible fiberoptic bronchoscopy as part of the diagnostic process, as reported previously (16). Supernatants were kept at 4°C for 72 h until use.

BAL samples from 21 IPF patients and 17 healthy controls were used in this study. Quantitative sandwich enzyme immunoassay for human MMP-1 was performed as recommended by the manufacturer (R&D Systems).

Statistics—The results are expressed as mean ± S.D. Differences were analyzed by Student’s t test or the Mann-Whitney U test. Values of p < 0.05 were considered statistically significant.

RESULTS

MMP-1 Is Overexpressed in IPF Lungs and Localizes to AECs—To corroborate the cellular localization of MMP-1 in IPF lungs, IPF (n = 5) and control tissues (n = 3) were examined. As illustrated in Fig. 1, A, C, and D, strong MMP-1 immunoreactivity was localized predominantly in the AECs (identified by cytokeratin, Fig. 1B). Occasionally endothelial cells were also stained, whereas MMP-1 was almost negligible in normal control tissues (Fig. 1E). Confirming the up-regulation of MMP-1 in IPF lungs, ELISA for MMP-1 performed in BAL fluids showed a significant overexpression of the enzyme in IPF patients (4.0 ± 2.4 versus 1.1 ± 1.4 ng/ml; p < 0.01; Fig. 1G).

MMP-1 Transfection to Mouse Lung Epithelial Cells—To investigate the functional consequences of MMP-1 expression in epithelial cells, full-length MMP-1 cDNA was transfected into the MLE12 cell line, and clones selected with puromycin were isolated. Successful transfection was confirmed by RT-PCR (Fig. 2A), Western blotting of conditioned medium (Fig. 2B) and total extracts (Fig. 2C), and by immunofluorescence (Fig. 2D). Collagenolytic activity measured in conditioned media using the SensoLyte 520 MMP-1 assay showed an increase of relative fluorescent units from 54.7 ± 2 to 79.0 ± 11 (p < 0.05).

Silencing MMP-1 with Short Hairpin RNA of Human A549 Epithelial Cells—Stable transfection of A549 cells with MMP-1-shRNA resulted in ~3-fold knockdown of the mRNA expression by quantitative RT-PCR (Fig. 3A) and in the reduction of MMP-1 protein evaluated by Western blotting (Fig. 3B). For some experiments, epithelial cells were stimulated with PMA, which resulted in a 300-fold increase of MMP-1 expression. Under these conditions, shRNA induced a 50% MMP-1 reduction (Fig. 3C).

MMP-1 Induces Epithelial Cell Proliferation—The WST-1 assay, which measures the metabolic activity of viable cells, was used to evaluate the effect of MMP-1 on MLE12 and A549 cell growth. As shown in Fig. 4A, MMP-1-transfected MLE cells exhibited a significant increase of cell growth at 36 and 48 h compared with wild type and mock cells. Because WST-1 measurements represent the combined result of proliferation and cell death, we also analyzed directly the cell proliferation rate using the BrdU incorporation assay. Fig. 4B shows a pattern of similar response with a significant increase of cell proliferation at 36 and 48 h in the MMP-1-transfected cells compared with mock and wild type cells. By contrast, MMP-1 knockdown reduced A549 cell proliferation examined by WST-1 and BrdU compared with the shControl (p < 0.01; Fig. 4, C and D).
MMP-1 Promotes Epithelial Cell Migration—To examine the effect of MMP-1 on the migratory capacity of the epithelial cells, we investigated the effects of MMP-1 on the closure of a scratch wound. For this purpose, MMP-1-transfected MLE12, and mock cells were grown to confluence and scraped to create a wound track devoid of cells. As shown in Fig. 5A, transfection of MMP-1 consistently accelerated wound closing compared with mock cells at 24 h, with an almost complete wound closure at 48 h (Fig. 5A). Treatment with mitomycin C, an irreversible inhibitor of mitosis, decreased the wound closure rate in mock- and MMP-1-transfected cells. However, the rate of closure of MMP-1-transfected cells was still significantly higher compared with controls as shown at 36 and 48 h in Fig. 5B.

MMP-1-transfected MLE12 and mock cells were subjected to a migration assay in collagen-coated Boyden chambers in the presence or absence of EGF, a potent epithelial chemotactic agent. As illustrated in Fig. 5C, a significant increase in the migration of MMP-1-transfected cells was observed compared with control cells (84 ± 3% in MMP-1 versus 57 ± 11% in mock; p < 0.01). Treatment of the cells with mitomycin C had no effect on transmigration. The effects of MMP-1 knockdown were also examined on migration using the Transwell system. Our results showed that cell migration of A549 cells treated with shMMP-1 was markedly reduced compared with the cells transfected with control shRNA lentiviral particles (p < 0.01; Fig. 5D).

MMP-1 Protects MLE12 Epithelial Cells from Apoptosis—We first examined the effect of human MMP-1 transfection on MLE12 cell survival during staurosporine induction of apoptosis. Staurosporine is a broad range protein kinase inhibitor that inhibits numerous Ser/Thr and Tyr kinases, triggering cell death. As shown in Fig. 6A, staurosporine treatment increased the percentage of annexin-V-labeled cells which was significantly reduced in the MLE12 cells transfected with MMP-1 (wild type MLE12 cells, 2.7 ± 0.1 to 7.3 ± 0.1; mock, 2.4 ± 0.1 to 6.7 ± 1.0; MLE12-MMP-1, 2.05 ± 0.1 to 3.6 ± 0.5; p < 0.01). Likewise, exposure of mock cells to bleomycin (a drug that causes damage by inducing apurinic/apyrimidinic sites and strand breaks in the DNA of target cells) for 3 h caused a significant increase of apoptosis in the mock cells (from 1.92 ± 0.04 to 16.5 ± 3.7) compared with the MMP-1-transfected MLE12 cells (from 1.7 ± 0.16 to 5.33 ± 1.9), p < 0.01; Fig. 6B).

MMP-1 in Epithelial Cells Influences Fibroblast Migration—To address whether MMP-1 could have a migratory effect to adjacent fibroblasts, MMP-1-transfected MLE12 or shRNA MMP-1 A549 cells were co-cultured with fibroblasts using Transwell inserts. As shown in Fig. 7A, MMP-1-transfected cells induced a significant increase of fibroblasts migration compared with mock (p < 0.01). Supporting a role of epithelial MMP-1 on fibroblast migration, MMP-1-knock-out A549 cells stimulated with PMA provoked a decrease in fibroblast migration compared with the control cells (p < 0.01) (Fig. 7B).

FIGURE 2. Transfection of human MMP-1 in mouse alveolar epithelial cells (MLE). A–C, detection of MMP-1 by RT-PCR (A) and Western blotting from conditioned media (B) and cell lysates (C). Positive expression was observed only in cells transfected with MMP-1 and in human fibroblasts (HF) stimulated with FGF1 that were used as a positive control. No expression was detected in wild type (WT) or mock-transfected cells. β-Actin was used as loading control. D, detection of MMP-1 by immunofluorescence. Immunoreactive MMP-1 was detected with Alexa Fluor 546-conjugated antibody, and nuclei were stained with DAPI. Images from differential interference contrast microscopy (DIC; Nomarski) are included. Scale bars, 16 μm.
MMP-1 Represses Mitochondrial Respiration—OCR was analyzed by treating the cells with oligomycin, FCCP and antimycin/rotenone. Basal, oligomycin A, and FCCP data were calculated by subtracting antimycin/rotenone values. Antimycin and rotenone are inhibitors of the mitochondrial complex III and I, respectively, so under exposure to both inhibitors any respiration left is not due to mitochondrial respiration. Then basal, coupled, maximal, and uncoupled respiration were analyzed. Basal respiration of the cells was that corresponding to the untreated cells. Oligomycin A is an inhibitor of the mitochondrial F$_{1}$F$_{0}$-ATPase, so the difference between the basal respiration and the respiration left when oligomycin is present...
represents the coupled respiration of the cell, that is the respiration directed to the production of ATP. Therefore, the respiration in the presence of oligomycin will be the uncoupled respiration, due to the proton leak. Finally, the addition of FCCP, an agent that uncouples mitochondria, allowed us to know the maximal respiration of the cells. To understand the role of MMP-1 in regulating mitochondrial respiration we compared the mitochondrial profile of the MLE epithelial cells transfected with an empty vector or with MMP-1. Fig. 8A shows that the presence of MMP-1 repressed mitochondrial respiration. Basal but also coupled respiration and maximal respiration were diminished, whereas only uncoupled respiration was maintained. These data indicated that the mitochondrial respiration directed into ATP turnover was reduced in the presence of MMP-1. To confirm these data we measured the OCR of A549 cells in the presence of shControl or shRNA against MMP-1. Fig. 8B confirms that MMP-1 acted as a repressor of mitochondrial respiration because inhibition of MMP-1 expression increased basal and coupled mitochondrial respiration in the A549 cells.

**MMP-1 Expression Stimulates HIF-1α Activation**—Because the respiratory rate was diminished in MMP-1-transfected cells, we aimed to analyze the activation of HIF-1α under normoxic and hypoxic conditions. As observed in a representative experiment shown in Fig. 9A, HIF-1α was already activated in total extracts of mock cells under normoxia and increased as expected under hypoxia. However, in MMP-1-transfected cells HIF-1α expression was elevated under both normoxic and hypoxic conditions compared with control cells (Fig. 9A). This effect was more clearly observed in the nuclear extracts of these cells (Fig. 9B). These results were confirmed by immunofluorescence of HIF-1α as shown in Fig. 9C. HIF-1α was most highly expressed in MMP-1-transfected cells under normoxic and hyperoxic conditions both in cytoplasm and nuclei. In contrast, MMP-1 knockdown by shRNA in A549 cells decreased the activation of HIF-1α under normoxic conditions (Fig. 9D).

**Hypoxia Up-regulates MMP-1 Expression in Human AECs**—A549 cells were exposed to hypoxia, and the levels of MMP-1 expression were analyzed. As observed in Fig. 10, A and B, MMP-1 gene and protein expression were up-regulated under hypoxic conditions. MMP-1 was localized mainly in mitochondrial fraction as illustrated in Fig. 10C.

**MMP-1 Decreases ROS Levels**—The levels of ROS were determined in mock- and MMP-1-transfected cells using CMH2DCFDA and MitoSOX red, a selective indicator of mitochondrial superoxide. As illustrated in Fig. 11, histograms of FACS analysis showed a significant decrease of mean fluorescence intensity of both total and mitochondrial ROS in MMP-1-transfected cells compared with control cells. ROS level measurements under normoxic conditions demonstrated that overexpression of MMP-1 led to a 3.6-fold decrease of mitochondrial ROS. When cells were cultured under hypoxia, mitochondrial ROS levels were duplicated in both mock and MMP-1 cells. Similar to what happened under normoxia, a 3.5-fold decrease of mitochondrial ROS levels was observed in cells overexpressing MMP-1 under hypoxic conditions compared with control cells. The effect of MMP-1 transfection on total ROS was similar under both normoxic and hypoxic conditions, resulting in a decrease of ROS levels.

**DISCUSSION**

IPF is a chronic, progressive, and usually lethal lung disorder of unknown etiology. Although the pathogenic mechanisms have not been elucidated, aberrantly activated alveolar epithe-
intestinal cells, which produce a variety of mediators during the development of the disease, seem to play a key role (1–3). Different transcriptional studies have shown that MMP-1 is one of the most up-regulated genes in the IPF lungs and that the protein, as confirmed in the present study, is strongly expressed by the AECs (6). However, the putative role(s) of the epithelial expressed MMP-1 is unknown. MMP-1 is an archetypical vertebrate collagenase that by the contribution of its catalytic and hemopexin domains is able to degrade fibrillar collagen type I, II, or III. However, multiple studies have shown that MMP-1 is also involved in liberating signaling molecule precursors, such as pro-TGF-α, other EGF-like ligands, and TGF-β from cell surfaces or extracellular matrix, and it can process several important mediators such as pro-TNF-α, IL-1β, L-selectin, α1-antiprotease inhibitor, C1q, connective tissue growth factor, and insulin growth factor-binding proteins 1 and 3 (12–14, 17). Elevated expression of MMP-1 has been documented in other lung disorders associated with cigarette smoke exposure, including emphysema and lung cancer (7, 18), and it has been shown that cigarette smoke up-regulates MMP-1 expression in lung epithelial cells through a MAPK-driven pathway (7).

Epithelial expression of MMP-1 has been also reported in the skin, where it seems to be required for keratinocyte migration on type I collagen matrix likely regulating migratory activities by binding to α2β1 integrin on the cell membrane of keratinocytes (19–21). Down-regulation of MMP-1 seems to be important for normal tissue remodeling whereas high levels of MMP-1 have been reported in chronic nonhealing wounds (22). However, studies related to the putative effects of this enzyme in AEC functions are scanty.

MMP-1 has not been demonstrated to play a similar role in mice and humans, and the murine orthologue of MMP-1, Mcol-A, shows lower identity (74% in nucleotides and 58% in amino acids) than those shared by mouse and human orthologues of most MMPs (23). Therefore, in the present study we evaluated several functions of MMP-1 expression in mouse AECs transfected with the human MMP-1. Our results showed that MMP-1 increases the migratory and proliferative capaci---
ties of the AECs and protects them from cell death. Paralleling these findings, knocking down MMP-1 in A549 human AECs resulted in a significant decrease of migration and proliferation. Moreover, using co-cultures of epithelial cells and fibroblasts, the two key cell types in the pathogenesis of IPF, we observed that MMP-1-transfected MLE cells induced fibroblast migration.

However, the specific mechanisms by which MMP-1 impact on epithelial cell migration, proliferation, and apoptosis are unknown. Regarding migration, it has been shown that type I collagen degradation mediated by MMP-1 is necessary for keratinocyte migration and wound healing in culture models supporting a typical extracellular function of this protease (19). However, in the last years, intracellular functions of MMPs have also been documented (24). Concerning MMP-1, intracellular localization in mitochondrial membranes and nuclei has been demonstrated in various cell types, such as glial Müller cells, Tenon’s capsule and corneal fibroblasts, and retinal pigment epithelial cells (15). In this context, the finding that intracellular MMP-1 accumulates during the mitotic phase of the cell cycle has suggested its participation in the temporary dissociation of nuclear membrane proteins promoting cell growth (15), as we found in our study. Importantly, evidence also indicates that intracellular association of MMP-1 with mitochondria and nuclei confers resistance to apoptosis which is also similar to our findings in MMP-1-transfected MLE cells. Thus, it has been also shown that after exposure to staurosporine, MMP-1 co-localizes with mitochondria clustered around the nucleus shortly after the induction of apoptosis. At later stages, it accumulates around the nuclei and nuclear fragments, indicating that it contributes to the breakdown of the nuclear envelope (15). In our study we also found that in lung AECs MMP-1 localized mainly in mitochondria.

An intriguing finding of the present study was the observation that MLE12 cells transfected with MMP-1 exhibited a repressed OCR, suggesting that MMP-1 could inhibit mitochondrial function. It has been proposed that a major advantage of decreasing mitochondrial respiration during hypoxia is to limit the production ROS. In this context, it was interesting to find that MMP-1 reduced OCR and the production of both mitochondrial and total ROS. Strong evidence supports that HIF-1 reduces ROS production under hypoxic conditions by multiple mechanisms. Therefore, our data indicate that the epithelial expression of MMP-1 represses the OCR, induces the expression of HIF-1α under normoxic conditions, and decreases the production of ROS. This finding is similar to that described in cancer cells which have increased glucose metabolism (25).

Interestingly, it has been reported recently that tumor cell lines expressing MT1-MMP (MMP-14), a membrane-type
MMP, exhibit increased glycolytic activity and moreover, that transfection of this enzyme in MT1-MMP-negative tumor cells induces the Warburg effect by activating HIF-1α (26). Similarly, in our study we found that in addition to repressing oxygen consumption, MMP-1-transfected cells displayed a higher activation of HIF-1α importantly under normoxia and in hypoxic conditions.

The Warburg effect is characterized by increased activity of aerobic glycolysis, despite the presence of abundant oxygen, and is accompanied by activation of HIF-1α during normoxia (27, 28). The transcription factor HIF-1α, a master regulator of oxygen homeostasis and hypoxic signaling, promotes glycolysis by increasing the expression of genes that encode glycolytic enzymes and glucose transporters while inhibiting oxidative phosphorylation (27–29). Importantly, HIF-1α also activates transcription of genes encoding proteases that degrade the extracellular matrix including several MMPs, such as MMP-2, MMP-9, and MMP-14 (30). In the present study, we showed that MMP-1 is also up-regulated when HIF-1α is induced by hypoxia in A549 epithelial cell line. While this manuscript was under revision it was reported that HeLa cells exposed to prolonged hypoxia show an induction of MMP-1 through CREB and NF-κB (31).

Taken together, our findings indicate a likely relationship between MMP-1 and HIF-1α, because the forced expression of the enzyme provokes the up-regulation of the transcription fac-

**FIGURE 9.** MMP-1 induces HIF-1α in mouse alveolar epithelial cells. A and B, MLE cells were maintained at 37 °C in a chamber under 21% O2 (normoxia, Nx) or 1% O2 (hypoxia, Hx) conditions. After 24 h, HIF-1α protein was analyzed in the whole cell extracts (A) and in the nuclear fraction (B). C, Western blot showing HIF-1α levels in control and MMP-1-silenced A549 cells under normoxic and hypoxic conditions. D, detection of HIF-1α by immunofluorescence. Immunoreactive HIF-1α was detected with Alexa Fluor 546-conjugated antibody and nuclei were stained with DAPI. Images from differential interference contrast microscopy (Nomarski) are included. Scale bars, 16 μm.

**FIGURE 10.** Hypoxia induces MMP-1 and HIF-1α in human alveolar epithelial cells. A549 cells were plated on 100-mm culture dishes and exposed to normoxic (Nx) or hypoxic (Hx) conditions for 24 h. A, MMP-1 gene expression by real-time PCR. Error bars, S.D. B, detection of MMP-1 and HIF-1α by Western blotting in whole lysates. C, Western blot of mitochondrial and cytosol fractions of A549 cells. *, p < 0.01.
tor whereas the activation of HIF-1α correlates with the over-
expression of MMP-1. This observation may be relevant for the
understanding of the pathogenesis of IPF because several stud-
ies involving the transcriptional profiling of IPF lungs, includ-
ing a comparative expression profiling and meta-analysis, have
revealed hypoxia signaling among the most relevant deregu-
lated pathways (32). Furthermore, HIF-1α was found increased
in IPF lungs and expressed almost exclusively in hyperplastic
type II AECs overlying areas of fibroblast foci (30). Likewise,
MMP-1 is also highly up-regulated in IPF and is expressed pri-
marily in hyperreactive AECs (4, 5).

In summary, we demonstrated for the first time that the
expression of MMP-1 in AECs inhibits mitochondrial function,
increases HIF-1α expression, decreases ROS production, and
contributes to a proliferative, migratory, and anti-apoptotic
phenotype.

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