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Antifibrotic Effect of Decorin in a Bleomycin Hamster Model of Lung Fibrosis*

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ABSTRACT. We reported previously that treatment with antibody to transforming growth factor-β (TGF-β) caused a marked attenuation of bleomycin (BL)-induced lung fibrosis (LF) in mice. Decorin (DC), a proteoglycan, binds TGF-β and thereby down-regulates all of its biological activities. In the present study, we evaluated the antifibrotic potential of DC in a three-dose BL-hamster model of lung fibrosis. Hamsters were placed in the following groups: (1) saline (SA) + phosphate-buffered saline (PBS) (SA + PBS); (2) SA + DC; (3) BL + PBS; and (4) BL + DC. Under pentobarbital anesthesia, SA (4 mL/kg) or BL was instilled intratracheally in three consecutive doses (2.5, 2.0, 1.5 units/kg/4 mL) at weekly intervals. DC (1 mg/mL) or PBS was instilled intratracheally in 0.4 mL/hamster on days 3 and 5 following instillation of each dose of SA or BL. In week 4, hamsters received three doses of either DC or PBS every other day. The hamsters were killed at 30 days following the first instillation, and their lungs were appropriately processed. Lung hydroxyproline levels in SA + PBS, SA + DC, BL + PBS, and BL + DC groups were 965, 829, 1854, and 1387 μg/lung, respectively. Prolyl hydroxylase activities were 103, 289, and 193% of SA + PBS control in SA + DC, BL + PBS, and BL + DC groups, respectively. The myeloperoxidase activities in the corresponding groups were 222, 890, and 274% of control (0.525 units/lung). Intratracheal instillation of BL caused significant increases in these biochemical markers, and instillation of DC diminished these increases in the BL + DC group. DC treatment also caused a significant reduction in the infiltration of neutrophils in the bronchoalveolar lavage fluid (BALF) of hamsters in the BL + DC group. However, DC treatment had little effect on BL-induced increases in lung superoxide dismutase activity and lipid peroxidation and leakage of plasma proteins in the BALF of the BL + DC group. Hamsters in the BL + PBS group showed severe multifocal fibrosis and accumulation of mononuclear inflammatory cells and granulocytes. In contrast, hamsters in the BL + DC group showed mild multifocal septal thickening with aggregations of mononuclear inflammatory cells. Hamsters in both control groups (SA + PBS and SA + DC) showed normal lung structure. Frozen lung sections following immunohistochemical staining revealed an intense staining for EDA-fibronectin and collagen type I in the BL + PBS group as compared with all other groups. It was concluded that DC potentially offers a novel pharmacological intervention that may be useful in treating pulmonary fibrosis. BIOCHEM PHARMACOL **54**;11:1205–1216, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. pulmonary fibrosis; fibronectin; collagen; transforming growth factor-β; decorin

Pulmonary fibrosis, the final pathway of many interstitial lung diseases, is initially accompanied by inflammation followed by a massive production of fibrous connective tissue in the interalveolar septa [1]. The fibrotic process is characterized by an excess number of fibroblasts [2], an absolute increase in lung collagen content, and an abnormality in the ultrastructural appearance and spatial distribution of collagen types [3–5]. The mechanisms responsible for the inflammatory/immune processes and the ensuing alterations in the connective tissue in lung fibrosis are not well understood.

BL¶ is used as a chemotherapeutic agent for the treatment of cancer [6]. However, its use as an antineoplastic drug is limited because it produces a dose-dependent pneumonitis that often progresses to interstitial lung fibrosis in humans [6]. IT instillation of BL in rodents is used frequently as an animal model of lung fibrosis, as it resembles that seen in humans [7, 8].

There are several lines of evidence which suggest that

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Abbreviations: BL, bleomycin; TGF-β, transforming growth factor-β; ECM, extracellular matrix; DC, decorin; SA, saline; BALF, bronchoalveolar lavage fluid; MPO, myeloperoxidase; SOD, superoxide dismutase; MDAE, malondialdehyde equivalent; IT, intratracheal; and ROS, reactive oxygen species.

the increased production of TGF-β is an important component in the pathophysiology of fibrosis including lung fibrosis induced by BL [9, 10]. In this regard, the two most convincing findings of potential clinical significance are: (1) treatment with a TGF-β neutralizing antibody causes a significant reduction in the accumulation of ECM in the glomeruli of anti-thymocyte serum (ATS)-treated animals [11]; and (2) treatment with TGF-β antibody diminishes the BL-induced lung fibrosis in mice as demonstrated in our laboratory [12].

DC, a small proteoglycan and a component of collagen fibrils in most ECM, is known to bind and reduce certain biological activities of all isoforms of TGF-β [13, 14]. In fact, the systemic administration of DC was found to prevent TGF-β-mediated accumulation of ECM in the glomeruli of ATS-treated animals [15]. The present study was designed to find out whether or not treatment with DC will have an antifibrotic effect in the BL-hamster model of lung fibrosis. The evidence presented in this paper suggests that DC has potential as a therapeutic agent for the treatment of lung fibrosis.

MATERIALS AND METHODS

Animals and Chemicals

Male Golden Syrian hamsters weighing 115–130 g were obtained from Simonsen, Inc. (Gilroy, CA). BL sulfate (Blenoxane®) was donated by Bristol Laboratories (Syracuse, NY). Rat DC was supplied by Teliós Pharmaceuticals, Inc. (San Diego, CA). All other reagents were of reagent grade or higher purity and were obtained from standard commercial sources.

Treatment of Animals

Hamsters were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. The animals were allowed to acclimate for 1 week to laboratory conditions before starting any treatment. A 12 hr light/12 hr dark cycle was maintained throughout the study. The hamsters had access to water and Rodent Laboratory Chow 5001 (Purina Mills, Inc., St. Louis, MO) *ad lib.*

The hamsters were placed randomly into four experimental groups: (1) SA + PBS; (2) SA + DC; (3) BL + PBS; and (4) BL + DC. BL was freshly dissolved in isotonic saline and DC in PBS prior to IT instillations. Under pentobarbital anesthesia, saline (4 mL/kg) or BL was instilled IT by the transoral route in three consecutive doses (2.5, 2.0, and 1.5 units/kg/4 mL) at weekly intervals. DC (1 mg/mL) solution or its vehicle, PBS, was instilled IT in 0.4 mL/hamster on days 3 and 5 following IT instillation of each dose of BL or SA. In week 4, hamsters received IT either three doses of DC or PBS every other day. The hamsters were killed at 30 days following the first instillation of either BL or SA, and their lungs were appropriately processed for biochemical studies, bronchoalveolar lavage collection, and histopathologic and immunohistochemical studies.

Processing of Lungs for Biochemical Studies

The hamsters designated for biochemical studies in each group were first anesthetized by an overdose of sodium pentobarbital followed by exsanguination of the descending abdominal aorta. After opening the thoracic cavity, the lungs were perfused *in situ* via the right ventricle with ice-cold isotonic saline. The lungs were quickly dissected free of nonparenchymal tissue and immediately frozen in liquid nitrogen and later stored at −80°. Subsequently, the frozen lungs were thawed and homogenized in 0.1 M KCl, 0.02 M Tris (pH 7.6) with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was mixed thoroughly by repeated inversions. After recording the volume (9–10 mL), the homogenate was divided into several aliquots and stored at −80° until measurement of various biochemical parameters, as described below.

DETERMINATION OF HYDROXYPROLINE. One milliliter of lung homogenate was precipitated with 0.25 mL of ice-cold 50% (w/v) trichloroacetic acid and centrifuged. The precipitate was hydrolyzed in 2 mL of 6 N HCl for 18 hr at 110°. The hydroxyproline content was measured by the technique described by Woessner [16].

DETERMINATION OF PROLYL HYDROXYLASE (EC 1.14.11.2) ACTIVITY. After thawing the lung homogenate, it was centrifuged at 12,000 × *g* for 20 min at 4°. The supernatant was used for the measurement of prolyl hydroxylase activity. The procedures for making prolyl hydroxylase substrate and assaying its activity were essentially the same as described in one of our previous papers [17]. During the reaction, tritium (^3H) is released in stoichiometric proportion to prolyl hydroxylation and ^3H_2O is collected and used as a measure of the enzyme activity [18]. The activity is expressed as dpm/total lung/30 min and reported as percent of the control.

DETERMINATION OF MPO ACTIVITY. The MPO activity of the lung homogenate was assayed according to the technique described by Goldblum and Wu [19]. The MPO was extracted in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer (pH 6.0). The samples were freeze–thawed (20 min at −70°) three times followed by homogenization each time on ice with a Polytron. The homogenate was then centrifuged at 40,000 × *g* for 15 min, and the resulting supernatant was used to assay the MPO activity spectrophotometrically as described in an earlier paper [20]. One unit of MPO activity was defined as the volume of the supernatant that degrades 1 μmol peroxide/min at 25°.

DETERMINATION OF MDAE. Lung MDAE level, as an index of lipid peroxidation, was measured from the amount

of thiobarbituric acid-reacting products present in the lung homogenate by the method of Ohkawa *et al.* [21].

DETERMINATION OF SOD (EC 1.15.1.1) ACTIVITY. The supernatant (12,000 × *g*) of lung homogenate was used to assay SOD activity from the rate at which it inhibits the autoxidation of epinephrine to adrenochrome as described by Misra and Fridovich [22]. The rate of formation of adrenochrome was 0.025 absorbance units/min at 480 nm in a Varian Cary 219 spectrophotometer (Varian Instrument Group, Sugarland, TX). Under these defined conditions, the volume of the supernatant required to inhibit the rate of formation of adrenochrome by 50% (i.e. rate of 0.0125 absorbance units/min) was defined to contain 1 unit of SOD activity.

Preparation of Lung for BALF Collection and Histopathological and Morphometric Studies

Four to six animals in each group were first anesthetized, and then BALF was collected according to the technique described in our earlier paper [23]. The BALF was centrifuged at 1500 × *g* for 20 min at 4°. After aspirating the supernatant, the pellet was suspended in 1 mL of isotonic saline. Total leucocyte count of the suspension was determined in a Coulter Counter (Coulter Electronics, Hialeah, FL), and cytological examination of cells in the suspension was done after cytocentrifugation and staining with Leuko-Stat Fisher (Diagnostics, Orangeburg, NY), a modification of the Wright's stain technique. The relative proportions of various leukocyte subpopulations were determined by a cell differential count of 200 cells for each animal. The protein content of the BALF-supernatant was determined by the technique of Lowry *et al.* [24].

After bronchoalveolar lavage and thoracotomy, the heart was ligated at the base for isolation of the pulmonary vasculature. The trachea was cannulated, and then both heart and lungs were removed *en bloc* and weighed. The lungs were fixed via the trachea at a pressure of 30 cm of water with a cacodylate-buffered glutaraldehyde-paraformaldehyde fixative (400 mOsM). The cannula was removed, the trachea was tied off, and the lung and heart were stored in this fixative. Before embedding, the lung was isolated from the heart and all nonpulmonary tissue by blunt dissection and removed. Blocks of tissue were cut from at least two sagittal slabs (2–3 mm thick) from the right cranial, right caudal, and left lung lobes of each lung. Each block was cut with about a 1 cm^2 face. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin. Sections (5 μm thick) were cut from the paraffin blocks and stained with hematoxylin and eosin for histological and morphometric evaluations.

Lesions were morphometrically evaluated in sections (N = 4–6) from each of the sampled lungs according to the technique of Bolender *et al.* [25]. We defined parenchymal lesions as thickening of interalveolar septa due to edematous swelling, inflammatory cells, or fibrosis associated with hyperplastic epithelial cells, and/or clusters of airway inflammatory cells in either airways or interstitium. The volume density of lesion, V_V, was determined by point-counting techniques using the formula $V_V = P_P = P_N/P_T$, where P_P is the point fraction of P_N, the number of points hitting a lesion divided by P_T, the total number of points hitting the section. Because of the patchy distribution of the lesion, each section was scanned systematically at a final magnification of 20× with a 42-point lattice test system until all fields (5–25 fields) in the section were evaluated. A mean volume density of lesion was obtained by averaging the V_V values contributed by each lung.

Preparation of Lung for Immunohistocytochemistry

Four to five hamsters in each group were designated for immunohistocytochemical evaluation. After decapitating the hamsters, their lungs were removed and immediately trimmed. The lobes were put into cryomolds containing Tissue-tek O.C.T. compound. The lobes were quick-frozen in an isopentane chamber submerged in liquid nitrogen. The cryomolds, after being wrapped in aluminum foil, were kept on dry ice and then were stored at −80° until microsection and staining.

Immunohistochemistry evaluations on the frozen lung sections were carried out as described previously [26]. The frozen sections were stained immunohistochemically using mouse monoclonal antibodies to EDA-fibronectin. The monoclonal antibodies were detected using goat anti-mouse IgG-horseradish peroxidase (HRP) followed by development with diaminobenzidine. Collagen type I immunohistochemistry was performed using goat polyclonal antibodies. The goat anticollagen antibodies were detected with rabbit anti-goat-HRP followed by diaminobenzidine. All tissue sections were counterstained with hematoxylin and eosin, dehydrated, mounted on glass slides, and evaluated by light microscopy. The slides were evaluated for intensity of staining by someone who was not familiar with any of the treatments and scored as follows: 0, no staining; 1, diffuse light staining; 2, higher intensity; and 3, highest intensity.

Statistical Analysis of Data

The biochemical data are expressed on a per lung basis. The data are presented in this manner to avoid the artificial lowering of the values in BL-treated animals due to the presence of proteins of extrapulmonary origin [27]. All values are reported as means ± SD and analyzed by one-way analysis of variance and Duncan's Multiple Range test. A value of $P < 0.05$ was considered significant.

RESULTS

Mortality

Out of twenty hamsters treated with BL in the BL + PBS group, four died. This 20% cumulative mortality in the latter group was reduced to 5% by DC treatment in the

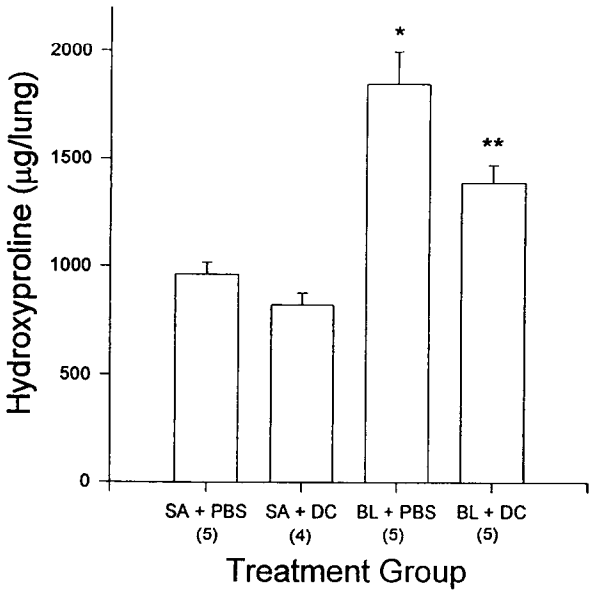

FIG. 1. Effects of repeated IT instillations of DC on BL-induced increases in lung hydroxyproline content. Data are expressed as means ± SD. The number of animals in each group is shown in parentheses below each bar, and treatment groups are shown along the x-axis and explained in "Materials and Methods." Key: (*) significantly higher ($P < 0.05$) than the remaining three groups, and (**) significantly higher ($P \leq 0.05$) than the SA + PBS and SA + DC groups.

BL + DC group since only one hamster died out of twenty in this group. None of the sixteen hamsters treated with SA in the SA + PBS group or the SA + DC group died over the entire duration of the study.

Hydroxyproline

The hydroxyproline content of the lung, as an index of collagen, for the various treatment groups is summarized in Fig. 1. BL treatment significantly increased the hydroxyproline content in the BL + PBS group to 192% of the SA + PBS control group. Although treatment with DC caused a significant reduction in the BL-induced increases in the lung hydroxyproline content of the BL + DC group, the hydroxyproline content in the latter group was still significantly higher than in the two control groups. There was no difference in the hydroxyproline content between the two control (SA + PBS and SA + DC) groups.

Prolyl Hydroxylase Activity

BL treatment significantly increased the lung prolyl hydroxylase activity in the BL + PBS group to 289% of the SA + PBS control, and treatment with DC caused a significant reduction in the BL-induced increases in the activity of this enzyme in the BL + DC group (Fig. 2). However, the activity in the latter group was still significantly higher than in the two control groups. There was no significant difference in the activity between the two control groups, SA + PBS and SA + DC.

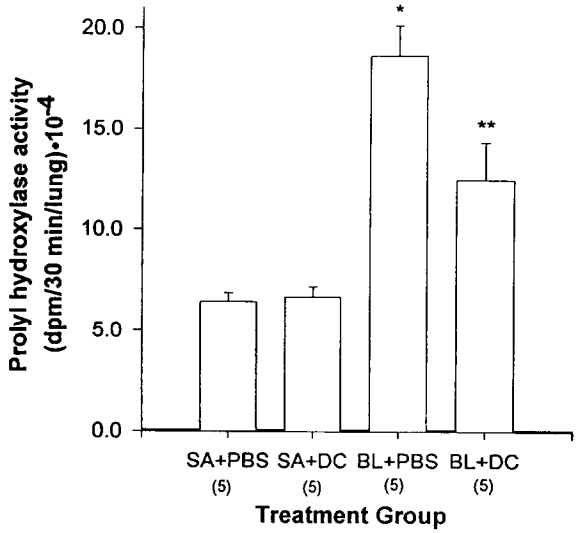

FIG. 2. Effects of repeated IT instillations of DC on BL-induced increases in lung prolyl hydroxylase activity. Data are expressed as means ± SD. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis and explained in "Materials and Methods." Key: (*) significantly higher ($P \leq 0.05$) than the remaining three groups, and (**) significantly higher ($P \leq 0.05$) than the SA + PBS and SA + DC groups.

MPO Activity

BL treatment dramatically increased the lung MPO activity in the BL + PBS group to 890% of the SA + PBS control group, and treatment with DC completely blocked the BL-induced increases in the activity of this enzyme in the BL + DC group (Fig. 3). Although the lung MPO activity in the SA + DC group was somewhat elevated as compared

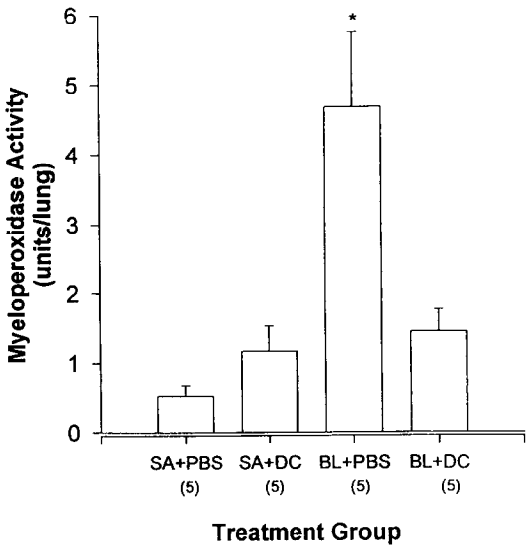

FIG. 3. Effects of repeated IT instillations of DC on BL-induced increases in lung MPO activity. Data are expressed as means ± SD. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis and explained in "Materials and Methods." Key: (*) significantly higher ($P \leq 0.05$) than the remaining three groups.

TABLE 1. Effects of repeated IT instillations of DC on BL-induced increases in various biochemical parameters of hamster lungs

Treatment group*	Biochemical parameters		
	MDAE Level (nmol/lung)	SOD activity (units/lung)	BALF-supernatant protein (μg/lung)
SA + PBS	47.7 ± 1.1 (6)	728.0 ± 53.1 (5)	2551.6 ± 17.4 (5)
SA + DC	45.9 ± 7.0 (6)	678.4 ± 23.3 (5)	2327.5 ± 101.8 (5)
BL + PBS	86.4 ± 7.9† (6)	1191.6 ± 92.8† (5)	9026.0 ± 878.9† (5)
BL + DC	81.5 ± 6.8† (6)	1028.2 ± 83.4† (5)	9268.4 ± 665.6† (5)

* See "Materials and Methods" for treatment details. The number in parentheses represents the number of animals used for the measurement. Each value is the mean ± SD.

† Significantly higher ($P \leq 0.05$) than the corresponding SA + PBS and SA + DC control groups.

with that of the SA + PBS group, there was no significant difference between these two groups.

Levels of MDAE and SOD Activity

BL treatment significantly increased the lung MDAE in the BL + PBS group as compared with the SA + PBS control group. However, treatment with DC failed to block the BL-induced increases in the lung MDAE content in the BL + DC group (Table 1). Similarly, treatment with BL caused a significant increase in the lung SOD activity in the BL + PBS group, and treatment with DC also failed to abolish the BL-induced increases in the SOD activity in the BL + DC group (Table 1).

Protein Content of BALF

The protein content of the BALF supernatant for the various treatment groups is summarized in Table 1. BL treatment significantly increased the protein content in the BL + PBS group to 354% of the SA + PBS control. Treatment with DC had no effect on the BL-induced increases in the BALF-supernatant protein in the BL + DC group.

Total and Differential Cell Counts

The recovered total and differential cell counts in the BALF of all four groups are summarized in Table 2. The hamsters treated with BL had four times as many cells in both the BL + PBS and BL + DC groups as hamsters in the SA + PBS and SA + DC control groups. The hamsters treated with BL in both the BL + PBS and BL + DC groups had three times as many macrophages as hamsters in the SA + PBS and SA + DC control groups. There was no difference in the number of macrophages between the two BL-treated groups. There was also no difference in the number of macrophages between the two saline control groups. The hamsters treated with BL in both the BL + PBS and BL + DC groups had a significantly higher number of neutrophils than hamsters in the SA + PBS and SA + DC control groups, and treatment with DC caused a significant reduction in the number of neutrophils from 0.89×10^6 in the BL + PBS group to 0.32×10^6 in the BL + DC group.

Histopathological and Morphometric Studies

Lungs from control hamsters (SA + PBS and SA + DC) showed thin interalveolar septa and no airway inflammatory cells (Fig. 4, A and B). Lungs from BL + PBS hamsters showed severe multifocal fibrosis and accumulations of mononuclear inflammatory cells and granulocytes (Fig. 4C). In contrast, lungs from BL + DC hamsters showed mild multifocal septal thickening with aggregations of mononuclear inflammatory cells (Fig. 4D), which comprised a small percentage of the lung.

Morphometric studies revealed that the volume density of lesions in the lungs of BL-treated hamsters averaged 0.43 in the BL + PBS group, and DC treatment caused a

TABLE 2. Effects of repeated IT instillations of DC on BL-induced changes in total and differential cell counts of BALF and volume density of lesion in the lungs

Treatment group*	Total cells ($\times 10^{-3}$)	Neutrophils ($\times 10^{-3}$)	Macrophages ($\times 10^{-3}$)	Monocytes ($\times 10^{-3}$)	Eosinophils ($\times 10^{-3}$)	Lymphocytes ($\times 10^{-3}$)	Volume density of lesion
SA + PBS	784 ± 2	14 ± 3	700 ± 109	15 ± 5	49 ± 25	6 ± 2	0
SA + DC	963 ± 9	28 ± 10	760 ± 178	24 ± 11	128 ± 91	23 ± 9	0
BL + PBS	3572 ± 35†	893 ± 352†	2300 ± 663†	156 ± 74	147 ± 69	76 ± 35	0.43 ± 0.13
BL + DC	3750 ± 48†	323 ± 115†‡	2824 ± 557†	225 ± 82	274 ± 135	105 ± 48	0.33 ± 0.14‡

* See "Materials and Methods" for treatment details. Each value is the mean ± SD of 5 animals.

† Significantly ($P < 0.05$) higher than the corresponding SA + PBS and SA + DC groups.

‡ Significantly ($P < 0.05$) lower than the corresponding BL + PBS group.

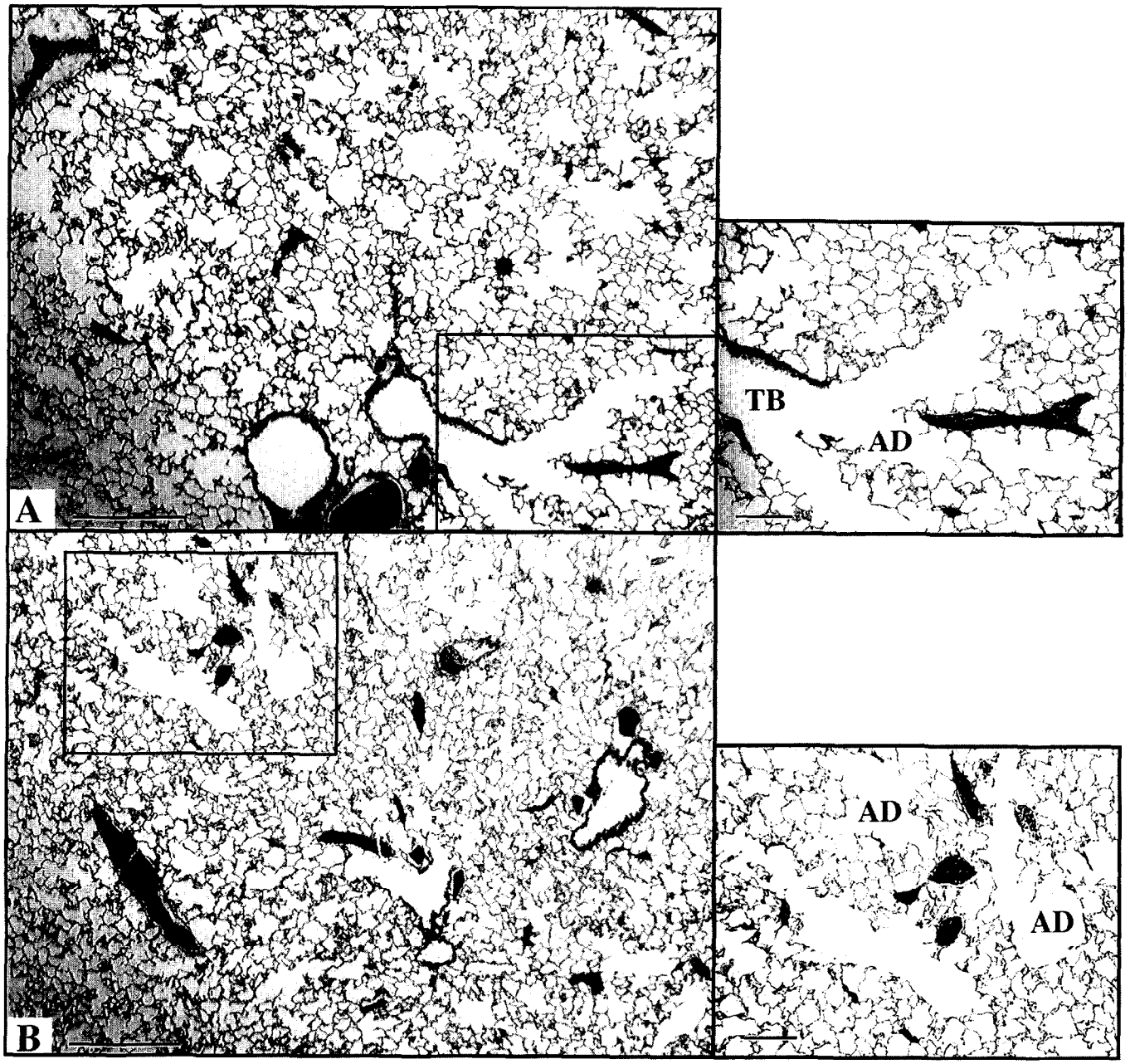

FIG. 4. Representative photomicrographs of four groups: (A) SA + PBS; (B) SA + DC; (C) BL + PBS; and (D) BL + DC. Boxes in the micrographs are enlarged areas to the right. Arrows in the enlargement in C identify fibroblasts and collagenous fibers and in D identify aggregations of mononuclear phagocytes. Bars in the micrographs = 500 μm and in the enlarged areas = 100 μm. AD = alveolar duct; and TB = terminal bronchiole.

significant ($P < 0.05$) reduction (by 23%) in the BL + DC group (Table 2). There were no discernable lung lesions in hamsters treated with SA in either the SA + PBS or the SA + DC control group.

Immunohistochemical Study

The immunostaining intensity scores graded on a scale of 0 through 3 (0 = no staining, 1 = diffuse light staining, 2 = higher staining, 3 = highest staining) revealed that hamsters treated with BL in the BL + PBS group had the highest immunostaining intensity for EDA-fibronectin and collagen type I, and treatment with DC significantly reduced the immunostaining intensity of these matrix proteins in the BL + DC group (Table 3). Representative photomicrographs of immunohistochemical staining for EDA-fibronectin (Fig. 5) and collagen type I (Fig. 6) provide additional evidence for the highest staining in the BL + PBS group and a markedly reduced staining by DC treatment in the BL + DC group.

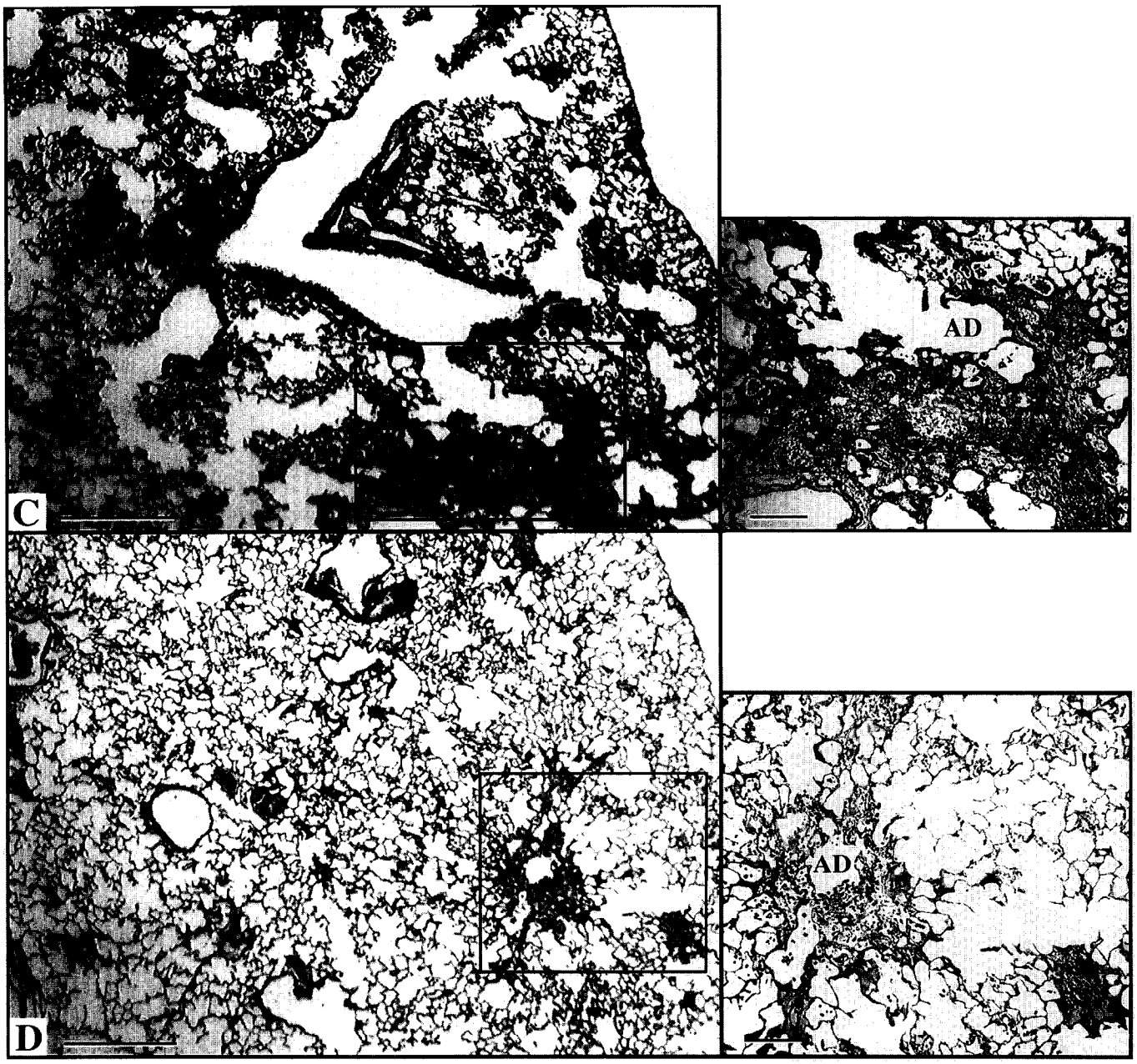

FIG. 4. Continued

TABLE 3. Effects of repeated IT instillations of DC on BL-induced increases in immunostaining intensity scores of EDA-fibronectin and collagen type I of hamster lungs

	Immunostaining intensity†	
Treatment group*	EDA-fibronectin	Collagen type I
SA + PBS (5)	1.00 ± 0.000	1.00 ± 0.000
SA + DC (4)	1.63 ± 0.125	1.00 ± 0.000
BL + PBS (5)	2.75 ± 0.250‡	2.80 ± 0.200‡
BL + DC (5)	1.70 ± 0.490	1.40 ± 0.292

* See "Materials and Methods" for treatment details and staining procedures. Each value is the mean ± SD. The number in parentheses represents the number of animals.

† Intensity score: 0, no staining; 1, diffuse light staining intensity; 2, higher staining intensity; 3, highest staining intensity.

‡ Significantly higher ($P \leq 0.05$) than the corresponding SA + PBS, SA + DC, and BL + DC groups.

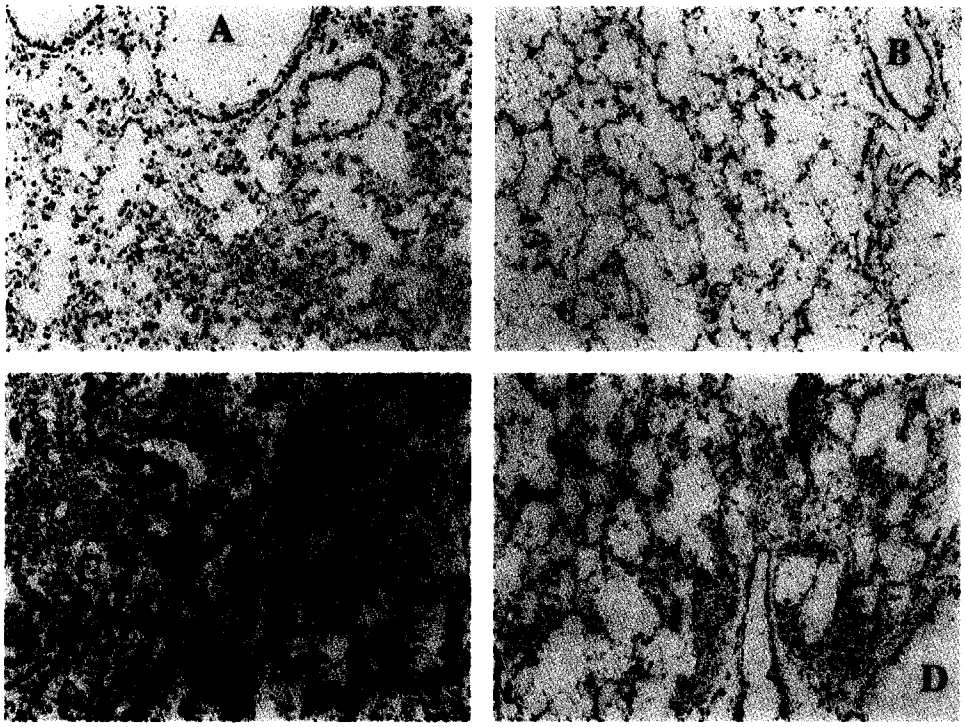

FIG. 5. Effects of repeated IT instillations of DC on BL-induced increases in immunostaining intensity of EDA-fibronectin in lung slices: (A) SA + PBS; (B) SA + DC; (C) BL + PBS; and (D) BL + DC. The highest immunostaining intensity is in the C group. See "Materials and Methods" for treatment details.

DISCUSSION

BL-induced lung toxicity is usually accompanied by an increased sequestration of neutrophils [28] that is measured by the lung level of MPO activity [19], increased lipid peroxidation measured by malondialdehyde content [28], increased collagen measured by hydroxyproline content [7], increased prolyl hydroxylase activity [29], an enzyme responsible for post-translational modification of collagen before its maturation [30], and increased pulmonary vascular permeability measured by the degree of leakage of plasma proteins in the alveoli [28]. In the present study, the hamsters treated with BL had significantly higher MPO and prolyl hydroxylase activities, malondialdehyde and hydroxyproline content in the lung and plasma protein in the BALF-supernatant in the BL + PBS group than in any other group. These findings are consistent with our earlier findings [31] and support the hypothesis that generation of ROS is involved in BL-induced lung toxicity, which subsequently leads to lung fibrosis.

The battery of biochemical markers that we employ while evaluating the antifibrotic potential of an exogenous compound in the BL-hamster model of lung fibrosis includes: lung MPO and prolyl hydroxylase activities, collagen and MDA content, and protein content of the BALF-supernatant. It is interesting that administration of DC attenuated the BL-induced increases in the MPO and prolyl hydroxylase activities and the hydroxyproline content of the lungs but had no effect on the lipid peroxidation and pulmonary vascular permeability in the BL + DC group. This suggests that DC treatment perhaps does not interfere with the ability of BL to generate ROS. It is conceivable that ROS generated by BL are involved in the production of TGF-β, and treatment with DC suppresses the TGF-β-mediated effects (to be discussed later) but not the ROS-mediated lipid peroxidation and pulmonary vascular permeability.

MPO is a heme-containing enzyme that is present primarily in the azurophilic granules of neutrophils [32]. The activity of this enzyme in the lung provides an index of intrapulmonary sequestration of neutrophils [19]. Several laboratories including our own have found sequestration of neutrophils in the lungs of BL-treated animals [20, 28]. Infiltration of an excess number of neutrophils in vascular, interstitial, and alveolar spaces would have deleterious effects on lung by generating ROS, releasing proteolytic enzymes and MPO [33, 34]. MPO released from the neutrophils is known to oxidize chloride ions to hypochlorous acid in a reaction with hydrogen peroxide [34]. Hypochlorous acid is capable of oxidizing all biologically vital constituents of cells and depletes cellular NAD and ATP, leading to cell death [35]. The results reported in this paper are consistent with the findings of our earlier paper that treatment with TGF-β antibodies reduced both the BL-induced increases in the lung collagen accumulation and MPO activity [12]. It is possible that DC, which is known to bind all isoforms of TGF-β, neutralizes all of their biological activities [13, 14] including the potent chemoattractant effect for neutrophils [36]. This may help explain a significant reduction in the infiltration of neutrophils and lung MPO activity in the BL + DC group as compared with animals in the BL + PBS group.

In the present study, we have demonstrated the beneficial effects of DC against BL-induced lung fibrosis in hamsters. We have employed a multi-dose BL model of

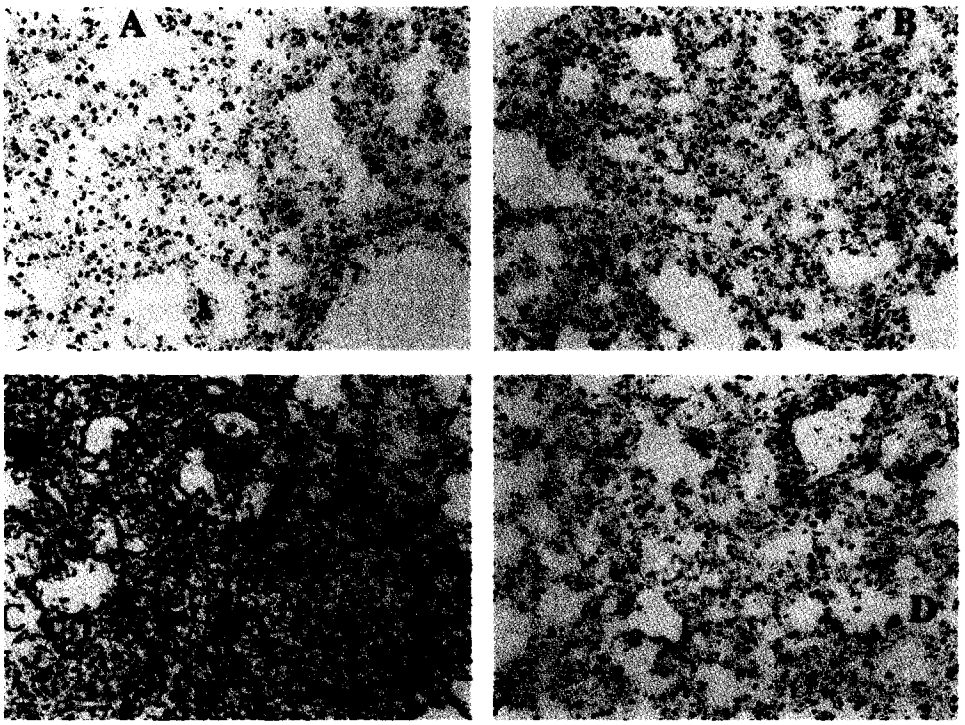

FIG. 6. Effects of repeated IT instillations of DC on BL-induced increases in immunostaining intensity of collagen type I in lung slices. (A) SA + PBS; (B) SA + DC; (C) BL + PBS; and (D) BL + DC. The highest immunostaining intensity is in the C group. See "Materials and Methods" for treatment details.

pulmonary fibrosis since this model appears to produce changes in ECM proteins resembling those seen in human idiopathic pulmonary fibrosis [37]. The repeated administration of DC directly into the lung by IT instillation was necessary to ensure an adequate level of this proteoglycan at the site of injury. Border *et al.* [15] found that two daily intravenous injections of DC given during the early phase of glomerulonephritis in rats had little effect on fibronectin accumulation and required 4–6 daily injections to suppress the deposition of this ECM protein to a level not significantly different from control. We administered DC twice a week for 3 weeks and three times during week 4 following the first IT instillation of BL or SA. This treatment regimen, although cumbersome, ensured the presence of DC at the site of injury and suppressed the BL-induced increases in the connective tissue reactivity of the lung in the BL + DC group, as revealed by biochemical, immunohistochemical, histopathological, and morphometric findings. It is understandable that the repeated instillations of DC by the IT route for treatment of pulmonary fibrosis in humans will face the problems of impracticability and noncompliance on the part of the patients. However, these problems could be overcome by administering DC in an aerosol form. It is possible that this mode of delivery will allow an adequate level of DC to reach the distal region of the lungs that would be effective in suppressing the development of lung fibrosis without the risk of producing systemic toxicity.

It appears that the mechanism for the antifibrotic effect of DC in the BL-hamster model of lung fibrosis resides in its ability to bind to all isoforms of TGF-β and inactivate their biological activities [13, 14]. Furthermore, there is a reciprocal relationship between the tissue levels of TGF-β and DC during the course of development of BL-induced lung fibrosis. As opposed to TGF-β, the lung level of DC was down-regulated in BL-induced pulmonary fibrosis in rats [38]. This *in vivo* result corroborates the *in vitro* result since fibroblasts incubated with TGF-β for an extended period have a lowered level of DC mRNA [39]. It is likely that TGF-β is an important component of the pathophysiology of BL-induced lung fibrosis for the following reasons. First, there is an increased expression of TGF-β gene and cell proliferation in lungs undergoing BL-induced pulmonary fibrosis [9, 40, 41]. Second, Raghu *et al.* [42] found that TGF-β caused a 2- to 4-fold increase in collagen production and collagen mRNA in fibroblasts cultured from normal and fibrotic human lungs. Third, TGF-β is a potent stimulator of ECM synthesis, although its effects on cell proliferation depend on the presence of other growth factors and culture conditions [43, 44]. Fourth, there is an abundant amount of TGF-β mRNA in alveolar macrophages in lungs from patients with idiopathic pulmonary fibrosis [45]. Sixth, TGF-β injected into wound chambers [46], into incisions [47], or subcutaneously [48] induces an influx of inflammatory cells and an increase in fibroblast number as well as an increase in collagen synthesis; TGF-β *in vitro* also induces an increased expression of level of connective tissue components including collagen and fibronectin [49]. Seventh, amelioration of BL-induced lung fibrosis in mice by treatment with TGF-β antibodies has been demonstrated in our laboratory [12].

The level of fibronectin, an adhesive ECM glycoprotein, has been found to be increased markedly during the course of development of pulmonary fibrosis [50] including the

BL-rat model [51]. The finding reported in this paper that BL-treated hamsters in the BL + PBS group had the highest immunohistochemical staining intensity of fibronectin than any other group, as evidenced by intensity score and photomicrograph, is consistent with the findings reported by other investigators [50, 51]. DC treatment completely blocked the BL-induced increases in the fibronectin staining intensity of the lungs in hamsters in the BL + DC group. Similarly, BL-induced increases in the lung collagen type I, as reported earlier by other investigators [52] and found in this study in the BL + PBS group, were also blocked markedly by DC treatment in the BL + DC group. These findings further suggest that the beneficial effects of DC in minimizing the deposition of ECM proteins in the BL-hamster model of lung fibrosis is probably coupled with its ability to bind and, thereafter, neutralize the growth-promoting effects of TGF-β on various connective tissue components including fibronectin and collagen [49].

Regardless of the mechanisms, our *in vivo* results demonstrated a beneficial effect of DC in preventing the deposition of ECM protein in the fibrotic lung induced by BL. These findings indicate that repeated IT instillations of DC suppress the *in vivo* effects of TGF-β, which is released in response to BL treatment. These results suggest the potential of DC therapy as a novel approach to the treatment of numerous human fibrotic disorders that are linked to the overproduction of TGF-β. Although we have not carried out experiments to demonstrate the TGF-β neutralizing activity of DC in the present study, its antifibrotic effect cannot be attributed to nonspecific proteoglycan or protein effects for two reasons. First, IT instillation of an equal amount of BSA did not reduce the BL-induced increased accumulation of lung collagen (data not shown). Second, Border *et al.* [15] have shown that, unlike DC, administration of proteoglycan aggregant or BSA to glomerulonephritic rats did not affect the disease, which is characterized by an excess deposition of ECM proteins in the kidney. It is very likely that the antifibrotic effect of DC is coupled with the suppression of TGF-β activity, since the beneficial effects of DC against BL-induced lung fibrosis are very similar to those we reported previously with intravenous injections of neutralizing antibodies to TGF-β [12]. However, other mechanistic possibilities for antifibrotic effects of DC as found in the present study cannot be ruled out for the following two reasons. First, DC has a very well-established role in binding to collagen fibers and in the regulation of fibril assembly, size of fibrils, and fibril–fibril interactions [53]. It is possible that this might be a pathway by which DC is affecting fibrosis. Second, the sulfated glycosaminoglycan chain of DC has many sulfate groups, which confer the ability on DC to bind to many proteins and cytokines with positive charges involved in BL-induced lung fibrosis.

While amelioration of fibrosis in a number of animal models of fibrotic diseases by blocking TGF-β with antibodies helps to identify the role of this cytokine in the pathogenic process of these diseases, a long-term treatment with antibodies to control a progressively advancing chronic disease such as fibrosis would be of limited therapeutic value for the following reasons: (1) the body develops its own immune reaction against the antibodies, and (2) extreme reductions of the TGF-β level may lead to autoimmune-like illness such as that seen in TGF-β_1 gene knockout mice which succumb to death soon after birth [54]. Our results show that treatment with DC inhibited the BL-induced increased production of collagens and EDA-fibronectin without producing any adverse effects even after multiple IT instillations of DC. Instillation of DC, *per se*, had no effect on the basal level of these ECM proteins in the SA + DC group. In addition, DC is a natural human compound that can be produced as a recombinant molecule and used for treatment of fibrotic conditions of any organ with little risk of initiating adverse immunological reactions, as opposed to TGF-β antibodies.

It is clear that repeated IT instillations of DC minimized the BL-induced lung fibrosis as evaluated by biochemical, histopathological, immunohistochemical, and morphometric studies. Although IT instillation for drug delivery is the least desirable route, it has nevertheless uncovered a potentially novel compound that may have clinical application and even may obtain patient compliance when delivered in an aerosol form for treatment of a life-threatening chronic disease such as lung fibrosis, which has thus far defied any effective therapeutic modality.

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