Impairment of Alveolar Macrophage Transcription in Idiopathic Pulmonary Fibrosis

Online Data Supplement

Ping Ren, Ivan O. Rosas, Sandra D. MacDonald, Hai-Ping Wu,

Eric M. Billings, and Bernadette R. Gochuico

MATERIALS AND METHODS

Subject selection: For each figure, oxygen use and/or medical treatment for subjects with IPF are noted in Supplemental Table E1.

Supplemental Table E1.

Oxygen or medical treatment for subjects with IPF

Figure	oxygen	prednisone	immunosuppressant	interferon-gamma	other*	none
1	3	1	1	0	4	7
2A	3	3	2	0	3	5
2C	3	3	0	1	0	3
2D,E	3	3	0	1	0	4
3A	3	3	2	0	3	5
3B	2	2	0	0	0	3
4	3	3	2	0	3	5

* colchicine and/or n-acetylcysteine

Isolation of alveolar macrophages and peripheral blood mononuclear cells (PBMCs): For bronchoscopy, subjects received topical 1% lidocaine, intravenous midazolam with or without fentanyl, and supplemental oxygen. Four 30-ml portions of 0.9% sterile saline were instilled in up to three lung segments through a fiberoptic bronchoscope (Olympus America, Melville, NY). Bronchoalveolar lavage (BAL) fluid was collected into specimen containers, filtered through gauze, and centrifuged (1000 x g, 5 min, 4°C). Pelleted cells were washed twice in RPMI 1640, centrifuged at 1000 x g for 5 min at 4°C, dispersed in RPMI 1640, transferred to sterile 100-mm diameter polystyrene tissue culture dishes, and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Adherent cells (about 95% alveolar macrophages) were harvested after 2 h.

Peripheral blood mononuclear cells (PBMCs) were purified from blood obtained by venipuncture. Blood samples were layered on top of Ficoll-Paque Plus™ solution (Amersham Biosciences, Piscataway, NJ), and centrifuged (1200 x g, 30 min, 4°C). PBMCs were gently aspirated and transferred into conical polypropylene tubes. Cells were washed twice with ice-cold PBS, dispersed in RPMI 1640, transferred to sterile 100-mm diameter polystyrene dishes, and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Adherent cells were harvested after 2 h.

Large-scale cDNA analyses: 5 μg of total RNA from alveolar macrophages or PBMCs were reverse-transcribed in the presence of $^{\alpha\text{-}33P}$dCTP. The [$^{\alpha\text{-}33}$P]-labeled nucleotides were purified through a Bio-Spin 6 Chromatography Column, added to 10 ml of hybridization solution, and incubated with cDNA filters overnight at 42°C. Filters were

washed twice with 2X SSC plus 1% SDS, and once with 0.5X SSC plus 1% SDS. Filters were exposed to Phosphorimager screens for 96 h at -80°C.

Northern blot analyses: Samples (10 μg) of total RNA were separated by electrophoresis in 1% agarose formaldehyde gel and transferred to nylon membranes in 10X SSC. Samples (10 ng) of human macrophage inflammatory protein (MIP)-1α, human leukocyte antigen (HLA)-C, fibronectin, and β-actin cDNA were labeled with $^{\alpha-32P}$d-CTP using a DECAprime II DNA labeling kit (Ambion, Inc., Austin, TX). Membranes were pre-hybridized with NorthernMax buffer for 1 hr at 42°C, hybridized with [$^{\alpha-32}$P]-labeled probe overnight at 42°C, washed, and exposed to film using an intensifying screen. RNA levels are reported relative to the amount of β-actin RNA in the same samples.

Isolation and quantification of total RNA and mRNA: Total RNA and mRNA were isolated from alveolar macrophages according to manufacturer's instructions (RNAgents® and PolyATract® System 1000, Promega) and were quantified using mass spectroscopy. Data are normalized for total number of cells.

Nuclear run-on experiments: 3.0 x 10^6 nuclei in 200 μl of transcription buffer (10 mM TrisCl, pH 8.0, 5 mM $MgCl_2$, 0.3 mM KCl, 10 μM NTP) and 200 μCi of [$^{\alpha-32}$P]-UTP were incubated for 1 h at 30°C. Total RNA and mRNA were isolated from nuclei according to manufacturer's instructions (RNAgents® and PolyATract® System 1000, Promega). mRNAs labeled with [$^{\alpha-32}$P]-UTP were separated by electrophoresis using a 6% TBE-urea gel, then exposed to film for 2 hours. Densities in 1 cm x 5 cm regions were quantified using a Storm 860 image analyzing system (Amersham Biosciences).

Western blot analyses and immunohistochemistry: Total extracts from alveolar macrophages or PBMCs were suspended in SDS-lysis buffer (0.4% SDS, 6.25% mercaptoethanol, 62 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM 4-[2-aminoethyl]-benzene-sulfonyl fluoride). Proteins (20 μg) were diluted in Novex Tris-Glycine SDS sample buffer (2X) (Invitrogen), heated at 90^{o}C for 5 min, separated using SDS-PAGE and 4-20% polyacrylamide gradient gels, transferred to nitrocellulose membranes, and incubated with primary antibodies against TFII-A, TFII-B, TFII-D, TFII-E, TFII-F, TFII-G, TFII-H, TFII-H p89, TFII-H p62, TFII-I, or TFII-J, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

5 x 10^5 BAL cells adhered to glass slides were prepared by spinning cells at 40 x g for 10 min using a Cytospin apparatus (Shandon, Pittsburgh, PA), immersed in 3% formaldehyde in PBS, permeabilized by incubation with 0.2% Triton X-100 in PBS for 10 min, blocked with 5% normal goat serum and 3% BSA in PBS, then incubated with rabbit anti-TFII-H p89 and anti-CD14, respectively (Santa Cruz Biotechnology, Inc.). Immunofluorescence was inspected and recorded using a confocal microscope (Nikon, Inc., Melville, NY).

Immunoprecipitation and 2-D gel electrophoresis: Total cell protein (1μg/μl) was incubated with 2 μg of goat anti-TFII-H p44 (Santa Cruz Biotechnology) overnight at 4°C. Proteins precipitated with protein A/G PLUS-Agarose (Santa Cruz Biotechnology) were solubilized in 2-D gel lysis buffer (8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, 40 mM Tris), and samples were applied to non-linear pH 3-10 IPG strips (Amersham Biosciences). Proteins were focused for 8000 v in the first dimension, separated in vertical 4-20% Tris-Glycine Zoom

gels in the second dimension (Invitrogen), and transferred to nitrocellulose, which was immunoblotted with rabbit anti-TFII-H p89 and anti-TFII-H p62. Proteins were quantified using ImageMaster 2D, version 4.1 (Amersham Biosciences).