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Effect of Endostatin on Bleomycin-Induced Pulmonary Fibrosis in Mice

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ABSTRACT

Background and objective : Endostatin has been isolated from the culture supernatant of mouse angioendothelioma during screening of endogenous angiogenesis inhibitors. We administered endostatin to mice with bleomycin-induced pulmonary fibrosis, and the pharmacological effect of endostatin on fibrosis was investigated.

Methods : Bleomycin was administered into the tracheas of 8-week-old male C57BL/6J mice on day 0 to induce pulmonary fibrosis. Endostatin was administered intraperitoneally daily on days 0 to 14. As a control, phosphate-buffered saline (PBS) was administered. The mice were killed on day 14, and their lungs were excised. The degree of fibrosis was evaluated with histological scores and hydroxyproline assays. Single-cell suspensions of lung tissue were prepared, and cell populations in the lungs were analyzed with flow cytometry.

Results : Bleomycin reduced body weight, but the body weight reduction was less in the endostatin group than in the PBS group at any time point during the course of observation. Endostatin significantly reduced hydroxyproline levels in the lungs, the histological score on day 14, and the numbers of endothelial cells and inflammatory cells in the lungs on day 7.

Conclusions : These results demonstrate that administration of endostatin attenuates the fibrotic response to bleomycin, possibly through a mechanism that decreases the numbers of endothelial cells and inflammatory cells, and suggest that endostatin is an option for the treatment of pulmonary fibrosis. (Jikeikai Med J 2010 ; 57 : 11-9)

Key words : endostatin, bleomycin, angiogenesis, pulmonary fibrosis

INTRODUCTION

Endostatin has been isolated from the culture supernatant of mouse angioendothelioma during the screening of endogenous angiogenesis inhibitors produced by tumor cells using the inhibition of vascular endothelial cell proliferation as an index[1]. On the basis of its amino acid sequence, endostatin has been identified as a fragment of the C-terminal noncollagen portion of collagen type XVIII[1]. Endostatin inhibits proliferation and growth-factor-induced migration of vascular endothelial cells *in vitro*[1]. Reported pharmacological actions of endostatin include the inhibition of tumor vascularization and of inflammatory vascularization[1,2]. Abdollahi et al. have recently found in a DNA chip experiment that endostatin affects the expression of many genes in vascular endothelial cells[3]. On the basis of these findings, new pharmacological actions of endostatin are expected to be discovered.

Idiopathic pulmonary fibrosis (IPF) is a treatment-resistant disease of unknown cause that usually develops in adults older than 50 years[4]. In IPF, fibrosis does not occur merely as scarring or fibrosis of

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the inflammatory injury repair system but may result from abnormal wound healing of repeated alveolar epithelial injury[4]. The involvement of vascularization in the fibrosis of IPF has long been controversial. Vascular density varies among IPF lesions[5,6]. Increased vascular density in areas of minimal fibrosis has been reported, whereas decreased vascular density has been reported in fibrotic areas, indicating that further investigation is necessary with regard to the degree of involvement of vascularization in fibrosis. However, vascular endothelial cells with various functions are likely involved in the pathology of IPF. Because endostatin markedly affects endothelial cell function, how endostatin acts on bleomycin-induced pulmonary fibrosis in a mouse model of lung injury and fibrosis is of interest.

In this study, we administered endostatin to a mouse model of bleomycin-induced pulmonary fibrosis and investigated the pharmacological effects of endostatin on fibrosis.

METHODS

1. Preparation of mouse model of bleomycin-induced pulmonary fibrosis

All animal experiments were approved by the Animal Care Committee of The Jikei University School of Medicine and were performed according to the Guidelines on Animal Experimentation of The Jikei University School of Medicine. Eight-week-old male C57BL/6J mice (Oriental Yeast Co., Tokyo, Japan) were anesthetized with intraperitoneal injections of pentobarbital. A longitudinal incision was aseptically made in the neck, and the trachea was exposed. The dose of bleomycin hydrochloride (Nippon Kayaku Co., Tokyo, Japan) was 50 μg/body. The dosage volume was adjusted to 50 μl and intratracheally administered with a 29-G needle. In the control group, 50 μl of normal saline was administered into the trachea. Each experimental group consisted of 5 or 6 mice. Experiments were performed at least in duplicate.

2. Purification and administration of endostatin

Human kidney cells (293-EBNA) expressing mouse endostatin were cultured[7]. When the cells had grown to confluence, they were cultured in serum-free medium, and mouse endostatin was purified from conditioned medium as previously described[7]. Two hundred microliters of 1,000 μg/ml endostatin was intraperitoneally administered once daily from day 0 to 14. Because the body weight of mice was maintained at 20 g during the study period, this amount corresponded to a dosage of 10 mg/kg/day. In the control mice, 200 μl of phosphate-buffered saline (PBS) was administered with the same procedure. The following 3 groups were established: an intratracheal saline group, an intratracheal bleomycin+intraperitoneal PBS group, and an intratracheal bleomycin+intraperitoneal endostatin group.

3. Hydroxyproline assay

The mice were killed on day 14, and both lungs were excised and homogenized with a homogenizer (PT1300D, Kinematica AG, Lucerne, Switzerland). Hydroxyproline was measured with the method of Reddy et al[8]. To the homogenate, 2 N sodium hydroxide was added. The sample was then hydrolyzed at 120°C for 20 minutes in an autoclave, followed by the addition of 0.056 M chloramine-T solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at room temperature for 25 minutes for oxidation. Ehrlich's aldehyde reagent (dimethylaminobenzaldehyde, Wako Pure Chemicals, Ltd.) was then added, and the mixture was incubated at 65°C for 20 minutes. After the mixture was cooled in ice, the absorbance at 550 nm was measured. Hydroxyproline content (μg)/bilateral lung weight (g) was calculated, and mean values were compared among the groups.

4. Histopathologic examination

The mice were killed on day 14, and both lungs were excised and infused with 10% neutral formalin through the trachea to distend the lungs. The distended lungs were fixed by immersion for 3 days. The lungs were embedded in paraffin, and the block was sectioned into 3-μm-thick slices. The sections were stained with hematoxylin-eosin and Masson's trichrome for histological examination. For quanti-

tative analysis of the degree of fibrosis, the Ashcroft scoring system was used[9].

5. Flow cytometric analysis

The mice were killed on day 7, and both lungs were excised. Lungs were minced with scissors to a fine slurry in 10 ml of digestion buffer (RPMI, 5% fetal calf serum, 1 mg/ml collagenase [Roche Diagnostics, Basel, Switzerland]), and 30 μg/ml DNase (Wako Pure Chemical Industries, Ltd.)[10]. The lung slurry was enzymatically digested for 60 minutes at 37°C. Any undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total lung cell suspension was pelleted, resuspended, and hemolyzed. Cell counts were determined with 4% acetic acid on a hemocytometer. To evaluate the effects of endostatin on lung inflammation and angiogenesis, we repared single-cell suspensions of the lungs, which were analyzed with flow cytometry. We defined flk-1 as an endothelial cell marker, CD45 as a leukocyte marker, CD4 and CD8 a T-cell markers, CD19 as a B-cell marker, Ly6G as a granulocyte marker, and F4/80 as a macrophage marker. Single-cell suspensions were stained with an allophycocyanin (APC)-conjugated mouse anti-flk-1 antibody (eBioscience, San Diego, CA, USA), a fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD4 antibody (eBioscience), a phycoerythrin (PE)-conjugated mouse anti-CD8 antibody (eBioscience), an APC-conjugated mouse anti-CD19 antibody (eBioscience), an FITC-conjugated mouse anti-F4/80 antibody (eBioscience), a PE-conjugated mouse anti-Ly6G antibody (eBioscience), and a peridinin chlorophyll protein complex (PerCP)-conjugated mouse anti-CD45 antibody (eBioscience). Cells were analyzed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). We examined the kinetics of CD45-positive cells and flk-1-positive cells in the bleomycin group.

6. Statistical Analysis

All results are expressed as means±SEM. For comparisons of the body weight changes, repeated measures ANOVA, followed by the Bonferroni test, was used. For pairwise comparisons of the number of CD45- and flk-1-positive cells, Student's t-test was used. For comparisons of the fibrosis score, hydroxyproline content, and the numbers of cells positive for CD4, CD8, CD19, Ly6G, F4/80, and flk-1, one-way ANOVA, followed by the Bonferroni test, was used. Differences with $p<0.05$ were considered significant. Statistical analysis was performed with the Prism 4 program (GraphPad Software, Inc., San Diego, CA, USA).

Results

1. Body weight changes

In the saline group, body weight increased slightly during the course of the experiment (Fig. 1). In the bleomycin+PBS group, body weight decreased from days 6 to 8, and the reduced body weight was maintained from days 8 to 14. In the bleomycin+endostatin group, body weight decreased from days 6 to 8, but increased again from days 8 to 14. The body weight reduction was smaller in the bleomycin+en-

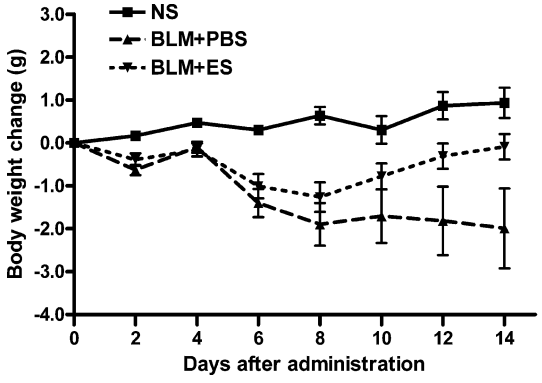


Fig. 1. Comparison of body weight changes.
Bleomycin (BLM) was intratracheally administered on day 0 to induce pulmonary fibrosis. As a control, normal saline (NS) was intratracheally administered. Endostatin (ES) was intraperitoneally administered from days 0 to 14. As a control, phosphate-buffered saline (PBS) was intraperitoneally administered. Body weight changes are presented as means±SE in each group, with the body weight on day 0 as the baseline. Statistical comparisons were made using repeated measures ANOVA followed by the Bonferroni test. NS: intratracheal NS group. BLM+PBS: intratracheal BLM+intraperitoneal PBS group. BLM+ES: intratracheal BLM+intraperitoneal ES group.

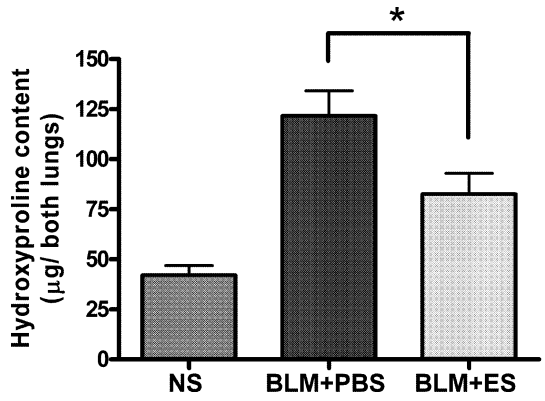


Fig. 2. Comparison of hydroxyproline content.
Mice were killed on day 14. Hydroxyproline was measured in lung tissue and normalized to micrograms per lung. Means±SE were compared among the groups. Statistical comparisons were made using one-way ANOVA followed by the Bonferroni test. *, $p<0.05$. NS: intratracheal normal saline (NS) group. BLM+PBS: intratracheal bleomycin (BLM)+intraperitoneal phosphate-buffered saline (PBS) group. BLM+ES: intratracheal BLM+intraperitoneal endostatin (ES) group.

dostatin group than in the bleomycin+PBS group at all time points during the observation period.

2. Comparison of hydroxyproline content

The hydroxyproline content was significantly higher in the bleomycin groups than in the saline group but was significantly lower in the bleomycin+endostatin group than in the bleomycin+PBS group (Fig. 2).

3. Histopathology

No apparent fibrosis, alveolar wall hypertrophy, or inflammatory cell infiltration was noted in the saline group (Fig. 3A, B). In the bleomycin+PBS group, severe fibrosis was noted immediately below the pleura and peribronchial and perivascular regions. Focal alveolar wall hypertrophy was also noted, partially forming a mass that had destroyed the original tissue. Severe peribronchial and perivascular infiltration of lymphocytes was noted, partially forming lymph follicles. Moderate numbers of neutrophils and macrophages had infiltrated into the alveolar spaces (Fig. 3C, D). In contrast, in the bleomycin+endostatin group, peribronchial and perivascular fibrosis and alveolar wall hypertrophy were mild, as was the destruction of the original tissue. Only mild peribronchial and perivascular lymphocyte infiltration and alveolar space neutrophil and macrophage infiltration were noted (Fig. 3E, F). The mean fibrosis score was significantly lower in the bleomycin+endostatin group than in the bleomycin+PBS group (Fig. 3G).

4. Cell analysis of lung tissue

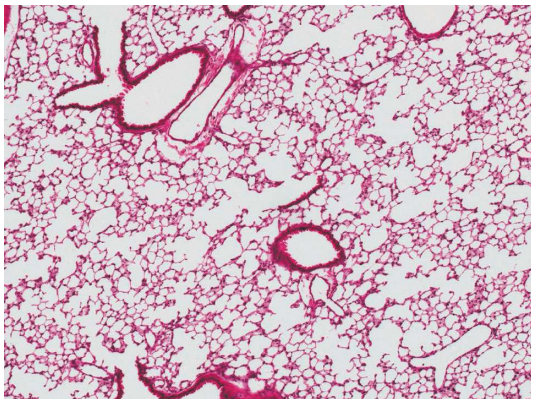
Treatment with bleomycin elevated the numbers of CD45-positive cell on days 7 and 14 (Fig. 4A) and the number of flk-1-positive cells on day 7 (Fig. 5A). On the basis of these findings, we examined the effect of endostatin on day 7. Treatment with endostatin decreased the numbers of cells positive for flk-1, CD45, CD4, CD8, CD19, Ly6G, and F4/80 compared with treatment with PBS (Fig. 4B, 4C, 5B).

DISCUSSION

The mouse model of bleomycin-induced pulmonary fibrosis is most commonly used as a model of human pulmonary fibrosis. We administered endostatin to mice with bleomycin-induced pulmonary fibrosis. The disease activity in the lung correlates with body weight changes in this mouse model. Body

Fig. 3. Histopathology.
Mice were killed on day 14, the lungs were excised, and tissue sections were prepared. A typical histopathological view of each group is presented (hematoxyline-eosin stain (A, C, E); original magnification×50 Masson's Trichome stain (B, D, F); original magnification×100). (A, B) Intratracheal normal saline (NS) group. (C, D) Intratracheal bleomycin (BLM)+intraperitoneal phosphate-buffered saline (PBS) group. (E, F) Intratracheal BLM+intraperitoneal endostatin (ES) group. (G) Fibrosis scores with the Ashcroft scoring system. Fibrosis scores were determined with the Ashcroft scoring system, and the means±SE of the scores were compared among the groups. Statistical comparisons were made with the one-way ANOVA followed by the Bonferroni test. *, $p<0.05$. NS: intratracheal NS group. BLM+PBS: intratracheal BLM+intraperitoneal PBS group. BLM+ES: intratracheal BLM+intraperitoneal ES group.

(A)



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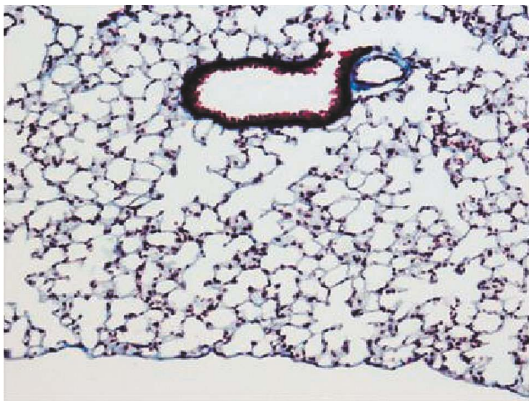

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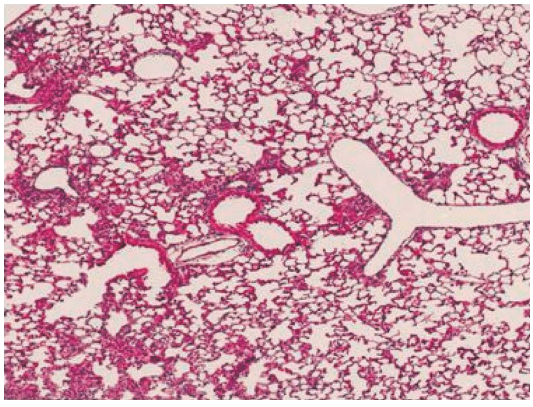

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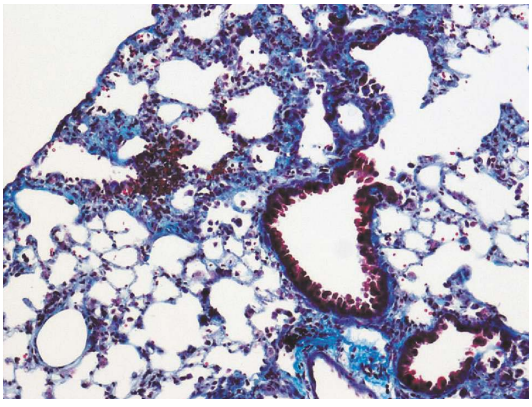

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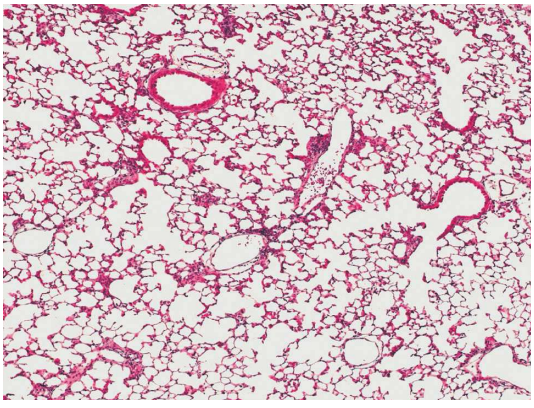

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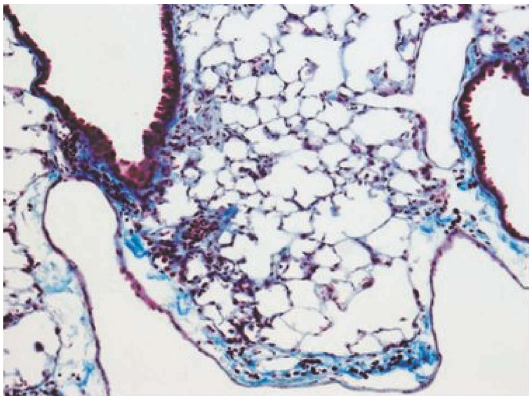

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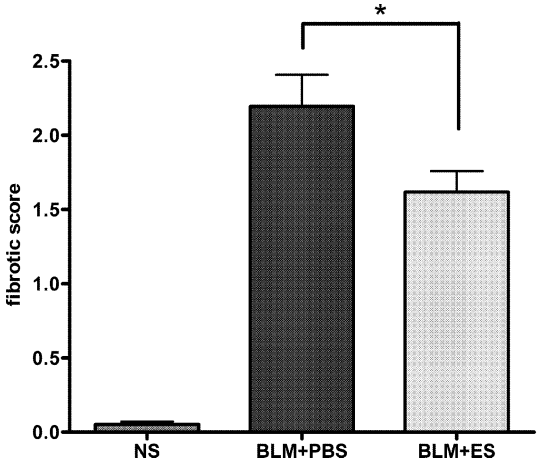

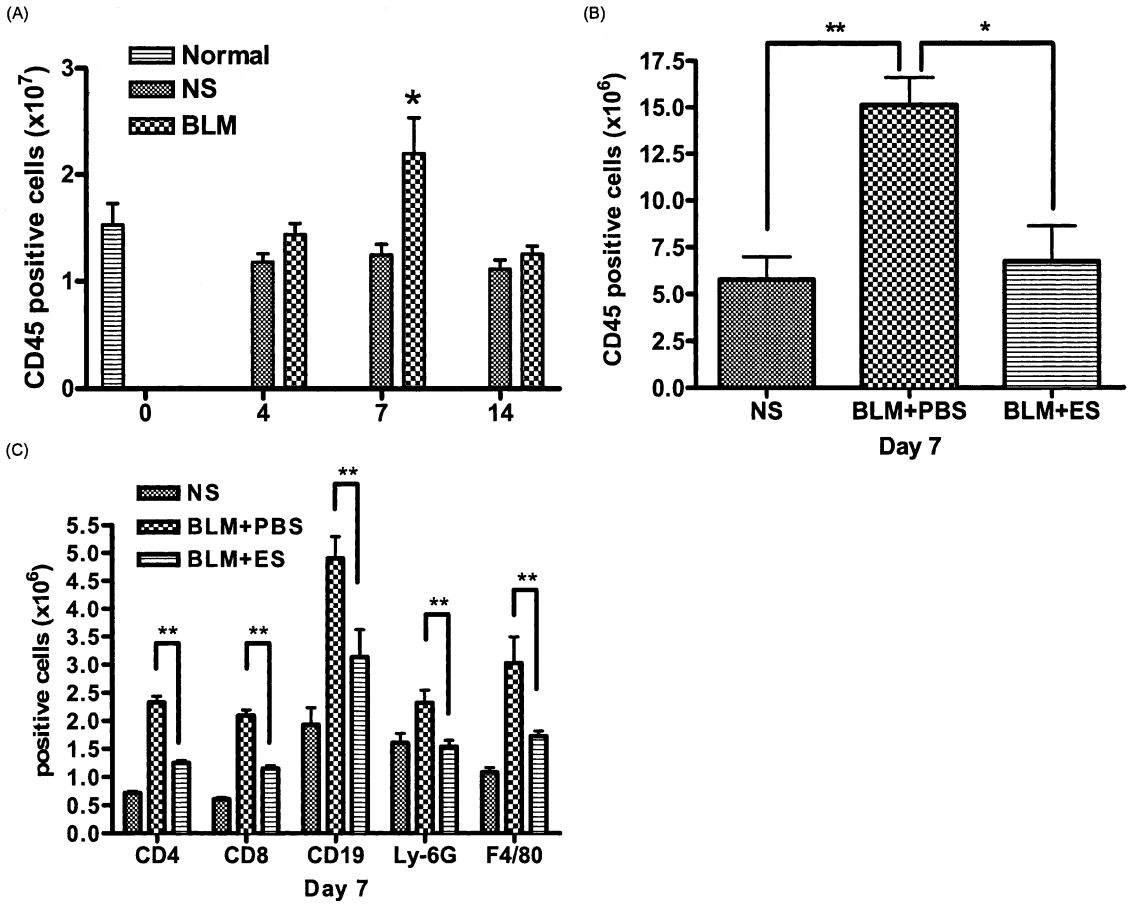

Fig. 4. Anti-inflammatory response of endostatin in a model of bleomycin-induced lung fibrosis.
Single cell suspensions were isolated from bleomycin- or saline-treated lungs, stained for CD45 (A and B), CD4, CD8, CD19, Ly6G, and F4/80 (C), and then examined with flow cytometric analysis. (A) Time course of lung CD45-positive cells in a model of bleomycin-induced lung fibrosis. The number of CD45-positive cells was significantly increased on day 7 after bleomycin administration. Endostatin had significantly decreased the numbers of cells positive for CD45 (B), CD4, CD8, CD19, Ly6G, and F4/80 (C) on day 7. The means±SE were compared among the groups. Statistical comparisons were made with Student's t-test (A), one-way ANOVA followed by the Bonferroni test (B, C). *, $p<0.05$, **, $p<0.01$. Normal: naïve control. NS: intratracheal normal saline (NS) group. BLM+PBS: intratracheal bleomycin (BLM)+intraperitoneal phosphate-buffered saline (PBS) group. BLM+ES: intratracheal BLM+intraperitoneal endostatin (ES) group.

weight did not change until day 3 after bleomycin administration and rapidly decreased after day 4. This body weight reduction was marked in the PBS group. In the endostatin group, body weight reduction was less than that in the PBS group and recovered from about day 8, suggesting that disease activity in the lung was more mild over time in the endostatin group than in the PBS group. In fact, the hydroxyproline content by lung weight on day 14 was significantly lower in the endostatin group than in the PBS group. Histopathological examination of the lung on day 14 also found that fibrosis was milder in the endostatin group than in the PBS group.

Vascularization in human IPF has recently been reported and has attracted attention[11–14]. Interferon-γ-1b is expected to be used to treat IPF, and its antifibrosis action has been reported to be partially mediated by chemokine (C-X-C motif) ligand 11, which has a vascularization-inhibitory action[14]. Vascularization has been reported to be involved in bleomycin-induced pulmonary fibrosis in mice[15]. Furthermore, administration of a substance with a

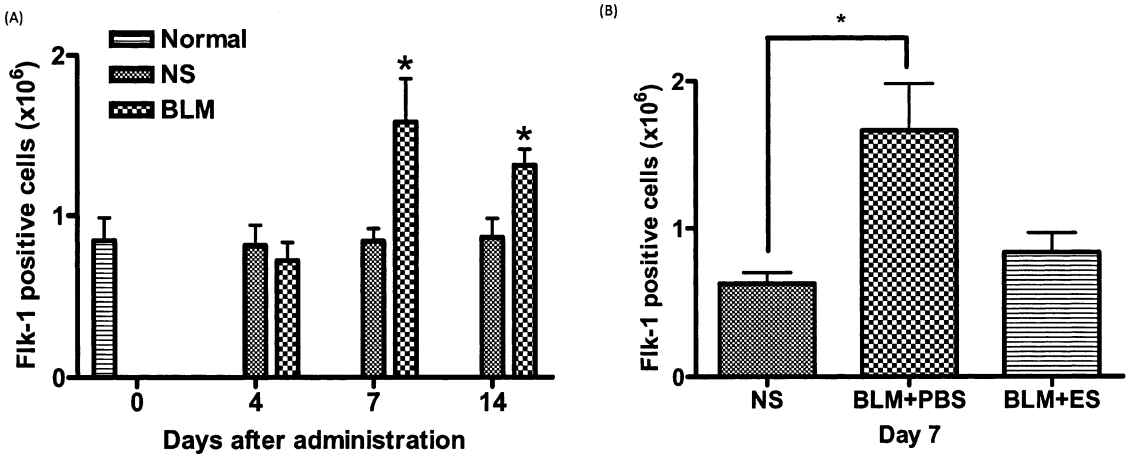

Fig. 5. Antiangiogenic effect of endostatin in a model of bleomycin-induced lung fibrosis.
Single-cell suspensions were isolated from bleomycin- or saline-treated lungs, stained for flk-1 (A and B), and then examined with flow cytometric analysis. (A) Time course of lung flk-1-positive cells in a model of bleomycin-induced lung fibrosis. The number of flk-1-positive cells had increased significantly on day 7 after bleomycin administration. (B) Endostatin showed a trend toward decreased levels of flk-1-positive cells on day 7. The means±SE were compared among the groups. Statistical comparisons were made with the Student t-test (A), one-way ANOVA followed by the Bonferroni test (B). *, $p<0.05$, **, $p<0.01$. Normal: naïve control. NS: intratracheal normal saline (NS) group. BLM+PBS: intratracheal bleomycin (BLM)+intraperitoneal phosphate-buffered saline (PBS) group. BLM+ES: intratracheal BLM+intraperitoneal endostatin (ES) group.

vascularization-inhibitory action inhibits bleomycin-induced pulmonary fibrosis[16-18]. Endostatin has a potent vascularization-inhibitory action. In fact, in the present study treatment with endostatin decreased the number of endothelial cells in the lung compared with that in the PBS group. Therefore, we speculate that inhibition of vascularization may be the mechanism by which bleomycin-induced pulmonary fibrosis is inhibited.

The antifibrosis effect exhibited by endostatin in this study may be partially due to an unknown action other than the vascularization-inhibitory action. The first 7 days after bleomycin administration is considered to be the acute alveolitis stage[19,20]. We analyzed the anti-inflammatory effect of endostatin in bleomycin-induced pulmonary fibrosis. Indeed, we showed that treatment with endostatin decreased the number of inflammatory cells in the lung compared with that in the PBS group on day 7. The decrease in the number of inflammatory cells is suspected to lead to the antifibrosis effect of endostatin. However, it is not clear whether endostatin and inflammatory cells interact directly. The regression of neovascularization may decrease the number of inflammatory cells infiltrating due to neovascularization. According to Abdollahi et al., endostatin affects the expression of many genes in vascular endothelial cells. Among them, chemokine-encoding genes have been described[3]. Chemokines play an important role in fibrosis. Further investigation of this point is necessary.

An elevation of the blood endostatin level in patients with IPF has recently been reported[21], and a similar finding in patients with scleroderma, particularly in patients with the complication of pulmonary fibrosis, has also previously been reported[22]. Fibroblast activation has been discussed as a possible mechanism of the increased endostatin levels in blood[23]. Kisker et al. have reported that the steady-state blood endostatin level was 990 ng/ml after endostatin was intraperitoneally administered to mice at 20 mg/kg/day[24]. Although direct comparison between our findings and those of Kisker et al. is not possible because of differences in animal species and measurement method, the blood level in our mouse experimental system may be much higher than that in human diseases. The blood endostatin level in mice with bleomycin-induced pulmonary fibrosis may be an

interesting subject for future studies.

Although endostatin inhibited bleomycin-induced pulmonary fibrosis in this experiment, many aspects of its mechanism of action remain unclear, and further investigation is necessary.

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