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Effect of Antibody Against Integrin α4 on Bleomycin-Induced Pulmonary Fibrosis in Mice

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ABSTRACT. Integrins are a family of transmembrane glycoproteins that can interact with components of the extracellular matrix. The α4β1 and α4β7 integrins are heterodimeric leukocyte cell surface molecules critical to their cell and matrix adhesive interactions. Evidence for a central role for the α4 integrins in leukocyte pathophysiology in the lung is well documented. In this study, we tested the hypothesis that neutralizing antibody for integrin α4 (PS2) may reduce bleomycin (BL)-induced lung fibrosis *in vivo*. Male C57BL/6 mice were injected intratracheally with saline (SA) or BL (0.08 U/mouse) followed by intraperitoneal injection of SA, isotype control antibody (1E6), or PS2 (100 μg) three times a week. Twenty-one days after the intratracheal instillation, mice were killed for bronchoalveolar lavage (BAL), biochemical, histopathological, and immunohistological analyses. Treatment with PS2 significantly reduced BL-induced increases in lung lipid peroxidation and hydroxyproline content. Lung histopathology also showed reduced fibrotic lesions in the BL-treated lungs by treatment with PS2. BL-treated mouse lungs also showed induction of cells with the myofibroblast phenotype, as indicated by the increased expression of α-smooth muscle actin (αSMA), whereas treatment with PS2 minimized the BL-induced αSMA expression. Furthermore, treatment with PS2 reduced the BL-induced increase in the BAL total cell number, and attenuated the BL-induced increase in the BAL protein level. It is concluded that integrin α4 may play an important role in BL-induced pulmonary fibrosis, and the use of anti-α4 antibody offers therapeutic antifibrotic potential *in vivo*. BIOCHEM PHARMACOL **60**;12:1949–1958, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. lung fibrosis; integrin; collagen; myofibroblast; mouse

IPF¶ is the final pathway of many interstitial lung diseases that leads to a reduction of lung compliance and impairment of the vital gas exchange function. Regardless of the etiology, IPF is characterized by inflammatory and fibroproliferative changes of the lung and an excess accumulation of collagen in the interstitium [1, 2]. Patients with IPF generally demonstrate the presence of recruited immune and inflammatory cells during active pulmonary fibrosis, indicating that pulmonary fibrosis is the result of aberrant repair after an initial inflammatory insult. Recruited inflammatory cells are likely to be involved in the initial insult in many instances. In addition, these cells may play a complex role in regulating the repair process. In either case, the recruitment of immune and inflammatory cells into the lung may play an important role in the determination of the fibrotic response.

Much evidence has documented the involvement of inflammatory cells and mediators in pulmonary fibrosis in widely used BL-rodent models [3, 4]. This model is appealing because it produces a characteristic picture of fibrosis with many of the components of human disease, and because BL-induced pulmonary fibrosis is a well-recognized adverse effect in human chemotherapy. Instillation (i.t.) of BL in rodents has been widely used for studying mechanisms of fibrogenesis and for screening potentially desirable antifibrotic reagents [5, 6]. Although the initial cause of BL-induced pulmonary toxicity is attributed to the generation of ROS once it binds to iron and DNA, the process leading to the final manifestation of pulmonary fibrosis involves release of various inflammatory mediators [4, 7]. The pathogenesis of BL-induced lung injury is ushered in initially by edema, hemorrhage, and a cellular infiltrate predominated by neutrophils and macrophages [8, 9]. An excess accumulation of the inflammatory leukocytes in vascular, interstitial, and alveolar spaces of the lung could inflict vascular and parenchymal injury by the generation of ROS and proteolytic enzymes [10]. Neutrophils contain

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¶ *Abbreviations*: IPF, interstitial pulmonary fibrosis; BALF, bronchoalveolar lavage fluid; ECM, extracellular matrix; i.t., intratracheal(ly); BL, bleomycin; 1E6, control antibody; PS2, antibody against integrin α4; TGF, transforming growth factor; mAb, monoclonal antibody; SA, saline; ROS, reactive oxygen species; and SMA, smooth muscle actin.

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substantial amounts of myeloperoxidase that can oxidize Cl^- to hypochlorous acid (HOCl) in a reaction with H_2O_2 that is known to cause cellular toxicity [11]. Macrophage- and neutrophil-derived ROS is able to stimulate the production of proinflammatory and fibrogenic cytokines that mediate enhanced fibroproliferative response [12, 13]. Besides a large inflammatory cell infiltration, the fibrotic process is characterized further by a hyperproliferative response of activated fibroblasts [14]. The fibroblast-like cells are primarily responsible for an absolute increase in lung collagen content, and an abnormality in the ultrastructural appearance and spatial distribution of collagen types [15].

The influx of inflammatory cells and activated fibroblasts into the injured lung depends on the ability of these cell types to interact with components of the ECM, comprised primarily of collagens. Integrins, heterodimeric transmembrane cell surface receptors, are required for the cell movement through and communication with the ECM [16]. The traffic and state of activation of leukocytes are modulated by various integrins. Since the initial lung response to BL is the influx of inflammatory cells including neutrophils, which exacerbate BL-induced damage by releasing more ROS, the prevention of the influx of inflammatory cells into lungs may be critical in containing the subsequent fibrotic response. We have investigated the possible role of the α4 integrin in the pathogenesis of fibrosis by administering an anti-α4 integrin antibody to mice with BL-induced lung fibrosis. The beneficial effect that this antibody has on both total collagen accumulation and the extent of pulmonary fibrotic lesions as shown in the present study suggests that the α4 integrin may be a reasonable target for anti-fibrotic therapy.

MATERIALS AND METHODS

The antibody against integrin α4 (PS2) and control antibody (1E6) were characterized previously [17] and produced by Biogen Inc. Chronic respiratory disease-free male C57BL/6 mice weighing 25–30 g were purchased from Charles River Laboratories. Bleomycin sulfate (Blenoxane^R) was a gift from Bristol Laboratories. The l-[3,4-^{3}H]proline for labeling the procollagen substrate of prolyl hydroxylase was obtained from NEN Life Science Products. Z-fix, an aqueous buffered zinc formalin, was purchased from Anatech, LTD. All other reagents were of reagent grade or higher purity and were obtained from standard commercial sources.

Treatment of Animals

Mice were housed four per cage and cared for in accordance with the NIH Guidelines for Animal Welfare. The mice were allowed to acclimate in the facilities for 1 week prior to all treatments. A 12-hr/12-hr light/dark cycle was maintained, and mice had access to water and Rodent Laboratory Chow *ad lib*. Animals were divided randomly into four experimental groups: (1) SA+SA; (2) BL+1E6; (3) BL+SA; and (4) BL+PS2. Mice were injected i.t. with a single dose of saline or BL at 0.08 U/100 μL/mouse under xylazine and ketamine anesthesia. After i.t. instillation, the mice received i.p. injection of 1E6, SA, or PS2 (100 μg in 0.2 mL/mouse) three times a week. Twenty-one days after the BL instillation, mice were killed under pentobarbital anesthesia for BALF, biochemical, and histopathological analyses.

Preparation of BALF and Lung Tissue

After anesthesia, the abdominal cavity was opened followed by exsanguination of the descending abdominal aorta. The lungs were prepared for lavage by cannulating the trachea with a blunt needle attached to a syringe. The lung lavage was carried out with 3 mL of cold isotonic saline delivered in 1-mL aliquots. An aliquot of the BALF was portioned for total cell count. The remaining BALF was centrifuged at 1500 *g* for 20 min at 4°; the resulting supernatant was aliquoted and then was stored at −70°. After BALF, the lung lobes were quickly dissected free of non-parenchymal tissue, immediately frozen in liquid nitrogen, and stored at −70°. Later, 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.). The homogenate was mixed thoroughly by repeated inversions, and the final homogenate volumes (4–5 mL) were recorded. The homogenate was divided into several aliquots and stored at −70° for biochemical measurements.

Determination of Lung Malondialdehyde Equivalent and Hydroxyproline Content

Lung malondialdehyde equivalent was estimated from the total amount of thiobarbituric acid-reacting products in unfractionated homogenate by the method of Ohkawa *et al.* [18]. For lung hydroxyproline assay, 1 mL of homogenate was precipitated with 0.25 mL of ice-cold 50% (w/v) trichloroacetic acid and centrifuged, and 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 [19].

Determination of Prolyl Hydroxylase (EC 1.14.11.2) Activity

The preparation of prolyl hydroxylase substrate (procollagen) and the method for prolyl hydroxylase assay were described in our previous paper [20]. Briefly, tibias freshly isolated from 10-day-old chicken embryos were labeled with [^{3}H]proline in proline-free culture medium at 37° for 6 hr. After removing the unincorporated label by washing, the tissue was homogenated and centrifuged at 3000 *g* for 20 min at 4°. The resulting supernatant was dialyzed extensively to remove the unincorporated label. The labeled procollagen substrate was aliquoted and stored at −70°. The incubation mixture for the enzyme assay in a

total volume of 2 mL consisted of ferrous ammonium sulfate (0.1 mmol/L), α-ketoglutaric acid (0.1 mmol/L), [^{3}H]proline procollagen (200,000 dpm), lung homogenate (0.2 mL), ascorbic acid (0.5 mmol/L), and Tris-hydrochloric acid buffer (0.1 mol/L, pH 7.8). The reaction was started by the addition of ascorbic acid and stopped by adding 0.2 mL of 50% trichloroacetic acid after 30 min at 37° in a Dubnoff metabolic shaker. During the reaction, tritiated water is released in stoichiometric proportion to prolyl hydroxylation and is used as a measure of the enzyme activity. The tritiated water of the reaction system was separated by vacuum distillation of the whole reaction mixture and counted for radioactivity. The enzyme activity was expressed as dpm of ^3H_2O released per total lung per 30 min.

Determination of Cell Counts in BALF

The total and differential cell numbers in the BALF were determined as previously described [21]. Total leukocyte number in the BALF was estimated by a Coulter counter (model F, Coulter Electronics, Inc.), according to the User's Manual. Slides for differential cell counts were prepared on a Shandon cytospin using 100 μL of lavage fluid and stained with Diff-Quik (American Scientific). Differential cell counts were done on a Zeiss microscope at X630. Macrophages, neutrophils, and lymphocytes were counted based on 400 cells per slide.

BALF Protein Assay

Protein content in the BALF supernatant was determined using the Bio-Rad protein assay (Bio-Rad laboratories), and BSA was used as the standard.

Histopathologic and Immunohistochemical Analysis

Three to four animals from each treatment group were chosen randomly for histopathologic and immunohistochemical evaluation at the end of the experiment. The abdominal cavity of the animal was opened followed by exsanguination of the descending abdominal aorta. Immediately thereafter, the lung tissue was prepared for histologic analysis as described [21]. After cannulating the trachea with a blunt needle, the thoracic cavity was opened, and then both heart and lung were removed *en bloc*. The lungs were fixed with Z-fix solution via the trachea at a pressure of 30 cm of water. The right cranial and caudal lobes and the left lobe were later blocked, embedded in paraffin, cut in 7-μm sections, and stained with hematoxylin and eosin. For immunohistochemical staining for αSMA, frozen lung sections of pulmonary tissue were fixed in -20° acetone for 10 min, briefly dried, and blocked with 3% BSA in PBS. A monoclonal antibody to αSMA, conjugated to Cy3 (Sigma No. F3777), was diluted 1:300 in 3% BSA/PBS and applied to the slides for 1 hr at room temperature. The slides were washed three times for 10 min per wash in PBS. Movoil (Calbiochem No. 475904) and coverslips were then applied to the sections.

Statistical Analysis of Data

Animal data were expressed on the basis of per total lung and were reported as means ± SEM. The data were compared within the four groups using two-way analysis of variance (SIGMASTAT) and the Student–Newman–Keuls method. A value of $P \leq 0.05$ was considered significant.

RESULTS

Lipid Peroxidation in the Mouse Lung

Lung malondialdehyde equivalent content as an index of lipid peroxidation in various groups of mice is shown in Fig. 1. BL instillation significantly increased malondialdehyde equivalent content in mouse lungs in both BL+1E6 and BL+SA groups as compared with the SA+SA and BL+PS2 groups, and the malondialdehyde equivalent content in the BL+1E6 group was higher than that in the BL+SA group. The treatment with PS2 effectively blocked BL-induced lung lipid peroxidation since the lung malondialdehyde equivalent level in the BL+PS2 group was no different from that of the SA+SA group.

Lung Hydroxyproline Content in the Mouse Lung

Lung hydroxyproline, the key index of lung collagen level, was determined for the four groups of mice, and the results are presented in Fig. 2. The i.t. instillation of BL elevated the lung hydroxyproline level in the BL+1E6 and BL+SA groups significantly to 185 and 205% of the SA+SA control group (178 ± 9.4 μg/lung), respectively. The BL-induced increase in the lung hydroxyproline level in the BL+PS2 group was decreased significantly by 35% by the treatment with PS2 compared with the BL+SA group. The lung hydroxyproline level in the BL+PS2 group was not significantly higher than that of the control (SA+SA) group.

Prolyl Hydroxylase Activity in the Mouse Lung

The prolyl hydroxylase activities in the mouse lungs of various groups are shown in Fig. 3. BL alone increased the lung prolyl hydroxylase activity in the BL+SA group significantly to 207% of the SA+SA control group (34,500 ± 900 dpm/30 min/lung). PS2 decreased the prolyl hydroxylase activity elevated by the BL treatment in the BL+SA group significantly. However, the prolyl hydroxylase activity in the BL+1E6 group was also significantly lower than that in the BL+SA group.

Total and Differential BALF Cell Counts in the Mouse Lung

The numbers of total cells in the BALF of various groups at 21 days after i.t. instillation of saline or BL are summarized in Fig. 4. BL treatment increased the total cell counts in the BALF from the BL+1E6 and BL+SA groups significantly

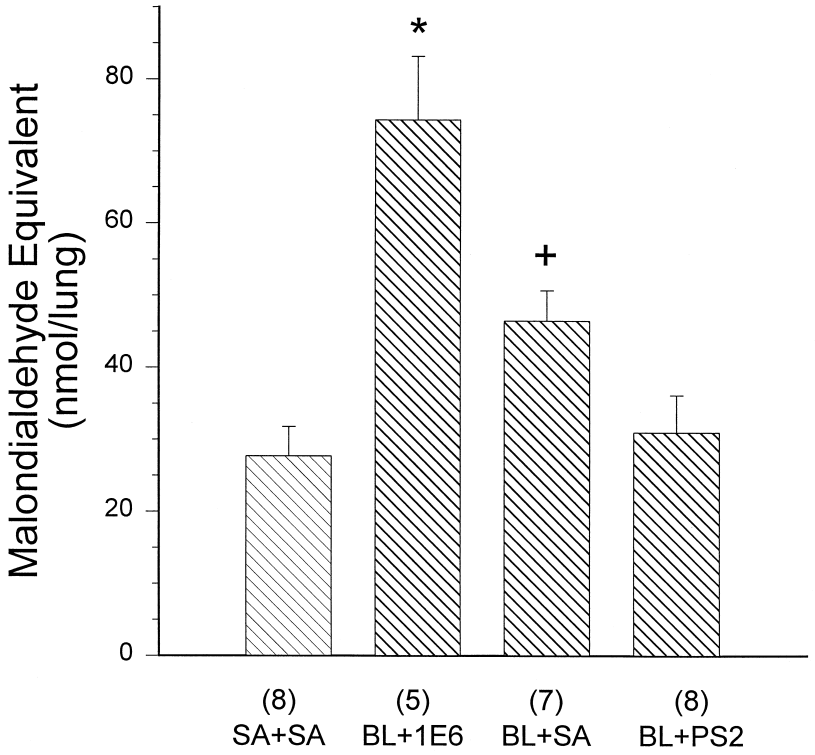

FIG. 1. Effect of PS2 treatment on BL-induced increase in lipid peroxidation of mouse lungs. The lungs were processed for lipid peroxidation assay as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis. SA+SA: saline + saline; BL+1E6: bleomycin + antibody control; BL+SA: bleomycin + saline; and BL+PS2: bleomycin + anti-α4 antibody. Values are expressed as means ± SEM. Key: (*) significantly ($P \leq 0.05$) higher than all other groups; and (+) significantly ($P \leq 0.05$) higher than the SA+SA and BL+PS2 groups.

compared with the saline control group (SA+SA). The BALF cell count in the BL+PS2 group was not different from that of the SA+SA group. The differential cell counts for macrophages, neutrophils, and lymphocytes are presented in Table 1. There were higher numbers of macrophages and lymphocytes in the BL-treated lungs, but there was no significant difference in the number of neutrophils among all four groups at 21 days after BL treatment.

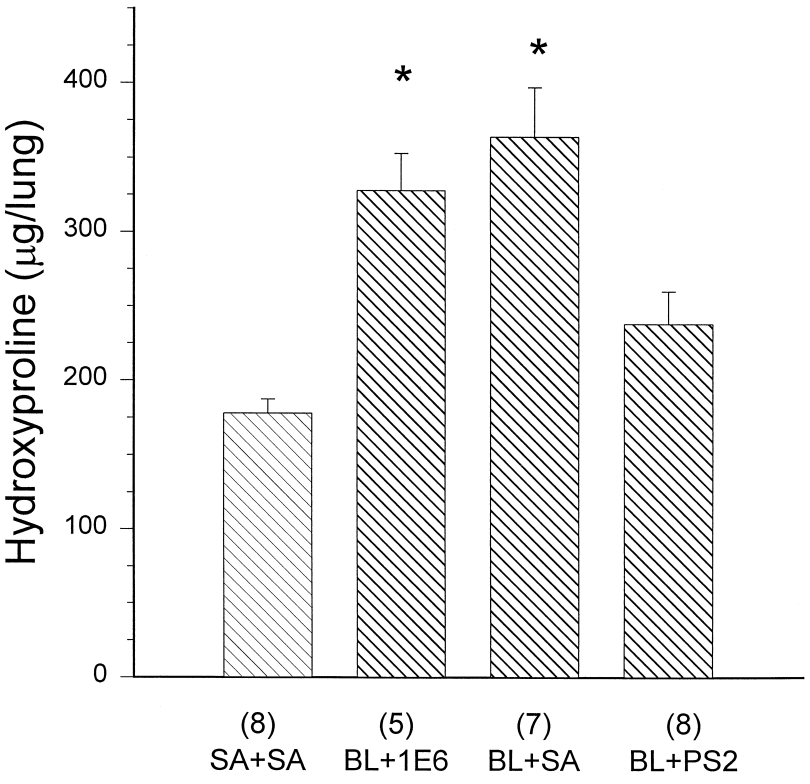

FIG. 2. Effect of PS2 treatment on BL-induced increase in hydroxyproline content of mouse lungs. The lungs were processed for hydroxyproline assay as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis. SA+SA: saline + saline; BL+1E6: bleomycin + antibody control; BL+SA: bleomycin + saline; and BL+PS2: bleomycin + anti-α4 antibody. Values are expressed as means ± SEM. Key (*) significantly ($P \leq 0.05$) higher than the SA+SA and BL+PS2 groups.

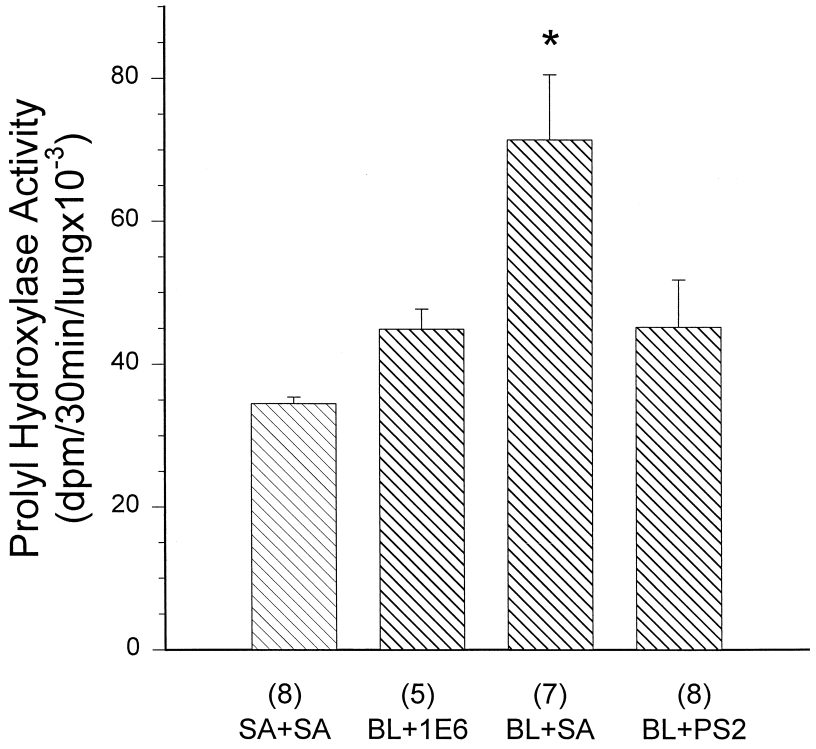

FIG. 3. Effect of PS2 treatment on BL-induced increase in prolyl hydroxylase activity of mouse lungs. The lungs were processed for prolyl hydroxylase activity assay as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis. SA+SA: saline + saline; BL+1E6: bleomycin + antibody control; BL+SA: bleomycin + saline; and BL+PS2: bleomycin + anti-α4 antibody. Values are expressed as means ± SEM. Key: (*) significantly ($P \leq 0.05$) higher than all other groups.

Protein Content in the BALF

The protein content of the BALF supernatant for the four experimental groups is presented in Fig. 5. Instillation (i.t.) of BL significantly increased the BALF protein of all the BL-treated groups as compared with the SA+SA group. However, treatment with PS2 in the BL+PS2 group

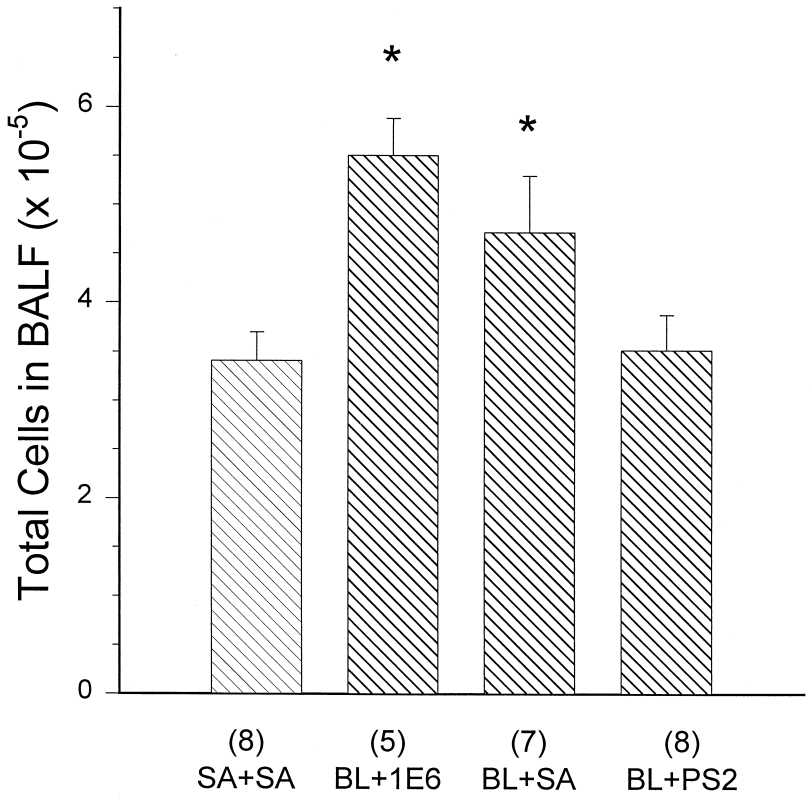

FIG. 4. Effect of PS2 treatment on BL-induced increase in BALF total inflammatory cells of mouse lungs. The lungs were lavaged and total cells in the BALF were counted as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis. SA+SA: saline + saline; BL+1E6: bleomycin + antibody control; BL+SA: bleomycin + saline; and BL+PS2: bleomycin + anti-α4 antibody. Values are expressed as means ± SEM. Key: (*) significantly ($P \leq 0.05$) higher than the SA+SA and BL+PS2 groups.

TABLE 1. Effects of PS2 treatment on BL-induced changes in differential cell counts in BALF from mice

Treatment groups* (N)	Macrophages ($\times 10^{-3}$/lung)	Neutrophils ($\times 10^{-3}$/lung)	Lymphocytes ($\times 10^{-3}$/lung)
SA + SA (8)	319.5 ± 3.8	1.28 ± 0.9	20.5 ± 3.0
BL + 1E6 (5)	437.3 ± 52.7†	8.81 ± 5.7	104.6 ± 47.1
BL + SA (7)	340.0 ± 50.7	8.66 ± 2.4	123.5 ± 48.7‡
BL + PS2 (8)	268.0 ± 22.6	3.52 ± 2.7	80.5 ± 20.5

* The determination of total and differential cell counts is described in Materials and Methods. The number of animals in each group is shown in parentheses following the treatment group. SA + SA: saline + saline; BL + 1E6: bleomycin + antibody control; BL + SA: bleomycin + saline; and BL + PS2: bleomycin + anti-α4 antibody. Values are expressed as means ± SEM.

† Significantly higher than the SA + SA and BL + PS2 groups.

‡ Significantly higher than the SA + SA group.

significantly decreased the BL-induced increase in the BALF supernatant protein, although it was still significantly higher than that in the SA+SA group.

Histopathology of the Mouse Lung

Histological examination of the mouse lungs revealed normal pulmonary parenchymal tissue in the SA+SA group (Fig. 6A). However, as shown in panels B and C of Fig. 6, the lungs from the BL+1E6 and BL+SA groups showed a patchy alveolitis and multifocal interstitial fibrosis containing an accumulation of extracellular fibers. The lungs of mice in these groups had thickened interalveolar septa and inflammatory cells in adjacent airspaces. Compared with the BL+1E6 and BL+SA groups, lungs from the BL+PS2 group had much less fibrotic lesions, although some lobes still showed a mild degree of interstitial fibrosis (Fig. 6D).

Immunohistochemical Staining for αSMA in the Mouse Lung

To determine the accumulation of fibroblasts and fibroblast-like cells in mice after BL treatment, we examined the expression of αSMA in the lung tissue using a monoclonal antibody against αSMA. In the control lungs, the immunopositivity occurred in the vascular and bronchial smooth muscle layers (Fig. 7A). In lungs treated with BL and control antibody (Fig. 7B) or BL and saline (Fig. 7C), there was extensive and intense immunostaining within fibrotic areas, both in the interstitium and pleura. However, lungs treated with BL and PS2 showed much reduced immuno-

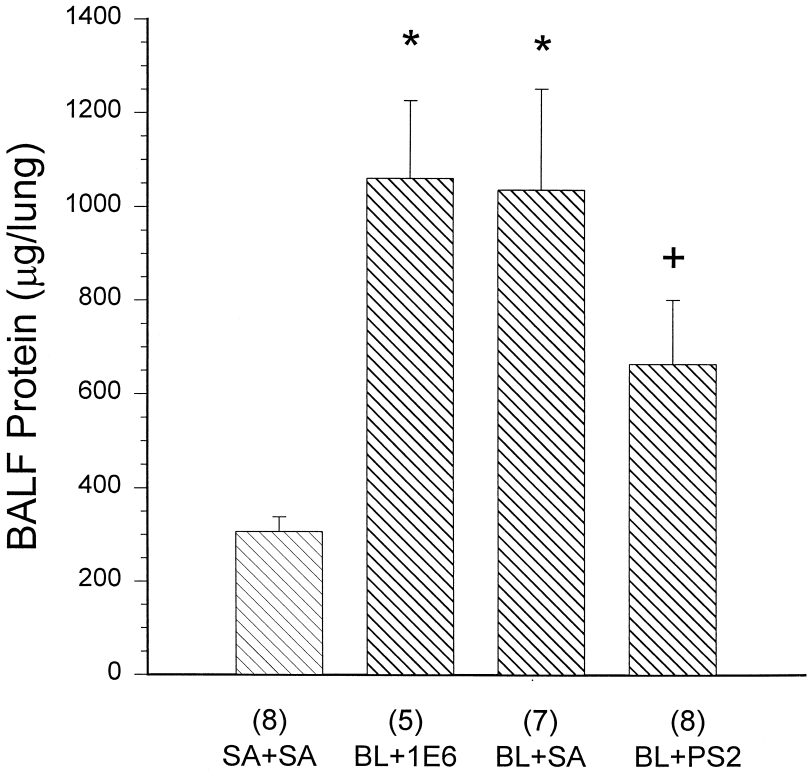

FIG. 5. Effects of PS2 treatment on BL-induced increases in BALF protein content of mouse lungs. The lungs were lavaged and the BALF was processed for protein assay as described in Materials and Methods. The number of animals in each group is shown in parentheses below each bar, and treatment groups are indicated along the x-axis. SA+SA: saline + saline; BL+1E6: bleomycin + antibody control; BL+SA: bleomycin + saline; and BL+PS2: bleomycin + anti-α4 antibody. Values are expressed as means ± SEM. Key: (*) significantly ($P \leq 0.05$) higher than the SA+SA and BL+PS2 groups; and (+) significantly ($P \leq 0.05$) higher than the SA+SA group.

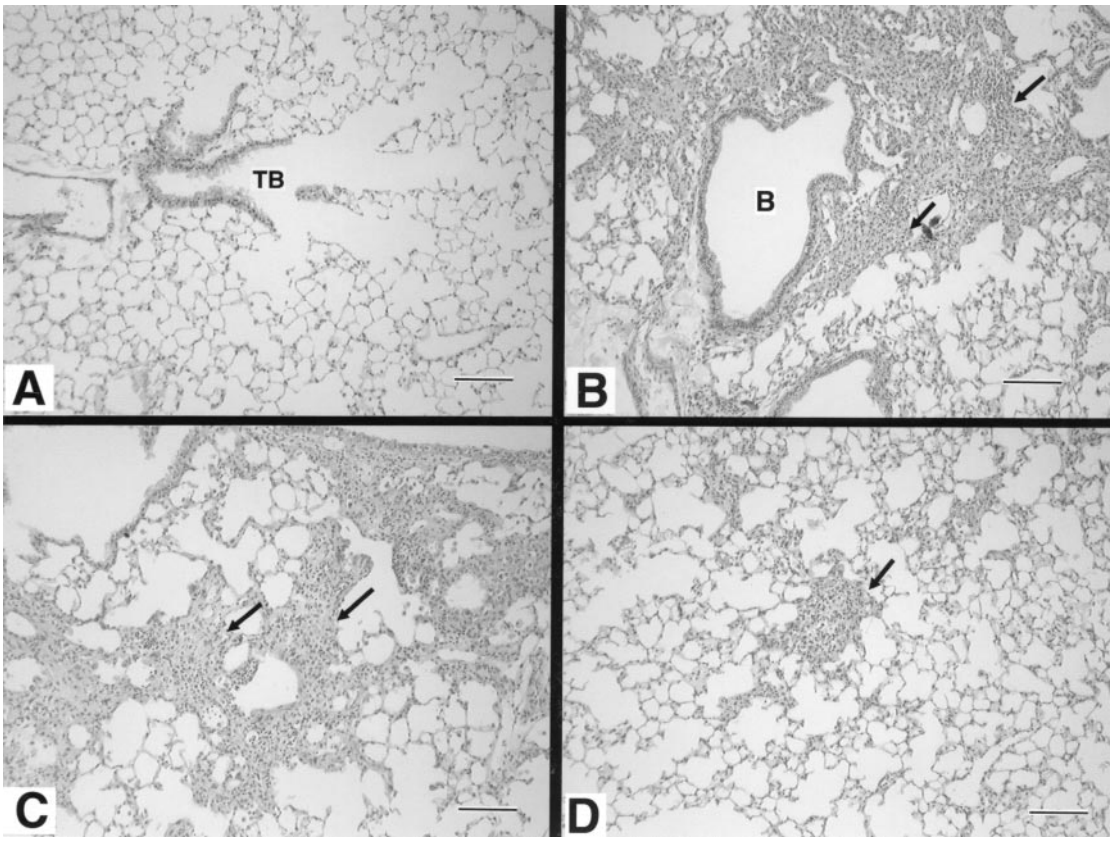

FIG. 6. (A) Representative photomicrograph of pulmonary proximal acini from a mouse in the SA+SA group. Note the normal appearance of the thin interalveolar septa acinus and lack of inflammatory cells and fibrosis. (B) Representative photomicrograph of pulmonary proximal acini from a mouse in the BL+1E6 group. Note the fibrotic lesions and multifocal thickening of interalveolar septa (arrows). (C) Representative photomicrograph of pulmonary proximal acini from a mouse in the BL+SA group. Note the fibrotic lesions and multifocal thickening of interalveolar septa (arrows). (D) Representative photomicrograph of pulmonary proximal acini from a mouse in the BL+PS2 group. Note the largely normal appearance of parenchyma and only moderate thickening of interalveolar septa (arrow). Paraffin sections, hematoxylin and eosin stain. B = bronchus; TB = terminal bronchiole. Bar = 100 μm.

staining of αSMA in the lungs (Fig. 7D), compared with the BL+1E6 and BL+SA groups.

DISCUSSION

IPF is a crippling disease and responds poorly to current therapy. Consequently, a great deal of effort has been devoted to searching for a more effective therapeutic modality as exemplified by targeting fibrogenic growth factor TGF-β in our laboratory [5, 6, 22]. In the present study, we provide evidence that integrin expressed in leukocytes is another possible therapeutic target in managing IPF. The antifibrotic effect of PS2 antibody was demonstrated by its ability to decrease an excessive amount of collagen and prolyl hydroxylase activity in the lungs of the BL+SA group. Increases in the prolyl hydroxylase activity in various animal models of lung fibrosis including BL-treated rodents usually but not always precede or parallel the accumulation of collagen in the lung [20, 23]. The enzyme-catalyzed hydroxylation of proline is an important posttranslational event in the processing of highly cross-linked mature collagen fibers [24]. However, the prolyl hydroxylase activity in the BL+1E6 group was not significantly higher than that of the SA+SA group. This discrepancy might result from some unexpected biological effect of the "irrelevant" control 1E6 antibody. Our data are consistent with other models of tissue injury, since immunosuppression of the α4 integrin was found to reduce the myocyte dysfunction caused by the emigrated neutrophils [25].

It is generally assumed that the leukocytes of the lung are involved in the evolution of pulmonary fibrosis by the secretion of ROS, fibrogenic cytokines, and growth factors [26]. During the acute inflammatory process of BL-induced lung injury, neutrophils migrate to and accumulate in the lung tissues, and the accumulation of neutrophils lasts as long as the fibrotic lesions persist [27]. Neutrophil sequestration within the pulmonary microvasculature contributes to the pathogenesis of various types of acute lung injury [28, 29]. It has been documented that neutrophil-derived ROS, proteolytic enzymes, and HOCl via myeloperoxidase are able to mediate cellular toxicity [10, 11]. The biocidal property of HOCl in oxidizing a variety of biologically significant substances including carbohydrates, nucleic acids, peptide linkages, and amino acids is well known. The release of HOCl from activated neutrophils was found to cause depletion of cellular NAD and ATP, leading to cell

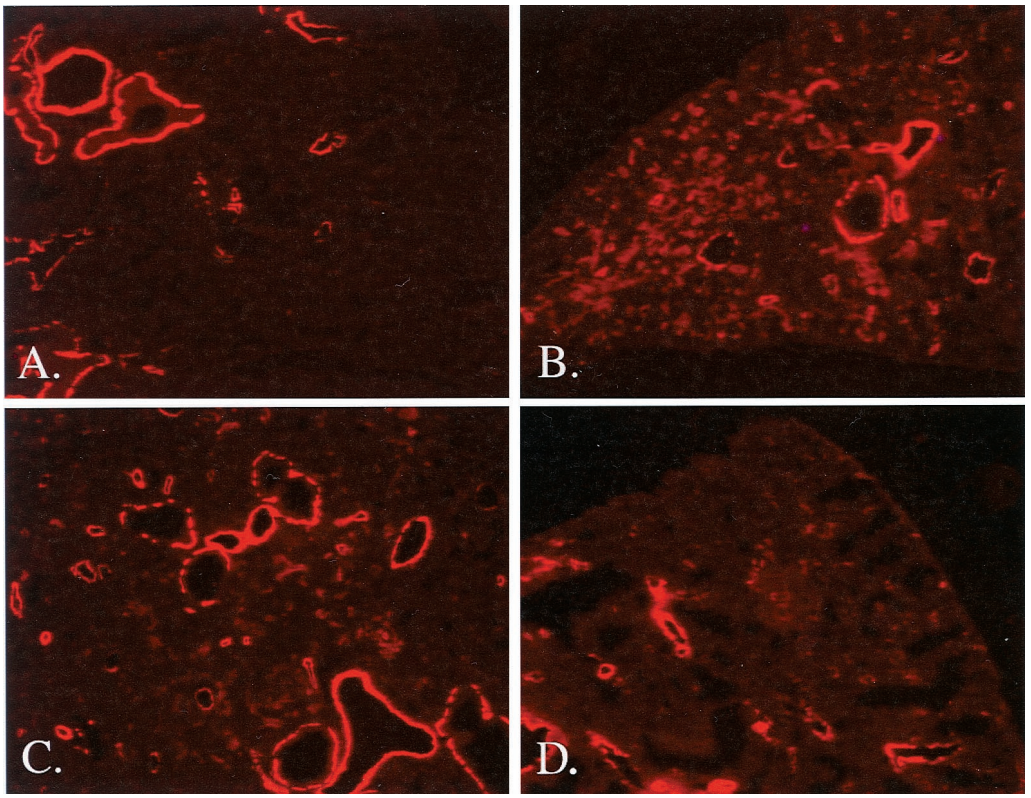

FIG. 7. (A) Representative immunohistochemical αSMA staining of pulmonary proximal acini from a mouse in the SA+SA group. Note that the αSMA expression occurred mainly at the vasculature and bronchiole smooth muscle. (B) Representative immunohistochemical αSMA staining of pulmonary proximal acini from a mouse in the BL+1E6 group. Note the increased αSMA expression in the lung parenchyma. (C) Representative immunohistochemical αSMA staining of pulmonary proximal acini from a mouse in the BL+SA group. Note the increased αSMA expression in the lung parenchyma. (D) Representative immunohistochemical αSMA staining of pulmonary proximal acini from a mouse in the BL+PS2 group. Note the relatively less αSMA expression in the lung parenchyma compared with that in the BL+1E6 and BL+SA groups.

injury [30]. Neutrophils generate ROS that have deleterious effects on parenchymal cells [31] and endothelial cells [32]. These inflammatory cells also secrete a variety of potent degradative lysosomal enzymes such as acid phosphatase. Macrophages [33] and possibly lymphocytes [34] are capable of producing various cytokines, including intrinsically active TGF-β. TGF-β-producing macrophages are a prominent feature in a variety of fibrotic pulmonary pathologies [35]. It has been demonstrated that alveolar macrophages isolated from animals with BL-induced fibrosis are capable of producing significant quantities of both active and latent TGF-β protein [36].

The traffic and state of activation of leukocytes are modulated by various surface proteins such as the integrins. It is clear that cell–cell interactions as well as cell–ECM interactions are critical for the pathogenesis of pulmonary fibrosis. A consistent finding in patients with active pulmonary fibrosis and in animal models of fibrotic lung diseases is the accumulation of increased numbers of immune and inflammatory cells in areas undergoing fibrosis [37]. The α4 integrin subunit CD49d associates with either the β1 (CD29) or β7 subunit to form the integrin heterodimers called very late antigen (VLA)-4 (α4β1; CD49d/CD29) and α4β7 [38, 39]. The α4 integrins are heterodimeric leukocyte cell surface molecules central to their cell and matrix adhesive property. The integrin α4β1 interacts with the immunoglobulin superfamily member, vascular cell adhesion molecule-1 (VCAM-1), and with an alternatively spliced form of fibronectin [40]. VLA-4 is expressed on all circulating leukocytes, and binds to VCAM-1 (CD106), a member of the Ig gene superfamily that is expressed on cytokine-activated endothelial cells, and to the matrix protein fibronectin. α4β7 is expressed on a subset of T and B cells, natural killer cells, and eosinophils. It binds to the mucosal vascular addressin (MAdCAM-1), a member of the Ig and mucin-like families of adhesion molecules, as well as to VCAM-1 and fibronectin [39, 41]. Studies *in vitro* have demonstrated that VLA-4 interaction with VCAM-1 is involved in mononuclear leukocyte and eosinophil adherence to endothelium and transendothelial migration [38, 42]. α4β7 is thought to be involved primarily in leukocyte recruitment to gut-associated lymphoid tissue [41]. Studies *in vivo* using blocking mAbs have established a role for CD49d in leukocyte recruitment in a variety of inflammatory and immune disorders [42]. CD49d appears to play a particularly important role in inflammatory cell recruitment in allergic disorders. In a rat model of nephrotoxic nephritis, mAbs to VLA-4 significantly attenuated renal injury [43]. Evidence for a central role for the integrins α4β1 and α4β7 in

leukocyte pathophysiology is accumulating rapidly. Five distinct α4 mAbs, each able to block α4-dependent adhesion *in vitro,* show beneficial effects *in vivo* in six different species, and in several organs. α4 integrins will likely be critical to both the normal physiology and pathology of the lung in humans [44].

In the present study, treatment with PS2 decreased BL-induced increases in total leukocytes in the BALF. However, the number of neutrophils was not significantly different among the four groups. It should be noted that at the time point (21 days after BL) we chose to study, an advanced degree of lung fibrosis was not ideal for inflammatory cell analysis since at this time the number of neutrophils in the lung has been reported to be decreased or back to the levels found in control-treated animals [14, 45]. In a previous study, analysis of inflammatory cell counts at different times after BL treatment revealed that total cells peak at day 2 by a factor of 8-fold. However, the number dropped to only half of the peak value at day 4 and to about one-fourth of the peak number at days 14 and 21 [27]. If early time points were included, the PS2 antibody might have shown a more marked effect on inflammatory cell influx into the alveoli. It has also been shown that mAbs specific for the leukocytic integrins CD-11a or CD-11b prevent lung fibrosis in a BL-mouse model of lung fibrosis without effect on the BALF cellularity [46]. The decreased total leukocytes in the lungs of BL+PS2 mice may be responsible for the diminished inflammatory injury and fibrosis in the lungs of BL-treated animals. The BL-induced lung injury was reduced significantly by the treatment of PS2, as indicated by measuring the lipid peroxidation and vascular permeability in the lung. Malondialdehyde equivalent, as indicated by the amount of thiobarbituric acid-reacting products, has been used as an index of lipid peroxidation [47]. The increase in lipid peroxidation in the lungs of BL-treated animals was due at least partly to the ROS generated by infiltrating leukocytes. PS2 could have minimized tissue lipid peroxidation by blocking the emigration of leukocytes into the lung initiated by the BL-induced injury. Similarly, the decreased injury was reflected by a decreased level of total protein in BALF supernatant, an index of vascular permeability, in the BL+PS2 group.

The increased collagen in the lung has been associated with increased numbers of fibroblasts in the interstitium and in the alveolar space itself [14]. Many of these fibroblast-like cells are myofibroblasts, which have a distinctive phenotype that includes the expression of αSMA, a contractile protein commonly found in smooth muscle cells [48], and thought to be important in fibrogenesis and wound healing [49]. In another BL model of fibrosis, most cells expressing procollagen mRNA have been demonstrated to be myofibroblasts [50]. The origin of these myofibroblasts is uncertain, although currently the fibroblast is the most favored cellular precursor. The emergence of αSMA-expressing fibroblast-like cells also suggests that BL induced a new cellular phenotype, the myofibroblast. Myofibroblasts have been demonstrated in a variety of fibrotic and healing tissues, and they are likely important in repair and fibrogenesis [49, 51]. One of the significant findings of the present study was that PS2 treatment attenuated the BL-induced myofibroblast proliferation. The mechanism for the inhibitory effect of myofibroblast proliferation by PS2 is not clear. However, the treatment with antibody against α4 integrin may have reduced the level of growth factors in the lung released by infiltrating leukocytes or have directly affected the behavior of myofibroblasts. In either case, the reduced proliferating myofibroblasts could lead to a decrease in the lung collagen accumulation in the BL-treated animals in the BL+PS2 group.

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