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Supplementary material

A neutrophil elastase inhibitor prevents bleomycin-induced pulmonary fibrosis in mice

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MATERIALS AND METHODS

Animal treatment

Male C57BL/6 mice (8-10 weeks old), purchased from Japan Clea (Tokyo, Japan), were maintained in specific pathogen-free conditions and provided with food and water ad libitum. To generate the bleomycin-induced pulmonary fibrosis model, bleomycin (5 mg/kg, Nippon Kayaku Co., Tokyo, Japan) was intratracheally administered in 75 μl saline to the C57BL/6 mice on day 0. Immediately after sacrificing the animals on day 7 and 14 after bleomycin instillation, bronchoalveolar lavage (BAL) was performed, and the lungs were removed en bloc. Animals were allocated to the following four groups: (*1*) intratracheal saline + saline intraperitoneally (Control group), (*2*) intratracheal bleomycin + saline intraperitoneally (BLM group), (*3*) intratracheal bleomycin + 100 mg/kg of sivelestat (ONO-5046; N-[2-(4-[2,2-dimethylpropionyloxy] phenylsulphonyl-amino) benzoyl] amino acetic acid; Ono Pharmaceuticals, Osaka, Japan) in saline intraperitoneally (BLM+Sivelestat group), (*4*) intratracheal saline + 100 mg/kg of sivelestat in saline intraperitoneally (Sivelestat group). Sivelestat and saline was given intraperitoneally, once a day for 7 to 14 days. Sivelestat suspension 0.2 ml (prepared immediately before administration in saline solution) was given intraperitoneally, once a day after bleomycin instillation for 7 to 14 days. Immediately after sacrifice on day 7 and day 14, bronchoalveolar lavage (BAL) was performed and the lungs were removed en bloc. Some lung samples were used for histopathological examination, collagen assay, and quantitative real-time polymerase chain reaction (PCR). The rest were used as homogenate samples with sonication. All experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Dokkyo Medical University School of Medicine Subcommittee on Research Animal Care.

Bronchoalveolar lavage and homogenate lung samples

Mice were anesthetized and a soft cannula (23G) was inserted into the trachea. BAL was performed five times by the instillation and withdrawn of 0.8 ml of saline at various time points. The total cell count of the bronchoalveolar lavage fluid (BALF) was determined using trypan blue staining solution. BALF was centrifuged, and the cell pellets were re-suspended in saline and cytospun onto glass slides. These cells were stained with Diff-Quick staining solution (Baxter, Miami, FL), and 500 cells were counted for cell classification. Protein concentrations of BALF were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

The BALF supernatant was frozen immediately at -80 °C. Some of the homogenate samples and the BALF supernatant were freeze-dried by a lyophilizer. The lyophilized samples were dissolved in saline for determination of neutrophil elastase activity, the levels of the active form of TGF-β1 and total TGF-β1, and phospho-Smad2 measurement.

Neutrophil elastase activity

Neutrophil elastase activity was determined by a method using the highly neutrophil elastase specific synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (Calbiochem, San Diego, CA) (E2). Briefly, samples were incubated in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate for 24 h at 37 °C. After incubation, fluorescence was measured spectrophotometrically at wavelengths 360 nm for excitation and 460 nm for emission. Fluorescence was considered to be a measure of neutrophil elastase activity.

Quantitative determination of active form and total TGF-β1

The concentrations of murine active form and total TGF-β1 were determined using ELISA kits (R&D Systems, Minneapolis, MN). The concentrations of TGF-β1 in supernatants were measured with specific ELISA kits (R&D Systems) using the quantitative immunometric sandwich enzyme immunoassay technique. For the assay, the frozen samples were thawed at room temperature and were first activated by being incubated with 1 N HCl for 10 min and neutralized by 1.2 N NaOH/0.5 M *N*-2-hydroxyethylpiperazine-*N*''-ethane sulfonic acid. Activated samples were then transferred to the wells of rigid, flat-bottom microtiter plates coated with TGF-β1 soluble receptor Type II. After incubation and thorough washing, horseradish peroxidase (HRP)-conjugated antibodies directed against TGF-β1 were added to the test wells. After a second incubation, excess HRP-conjugated antibody was removed by washing. The HRP substrate was then added and the color intensity was measured with a microtiter plate reader.

Histopathological evaluation

The right lung was fixed in 10% buffered formalin. It was stained with haematoxylin-eosin and Masson's trichrome. Histopathological grading of fibrosis was performed by three experienced histopathologists using a blinded semiquantitative scoring system for extent and severity of fibrosis in lung parenchyma based on previous studies (E1) with modifications. Briefly, four lung sections from the right lung of each animal were systematically scanned and each successive field was scored using the following grading score according to the method of Ashcroft (E1), with minor modifications: Grade 0, normal lung; Grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; Grade 2, moderate thickening of walls without obvious damage to the lung architecture; Grade 3, increased fibrous with definite damage to lung architecture and formation of fibrous bands or small fibrous masses; Grade 4, severe distortion of architecture and large fibrous area; Grade 5, total fibrous obliteration of the field. The area of the

fibrosis field for each grade and the ratio to the entire field of the section were calculated using a film scanner and the NIH Image software. The sum of the product of ratio multiplied by the grade was used as the score for each section. The mean score of the 4 sections was considered as the fibrosis score for the animal.

Collagen assay

The left lung samples were immediately frozen at -80 °C. They were homogenized and some of the homogenate samples were used for measuring the collagen content. The collagen content was determined by the Sircol Collagen Assay kit (Biocolor Ltd., Belfast, Northern Ireland) (E1). This assay kit is based on the specific binding of Sirius Red dye with the [Gly-X-Y]n helical structure found in collagen. Collagen-bound dye was quantitatively analyzed using a spectrophotometer set at a wavelength of 540 nm.

Real-time PCR analysis

Total RNA was extracted using TRI REAGENT (Life Technologies, Frederick, MD) as described previously (E3) from the lung samples. Reverse transcription was performed using 1 μg of total RNA according to the manufacturer's protocol (PE Applied Biosystems, Foster City, CA). Quantification of TGF-β1 mRNA expression was performed by real-time PCR. Real-time PCR was performed on an ABI Prism 7700 sequence detection system (PE Applied Biosystems), as described previously (E3), by using SYBR green (Roche Diagnostics, Somerville, NJ) as a double-stranded DNA–specific binding dye. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. The primers for real-time quantitative reverse transcription (RT)-PCR were as follows: mouse TGF-β1 (Forward primer; 5'-CGGGGCGACCTGGGCACCATCCATGAC-3', reverse primer; 5'-

CTGCTCCACCTTGGGCTTGCGACCCAC-3') (E4). Mouse GAPDH forward primer: 5'-GCACAGTCAAGGCCGAGAAT-3', Mouse GAPDH reverse primer: 5'-GCCTTCTCCATGGTGGTGAA-3' (E5). The PCR was cycled 40 times after initial denaturation (at 95.8 °C for 2 minutes) with the following parameters: denaturation, 95.8 °C for 15 seconds; and annealing and extension, 60.8 °C for 1 minute. The threshold cycle (CT) was recorded for each sample to reflect the mRNA expression level. A validation experiment proved the linear dependence of the CT value for both TGF-β1 and GAPDH concentrations and consistency of ΔCT (TGF-β1 average CT – GAPDH average CT) in a given sample at different RNA concentration. Therefore ΔCT was used to reflect the relative TGF-β1 expression levels. To determine the effects of different stimuli on TGF-β1 gene expression compared with that seen in unstimulated cells, ΔΔCT was calculated as follows: ΔΔCT = ΔCT stimulus - ΔCT unstimulated cells. TGF-β1 mRNA was indexed to the GAPDH by using the following formula: 1/(2ΔCT) 3 100%. The value of 2ΔΔCT was calculated to demonstrate the fold changes of TGF-β1 gene expression in stimulated lungs compared with that seen in unstimulated lungs.

Phospho-Smad2 measurement

Western blot analysis: Total cellular proteins (50 μg) of lung homogenate protein extracts were subjected to gradient (4~20%) SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was blocked overnight with 5% milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) and then probed at room temperature for 1 hour with primary antibodies (phospho-Smad2 (Ser465/467) antibody at 1:1000 dilution, anti-actin (sc-8432; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:10000) supplemented with 0.05% Tween-20 and 1% bovine serum albumin. After 3 washes with TBST, the membrane was incubated with HRP-conjugated secondary antibody (1:5000 dilution). Reactive protein bands were visualized using

ECL Plus Western blotting detection reagents (Amersham, Uppsala, Sweden) and the bands intensities were scanned and quantified using a densitometer.

ELISA analysis: The Sandwich ELISA PathScan® Phospho-Smad2 (Ser465/467) Kit (Cell Signaling Technologies, Danvers, MA) was used to measure the concentration of phospho-Smad2 measurement according to the manufacturer's instructions. The lyophilized lung homogenized samples were dissolved by 100 μl saline and measured protein concentration by Bradford-based Coomassie®-plus Protein Assay Reagent Kit (Pierce, Rockford, IL). The lung homogenized samples with 30mg of protein were applied for this measurement. This antibody may detect Smad3 phosphorylated at the equivalent sites. After incubation, read absorbance at 450 nm spectrophotometrically. Relative intensity was calculated by the fold changes of phosphor-Smad2 signals in stimulated lungs compared with that seen in unstimulated lungs.

Statistical analysis

Data are expressed as means ± standard error of the means (SEM). Statistical significance was determined by one-way ANOVA or *t* test. P-values less than 0.05 were considered significant.

References

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