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Effect of Salviae Miltiorrhizae Radix (Tanjin) on bleomycin-induced pulmonary fibrosis in mice

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Abstract

The time course of the pulmonary fibrosis development in ICR mice treated with 12.5 mg/kg of bleomycin (BLM) *i.p.* for 10 days was examined in parameters of weight, hydroxyproline content and histological changes of the lung. Then, the effect of Salviae Miltiorrhizae Radix was investigated on the fibrosis.

1) By the day after the final BLM-treatment, acute phase inflammation and slight fibrotic change were observed histologically. The hydroxyproline content in the lung increased only slightly. Two weeks after the final BLM-treatment, the hydroxyproline content increased clearly. Histologically, edema was decreased and fibrosis was developed. Five weeks later, the fibrosis became severe and the hydroxyproline content increased more.

2) When aqueous and methanol extracts of Salviae Miltiorrhizae Radix (50-500 mg/kg, *p.o.*) were given for 10 days of BLM-treatment and the following 5 weeks consecutively, both extracts suppressed the fibrosis.

3) The methanol extract suppressed the fibrosis even if the extract was given from 2 weeks after the final BLM-treatment, suggesting that the extract was effective on the cellular events in the later phase of the fibrosis.

4) The methanol extract was dissolved in water, and extracted with ether, chloroform, ethyl acetate and *n*-butanol, in this order. The ethyl acetate and the *n*-butanol soluble fractions and the water soluble fraction as the rest of extraction inhibited the pulmonary fibrosis, but neither ether nor chloroform soluble fraction did so. Thus, the active principle(s) of Salviae Miltiorrhizae Radix were substance(s) with low lipophilicity.

Key words pulmonary fibrosis, bleomycin, Salviae Miltiorrhizae Radix, 丹参.
Abbreviations BLM, bleomycin; IL-1, interleukin 1; TNF, tumor necrosis factor.

Introduction

Fibrosis is a result of over-proliferation of fibroblast, and damages functions of organs. Thus, fibrosis is related with many kinds of diseases such as hypertrophic scar, liver-cirrhosis, pulmonary fibrosis and so on. In such diseases, fibroblast should be regulated from the over-proliferation. There is, however, no therapeutically effective drug for fibrosis or to regulate the proliferation of fibroblast.

It has been reported that Salviae Miltiorrhizae Radix suppresses bleomycin (BLM)-induced pulmonary fibrosis in mice.[1] It is well known that BLM has a severe side-effect to induce

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pulmonary fibrosis, although it shows a good therapeutic effect on cephal-cervical flat epithelioma, malignant lymphoma and testicular carcinoma. BLM distributes to pulmonary tissue in higher concentration and damages the pulmonary capillary endothelium.[2] Thus, the enhanced permeability of the capillary causes pulmonary edema in the early stage of the fibrosis induction. This might be a trigger of the pulmonary fibrosis. However, inflammation does not always result in fibrosis. It has been suggested as a mechanism of BLM-induced development of fibrosis that BLM activates alveolar macrophages to release fibroblast stimulating factors such as interleukin 1 (IL-1)[3] and tumor necrosis factor (TNF).[4] Mast cells may also participate in the development of fibrosis,[5] although we[6] demonstrated an inconsequential role of mast cells in the induction of fibrosis because BLM (5 mg/kg, *i.v.*, 10 days) could induce pulmonary fibrosis in genetically mast cell deficient $WBB6F_1$-W/W^v mice as well as $WBB6F_1$-+/+ mice having mast cells normally, and there was not much difference in the histological changes of the lungs between the two strains of mice.

It takes several weeks to accumulate collagen in the lung and to form fibrosis after the BLM-treatment. There might be different stages until the final formation of the fibrosis, although the cellular mechanisms for the induction of fibrosis is not well known as described above. In the present paper, first we examined the time course for induction of pulmonary fibrosis by BLM, biochemically and histologically. Then, we examined if the aqueous and methanol extracts of Salviae Miltiorrhizae Radix could suppress the BLM-induced fibrosis. The suppressive effect of the methanol extract was also examined by administrating it on various stages of the development of the fibrosis. Furthermore, the effect of crude fractions from the methanol extract was examined on the fibrosis.

Materials and Methods

Animals : ICR female mice (Japan SLC Co., Ltd. Hamamatsu, Japan) were used at 15 weeks of age. They were maintained in filtered laminar-air-flow cages at 22± 1°C and 60 % humidity with free access to pellet food and water.

Drugs : Bleomycin hydrochloride (BLM, Nippon Kayaku Co., Ltd. Tokyo, Japan) was dissolved in sterilized normal saline. Dān-shēn (丹参), Salviae Miltiorrhizae Radix, *Salvia miltiorrhza* BUNGE (Sichan Porv., 四川省 ; China) was supplied by Matsuura Yakugyo Co., Ltd. Nagoya, Japan. Salviae Miltiorrhizae Radix was extracted with water or methanol by ordinary methods. The methanol extract was fractionated by the method described under the results. The extracts and the fractions were suspended or dissolved in water containing 2 % dimethyl sulfoxide (DMSO).

Induction of pulmonary fibrosis : BLM was given to mice *i.p.* in a dose of 12.5 mg/kg for 10 days. Mice were killed by bleeding under anesthesia with ether 5 weeks after the final administration of BLM unless otherwise indicated. To examine the time course of the fibrosis induction, mice were killed 0, 2 and 5 weeks after the final BLM-treatment. The left and right lungs were used for histological examination and hydroxyproline content measurements, respectively.

Administration of the extracts and its fractions : Mice were given the extracts and/or its fractions *p.o.* successively from the beginning of the BLM-treatment to the day before the killing, unless otherwise indicated. Mice in the control group were given 0.1 ml/10 g body weight of 2 % DMSO solution *p.o.* as a vehicle.

Histological examination of lung : The left lungs were inflated with 10 % buffered formalin through cannulated trachea at a pressure of 10 to 12 cm H_2O, and then fixed for 24 to 48 hr in the fixative solution. The fixed organs were cut into parasagittal slices of 5 blocks covering virtually the whole of each lung. Histological sections (3 μm) were obtained from each block after embedding in paraffin. Both hematoxylin-eosin and Azan-Mallory staining were carried out on the sections. The fibrotic area (%) was evaluated for each section from the 5 blocks as a unit as follows : Unit 0 : 0 %, Unit 0.5 : less than 1 %, Unit 1 : 1 to 5 %, Unit 2 : 5 to 10 %, Unit 3 : 10 to 20 %,

Unit 4 : 20 to 40 %, Unit 5 : more than 40 %. To express the fibrosis intensity of each animal, a mean of units of 5 sections was used.

Hydroxyproline content : The right lungs were weighed (wet weight), and cut into 1 mm thickness with a tissue chopper (Mickle, Laboratory Engineering Co., Gomshall, Surrey, England). The chopped lungs were dried using acetone (10 ml, 3 times) and weighed (dry weight). The dried samples of lung were kept at 120°C for 24 hr in tightly capped tubes containing 2 ml 6 N HCl for hydrolyzation. The amount of hydroxyproline in the hydrolysate was measured according to Kivirikko *et al*.[7]

Statistics : The data were expressed as the mean±S.E. Non-parametric Wilcoxon's U-test was used for statistical analysis of data of fibrotic area in the histological examination. The other data were evaluated with Student's or Welch's *t*-test after F-test. The difference of two groups at $p<0.05$ was considered as significant.

Results

Time course of induction for pulmonary fibrosis by BLM-treatment

Mice treated with BLM were killed on the day after the final BLM-treatment (D10), 2 weeks after (W2) and 5 weeks after (W5) to investigate the time course of fibrosis induction, where the wet and dry weights and the hydroxyproline content in the lung were examined as well as histological changes of the lung.

The wet and dry weights of the lung had already increased obviously by D10. Then, the wet weight maintained the level by W2, but decreased clearly on W5 (Fig. 1, top panel), while the dry weight did not decrease much by W5 (Fig. 1, middle panel). The hydroxyproline content on D10 had increased slightly compared with the normal level, and then increased gradually by W5 as time elapsed (Fig. 1, bottom panel). The histological findings of the lung in mice on D10 (W0) were as follows (Fig. 2, top picture): edema of interstitium and alveolar septa, tiny foci of fibrotic change, great increase of alveolar macrophages in number, lymphocytic cell infiltration

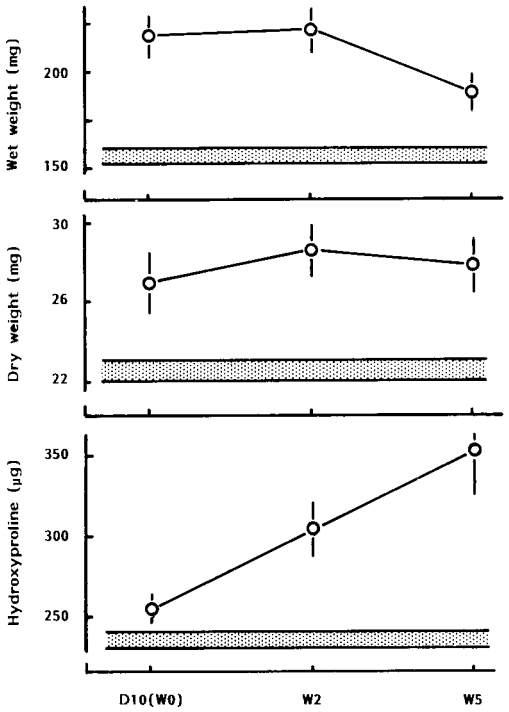

Fig. 1 Wet and dry weights as well as hydroxyproline content of right lung in mice treated with bleomycin (BLM).
Female mice, 15 weeks old, were given 12.5 mg/kg *i.p.* for 10 days. Animals were killed on the day after the final BLM-treatment (W0) and 2 and 5 weeks (W2 and W5) after. Each group includes 7 to 10 animals (Mean±S.E.). Dotted area : Normal level.

and proliferation of a bronchial-associated lymphoid tissue. Edema of the alveolar spaces was also found in some of the tissue sections. The fibrotic change was observed mainly in subpleural regions, showing a proliferation of fibroblast-like cells, a slight increase of matrix substance and infiltrations of mononuclear cells intermingled with lymphocytes. Such a fibrotic change was also observed at the surrounding area of the proliferated bronchial-associated lymphoid tissues. Macrophages in alveolar spaces were located at the surroundings of the fibrotic areas and subpleural regions, although they were found throughout the lung tissue. On 2W (Fig. 2, middle pictuer), edema was not so obvious. Fibrotic areas became wide and increased a collagenous matrix in comparison with W0. A cellurarity in

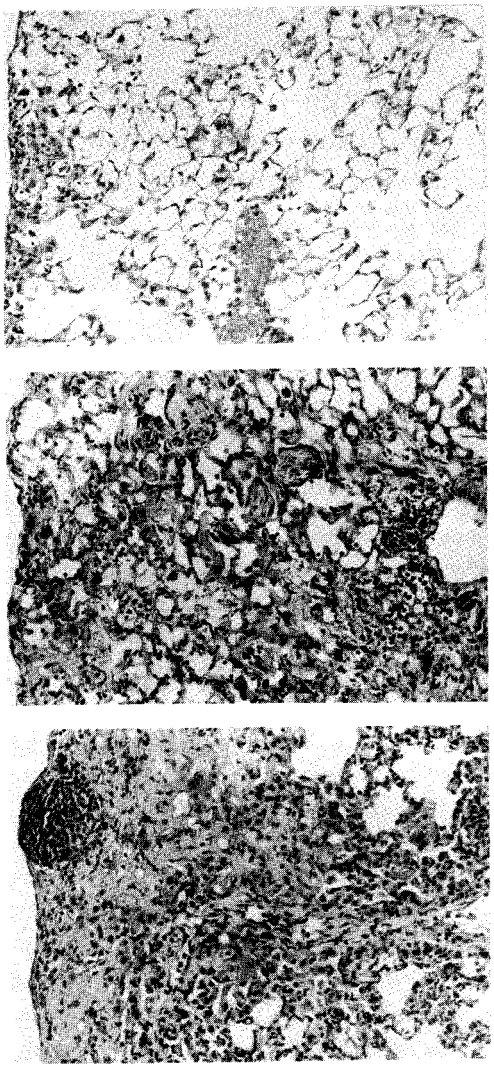

Fig. 2 Histological change in lung of mice treated with bleomycin.

See legend of Fig. 1. The top picture represents the tiny focus of fibrotic change in subpleural region on W0. The middle picture represents fibrotic area on W2. The bottom picture represents the fibrotic area with abundant collagenous matrix and lymphocyte aggregation. Hematoxylin-eosin stain, ×100.

the fibrotic areas decreased mainly as a result of a reduction of mononuclear cell infiltration. Macrophages showing foamy changes at varied degrees increased in number in alveolar spaces. Fibrocellular thickening of the pleura was often noted. On W5 (Fig. 2, bottom picture), fibrosis became severe, and the fibrotic subpleural regions concaved from the lung surface by increase of collagenous matrix. By W5, foamy alveolar macrophages increased in number and filled up many of the alveolar spaces, especially in subpleural regions. Aggregation of lymphocytes surrounded with or without fibrosis also appeared in the subpleural regions.

Effect of aqueous and methanol extracts of Salviae Miltiorrhizae Radix on BLM-induced pulmonary fibrosis

Mice treated with BLM were given the extracts (50-500 mg/kg) *p.o.* successively for 10 days of BLM-treatment and the following 5 weeks. The wet and dry weights of the right lung in the control group increased clearly as well as the hydroxyproline content of the right lung in comparison with the respective normal levels (Fig. 3)

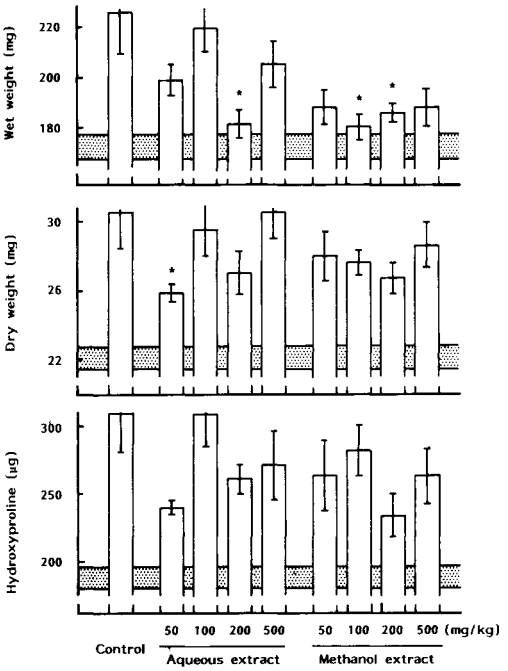

Fig. 3 Effect of aqueous and methanol extracts of Salviae Miltiorrhizae Radix on weight and hydroxyproline content of right lung in ICR mice treated with bleomycin (BLM).

Female mice, 15 weeks old, were given 12.5 mg/kg BLM *i.p.* for 10 days, and killed 5 weeks after the final BLM-treatment. Extracts were given *p.o.* every day from the beginning of BLM-treatment to the killing of mice. Each column represents the mean±S.E. of 6 to 10 mice.

*: Statistical significance from the control at $p<0.05$. Dotted area : Normal level.

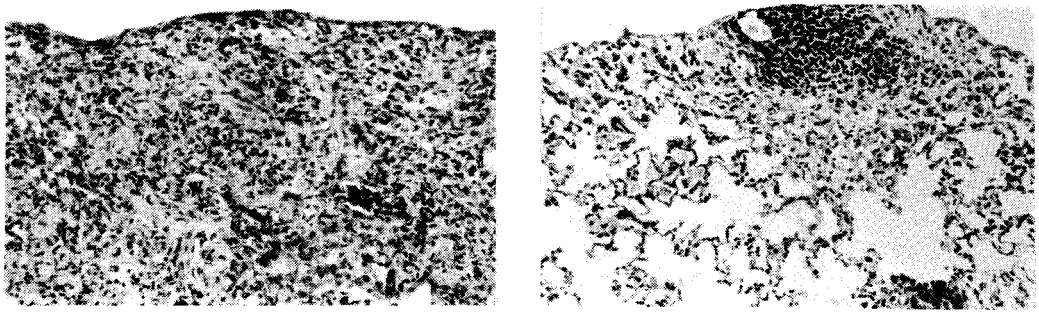

Fig. 4 Effect of the methanol extract (200 mg/kg) of Salviae Miltiorrhizae Radix on the fibrosis in mice treated bleomycin.
See legend of Fig. 3, The left picture : Control, The right picture : Treated with 200 mg/kg of the extract. Hematoxylineosin stain, ×100

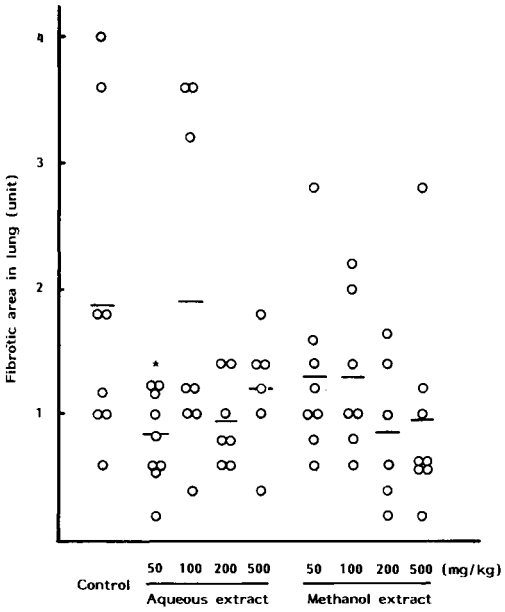

Fig. 5 Effect of aqueous and methanol extracts of Salviae Miltiorrhizae Radix on fibrosis of left lungs in ICR mice treated with bleomycin.
See legend of Fig. 3. Severity of fibrosis was evaluated from histological findings of sections from 5 blocks of the left lung as unit (0 : 0 %, 0.5 : less than 1 %, 1 : 1-5 %, 2 : 5-10 %, 3 : 10-20 %, 4 ; 20 - 40 %). Circles represent fibrotic area in the lung of individual mice. *: Statistical significance from the control at $p<0.05$.

The wet weight of the lung decreased or showed a tendency to decrease by the treatment with 50 and 200 mg/kg aqueous extracts and all doses of methanol extracts compared with the control (Fig. 3, top panel). The dry weight of the lung also decreased or showed a tendency to decrease by the treatment with 50 and 200 mg/kg of the aqueous extract and showed a tendency to decrease by all doses of the methanol extract (Fig. 3, middle panel). The hydroxyproline content showed no significant decrease by the treatment with 50mg/kg of the aqueous extract and 200mg/kg of the methanol extracts ($0.05<p<0.1$) and showed a tendency to decrease with other doses except for 100 mg/kg of the aqueous extract (Fig. 3, bottom panel).

In the histological examination of left lungs in the BLM-treated mice, the lungs were affected by the fibrosis clearly (Fig. 4, left picture). In severely affected animals, about 50 % of an area of a section was occupied by fibrosis. The treatment with the extract of Salviae Miltiorrhizae Radix suppressed the fibrosis (Fig. 4, right picture). When the histological data of the fibrotic area were expressed as the mean unit of 5 sections of a lung (Fig. 5), the fibrotic area of the lung decreased significantly by the treatment with 50 mg / kg of the aqueous extract and tended to decrease by the treatment with 200 mg/kg of the aqueous extract and 200 and 500 mg/kg of the methanol extract ($0.05<p<0.1$).

Effect of the methanol extracts given in varying timings and periods on BLM-induced fibrosis

The protocol of dosing the extracts is shown in Fig. 6. Namely, mice treated with BLM were given 100 or 200 mg/kg of the methanol extract *p.o.* successively in the 4 protocols, from the start of BLM-treatment to the killing of mice (D0 to W5 group), from the start to the end of the BLM-treatment (D0 to D9 group), from the day after the final BLM-treatment to the killing (W0 to W5

group) or from 2 weeks after the final BLM-treatment to the killing (W2 to W5 group). The mice treated with the extract were all killed 5 weeks after the final BLM-treatment. The methanol extract given in D0-W5 decreased significantly

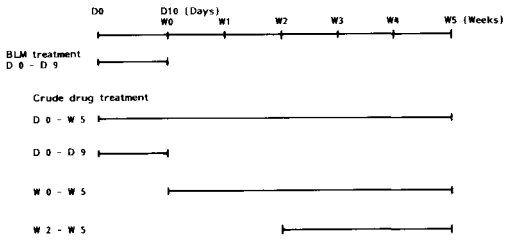

Fig. 6 Protocol for drug treatment.
Female mice, 15 weeks old, were given 12.5 mg/kg bleomycin (BLM) *i.p.* for 10 days, and killed 5 weeks after the final treatment. Crude drug (the methanol extract of Salviae Miltiorrhizae Radix) was given *p.o.* every day in doses of 100 and 200 mg/kg.

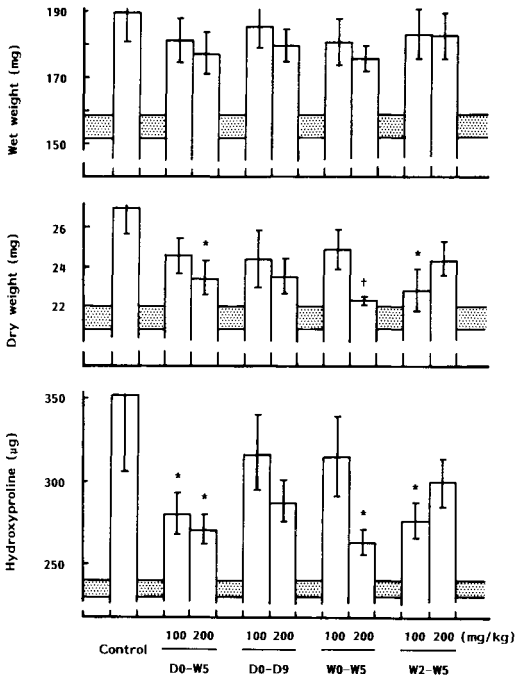

Fig. 7 Effect of methanol extract of Salviae Miltiorrhizae Radix given in varying schedule on weight and hydroxyproline content of right lung in ICR mice treated with bleomycin.
See Fig. 6 for the experimental protocol. Each column represents the mean±S.E. of 7 to 10 mice. *, †: Statistical significance from the control at $p<0.05$ and $p<0.01$, respectively. Dotted area : Normal ievel.

the dry weight in a dose of 200 mg/kg (Fig. 7, middle panel) and decreased the hydroxyproline content in both doses (Fig. 7, bottom panel). The D0-D9 administration of the extract showed only a tendency to decrease these parameters. The D10 (W0)-W5 administration of 200 mg/kg and the W2-W5 administration of 100 mg/kg decreased the dry weight and hydroxyproline content significantly. The W2-W5 administration of 200 mg/kg also showed a tendency to decrease the dry weight and the hydroxyproline content.

Effects of fractions from the methanol extract on BLM-induced lung fibrosis

The methanol extract was fractionated by the method shown in Fig. 8. The effect of the methanol extract and its fractions (Fr.1 to Fr. 5) were examined on the fibrosis. The fractions were administrated *p.o.* to BLM-treated mice in three doses of 50, 100 and 200 mg/kg except for Fr.2 in a dose of 100 mg/kg successively for 10 days of BLM-treatment and the following 5 weeks. The wet weight of lung decreased by the administration of Fr.3, Fr.4 and Fr.5 (Fig. 9, upper panel). Only Fr.3 decreased the dry weight (Fig. 9, lower panel). The hydroxyproline content decreased clearly by the administration of Fr. 3, 4 and 5 (Fig. 9).

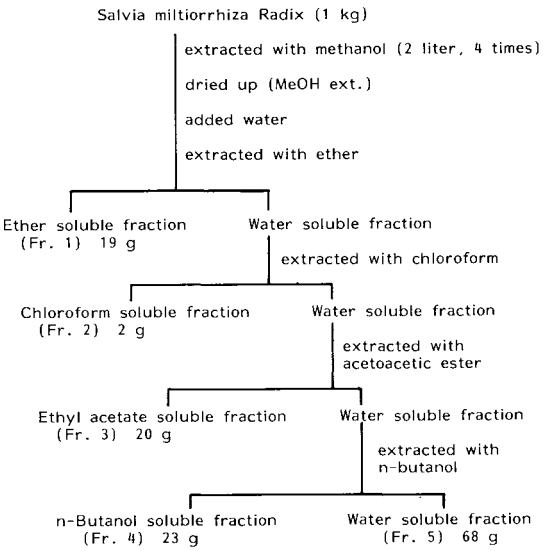

Fig. 8 Fractionation of the methanol extract of Salviae Miltiorrhizae Radix.

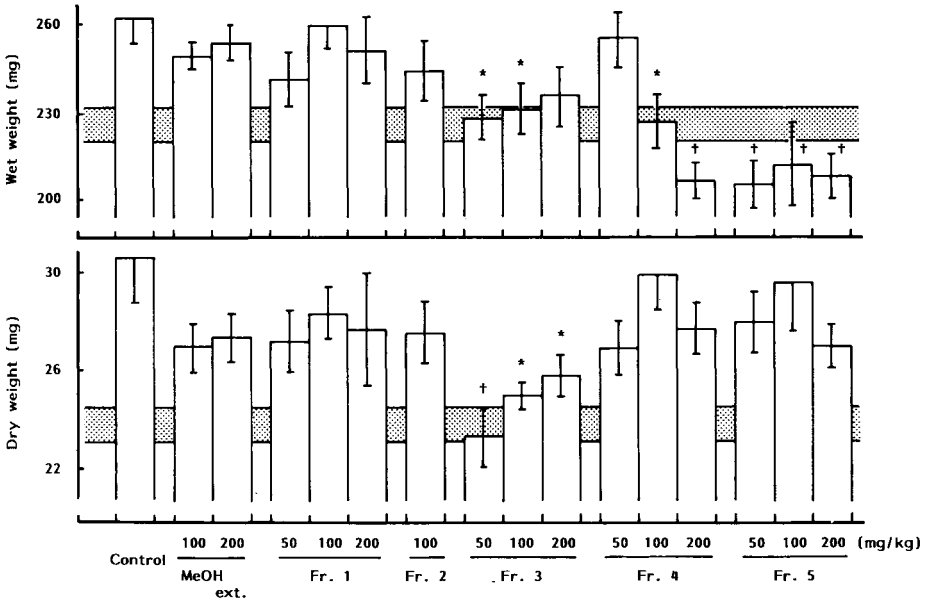

Fig. 9 Effect of methanol extract of Salviae Miltiorrhizae Radix and its fractions on weight of right lung in ICR mice treated with bleomycin (BLM).

Female mice, 15 weeks old, were given 12.5 mg/kg BLM *i.p.* for 10 days and killed 5 weeks after the final treatment. The extract and its fractions were given *p.o.* every day from the beginning of the BLM treatment to the killing of mice. Each column represents the mean±S.E. of 6 to 10 mice. *, †: Statistical significance from the control at $p<0.05$ and $p<0.01$, respectively. Dotted area : Normal level.

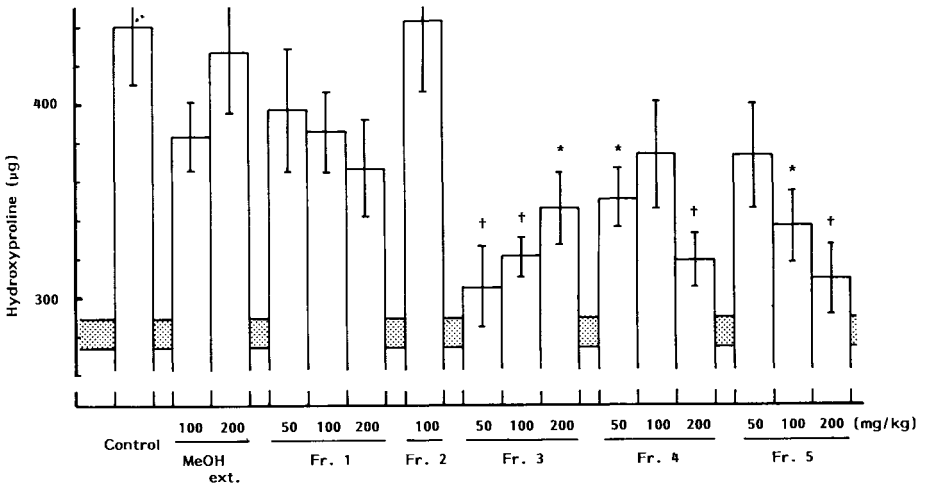

Fig. 10 Effect of methanol extract of Salviae Miltiorrhizae Radix and its fractions on hydroxyproline content of right lung in ICR mice treated with bleomycin.

See legend of Fig. 9. Each column represents the mean±S.E. of 6 to 10 mice. *, †: Statistical significance from the control at $p<0.05$ and $p<0.01$, respectively. Dotted area : Normal level.

Discussion

ICR mice were used in this study, because this strain of mice was a high responder to BLM for the induction of the pulmonary fibrosis.[8] Severe fibrosis was seen 5 weeks after 10 days-treatment with BLM (Figs. 1 and 2). The effect of the extracts of Salviae Miltiorrhizae Radix on the pulmonary fibrosis was evaluated 5 weeks after 10 days of BLM-treatment. When the aqueous and methanol extracts of Salviae Miltiorrhizae Radix were given throughout from the beginning of BLM-treatment to the day before the killing of mice, both extracts showed an inhibitive effect on the pulmonary fibrosis. Among the experiments that the methanol extract was given *p.o.* in doses 100 and 200 mg/kg from the beginning of BLM-treatment to the day before the killing of mice, the extract suppressed the wet weight of lung significantly in Fig. 3, but showed only a tendency to suppress it in Figs. 7 and 9. The dry weight of lung was also suppressed or showed a tendency to be suppressed as shown in Figs. 3, 7 and 9. The hydroxyproline content of lung suppressed significantly in Fig. 7. and showed a tendency to be suppressed in Figs. 3 and 10. Although there were some differences of the efficiency among the experiments, these results also suggest that the methanol extract suppressed the fibrosis.

In the time course of the induction of pulmonary fibrosis by BLM, by D10 (W0), the next day of the final BLM-treatment, an increase of the wet and dry lung weights were already observed. However, the hydroxyproline content increased only slightly. These results as well as histological findings indicate the lung was in acute phase inflammation at this time. By W2, the hydroxyproline content increased clearly. Histologically, edema was decreased, fibrosis was developed clearly and macrophages showed foamy changes. By W5, the changes of these parameters became obvious, and the aggregation of lymphocytes was notable. The decrease of the wet weight of lung on W5 may have resulted from absorption of the edematous fluid. As described under the introduction, lung tissue damage by BLM and the following inflammation and edema might be a trigger of the fibrosis. In general, proliferation or infiltration of fibroblasts as well as macrophages is necessary for the process of recovering from tissue injury. In the case of BLM-induced inflammation of lung, however, the fibroblast shows over-proliferation thereafter, resulting in fibrosis. Although precise cellular mechanism causing the over-proliferation of fibroblasts is not well known yet, participation of alveolar macrophages and mast cells in it has been considered as described in the introduction. Then, the methanol extract was given using varied timing and periods in the development of the fibrosis. The extract suppressed the fibrosis clearly by the administration for a period from W2 to W5, the later phase of the fibrosis development, as well as those from D0 through W5 and from W0 to W5. The administration for a period from D0 to D9, acute inflammation phase as described above, did not suppress it so much. Therefore, it is not likely that the extract suppressed the BLM-induced inflammation and edema in the lung as a mechanism for its inhibitive effect on the fibrosis. These results, rather, indicate that the extract was effective on cellular events in the late phase of the fibrosis induction, and was effective on the ongoing fibrosis. In the present study, however, we could not conclude whether the extract inhibited the fibrosis through suppressing the fibroblast proliferation directly or through actions to cells such as macrophages other than fibroblasts, or their factors such as IL-1 and TNF.

Next, we examined the effect of 5 fractions obtained from the methanol extract on the fibrosis. The ether and the chloroform soluble fractions (Fr, 1 and Fr.2) did not suppress the increase of hydroxyproline content, but the ethyl acetate, the *n*-butanol and the water soluble fractions (Fr. 3, Fr. 4 and Fr. 5) suppressed it. Fr.3 suppressed the wet and dry weights of the lung but Fr.4 and Fr.5 suppressed only the wet weight. These results suggest that the active component(s) of the methanol extract to suppress the fibrosis are substance(s) with low lipophilicity. A histological examination was not done in the

experiment of the fractions and the reason was unclear for the difference of the effects between Fr.3 and Fr.4 and 5 on the dry lung weight. The reasons were also unknown as to why Fr.4 in the highest dose and Fr.5 in all doses decreased the wet lung weigt to lower than the normal level and why a lower dose of Fr.3 showed the suppressive effect on the fibrosis more strongly than a higher dose did. One cause might be that each fraction used here was still crude and contained many components showing various activities. Now, we are under taking an investigation to isolate the active principle from the methanol extract. It will be helpful to know the mechanisms for Salviae Miltiorrhizae Radix to inhibit the fibrosis.

和文抄録

ICRマウスに12.5 mg/kgのブレオマイシン(BLM)を10日間腹腔内注射し，その後の肺の重量，hydroxyproline量および組織学的変化をパラメータとして，肺線維症誘発のtime courseを検討し，ついで，肺線維症に及ぼすSalviae miltiorrhizae Radix (丹参) の肺線維症に及ぼす影響を検討した。

1) BLMの最終注射翌日には，組織学的に急性炎症および軽度の線維性変化が観察された。Hydroxyproline量は極軽度の増加がみられるにすぎなかった。BLMの最終注射から2週間後にはhydroxyproline量は明らかに増加し，組織学的には浮腫は減少し，線維症の像がみられた。5週間後には線維症は重症化し，hydroxyproline量はさらに増加した。

2) 丹参の水性およびメタノールエキス（50-500 mg/kg）をBLM処置の10日間およびその後の5週間連続経口投与した場合，両エキスは線維症を抑制した。

3) メタノールエキスはBLMの最終注射の2週間後からの投与によっても線維症を抑制した。このことはメタノールエキスが比較的後期の線維症状発症過程に対しても有効であることを示している。

4) メタノールエキスを水に溶解し，ついで，エーテル，クロロホルム，酢酸エチルおよびn-ブタノールで順次抽出した。酢酸エチルおよびn-ブタノール可溶性画分ならびに残りの水可溶性画分は肺線維症を抑制した。しかし，エーテルおよびクロロホルム可溶性画分は抑制しなかった。したがって丹参の有効成分は脂溶性の低い物質である。

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