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Original

Exogenous S100A4 Protein Attenuates Bleomycin-induced Pulmonary Fibrosis in Mice by Reducing the Levels of Fibroblast Growth Factors

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SUMMARY

Background and objective : The calcium-binding protein S100A4 belongs to the S100 family and is involved in fibrotic and inflammatory processes, in which tissue remodeling, cell motility, and epithelial–mesenchymal transition play major roles. Cytoplasmic S100A4 is a marker of lung fibroblasts in pulmonary fibrosis ; however, the effects of exogenous S100A4 on fibrotic and inflammatory processes in pulmonary fibrosis are unclear. This study examined the effects of exogenous S100A4 protein in mice with bleomycin-induced pulmonary fibrosis.

Methods : Bleomycin was administered to mice by intratracheal instillation on day 1. Intratracheal S100A4 protein was administered 4 times after bleomycin treatment. Bronchoalveolar lavage fluid was obtained and lung histological examinations were performed on day 14 after bleomycin administration. Lung tissue was homogenized on the same day to assess the mRNA expression of cytokines, fibroblast growth factors, and S100A4.

Results : Unexpectedly, we observed that the administration of exogenous S100A4 protein apparently reduced lung fibrosis in bleomycin-treated mice. In addition, the levels of lymphocyte accumulation and insulin-like growth factor-1 mRNA were significantly reduced in bleomycin-treated lung by S100A4 administration.

Conclusions : Exogenous S100A4 protein attenuates bleomycin-induced pulmonary fibrosis in mice by reducing lymphocyte function and the levels of fibroblast growth factors.

Key words : S100A4 ; bleomycin-treated mice ; pulmonary fibrosis ; fibroblast growth factors

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INTRODUCTION

The calcium-binding protein S100A4, which belongs to the S100 family, promotes metastasis in animal models[1]. S100A4 is expressed in the nucleus, cytoplasm, and extracellular space, and its functions

include regulation of cell survival, motility, and invasion[2~4]. The human S100A4 gene is located at a frequently reorganized gene cluster on chromosome 1q21. It consists of four exons, the first two of which are noncoding[5]. Several studies have demonstrated roles for S100A4 in cancer advancement and the enhancement of metastasis[1,2]. Recent studies have reported that S100A4 is also associated with renal fibrosis, liver cirrhosis, pulmonary fibrosis, cardiac hypertrophy, rheumatoid arthritis, and neuronal injuries[6]. In these diseases, endogenous S100A4 is involved in fibrotic and inflammatory processes, in which tissue remodeling, cell motility, and epithelial-mesenchymal transition play major roles[6]. Furthermore, cytoplasmic S100A4 is a marker of lung fibroblasts in pulmonary fibrosis[7] ; however, the effects of exogenous S100A4 on the fibrotic and inflammatory processes of pulmonary fibrosis remain unclear.

This study examined the effect of exacerbations by exogenous S100A4 in mice with bleomycin-induced pulmonary fibrosis and found an unexpected effect of this protein on pathogenesis. We discuss a novel therapeutic role for S100A4 in fibrotic lung diseases.

MATERIALS and METHODS

Animals

C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). This study was approved by the Animal Ethics Committee of Dokkyo Medical University.

Bleomycin and S100A4 administration in mice

To investigate the direct synergic effect of S100A4 in lung tissues inflammations, we demonstrated by intratracheal administration with the protein in bleomycin-induced mice. Figure1 shows the study protocols. Groups 1, 3, and 4 were treated with bleomycin to induce pulmonary fibrosis. Saline (bleomycin [−]) or bleomycin sulfate (bleomycin [+] ; 5 mg/kg body weight ; Nippon Kayaku, Tokyo, Japan) was given by intratracheal administration to mice anesthetized with sodium pentobarbitone (50 mg/kg body weight) on day (D) 1. Bleomycin (+) mice, anesthetized by sodium pentobarbitone, were given 50 μL S100A4 (R&D, Minneapolis, MN) (500 ng/body to Group 3 or 50 ng/body to Group 4) or saline (Group 1) by intratracheal administration 4 times on D3, D4, D7, and D10. In addition, bleomycin (−) mice (saline ; Group 2) were administered 500 ng/mL S100A4. Using an aseptic technique, a single incision was made in the neck and the muscle tissue covering the trachea was snipped to expose the tracheal rings. Bronchoalveolar lavage (BAL) fluid was collected on D14 after bleomycin or saline administration, and histological examination of the lungs was performed on D14.

BAL

BAL and cell counting were performed as described previously[8]. The cells in the BAL fluid were harvested and counted.

Expression of cytokines, fibroblast growth factor, and S100A4 mRNA in lung tissue

The right lobes of the lung were harvested and stored at −80℃ for several days after washing with phosphate-buffered saline. The tissue was thawed at 0℃ in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA) and then homogenized with a microhomogenizer (10,000×g, 90s). Total RNA extraction was performed by a modified guanidine isothiocyanate-phenol-chloroform method, as described previously[9]. The resultant complementary DNA products were diluted in Tris buffer and used for real-time quantitative PCR.

Real-time quantitative PCR

Interleukin (IL)-4, IL-5, IL-13, interferon (IFN)-γ, transforming growth factor (TGF)-β1, insulin-like growth factor (IGF)-1, platelet-derived growth factor (PDGF)-AA, and S100A4 mRNA expression levels were quantified by real-time RT-PCR (ABI/PRISM 7000 ; Applied Biosystems, Foster City, CA). PCR data were analyzed using ABI/PRISM 7000 SDS v1.0 software (Applied Biosystems), with β-actin as the internal control. The PCR conditions were described previously[9]. The mouse mRNA primers were purchased from Takara Bio USA (Madison, WI).

Histopathology

Paraformaldehyde in phosphate buffer was perfused into the left lung at a constant pressure. After fixation, lung tissues were embedded in paraffin and sec-

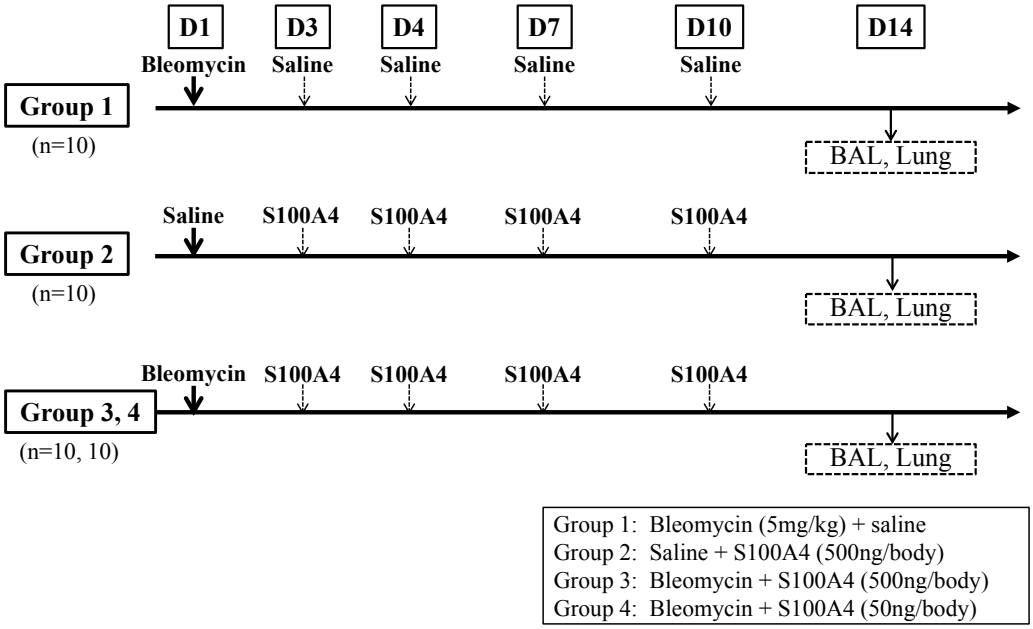


Figure 1 Schema of the study protocols.
Group 1, mouse model of bleomycin-treated pulmonary fibrosis with saline ; Group 2, mouse model of S100A4 only (500 ng/mouse) ; Group 3, mouse model of bleomycin-treated pulmonary fibrosis with S100A4 (500 ng/mouse) ; and Group 4, mouse model of bleomycin-treated pulmonary fibrosis with S100A4 (50 ng/mouse). BAL fluid was taken and lungs were analyzed for the expression of mRNA and histology on day 14 (D14).

tioned with a microtome at a thickness of 4 μm. The sections were mounted on slides and stained with hematoxylin and eosin (HE). For each mouse, four sections of the whole lung stained with HE were selected randomly. The severity of lung fibrosis was analyzed as described previously[9]. To obtain photomicrographs, a fluorescence microscope (model EX41 ; Olympus America, Melville, NY) with a ×20 lens and an Olympus Magna Fire camera was used.

Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical significance was determined by Student's *t*-test (two-tailed) for two groups or one-way analysis of variance (with Tukey's multiple comparisons test) for three or more groups. P values <0.05 were considered to be significant. JMP software (Version 7.0 for Mac ; SAS Institute, Cary, NC) was used for statistical analyses.

RESULTS

Cells in BAL fluid from bleomycin-treated mice with or without S100A4 administration

To examine the effect of S100A4, cell populations in the BAL fluid taken from each treated group and control mice administered saline alone were examined (Figure 2A). The number of each cell type following administration with bleomycin alone (Group 1) was compared with that in the control group (representing the basal level). The number of total cells and macrophages was not significantly different among each study group. Eosinophilia was not observed (data not shown). In contrast, treatment with S100A4 alone (Group 2) increased the number of neutrophils, which was decreased with pre-administration of bleomycin in Group 3 ($0.7 \pm 0.1 \times 10^5$ cells) and Group 4 ($0.1 \pm 0.01 \times 10^5$ cells), although the number was greater in Group 3 than in Group 1 ($0.2 \pm 0.2 \times 10^5$ cells). Notably, bleomycin-induced lymphocytosis was significantly attenuated by treatment with S100A4, the effect of which when administered alone was modest. On the

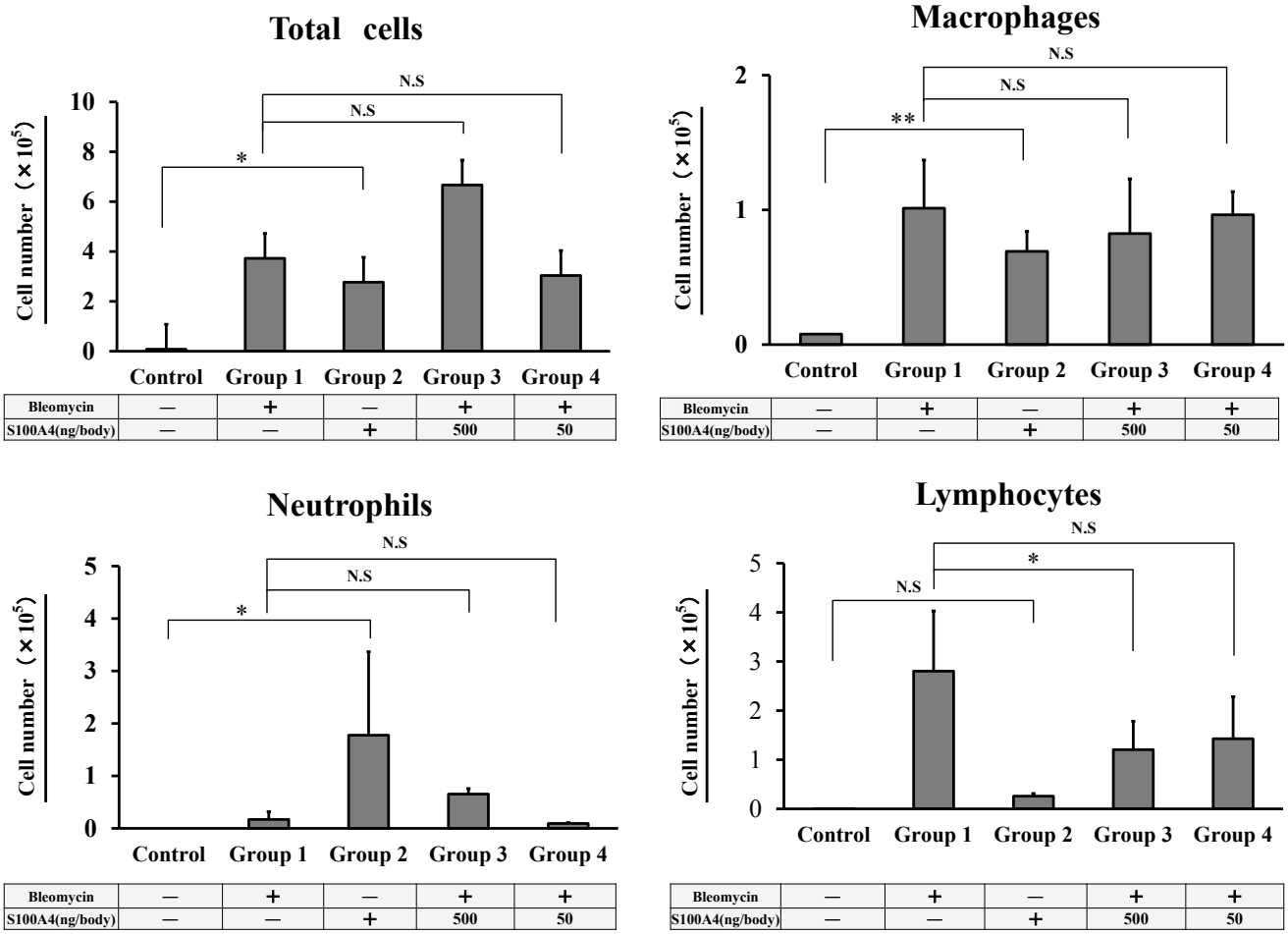


Figure 2 A

other hand, the number of total cells, macrophages, and neutrophils was significantly ($P<0.05$) greater in Group 2 ($2.8\pm2.0\times10^5$ cells, $0.7\pm0.1\times10^5$ cells, and $1.8\pm1.6\times10^5$ cells, respectively) than in saline alone ($0.1\pm0.1\times10^5$ cells, $0.1\pm0.1\times10^5$ cells , and $0.1\pm0.1\times10^7$ cells, respectively).

Histopathological analysis in bleomycin-treated mice with or without S100A4 administration

We performed histopathological analysis on D14 of the lungs of bleomycin-treated mice that had been administered (Groups 3 and 4) or not administered (Group 1) S100A4 (Figure 2B). The lung fibrosis score (Figure 2C) was significantly reduced in Group 3 (3.3 ± 0.6) compared with Group 1 (5.0 ± 0.8). These results indicated that bleomycin-induced pulmonary fibrosis could be reduced by treatment with S100A4. In contrast, the administration of S100A4 alone (Group 2) increased ($P<0.001$) pulmonary fibrosis (2.5 ± 0.6) when compared to saline alone (0.5 ± 0.1). These results indicated the unexpected effect that S100A4 attenuated bleomycin-induced pulmonary fibrosis.

Expression of cytokine and fibroblast growth factor mRNA in lung tissue of bleomycin-treated mice with or without S100A4

Since analysis for cytokines and fibroblast growth factors (IGF-1, PDGF, and TGF-β) at the protein level was affected by their widely varying concentrations (data not shown), mRNA expression in whole lung tissue was investigated on D14 after bleomycin administration. T helper (Th) cytokines as well as growth factors have been suggested to be involved in the pathogenesis of pulmonary fibrosis. Administration of S100A4 alone showed a tendency for increased mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) as compared with a Th1 cytokine (IFN-γ)

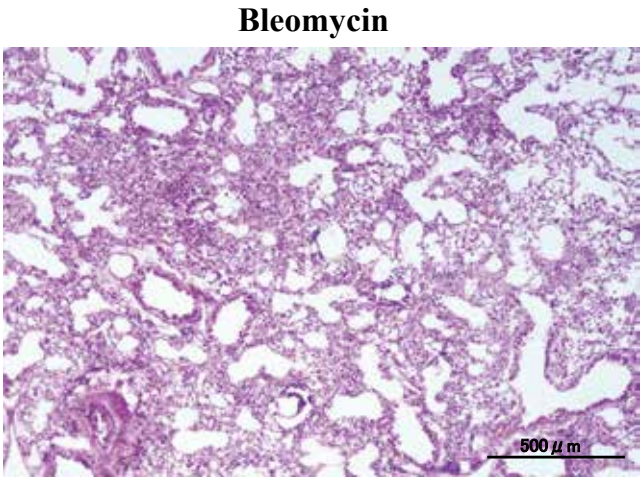
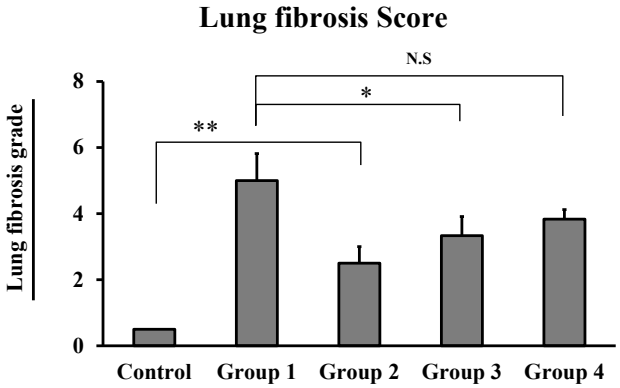


Figure 2 B



Bleomycin	−	+	−	+	+
S100A4(ng/body)	−	−	+	500	50

Figure 2 C

Figure 2 The types of cells (**a**) in BAL fluid in bleomycin-treated mice.

The number of cells ($\times 10^5$) in BAL fluid was assessed on day 14. Saline-treated mice (bleomycin [−]) were administered saline intratracheally four times as controls. Histopathological analysis (**b**) of the lungs of bleomycin-treated mice. For histopathological analysis, lung sections were stained with HE. The scale bar represents 500 μm. Original magnification, ×100. B : Quantification of lung fibrosis (**c**) in lung tissue by score. Data are presented as means ± SD for each group. *P<0.05 compared with bleomycin-treated mice administered saline. N.S., no significant difference.

(Group 2) (Figure 3A). Among the bleomycin-treated groups (Groups 1, 3, and 4), there was no significant difference in the expression levels of IFN-γ, IL-4, and IL-5, regardless of S100A4 administration. IL-13 mRNA expression in bleomycin-treated lungs (Group 1 : relative ratio, 3.3 ± 0.8) was significantly augmented by S100A4 (Group 3 : relative ratio, 19.0 ± 6.8). The expression of IL-4, IL-5, and IL-13 mRNA was significantly greater in Group 2 (14.6 ± 1.4, P<0.01, 17.3 ± 10.7, P<0.05, and 8.4 ± 6.6, P<0.05, respectively) than in saline only. There was no significant differences in the expression of IFN-γmRNA between Group 2 and saline alone. As for the fibroblast growth factors (Figure 3B), a modest increase in TGF-β1 mRNA expression was similarly observed in bleomycin-treated lungs (Groups 1, 3, and 4) as compared with the control, whereas S100A4 itself had no apparent effect on expression. S100A4 alone (Group 2) significantly induced PDGF-AA mRNA expression ; however, it did not influence the expression induced by bleomycin treatment (Groups 1, 3, and 4). Notably, the bleomycin treatment-induced expression of IGF-1 mRNA (Group 1 : relative ratio, 12.0 ± 0.5) was significantly reduced by S100A4, which showed clearly a

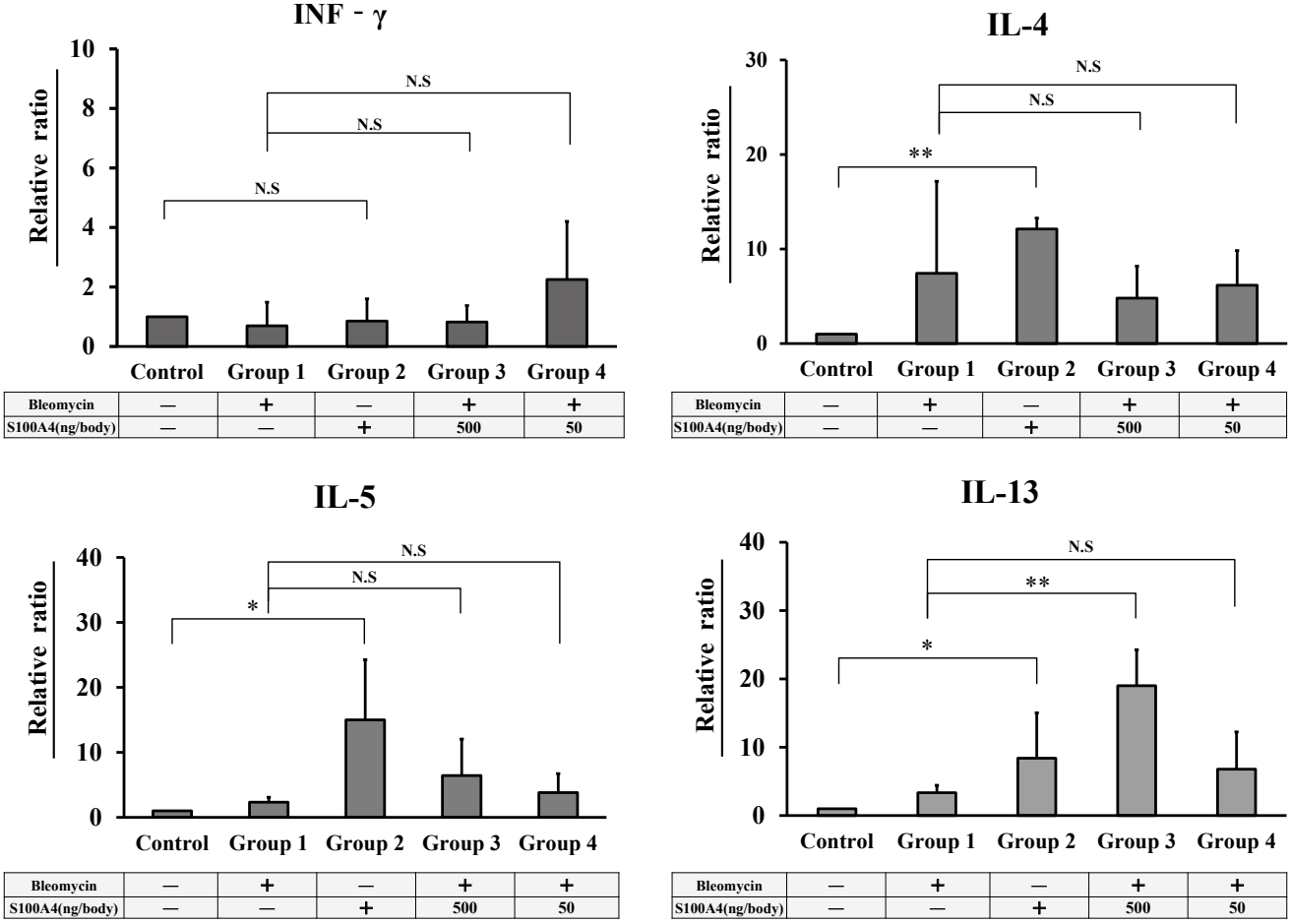
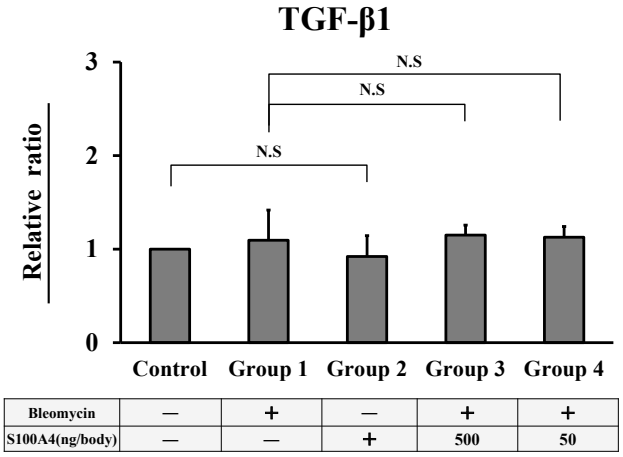


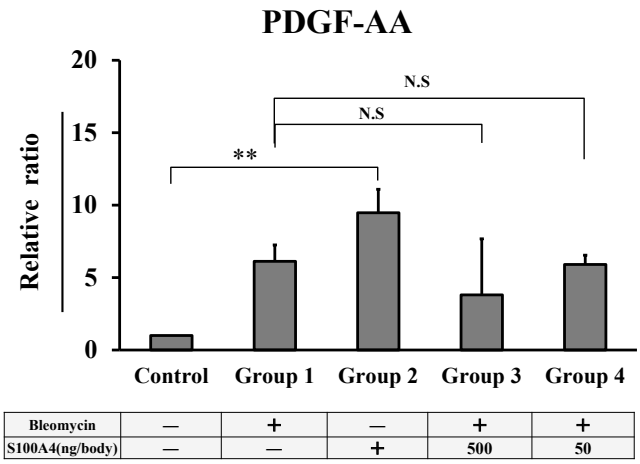
Figure 3 A

dose-dependent effect (Group 3 : relative ratio, 7.0± 0.2). Conversely, administration of S100A4 alone (Group 2) has a modest effect on the expression of IGF-1 mRNA. The expression of PDGF-AA and IGF-1 mRNA was significantly greater in Group 2 (9.5 ±1.6, $P<0.01$, and 1.8± 0.5, $P<0.05$, respectively) than in saline only. There was no significant differences in the expression of TGF-β1 mRNA between Group 2 and saline alone. Finally, we observed that bleomycin treatment significantly increased S100A4 mRNA expression (Group 1 : relative ratio, 2.6± 0.7) in lung tissue, which was significantly reduced in mice administered exogenous S100A4 at a high dose (Group 2 : relative ratio, 0.8± 0.5 ; Group 3 : relative ratio, 1.3± 0.9) compared with those administered S100A4 at a low dose (Group 4 : relative ratio, 1.6± 1.1) (Figure 3C). There was no significant differences in the expression of S100A4 mRNA between Group 2 (0.8± 0.5) and saline. These results demonstrated that the administration of exogenous S100A4 attenuated the expression of endogenous S100A4 mRNA by a negative feedback loop.

DISCUSSION

We demonstrated that exogenous S100A4 ameliorated bleomycin-induced lung fibrosis with a reduction of lymphocyte accumulation and expression of IGF-1 mRNA in lung tissue. S100A4 is expressed in the cytoplasm and exported extracellularly[3,4], is involved in cellular proliferation and the development of fibrosis[6], and is expressed by lung fibroblasts in interstitial pneumonia and malignant tumor cells[6]. The observed therapeutic effects of exogenous S100A4 administration was contrary to our expectations, although endogenous S100A4 mRNA expression was attenuated in whole lung tissue, such as observed for the negative feedback mechanism via receptor for advanced glycation end products (RAGE), a ligand of





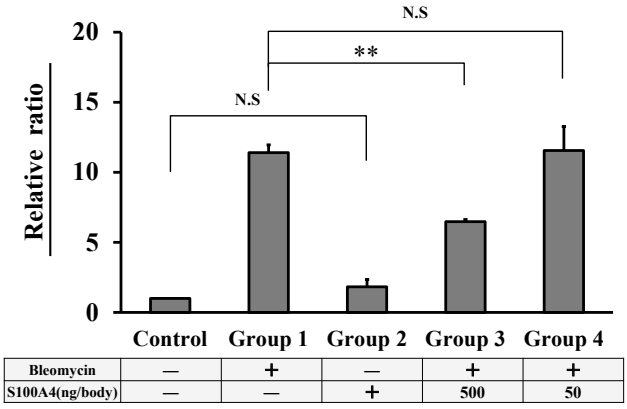


Figure 3 B

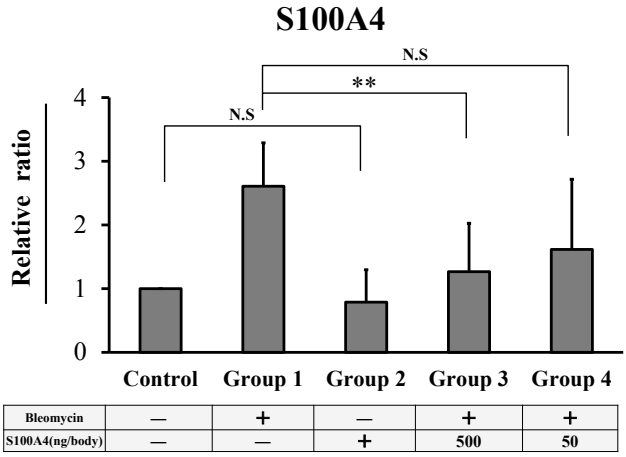


Figure 3 C

Figure 3 mRNA expression of cytokines and fibroblast growth factors in lung tissue.

The mRNA expression of Th1-type (IFN-γ) /Th2-type (IL-4, IL-5, and IL-13) cytokines (**a**) and fibroblast growth factors (TGF-β1, IGF-1, and PDGF-AA) (**b**) in lung tissue from each group is shown. Expression of S100A4 (**c**) mRNA in lung tissue from each group. Expression is shown as a ratio relative to expression in the lung of saline-treated mice (bleomycin[−]and S100A4[−]) as controls. Data are presented as means ± SD for each group. *P<0.05 and **P<0.01 compared with bleomycin-treated mice administered saline. N.S., no significant difference.

the S100 family. This endogenous down-regulation may be implicated in the effect of exogenous S100A4 on fibrogenesis ; however, other mechanisms may also be involved.

RAGE is expressed on a range of cells, including endothelial cells, smooth muscle cells, epithelial cells, lymphocytes, macrophages, and neutrophils[10]. The latter two inflammatory cells are involved in the pulmonary pathogenesis of both fibrogenic diseases and experimental models. Indeed, we observed an influence of S100A4 administration on the numbers of lymphocytes and neutrophils, but not eosinophils and macrophages, *in vivo*. Exogenous S100A4 reduced the pulmonary influx of lymphocytes in bleomycin-treated mice, which was coincident with an improvement of fibrogenesis. In contrast, neutrophil influx was more pronounced following S100A4 administration alone rather than with bleomycin. Epithelial-mesenchymal transition and bone marrow progenitors reportedly contribute to S100A4-positive fibroblasts in bleomycin-induced lung fibrosis. Furthermore, we observed that lung fibrosis could be induced by S100A4 alone to a modest but significant extent as compared with control, indicating a fibrogenic effect that might involve the observed neutrophil influx. However, in terms of the pathogenesis of bleomycin-induced lung

fibrosis, we focused on the therapeutic effect of S100A4, which was completely unexpected. To explain the mechanism underlying this effect, the attenuation of lymphocyte influx may be involved, which is suggested by the positive correlation between the levels of fibrogenesis and lymphocyte influx in the current study, although cell type was not analyzed in detail. However, we could not identify an apparent correlation between the expression of any cytokine and fibrogenesis, while Th2-type cytokines, such as IL-4 and IL-13, are reportedly involved in the pathogenesis of lung fibrosis. Several studies[8,9] stated that Th2-dominant inflammation was not required for the development, severity, or prognosis of bleomycin-induced pulmonary fibrosis. In our results, Th2-type inflammation did not seem to play a major role in the development of fibrosis. Thus, suppressing an unknown function of lymphocytes may be related to the therapeutic effect of S100A4 on the development of fibrosis.

The current study indicated another effect of S100A4 ; namely, the reduced expression of IGF-1 mRNA, which is produced from osteoclasts and fibroblasts[11] and plays an important role as a prosurvival factor for fibroblasts in bleomycin-induced lung injury[12]. A noteworthy fact is that the levels of IGF-1 mRNA were positively correlated with the level of fibrosis following bleomycin treatment. It has been already reported that IGF-1 promotes the survival of lymphocytes[13]. Thus, S100A4 may mediate cross-talk regulation between IGF-1 gene expression and the suppression of lymphocyte influx to ameliorate bleomycin-induced pulmonary fibrosis, although the precise mechanism is still unknown. Another growth factor, TGF-β, which is a major fibroblast growth factor, also plays important roles in the development of fibrosis in several organs[14]. TGF-β1 expression was not reduced by S100A4 administration in the current study, whereas Tomcik et al. demonstrated that S100A4 amplifies TGF-β-induced fibroblast activation in systemic sclerosis and that excess amounts of S100A4 activated TGF-β/Smad2/3 signaling in fibroblasts, resulting in increases in p-Smad3[15], which was suggested to activate collagen synthesis at the mRNA level. Conversely, they demonstrated that S100A4 also up-regulated Smad7 expression, which inhibits the initiation of TGF-β signaling[16]. Remarkably, the overexpression of Smad7 is known to prevent bleomycin-induced pulmonary fibrosis[17]. Furthermore, Tang et al. demonstrated that overexpressing latent TGF-β1 protected against bleomycin-induced lung injury via overexpressed Smad7-mediated inhibition of TGF-β/Smad2/3 signaling and enhancement of regulatory T cell responses[18]. Therefore, the ameliorating effect of S100A4 in this pulmonary fibrosis model might be a result of the reinforcement of the function of Smad7 to regulate TGF-β/Smad2/3 signaling in a negative feedback loop.

In conclusion, the present study demonstrates that exogenous S100A4 attenuates bleomycin-induced lung fibrosis. On the other hand, the functions of exogenous S100A4 might be regulated by the background of various inflammations in lung tissues. Although the exact details underlying the S100A4-mediated regulation are still unknown, further clarification of these complex mechanisms is needed to provide an understanding of the pathogenesis of pulmonary fibrosis and the development of new therapeutic strategies.

ABBREVIATIONS

BAL : bronchoalveolar lavage
IFN : interferon
IGF : insulin-like growth factor
IL : interleukin
N.S. : no significant difference
PDGF : platelet-derived growth factor
SD : standard deviation
TGF-β : transforming growth factor-β
Th : T helper
RAGE ; receptor for advanced glycation end products

COMPETING INTERESTS

The authors declare that they have no competing interests.

Authors' contributions

MW, HH, and MA : performed experiments, analyzed data, and wrote the manuscript. MT : performed experiments and analyzed data. KK : performed experiments, analyzed data, and helped write

the manuscript. KS : analyzed data, performed experiments, and helped write the manuscript. YF : conceived and performed experiments and helped write the manuscript. YI : conceived experiments and helped write the manuscript. TF : conceived experiments and reviewed the manuscript. All authors read and approved the final manuscript.

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