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Thalidomide Prevents Bleomycin-Induced Pulmonary Fibrosis in Mice

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Pulmonary fibrosis in humans can occur as a result of a large number of conditions. In idiopathic pulmonary fibrosis (IPF), pulmonary function becomes progressively compromised resulting in a high mortality rate. Currently there are no proven effective treatments for IPF. We have recently reported that IL-6 and TGF-β1 plays an important role in proliferation and differentiation of lung fibroblasts, and all-trans-retinoic acid (ATRA) prevented bleomycin-induced lung fibrosis through the inhibition of these cytokines. Thalidomide (Thal) has been used in the treatment of multiple myeloma through the inhibitory effect on IL-6-dependent cell growth and angiogenesis. In this study, we examined the preventive effect of Thal on bleomycin-induced pulmonary fibrosis in mice. We performed histological examinations and quantitative measurements of IL-6, TGF-β1, collagen type Iα1 (COL1A1), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) in bleomycin-treated mouse lung tissues with or without the administration of Thal. Thal histologically ameliorated bleomycin-induced fibrosis in mouse lung tissues. Thal decreased the expressions of IL-6, TGF-β1, VEGF, Ang-1, and COL1A1 mRNA in mouse lung tissues. In addition, Thal inhibited angiogenesis in the lung. In vitro studies disclosed that Thal reduced 1) production of IL-6, TGF-β1, VEGF, Ang-1, and collagen synthesis from human lung fibroblasts, and 2) both IL-6-dependent proliferation and TGF-β1-dependent transdifferentiation of the cells, which could be the mechanism underlying the preventive effect of Thal on pulmonary fibrosis. These data may provide a rationale to explore clinical use of Thal for the prevention of pulmonary fibrosis. The Journal of Immunology, 2007, 179: 708–714.

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3 Abbreviations used in this paper: IPF, idiopathic pulmonary fibrosis; PKC, protein kinase C; ATRA, all-trans-retinoic acid; Thal, thalidomide; COL1A1, collagen type Iα1; VEGF, vascular endothelial growth factor; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; CMC, carboxymethylcellulose; α-SMA, α smooth muscle actin.

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FIGURE 1. Effect of Thal on bleomycin-induced pulmonary fibrosis and the expressions of IL-6, TGF-β, and COL1A1 mRNA in lung mouse tissues. Eight-week-old mice were injected i.p. with bleomycin sulfate (2 mg/mouse/day) (Bleo, Bleo+Thal) on days 1, 8, and 15. Thal (4 mg/mouse/day) dissolved in 0.1 ml of 0.5% CMC (Bleo+Thal) or 0.1 ml of 0.5% CMC alone (control, Bleo) was administered i.p. five times per week during the time course. On day 28, mice (n = 3 in each experiments) were sacrificed. A Histological changes were demonstrated by H&E and Azan staining (original magnification, ×100). B–D. Real-time RT-PCR was performed to determine the changes in mRNA levels from lung tissues of mice for IL-6 (B), TGF-β1 (C), and COL1A1 (D) as described in Materials and Methods. The levels of mRNA are represented as the ratio to 18S rRNA. The results are indicated as the mean ± SD of three separate experiments.

Thal prevents bleomycin-induced pulmonary fibrosis in mice through the inhibition of the production of proinflammatory, profibrotic cytokines and the reduction of angiogenesis.

Materials and Methods

Cell culture

W138VA-13, a human embryonal lung fibroblastic cell line transformed by SV40, and IMR-90, a cell line derived from human fetal lung fibroblasts, were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS. All cells were cultured with antibiotics in a humidified incubator with 5% CO₂ at 37°C. In some experiments, cells were stimulated with human recombinant TGF-β1 (PeproTech) at a dose from 0.5 to 5 ng/ml for 24 h.

When cells were treated with Thal (Sigma-Aldrich), Thal was diluted in DMSO and added to the growth medium to yield the final DMSO solvent concentration <0.05% (v/v). Control cells were treated with the same concentration of DMSO. In preliminary experiments, this final concentration of DMSO had no gross effect on W138VA-13 and IMR-90 cells.

Animal studies

C57BL/6 female mice were purchased from Japan SLC and maintained in our specific pathogen-free animal facility. All animals were kept according to the Animal Protection Guidelines of Kyoto University. All protocols for animal use and euthanasia were reviewed and approved by the Institute of Laboratory Animals (Graduate School of Medicine, Kyoto University, Japan). Eight-week-old mice were injected i.p. with bleomycin sulfate (2 mg/mouse/day; Nippon Kayaku) on days 1, 8, and 15. In some experiments, mice were injected i.p. with 4 mg of Thal dissolved in 0.1 ml of 0.5% carboxymethylcellulose (CMC; Sigma-Aldrich) or 0.1 ml of 0.5% CMC alone (control, Bleo) on days 1, 8, and 15. Thal (4 mg/mouse/day) dissolved in 0.1 ml of 0.5% CMC (Bleo+Thal) or 0.1 ml of 0.5% CMC (Bleo alone) was administered i.p. five times per week during the time course. On day 28, mice (n = 3 in each experiments) were sacrificed. A Histological changes were demonstrated by H&E and Azan staining (original magnification, ×100). B–D. Real-time RT-PCR was performed to determine the changes in mRNA levels from lung tissues of mice for IL-6 (B), TGF-β1 (C), and COL1A1 (D) as described in Materials and Methods. The levels of mRNA are represented as the ratio to 18S rRNA. The results are indicated as the mean ± SD of three separate experiments.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described (7). The fixed cells were stained with mouse anti-human α-smooth muscle actin (α-SMA) mAb (1:100) (Sigma-Aldrich) followed by Alexa 488-conjugated donkey anti-mouse Ab (1:1000; Molecular Probes). Hoechst 33258 fluorochrome (Sigma-Aldrich) was used for nuclear staining.

Immunohistochemistry

Mice were sacrificed at day 28 after the first injection of bleomycin, and the lungs were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin and sectioned. Sections were immunostained as described (6) by using anti-mouse CD31 Ab (1:100; BD Pharmingen). TSA Biotin System (PerkinElmer Life and Analytical Sciences) was used to enhance staining, and peroxidase activity was envisioned with diaminobenzidine kit (DakoCytomation). The sections were counterstained with hematoxylin. The CD31-positive vessels were counted in 10 fields of vision (magnification; ×200) in each of three consecutive sections by three investigators.

Measurement of IL-6, sIL-6R, TGF-β1, IL-1β, VEGF, Ang-1, and Ang-2

The concentrations of IL-6, sIL-6R, TGF-β1, IL-1β, VEGF, Ang-1, and Ang-2 in the culture supernatants with or without Thal (50 µg/ml) for 24 h were measured by ELISA kit (BioSource; R&D Systems).

Cell proliferation assay

Cell proliferation assay was performed as described (6). Cells were cultured in 96-well flat-bottom culture plates for 4 days with or without IL-6 (1–10,000 pg/ml), Thal (50 µg/ml), and/or mouse anti-human IL-6R mAb (20 ng/ml) (DakoCytomation) to inhibit the binding of IL-6 to its receptor. Cell Counting Kit-8 (Dojindo, Tokyo, Japan) was used to characterize the growth of cells.

Statistical analysis

Results are given as the mean ± SD of values. Statistical analysis was performed using Bonferroni/Dunn multiple comparison tests.

Results

Prevention of bleomycin-induced pulmonary fibrosis by Thal

We examined the effect of Thal on bleomycin-induced lung fibrosis in mice. The histological changes at 28 days are shown in Fig. 1A. In the lung tissues from the bleomycin-treated mice without Thal, pulmonary interalveolar septa became thickened
and infiltrated by inflammatory cells, with collagen depositions in the interstitium disclosed by Azan staining. Intrapulmonary administration of Thal five times per week inhibited the collagen deposition in the bleomycin-treated mouse lungs. Mice treated by Thal without bleomycin-treatment showed no changes at all (data not shown).

Effect of Thal on the expressions of IL-6, TGF-β1, and COL1A1 mRNA in mouse lung tissues

We previously reported that IL-6 played an important role in pulmonary fibrosis (6). In this report, mRNA levels of IL-6 from lung tissues of mice 28 days after first injection of bleomycin were analyzed by real-time RT-PCR and shown to be ~21.8-fold elevated compared with control mice, which were significantly suppressed by the administration of Thal (p = 0.0002) (Fig. 1B). TGF-β1 is a well-known key cytokine in the process of human pulmonary fibrogenesis (27). We examined the effect of Thal on TGF-β1 production in bleomycin-treated mouse lung tissues. The mRNA levels of TGF-β1 (Fig. 1C) and COL1A1 (Fig. 1D), which reflects collagen synthesis, in the lung tissues of bleomycin-treated mice were highly elevated (~10.2- and 16.2-fold, respectively) compared with control mice without bleomycin treatment or Thal. Notably, in bleomycin-treated mice with Thal, the mRNA levels of TGF-β1 and COL1A1 were markedly decreased (p < 0.0001 and p < 0.0001, respectively).

Effect of Thal on IL-6 production from human lung fibroblasts and the IL-6-mediated proliferation of lung fibroblasts

In vitro, human lung fibroblasts secreted IL-6, and IL-6 stimulated the proliferation of the cells in an autocrine manner (6). Thus, we examined the effect of Thal on IL-6 production of lung fibroblasts and IL-6-mediated cell proliferation. W138VA-13 cells were cultured with or without Thal for 48 h and the concentrations of IL-6 in the culture supernatants were measured. The concentration of IL-6 in the culture supernatant was decreased by the addition of Thal (p = 0.006) (Fig. 2A). According to our previous study, addition of IL-6 stimulated cell growth in a dose-dependent manner, and reached a plateau at the concentration of 1000 pg/ml at 96 h of culture (6). 1000 pg/ml IL-6-mediated cell proliferation was blocked in the presence of neutralizing Ab against IL-6. Thal also inhibited the IL-6-mediated proliferation to a similar extent as the neutralizing Ab. An additive effect was observed when both the neutralizing Ab and Thal were simultaneously added to the culture (Fig. 2B). Similar examinations were studied at 48 and 72 h, but the apparent cell proliferation was not seen by the addition of IL-6 (data not shown).

Effect of Thal on TGF-β1 production and transdifferentiation of lung fibroblasts

We performed experiments to study the effect of Thal on TGF-β1 production of lung fibroblasts. IMR-90 cells were cultured with or without Thal for 24 h and the concentrations of TGF-β1 in the culture supernatants were measured. The concentration of TGF-β1 in the culture supernatant was decreased by the addition of Thal (p = 0.0267) (Fig. 2C). TGF-β1 is known to promote collagen synthesis by transdifferentiation of fibroblasts to myofibroblasts (28). We next investigated the impact of Thal on TGF-β1-induced transdifferentiation of IMR90 cells. To study whether Thal influenced this transdifferentiation...
process as well as cell proliferation, we examined the expression of α-SMA in TGF-β1 stimulated IMR-90 cells. IMR-90 cells were preconditioned overnight with Thal, and then stimulated with TGF-β1 (5 ng/ml) for 24 h. The cytoplasmic expression of α-SMA was significantly up-regulated by TGF-β1 treatment, which was significantly decreased by the addition of Thal (p < 0.0001) (Fig. 2D). The stimulative effect of TGF-β1 on transdifferentiation of IMR-90 cells was observed in a dose-dependent manner, and Thal suppressed the effect of TGF-β1 even at the concentration of 5 ng/ml (Fig. 2E).

**FIGURE 3.** Effect of Thal on bleomycin-induced angiogenesis and the expressions of VEGF, Ang-1, and Ang-2 mRNA in mouse lung tissues. The samples from mouse lung were common with the experiments shown in Fig. 1. A, Representative results of immunohistochemistry for CD31 in mouse lung tissues at 28 days after bleomycin instillation with or without Thal (original magnification; ×200). B, The numbers of vessels, which showed positive reactivity for CD31, in three separate sections were counted and the values represent the mean ± SD. C–E, Real-time RT-PCR was performed to determine the changes in mRNA levels of VEGF (C), Ang-1 (D), and Ang-2 (E) from lung tissues of mice with or without Thal. The levels of mRNA are represented as the ratio to 18S rRNA. The results are indicated as the mean ± SD of three separate experiments.

**FIGURE 4.** Effect of Thal on VEGF and Ang-1 productions from human lung fibroblasts and myofibroblast. A and B, IMR-90 cells were cultured for 24 h with or without Thal (50 μg/ml), IL-6 (10 ng/ml), or TGF-β1 (5 ng/ml) and the concentrations of VEGF (A) and Ang-1 (B) in the culture supernatants were measured by ELISA. C and D, IMR-90 cells were preconditioned with TGF-β1 (5 ng/ml) for 24 h, which were transdifferentiated to myofibroblasts as shown in Fig. 2, D and E, and then were cultured with or without Thal, IL-6, or TGF-β1 for 24 h. The concentrations of VEGF (C) and Ang-1 (D) in the culture supernatants were measured by ELISA. In the experiments A–D, all cultures contained the same concentration of DMSO. The results are indicated as the mean ± SD of three separate experiments.
Thal inhibited angiogenesis in bleomycin-treated mouse lung tissues

It is well established that Thal possesses antiangiogenic activities. To evaluate the effect of Thal on angiogenesis in bleomycin-treated lung tissues, we counted the number of vessels in the lung by immunostaining for pan-endothelial marker CD31. The number of CD31-positive vessels was increased in bleomycin-treated lung, which was decreased by the administration of Thal to the control levels (Fig. 3, A and B).

mRNA levels of VEGF, Ang-1, and Ang-2 in mouse lung tissues

To study a more precise mechanism for Thal-mediated inhibition of angiogenesis, we analyzed mRNA levels of VEGF, Ang-1, and Ang-2, which are known to be important regulators of angiogenesis (29–32), from lung tissues of mice 28 days after the first injection of bleomycin by real-time RT-PCR. All of their levels were ~12.0, 7.2, and 7.4-fold elevated, respectively, by bleomycin treatment compared with control mice, which were significantly suppressed by the administration of Thal (p = 0.0002, p < 0.0001, and p < 0.0001, respectively) (Fig. 3, C–E).

Effect of Thal on VEGF, Ang-1, and Ang-2 productions from human lung fibroblasts and myofibroblast

We performed in vitro experiments to examine whether the effect of Thal on VEGF, Ang-1, and Ang-2 production was related to cell differentiation. The angiogenic cytokines have been reported to be produced by myofibroblasts (33). IMR-90 cells were cultured with or without Thal, IL-6 (10 ng/ml), or TGF-β1 (5 ng/ml) for 24 h, and the concentrations of VEGF, Ang-1, and Ang-2 in the culture supernatants of lung “fibroblasts” were measured. The concentrations of VEGF and Ang-1 in the culture supernatant were increased by TGF-β1 stimulation (p < 0.0001, p < 0.0001, respectively), which were decreased by the addition of Thal (p = 0.0005, p = 0.0005, respectively) (Fig. 4, A and B). In contrast, preconditioned IMR-90 cells with TGF-β1 (5 ng/ml) for 24 h, which were transdifferentiated to myofibroblasts as shown in Fig. 4, were cultured with or without Thal, IL-6, or TGF-β1 for 24 h, and the concentrations of VEGF, Ang-1, and Ang-2 in the culture supernatants of lung “myofibroblasts” were measured. The concentrations of VEGF and Ang-1 in the culture supernatant were increased by TGF-β1 stimulation (p = 0.0001, p = 0.0001, respectively), which were decreased by the addition of Thal (p = 0.0028, p = 0.0054) (Fig. 4, C and D). Although the basal productions of VEGF and Ang-1 were increased after the lung fibroblasts were transdifferentiated to myofibroblasts by the addition of TGF-β1 (from 30 to 100 pg/ml and from 100 to 1000 pg/ml, respectively), the stimulant effects of TGF-β1 on their productions in myofibroblasts were less than in fibroblasts. IL-6 had almost no effect on VEGF and Ang-1 productions of these cells (Fig. 4). Ang-2 was not detected in the culture supernatants of both fibroblasts and myofibroblasts with or without Thal, IL-6, or TGF-β1 (data not shown).

Both early and late preventive effects of Thal on bleomycin-induced lung fibrosis

To study the “preventive” and “therapeutic” effects of Thal on lung fibrosis, we examined the early (probably the inflammatory responses are mainly demonstrated) and late (probably the postinflammatory, fibrotic changes are mainly observed) effects of Thal by transient administration to bleomycin-treated mice. The administration throughout the course of Thal most effectively prevented bleomycin-induced pulmonary fibrosis and the administration for the first 14 days or the last 14 days ameliorated it compared with control (Fig. 5). Taken together, these results demonstrate the

“late,” namely therapeutic effect of Thal in bleomycin-induced lung fibrosis models, in addition to the “early,” namely preventive effect.

Discussion

Several factors have been reported to be associated with pulmonary fibrosis, including TGF-β1, TNF-α, IL-1, and IL-6, platelet-derived growth factor, VEGF and fibroblast growth factor (7, 34–37). We previously demonstrated that both IL-6 and TGF-β1 played important roles in pulmonary fibrosis (7). In this study, we showed that markedly increased mRNA levels of IL-6, TGF-β1, and COL1A1, which represents collagen synthesis, in the bleomycin-treated mouse lung tissues were decreased by the addition of Thal. As we previously reported (6), human lung fibroblasts were proliferated by IL-6 in a dose-dependent manner. In this study, Thal inhibited both IL-6 production and IL-6-induced cell proliferation of lung fibroblasts. In contrast, it is well known that fibroblasts transdifferentiate to myofibroblasts, which express elevated levels of α-SMA and, consequently, display a markedly enhanced ability to contract extracellular matrix (38, 39). IL-6 did not induce transdifferentiation of fibroblasts (data not shown). In this study, we demonstrated that the expression of α-SMA was increased by TGF-β1 in a dose-dependent manner, with a maximum effect at 5 ng/ml, which was the dose used throughout this study. Thal decreased both TGF-β1 production from cultured fibroblasts and TGF-β1-induced α-SMA expression, namely transdifferentiation process. In addition, bleomycin treatment increased the level of TGF-β1 mRNA in mouse lung tissues, which was decreased by the addition of Thal. There have been some reports concerning the interaction between IL-6 and TGF-β1 (40–42). However, IL-6 failed to induce TGF-β1 production and vice versa in IMR-90 cells (data not shown). In addition, TNF-α and IL-1β, both of which are cytokines reported to stimulate IL-6 (43, 44) and TGF-β1 production (45–47), were undetected in culture supernatants of both WI38VA-13 and IMR-90 cells using ELISA (6). Because TGF-β1 does not stimulate the proliferation of α-SMA-positive myofibroblasts, we propose a dual inhibitory effect of Thal on IL-6-dependent proliferation and TGF-β1-dependent transdifferentiation of fibroblasts to myofibroblast, which may be the mechanism underlying the preventive and therapeutic effect of Thal on pulmonary fibrosis. Recently it has been reported that Thal reduced
the production of TNF-α, IL-8, and IL-18 from alveolar macrophages in pulmonary fibrosis (48). Especially as to TNF-α, Thal has been reported to reduce its production in damaged lung (49, 50). We mainly discussed IL-6 and TGF-β1 in the present study, because TNF-α was undetected in culture supernatants of lung fibroblasts as mentioned above. However, Thal may have effects on TNF-α production from other cells such as alveolar macrophages in vivo.

Currently the usefulness of Thal for the treatments of multiple myeloma or myelofibrosis has been reported. Some reports show that the mechanism underlying the preventive effect of Thal on multiple myeloma and myelofibrosis is related to the suppression of angiogenesis, which is associated with poor prognosis of these diseases (10, 37, 51–52). Furthermore, the association between pulmonary fibrosis and neovascularization has been recently demonstrated in the lung tissues of patients with IPF (53) or in a rat bleomycin-treated model (54). In addition to the effect on cytokine production such as IL-6 and TGF-β1, the suppression of angiogenesis might be one of the mechanisms for the inhibitory effect of Thal on pulmonary fibrosis. Therefore, in this study, we investigated whether Thal had the preventive effect on angiogenesis associated with pulmonary fibrosis and demonstrated that the increased number of vessels in bleomycin-treated fibrous mouse lung tissues was decreased by the administration of Thal. In other words, the association between pulmonary fibrosis and angiogenesis was demonstrated in our bleomycin-treated mouse model.

To elucidate further the precise mechanisms involved, we next examined the expression of several vascular-specific growth factors in pulmonary fibrosis. VEGF has an important role in endothelial cell proliferation, vascular permeability, and angiogenesis in several inflammatory lesions (31). VEGF expression is known to be up-regulated by various stimuli, such as low oxygen tension and cytokines. Ang-1 and Ang-2 are also important regulators of blood vessel growth, maturation, and function. Ang-1 promotes angiogenesis, induces vascular maturation, and decrease vascular permeability. Ang-2 has the ability to destabilize blood vessels, enhance vascular leak, and antagonize Ang-1 (29, 30, 33). Here we demonstrated that VEGF, Ang-1, and Ang-2 mRNA level were increased in the bleomycin-treated mouse lungs compared with control, which were inhibited by Thal. These vascular-specific growth factors are expressed by several cells such as alveolar epithelial cells, macrophages, smooth muscle cells, and myofibroblasts. To study the importance of these factors especially in fibroblasts, we examined the effect of Thal on VEGF, Ang-1, and Ang-2 productions of human lung fibroblasts and myofibroblasts cultured with or without IL-6 or TGF-β1. The concentrations of VEGF and Ang-1 in the culture supernatants of lung fibroblasts were increased by the addition of TGF-β1, which were decreased by Thal. Although the basal productions of VEGF and Ang-1 were more increased after the lung fibroblasts were transdifferentiated to myofibroblasts by the addition of TGF-β1 (from 30 to 100 pg/ml and from 100 to 1000 pg/ml, respectively), the stimulant effects of TGF-β1 on their productions in myofibroblasts were less than in fibroblasts. IL-6 had almost no effect on VEGF and Ang-1 production of these cells. Therefore, one of the possible mechanisms by which Thal exerts its inhibitory effects on angiogenesis in pulmonary fibrosis may be through the suppression of TGF-β1-mediated productions of both VEGF and Ang-1 in fibroblasts and myofibroblasts. Ang-2 was not detected in culture supernatants of fibroblasts and myofibroblasts with or without Thal, IL-6, or TGF-β1 (data not shown). Although the precise cellular suppressive mechanism of Thal in Ang-2 production has not been fully investigated, the inhibitory effect of Thal on Ang-2 mRNA expression in mouse fibrotic lung tissues might be dependent on other cells such as lung epithelial cells associated with pulmonary fibrosis.

It has been previously reported that a platelet-derived growth factor receptor/abl/c-kit kinase inhibitor, imatinib mesylate was beneficial in the inhibition of lung fibrosis both by anti-inflammatory and antifibrotic mechanism in rat bleomycin model (55). It is noteworthy that in this report we showed the late namely therapeutic effect of Thal in bleomycin-induced lung fibrosis models in addition to the early namely preventive effect, because in the clinical use, the therapeutic effect is often more important when the clinicians find that the fibrotic changes of various etiology are already apparent in their patients. The histopathological fibrosis in our animal studies was entirely interstitial and without the significant consolidation or architectural remodeling found in even the earliest example of human IPF. Whether the changes we have documented for bleomycin-induced injury after 28 days translate to potential benefits in patients with IPF remains to be shown in clinical trials. Furthermore, the oral administration of the drug results in good compliance. Our data may lead to the development of novel strategies incorporating Thal for the prevention and treatment of various types of lung fibrosis.

Disclosures

The authors have no financial conflict of interest.

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