Intratracheal gene transfer of decorin reduces subpleural fibroproliferation induced by bleomycin

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Shimizukawa, Minoru, Masahito Ebina, Ko Narumi, Toshiaki Kikuchi, Hiroshi Munakata, and Toshihiro Nukiwa. Intratracheal gene transfer of decorin reduces subpleural fibroproliferation induced by bleomycin. Am J Physiol Lung Cell Mol Physiol 284: L526–L532, 2003.—Decorin, a small leucin-rich proteoglycan, is a negative regulator of transforming growth factor-β, but the antifibrotic effect of decorin gene transfer has not been examined in a mouse model of usual interstitial pneumonia (UIP). We constructed a replication-defective recombinant adenovirus harboring human decorin gene (AdCMV.DC) and administered 1 × 10⁶ plaque-forming units of AdCMV.DC intratracheally or intravenously to C57BL/6 mice with intraperitoneal injection of bleomycin, which induces a subpleural fibroproliferation, mimicking UIP, by day 28. Only intratracheal administration of AdCMV.DC increased decorin mRNA expression in the lung and decreased the hydroxyproline content augmented in bleomycin-induced pulmonary fibrosis (1.13 ± 0.02 to 0.96 ± 0.02, P = 0.006). In contrast, intravenous administration of AdCMV.DC increased the decorin expression only in the liver, but not in the lung, and without reducing lung fibrosis. These results indicate that adenoviral decorin gene transfer is effective only by direct administration to fibrosing lungs.

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a specific form of chronic fibrosing interstitial pneumonia limited to the lung and associated with the histological appearance of usual interstitial pneumonia (UIP) on surgical lung biopsy (2). Because of the short survival of patients with IPF and the lack of curative therapy, new therapeutic strategies based on the molecular pathogenesis of IPF need to be established.

Among various cytokines and growth factors contributing to fibroproliferative disorders (5), transforming growth factor (TGF)-β is broadly accepted as a key factor because 1) it is upregulated in response to chemical or biological tissue injuries, 2) it inhibits immune responses, and 3) it synthesizes extracellular matrix proteins to deposit in various fibrous disorders, including pulmonary fibrosis (19). TGF-β is secreted in a latent form complex with latency-associated peptide and activated by proteolysis (8) or by contact with integrin α6β1 to bind with the cell-surface type II TGF-β receptor (22). An analysis of gene expression in bleomycin-induced pulmonary fibrosis in mice using an oligonucleotide microarray also supports the importance of activation of TGF-β by revealing the absence of an increase of TGF-β mRNA, regardless of the upregulation of TGF-β-inducible genes (14). The essential role of TGF-β in fibrogenesis in mouse lung injured by bleomycin is also revealed by recent evidence showing that mice deficient in Smad3, which transduces TGF-β signals from the cell membrane to the nucleus, have a reduction in hydroxyproline content in lungs compared with wild-type mice treated with bleomycin (40).

Decorin, a small chondroitin-dermatan sulfate proteoglycan composed of a core protein and a single glycosaminoglycan chain, distributes in the extracellular matrix in association with collagen (12). Yamaguchi et al. (38) reported first that decorin binds to TGF-β and neutralizes its activities through the leucine-rich repeats of its core protein. Yamaguchi and Ruoslahti (38a) also revealed that recombinant decorin inhibits the production of extracellular matrix and attenuates the manifestations of experimental glomerulonephritis. Thereafter, the therapeutic effect of decorin gene on antifibroproliferation was reported in a Thy-1 glomerulonephritis model (13) and in pulmonary fibrosis induced by intratracheal (IT) injection of bleomycin (16), which constitutes a parabronchial distribution of fibrosis.

We reported previously that continuous subcutaneous injection of bleomycin through a microosmotic pump for 7 days caused subpleural and heterogeneous fibroproliferation similar to UIP lesions, a pathological feature of human IPF (26, 38a). In the present study, we modified the method by Swiderski et al. (32) of intraperitoneal injection of bleomycin every other day for 7 days for a constant composition of subpleural fibroproliferation in the mouse lung.

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The purpose of this study is to elucidate the antifibrotic effect of adenoviral human decorin gene transfer using two different routes, IT and intravenous (IV) administration. We tried the IV route because adenovirus-mediated gene transfer to the liver can be efficiently achieved by IV administration (36a) and because decorin administered via the IV route accumulates in the liver, kidney, and lung (6, 13). We transfected human decorin gene because of its high homology across species (30).

MATERIALS AND METHODS

Cells and animals. The cell lines of A549 (ATCC CCL-185) and 293 (ATCC CRL-1573) were purchased from American Type Culture Collection (Rockville, MD). COS-7 (TKG 0514) was supplied by Cancer Cell Repository (Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan). MLEC-PAI/Luc (1), a mink lung epithelial cell line, was supplied by Cancer Cell Repository (Institute of Development, Aging, and Cancer, Tohoku University). All cells were maintained in DMEM supplemented with 10% fetal calf serum at 37°C in a 5% CO2–95% air atmosphere.

For the in vivo experiments, female C57BL/6 mice, 12 wk old and weighing ~20 g, were purchased from Japan Charles River (Yokohama, Japan) and maintained at a constant temperature, humidity, and light cycle with food and water ad libitum.

Recombinant adenoviral vectors. AdCMV.DC (Fig. 1A), an E1A- and partial E3-adenovirus type 5-based replication-deficient vector containing the cytomegalovirus promoter (7), and human decorin cDNA (18), a kind gift from Dr. Y. Yamaguchi (Burnam Institute, La Jolla Cancer Center, La Jolla, CA), was constructed as described previously (10, 24). An adenoviral vector without the transgene was also constructed as a control (AdCMV.Null). Both viruses were purified by cesium chloride density gradient ultracentrifugation, tittered by plaque-forming assay on 293 cells, and then demonstrated to be free of replication-competent adenovirus (20).

Administration of adenoviral vectors in vivo. For IT administration, vectors were dissolved in 30 μl of saline and injected directly into the trachea of the mouse under 2.5% tribromoethanol anesthesia, and for IV administration, vectors were dissolved in 100 μl of saline and injected into the jugular vein through a 29-gauge needle.

Western blot analysis. Subconfluent A549 cells were infected with AdCMV.DC at the various multiplicities of infection (MOI). After 20 h, the medium was replaced with serum-free medium, and the cells were incubated for a further 40 h. After being treated with or without chondroitinase ABC (Seikagaku Kogyo, Tokyo, Japan) digestion by incubation of 40 μl of culture medium with 5 μl of 4 M Tris–HCl, 5 μl of 4 M CH3COONa, and 5 μl of chondroitinase ABC for 90 min at 37°C (25), 10 μl of the medium were subjected to electrophoresis on a 10% Tris-glycine gel and transferred to a polyvinylidene difluoride membrane. The membrane was probed with monoclonal antibody (Seikagaku Kogyo) generated against the decorin core protein moiety and then with alkaline phosphatase-conjugated anti-mouse IgG (Promega, Madison, WI) and visualized with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system (KPL, Gaithersburg, MD). As a control, 100 μg/ml of bovine decorin (Sigma Chemical, St. Louis, MO) diluted with DMEM were used.

RT-PCR. To demonstrate the expression of decorin mediated by the AdCMV.DC, total RNA was extracted from the lungs or livers of C57BL/6 mice 3 days after the administration of AdCMV.DC or AdCMV.Null [1 × 109 plaque-forming units (pfu) via the IT or IV route], and 1 μg of RNA was subjected to reverse transcription using the RNA PCR kit (Takara, Kyoto, Japan) at 42°C in a total volume of 20 μl. One-tenth of the cDNA was amplified with the following primers specific for either vector-derived decorin or the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts: for decorin, 5′-GTAGGGACCTGCGAGTTGTC-3′ and 5′-TGCCACATTCTATGACAACTC-3′; for GAPDH, 5′-ATGGTGAAAGGTCCGTGAGACGGA-3′ and 5′-TACTCTTGGAGGGCATTGAGGC-3′. The amplification profile was 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. Each PCR was carried in a volume of 50 μl, and 5 μl of PCR products were resolved on a 1% agarose gel and stained with 0.5 μg/ml of ethidium bromide.

Luciferase assay. MLEC-PAI/Luc cells were seeded in a 96-well plate (2.5 × 104 cells/well) and incubated for 5 h. The medium was discarded, and both recombinant human TGF-β1 (rHTGF-β1; R&D Systems, Minneapolis, MN) and bovine decorin diluted at various concentrations with 0.1%
bovine serum albumin/DMEM were added to the plate and subsequently incubated for 14 h. The luciferase activity was measured with a luciferase assay system (Promega) and a luminometer (AB-210; ATTO, Tokyo, Japan).

**Immunohistochemistry** for β-galactosidase. Three days after the administration of AdCMV.LacZ (1 × 10^9 pfu), the mice were killed after anesthesia, and the lungs were infused through the trachea in the thoracic cage with 50% optimum cutting temperature compound embedding medium diluted with saline and snap-frozen at −80°C. For immunohistochemistry for β-galactosidase, 5-μm-thick frozen sections were prepared and fixed in 0.25% glutaraldehyde at 4°C for 10 min, rinsed in PBS, and incubated for 4 h at room temperature in 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, 1.3 mM MgCl₂, and 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in PBS.

**Bleomycin-induced pulmonary fibrosis.** Pulmonary fibrosis of 12-wk-old female C57BL/6 mice was induced by intraperitoneal instillation of bleomycin chloride (Nipponkayaku, Tokyo, Japan); 0.75 mg/body dissolved in 0.1 ml of saline every other day for 7 days, on days 0, 2, 4, and 6: AdCMV.DC or AdCMV.Null (1 × 10^9 pfu) was administered 24 h before the start of the bleomycin instillation under 2.5% trisbromoethanol anesthesia. On day 28, the mice were anesthetized and killed, and the lungs in the thoracic cages were infused through the trachea with 10% buffered formalin and fixed at room temperature for 16 h. The right lungs were used for analysis of the hydroxyproline content, as described below. The left lungs were embedded in paraffin, and hematoxylin- eosin- and elastica-Masson-stained sections were prepared.

**Hydroxyproline analysis.** The whole collagen content of the right lung was estimated by an assay of hydroxyproline (35). Briefly, after acid hydrolysis of the lung with 6 N HCl at 110°C for 16 h in a sealed glass tube, the hydroxyproline content was determined by high-performance liquid chromatography (11). To compare the results of different experiments, the IT and IV experiments, the content of hydroxyproline per dry lung weight was normalized to the relative ratio according to the mean level of the saline treatment control group of each experiment.

**Statistical analysis.** Statistical analysis was performed with Statview 4.5 (Abacus Concepts, Berkeley, CA). The statistical significance of difference in the results was evaluated using unpaired ANOVA, and P values were calculated by Mann-Whitney's U-test. A value of <0.05 was accepted as statistically significant. All values were represented as the means ± SE.

**RESULTS**

**Expression of decorin by AdCMV.DC in vitro and in vivo.** The decorin secreted by AdCMV.DC (Fig. 1A)-infected cells (1–100 MOI) in the culture medium was subjected to Western blot analysis after being treated with or without chondroitinase digestion (Fig. 1B), which degrades only glycosaminoglycans (25). As a result, the decorin core protein of ~45 kDa was detected after chondroitinase digestion, and the amount of expression was increased in an MOI-dependent manner. In vivo, RT-PCR analysis was used to examine the expression of human decorin mRNA in the lungs and the livers of the C57BL/6 mice, which were administered 72 h before with IT or IV transfection of AdCMV.DC or AdCMV.Null (1 × 10^9 pfu; Fig. 1C). Its expression was elevated only in lungs of the mice with IT administration of AdCMV.DC and without expression in the livers. In contrast, the mice with IV administration of AdCMV.DC or AdCMV.Null (1 × 10^9 pfu) had human decorin mRNA expression in the livers but not in the lungs.

**Anti-TGF-β activity of decorin in vitro.** The anti-TGF-β activity of decorin was examined by luciferase assay using MLEC-PAI/Luc cells (Fig. 2). We found that 100 μg/ml of decorin completely inhibited the luciferase expression levels of MLEC-PAI/Luc cells by rhTGF-β1, 0.1–10 ng/ml, and that there was partial inhibition by 10 μg/ml of decorin. However, a low dose of decorin, 10 ng–1 μg/ml, did not affect the activity of rhTGF-β1, even to 0.1 ng/ml. These results indicate that a large amount of decorin, more than 10^4 times the concentration of TGF-β1, was required to inhibit the transcriptional activity of TGF-β1 on MLEC-PAI/ Luc cells.

**Administration of AdCMV.LacZ in vivo.** To assess distribution of the exogenous transgene expression in the mouse lung by IT or IV administration of adenoviral vectors, we used AdCMV.LacZ, which contains LacZ genes (1 × 10^9 pfu) that code β-galactosidase instead of human decorin, because of the poor immunohistochemical reactivity of the available anti-human decorin antibodies. Expression of β-galactosidase-positive cells in the mouse lungs was observed only with IT administration, and the cells were distributed in the airway epithelial cells, the alveolar epithelial cells, and the alveolar macrophages (Fig. 3). In contrast, IV administration of AdCMV.LacZ yielded no positive cells in the mouse lungs (data not shown).

**Antifibrotic effect of human decorin transgene.** The hydroxyproline content per dry weight of the right lung was compared among four groups: one control group
treated with saline (saline/saline) and three bleomycin-treated groups with administration of AdCMV.DC (BLM/AdCMV.DC), AdCMV.Null (BLM/AdCMV.Null), or saline (BLM/saline) (Fig. 4). To compare results between the experiments using two different routes, IT and IV, the hydroxyproline content level was normalized according to the mean level of saline/saline of each route. The hydroxyproline content by saline/saline-IT was 29.17 ± 0.80 mmol/g dry tissue (n = 5) and that by saline/saline-IV was 27.74 ± 0.45 mmol/g dry tissue (n = 4), and was, therefore, without statistical difference (P = 0.227).

IT administration of AdCMV.DC significantly reduced the relative hydroxyproline content (0.96 ± 0.02, n = 9) compared with that of the BLM/AdCMV.Null-IT group (1.07 ± 0.04, n = 7, P = 0.030) and much more than that of the BLM/saline group (1.13 ± 0.02, n = 7, P = 0.006) (Fig. 4). On the other hand, there was no statistical difference by IV administration in the relative content of hydroxyproline between the BLM/AdCMV.Null-IV group (n = 8, 1.02 ± 0.02) and BLM/AdCMV.DC-IV group (n = 7, 1.04 ± 0.04).

Histological changes by administration of AdCMV.DC. The lungs of a mouse model of UIP examined in this study showed a heterogenous distribution of fibroproliferation with microhoneycomb-like lesions distributed in the subpleural regions by day 28 (Fig. 5, A and B), similar to the fibrous lungs of patients with idiopathic pulmonary fibrosis. Histological observation of these lungs showed that the subpleural distribution of bleomycin-induced fibroproliferation (Fig. 5B) was not changed by IT administration of AdCMV.Null (Fig. 5C) but was remarkably decreased by IT AdCMV.DC (Fig. 5D).

DISCUSSION

Decorin is a ubiquitous proteoglycan, with a core protein of 45 kDa, shown to have two binding sites for TGF-β to block its function (12). Hildebrand et al. (12) reported that the latent recombinant form of TGF-β1 does not bind decorin, whereas the activated form of TGF-β1 does, which suggests the regulatory effect of decorin on the activities of TGF-β1. We confirmed that decorin hampered the transcription activity of TGF-β1 in vitro using MLEC-PAL/Luc cells and found that a 10^4 times greater concentration of decorin was required to block this activity. In addition to TGF-β, the core protein of decorin is known to interact with several different matrix proteins, including fibronectin (29), thrombospondin (34), C1q (17), and types I, II, and IV collagen (4, 33), to influence matrix assembly. Moreover, decorin interacts with epidermal growth factor receptor to trigger a signaling cascade for the elevation of endogenous p21 and growth suppression (21).

Fig. 4. Relative content of hydroxyproline per dry weight of right lungs. IT administration of AdCMV.DC (BLM/AdCMV.DC-IT) reduced the hydroxyproline content (n = 9, 0.96 ± 0.02) in contrast to that of bleomycin (BLM/saline-IT) (n = 7, 1.13 ± 0.02, P = 0.006) and BLM/AdCMV.Null-IT (n = 7, 1.07 ± 0.04, P = 0.030) with statistical difference. No significant difference (NS) was found in the hydroxyproline content ratio between BLM/AdCMV.Null-IV group (n = 8, 1.02 ± 0.02) and BLM/AdCMV.DC-IV group (n = 7, 1.04 ± 0.04). IP, intraperitoneal.
AdCMV.LacZ caused β-galactosidase expression in airway epithelial cells, alveolar type II cells, and alveolar macrophages, these results indicate that only direct administration of AdCMV.DC to the lungs through the respiratory tract would attain a dose adequate to prevent bleomycin-induced fibrosis in the lung. Although IT administration of decorin protein has not been tried for bleomycin-induced pulmonary fibrosis, IV injection of a high dose of human decorin protein was revealed to inhibit the increased production of extracellular matrix in an experimental glomerulonephritis model of rats (6). Our results showing IV administration of AdCMV.DC did not improve bleomycin-induced pulmonary fibrosis suggest that the production of decorin in the lung was not adequate to reduce fibroproliferation.

We used an UIP model of mouse lung, which shows a heterogenous distribution of fibroproliferation, distributed mainly subpleurally, forming similar lesions to those observed in the lungs of patients with IPF (26), the mechanisms of which have not yet been elucidated. We did not clarify in this study how IT administration of decorin gene reduced the subpleural fibrogenesis. For entry of adenovirus into the cells, the process has at least two steps: 1) initial attachment of the viral fiber-knob protein to the high-affinity receptor coxsackievirus and adenovirus receptor (3), and 2) translocation of the virus into the cell cytoplasm via the coated-pit internalization process, mediated in part by an interaction of the viral penton base with integrin αvβ3/5, which was reported to be less widely distributed in the well-differentiated airway epithelial cells (28). Considering that alveolar macrophages eliminate adenovirus vectors administered via the respiratory tract (36) and that alveolar macrophages produce key cytokines or growth factors, including TGF-β, for fibroproliferation, alveolar macrophages would be more efficiently transfected by IT than by IV administration of AdCMV.DC. Therefore, our results suggest that, even though direct gene transfer to the subpleural lesions is not likely to happen, the fibrosing process of these lesions can be blocked through transfection of alveolar macrophages with AdCMV.DC.

We do not know the mechanisms for the relative reduction of hydroxyproline in bleomycin-injured lungs by AdCMV.Null administered via both IV and IT routes. A nonspecific immune response against adenoviral null vectors administered IT was previously reported (27) to induce anti-inflammatory cytokines, such as interferon-γ, a potent antifibrotic cytokine (15). Different effects might be caused on fibroproliferation and collagen deposition by our experimental system, which requires further study to explain these phenomena.
In conclusion, adenoviral gene transfer of decorin through IT administration of AdCMV.DC effectively reduces the subpleural fibroproliferation induced by bleomycin.

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