

ORIGINAL ARTICLE

Aqueous fraction of *Sauropus androgynus* might be responsible for bronchiolitis obliterans

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ABSTRACT

Background and objective: Bronchiolitis obliterans (BO) has been reported to develop following ingestion of *Sauropus androgynus* (SA), a leafy shrub distributed in Southeast Asia. Little is known about direct effects of SA on airway resident cells or haematopoietic cells *in vitro*. Identification of the SA component responsible for the development of BO would be an important key to elucidate its mechanism. We sought to elucidate the direct effects of SA on airway resident cells or haematopoietic cells and identify the SA element responsible for the pathogenesis of BO.

Methods: SA dry powder was partitioned into fractions by solvent extraction. Human and murine monocytic cells, epithelial cells and endothelial cells were cultured with SA solution or fractions eluted from SA. We also investigated the effect of SA *in vivo* using a murine BO syndrome (BOS) model.

Results: The aqueous fraction of SA induced significant increases of inflammatory cytokine and chemokine production from monocytic lineage cells. This fraction also induced significant apoptosis of endothelial cells and enhanced intraluminal obstructive fibrosis in allogeneic trachea allograft in the murine BOS model. We found individual differences in tumour necrosis factor α (TNF- α) production from monocytes of healthy controls stimulated by this aqueous fraction of SA, whereas it induced high-level TNF- α production from monocytes of patients with SA-induced BO.

Conclusions: These results suggest that an aqueous fraction of SA may be responsible for the pathogenesis of BO.

SUMMARY AT A GLANCE

Ingestion of *Sauropus androgynus* (SA) as dry powder or fresh juice has been reported to induce constrictive bronchiolitis obliterans (BO), but the pathogenesis of this condition is unknown. This study suggests that an aqueous fraction of SA could be responsible for development of SA-induced BO.

Key words: constrictive bronchiolitis obliterans, murine bronchiolitis obliterans syndrome model, solvent system, tumour necrosis factor α .

Abbreviations: BO, bronchiolitis obliterans; BOS, BO syndrome; KA, *kakkan-to*; PI, propidium iodide; SA, *Sauropus androgynus*; TNF- α , tumour necrosis factor α .

INTRODUCTION

Bronchiolitis obliterans (BO) is a rare but fatal disease that can occur in various clinical settings, such as collagen diseases, B cell malignancies or organ transplantation.¹ It is an obstructive lung disease characterized by inflammation in the respiratory bronchioles leading to irreversible fibrotic obstruction of small airways.²

In 1996, an outbreak of BO in individuals who had taken *Sauropus androgynus* (SA) was reported in Taiwan.^{3,4} The leaf of SA, a common nutritious vegetable in Thailand, was introduced to Taiwan and became a popular bodyweight-reducing vegetable.⁵ In 2003, several cases of SA-associated BO were also reported in Japan.⁶ We treated two cases of SA-associated BO. Both patients died 6–7 years after the onset of respiratory failure, and pathological findings on autopsy were consistent with BO (unpubl. data).

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The precise mechanism of SA-associated BO development has not been elucidated. The inflammatory process of BO was characterized by peribronchiolar lymphocyte and/or macrophage infiltration and disruption of both the submucosa and epithelium followed by irreversible fibroproliferation of airways.^{7,8} Many mediators, such as cytokines, chemokines and angiogenic factors, have been reported to play critical roles in the development of BO.^{7,9,10} Thus, it is worthwhile to investigate whether SA can influence pulmonary resident cells and inflammatory cells. Lai *et al.* have reported that serum concentration of tumour necrosis factor α (TNF- α) was higher in SA-BO patients than in healthy controls.³ We speculate that TNF- α plays an important role in the pathogenesis of SA-BO. In the present study, we extracted and fractionated SA into components using various solvents and investigated which SA component could induce inflammatory mediators (such as TNF- α) production from inflammatory cells and apoptosis of endothelial cells.

METHODS

Sauropus androgynus

Dry powder of SA was purchased from Kushi International (Tokyo, Japan). The dry powder is the same product that our SA-BO patients had actually taken. It is a concentrated dry powder made from leaves of SA, equivalent to 10 times the weight of raw leaves. Our patients had been taking a 75 g equivalent of SA leaves (7.5 g of dry powder) every day for 12–16 weeks. According to the report of the SA-BO outbreak in Taiwan,³ patients had taken uncooked SA juice (equivalent to 150 g of raw leaves) every day for a mean duration of 10 weeks. In Malaysia, people usually take 150–200 g of 'cooked' SA leaves per week without any reports of health hazard. The SA dry powder was suspended in dimethyl sulfoxide (10 mg/mL). After removal of microorganisms and endotoxins through an adsorptive-based membrane filter unit (CUNO Zeta Plus, Sumitomo3M Co., Ltd, Tokyo, Japan), the solution of SA was frozen and stored at -20°C . Zeta plus is a positively charged and 0.20- μm pore size membrane filter that is generally used to remove endotoxin and microorganisms, with a reported efficacy for endotoxin removal over 99.5%.¹¹

Kakkon-to

In some experiments, *kakkon-to* (KA) was used as a control. KA is one of the most popular Chinese herb medicines, which is widely used in Japan. It is prescribed for the relief of some symptoms related to the common cold. It is made from several plant leaves and stalks. As KA is fairly safe, at least for the Japanese population, and is often used for experiments in comparison with other herbal medicines, *in vitro* or *in vivo*,^{12,13} it was chosen as a control in our study.

Fractioning of *Sauropus androgynus*

SA was partitioned into four fractions by solvent extraction systems (Fig. 1). First, SA dry powder was

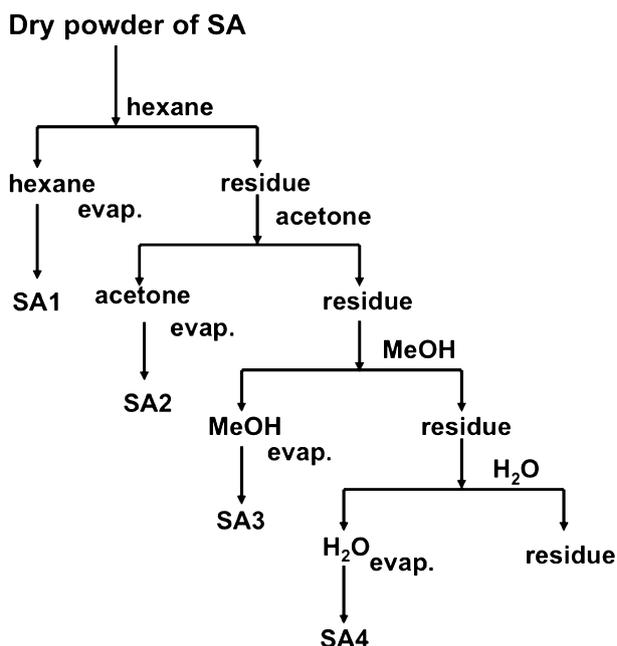


Figure 1 Extraction of *Sauropus androgynus* (SA) by various solvent systems. SA was extracted with hexane (SA1). The residue was extracted with acetone, methanol and H₂O, successively, and solvents evaporated to give four fractions, SA1–SA4, respectively. SA4 was considered to be an aqueous fraction of SA.

extracted with hexane. The hexane solution was concentrated under reduced pressure to dryness (SA1). Hexane non-soluble residue was then extracted with acetone, methanol and water successively. Next, the solvents of each extract were evaporated to give fractions (SA2), (SA3) and (SA4), respectively. The amount of each fraction from 1 g of SA was as follows: SA1, 5.4 mg; SA2, 0.3 mg; SA3, 15 mg; SA4, 9.4 mg. The solution of each SA fraction was preserved at -20°C after microorganisms and endotoxins had been removed through an adsorptive-based membrane filter unit (CUNO Zeta Plus).

Cells

The human acute monocyte leukaemia cell line (THP-1), the murine macrophage-like cell line (RAW 264.7), the human alveolar epithelial cell line (A549) and the murine endothelial cell line (MS1) were purchased from American Type Culture Collection (Manassas, VA, USA). Human monocytes were isolated from the peripheral blood of healthy volunteers and SA-BO patients by Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) density gradients and plastic adhesion method.¹⁴ Human alveolar macrophages were isolated from bronchoalveolar lavage fluids derived from 20 patients who underwent bronchoscopy for diagnosis (10 patients with idiopathic interstitial pneumonia, 10 patients with sarcoidosis). All healthy volunteers and patients provided written informed consent.

Cell cultures

In some experiments, cells were cultivated (cell lines or macrophages) with medium (RPMI1640, Sigma-Aldrich, St. Louis, MO, USA) supplemented with SA (whole SA), SA fractions (SA1-SA4) or KA. Whole SA or KA was added to the medium at a concentration of 20 µg/mL. In the following cell culture experiments, each of the four fractions (SA1-4) was used at the equivalent concentration to 20 µg of whole SA/mL (i.e. SA1, 1.12 µg/mL; SA2, 0.52 µg/mL; SA3, 3 µg/mL; SA4, 1.88 µg/mL).

Enzyme-linked immunosorbent assay

The concentration of cytokines/chemokines was measured using enzyme-linked immunosorbent assay kits according to manufacturer instructions (Quantikine enzyme-linked immunosorbent assay kit, R&D systems, Minneapolis, MN, USA). The lower limit of detection was 1.6 pg/mL.

Assessment of viability and apoptosis

The viability of cells (A549 and MS1) was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (Wako, Osaka, Japan) assay. Apoptosis of the MS1 was examined by flow cytometry analysis of cells stained with a combination of annexin V and propidium iodide (PI). After the MS1 cells were stained with annexin V biotin (0.5 µg/mL final concentration) and PI (1 µg/mL final concentration) according to the manufacturer's instructions (BioVision, Inc., Mountainview, CA, USA), samples were analysed by flow cytometry (excitation at 488 nm; emission at 530 nm) (FACS Calibur, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). PI was added to samples to distinguish necrotic and late apoptotic events (annexin V⁻, PI⁺; annexin V⁺, PI⁺) from early apoptotic events (annexin V⁺, PI⁻).

Murine bronchiolitis obliterans syndrome model

A well-established murine model of BO syndrome (BOS) involving heterotopic subcutaneous trachea transplantation was used.¹⁵ BALB/c mice (SLC, Shizuoka, Japan) were used as tracheal graft donors into major histocompatibility complex-incompatible C57BL/6 (SLC) recipients. The donor trachea was implanted into a subcutaneous pocket behind the neck of the recipient mouse. Mice were sacrificed at various time points, and the transplanted tracheas were removed for analysis airway rejection in this model, referred to as obliterative airway disease. It is preceded by substantial periairway/subepithelial mononuclear cell infiltration and peaks between days 10 and 14, followed by lumen obliteration comparable with human BO in days 21–28. The aqueous fraction of SA was administered by intraperitoneal injection into recipient mice from 14 days before transplantation every day at a dose of 100 µg of SA4 until sacrifice. Although oral administration of SA might be more suitable for evaluation of the pathogenic mechanism of SA-induced BO, it is technically difficult to admin-

ister a regular dose of SA to mice orally every day. Thus, we chose the intraperitoneal injection to achieve definite systemic administration of SA in mice.

The injected dose was deduced from consumption dose of SA by SA-BO patients. In brief, our SA-BO patients had been taking 75 g equivalent of SA leaves (7.5 g of dry powder) every day for 12–16 weeks. In Taiwan, SA-BO patients had taken 150 g equivalent dose of SA leaves. Assuming that the mean body weight of C57BL/6 mice was 30 g, mice should take 50–100 µg of SA4 (5–10 mg of SA dry powder) every day. To clarify the effect of SA4 in mice BO model in a rather shorter period, we chose 100 µg of SA4 as a daily dose of injection.

Normal saline was used as an injection control taking the following points into consideration. Although a different plant extract could be a candidate as a control, it was difficult to choose a plant that is perfectly safe and as widely used as a supplementary food. In addition, we confirmed that the aqueous part of KA administration showed no effect in tracheal allograft in our pilot experiments (data not shown).

Cyclosporine A (20 mg/kg/day) was also injected intraperitoneally into allograft recipients starting postoperative day 0 and every day thereafter. The rate of intraluminal occlusive area of transplanted tracheas was calculated using 'ImageJ' (<http://rsbweb.nih.gov/ij/download.html>). The Animal Ethics Committee of Nagoya University Graduate School of Medicine approved all experiments.

Statistical analysis

Data are expressed as mean ± standard error. Differences in mean values between two groups were evaluated with the Mann-Whitney *U*-test. Analysis of variance followed by Bonferroni's test was used for multiple comparisons. Statistical significance was considered when a *P*-value <0.05 was obtained.

RESULTS

Sauropus androgynus enhances TNF-α production from macrophages

We first analysed whether SA could influence TNF-α production from macrophages or monocytes. As shown in Figure 2, SA enhanced TNF-α production from human alveolar macrophages (Fig. 2a), murine human acute monocytic leukaemia cell line (Fig. 2b) more than KA, a Chinese herbal medicine used as a control. These data suggest some ingredients of SA directly influence monocytes or pulmonary resident cells and induce TNF-α production. KA, a representative traditional herb medicine, is mainly made from *Pueraria* root and is composed of seven medicinal herbs. We used human alveolar macrophages from patients with diffuse interstitial lung diseases and sarcoidosis because they were difficult to obtain from healthy volunteers or patients with other than diffuse pulmonary diseases. However, we could not find any

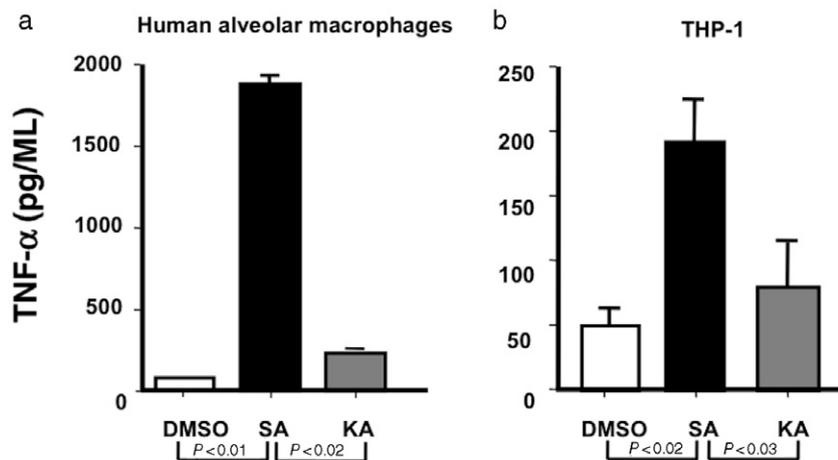


Figure 2 SA solution induces tumour necrosis factor α (TNF- α) production from monocytic lineage cells. Human alveolar macrophages (a) and human acute monocyte leukaemia cell line (THP-1) cells (b) were cultured in medium supplemented with dimethyl sulfoxide (DMSO) (open bars), *Sauropus androgynus* (SA) solution (solid bars), or *kakkon-to* (KA; assay control) solution (grey bars) for 24 h. The concentrations of TNF- α in the supernatants were measured by enzyme-linked immunosorbent assay. SA solution significantly enhanced TNF- α production compared with DMSO or KA. KA was used as an assay control. Data are means \pm standard error of the mean of duplicate values of five independent experiments.

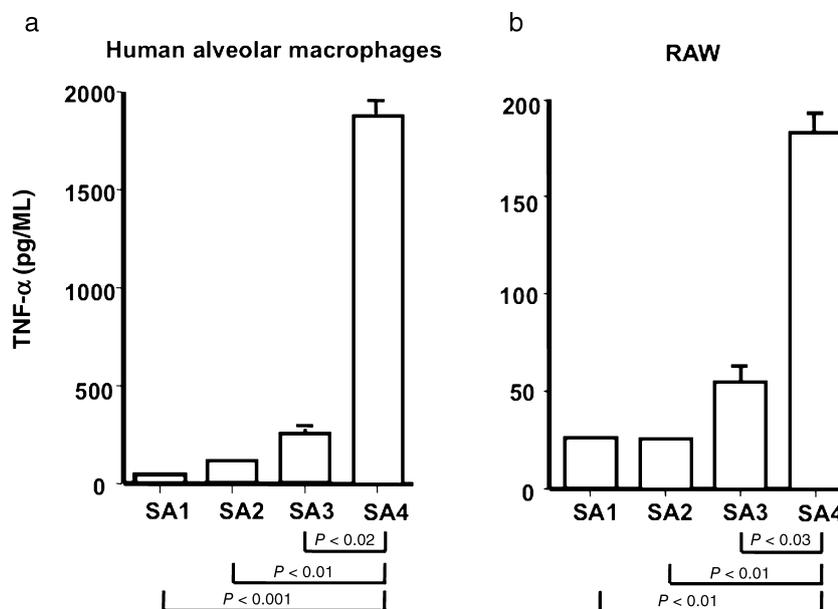


Figure 3 Different effects of SA fractions on tumour necrosis factor α (TNF- α) production from monocytic lineage cells. *Sauropus androgynus* (SA) was partitioned into four fractions: SA1 (hexane-soluble fraction), SA2 (acetone-soluble fraction), SA3 (methanol-soluble fraction) and SA4 (water-soluble fraction). Human alveolar macrophages (a) and murine monocytic cell lines (RAW) cells (b) were cultured in the medium supplemented with each fraction for 24 h, and TNF- α concentrations in the supernatants were measured. SA4 significantly enhanced TNF- α production from both cells compared with other fractions. Data are means \pm standard error of the mean of duplicate values of five independent experiments.

differences in cytokine or chemokine production between alveolar macrophages from interstitial lung diseases and those from sarcoidosis under SA stimulation.

Aqueous fraction of *Sauropus androgynus* enhances TNF- α production from macrophages

To investigate what SA component is responsible for TNF- α production from macrophages or monocytes,

we partitioned SA into four fractions (SA1–SA4) using a solvent extraction system (Fig. 1, and Material and Methods). Among these fractions, SA4 is an aqueous fraction of SA. As shown in Figure 3, SA4 induced significantly higher TNF- α production from human and murine macrophages compared with SA1, SA2 or SA3 ($P < 0.03$). SA4 also induced enhanced TNF- α production from a human acute monocyte leukaemia cell line. These data suggested that this aqueous fraction

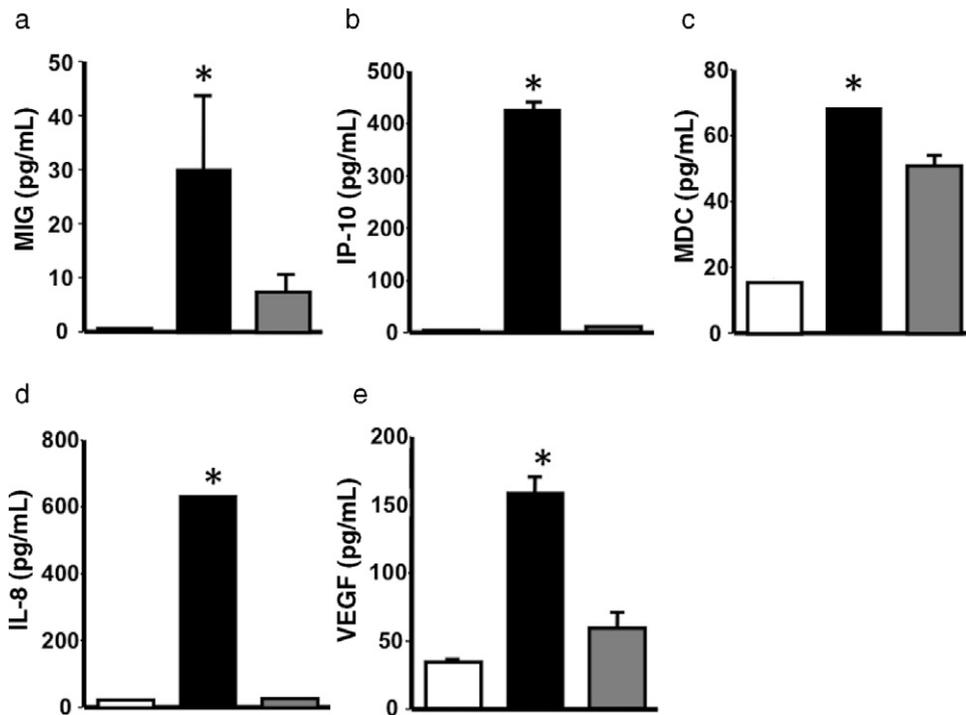


Figure 4 Effect of aqueous fraction of *Sauropus androgynus* (SA) on monocytic lineage cells. Human acute monocytic leukaemia cell line (THP-1) cells were cultured in medium supplemented with dimethyl sulfoxide (DMSO) (open bars; □), SA4 (aqueous fraction of SA) (solid bars; ■) or *kakkon-to* (KA) solution (grey bars assay control; ▒) for 24 h, and concentrations of chemokines/cytokines in supernatants were measured by enzyme-linked immunosorbent assay. SA4 significantly enhanced chemokine/cytokine production from THP-1. (a) CXCL9 (MIG), (b) CXCL10 (IP-10), (c) CCL22 (MDC), (d) interleukin (IL)-8 and (e) vascular endothelial growth factor (VEGF). Data are means \pm standard error of the mean of duplicate values of five independent experiments. * $P < 0.05$ versus KA.

might be responsible for the inflammatory change that occurred after intake of SA in SA-BO patients.

Aqueous fraction of *Sauropus androgynus* induces inflammatory chemokine production from macrophages

Human and mouse studies have revealed that CXCR3 ligand chemokines and ELR+ chemokines may play important roles for developing BO following lung transplantation.^{7,9,16,17} We further investigated whether the aqueous part of SA induces these chemokines from macrophages. SA4 significantly induces CXCL9 (MIG), CXCL10 (IP-10) and interleukin (IL)-8 production from human macrophages (Fig. 4a,b,d) compared with KA (assay control). In addition, CCL22 (MDC), which is predictive of post-transplant BOS onset,¹⁸ was also produced by macrophages under stimulation by SA4 (Fig. 4c). We also showed that SA4 induced vascular endothelial growth factor production by macrophages (Fig. 4e), which might indicate that SA could influence angiogenesis at a local lesion, such as a bronchiole. Both CCL22 and vascular endothelial growth factor production induced by SA were also significantly higher compared with those induced by KA.

Aqueous fraction of *Sauropus androgynus* suppresses endothelial cell proliferation

As pathological findings of SA-BO and post-transplantation BO have indicated obstruction of

arteries in small bronchi,¹⁹ we investigated whether SA could influence endothelial cell proliferation. As shown in Figure 5A, the aqueous part of SA (SA4) significantly inhibited proliferation of the endothelial cell line (MS1) compared with KA but not the alveolar cell line (A549). This inhibitory effect on the endothelial cell line could be enhanced in a dose-dependent manner (Fig. 5B). Further, SA4 induced apoptosis of MS1 cells in significantly higher numbers compared with the KA control (Fig. 5C,D).

Aqueous fraction of *Sauropus androgynus* enhances obstructive bronchiolitis in murine bronchiolitis obliterans syndrome model

Previous studies showed that simple feeding or injection of SA could not induce obstructive bronchiolitis in mice. We speculate that some unknown additional factors are needed to establish BO in SA-injected mice. Thus, we investigated the impact of the aqueous part of SA on BO development *in vivo* using a murine BOS model. In this murine model, inflammatory cell infiltration with fibrosis followed by luminal obstruction can be observed in the subcutaneous transplant allogeneic trachea.¹⁵ SA4 or normal saline was injected intraperitoneally daily in BOS model mice for 2 weeks before transplantation. Two weeks after transplantation, the trachea allograft in both control and SA4-injected mice showed significant inflammatory cell

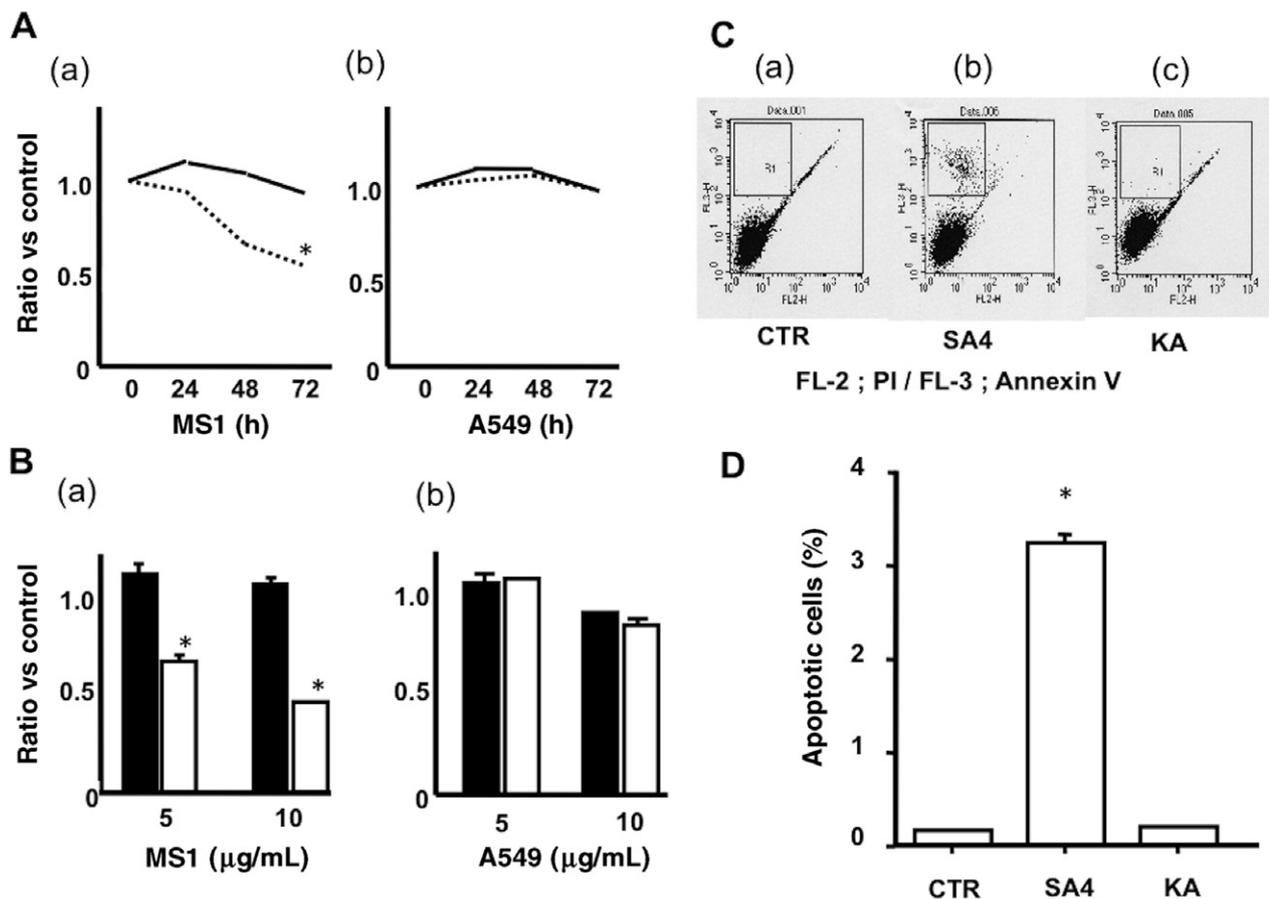


Figure 5 Aqueous fraction of *Sauropus androgynus* (SA) induces growth inhibition and apoptosis of endothelial cells. Murine endothelial cell line (MS1) cells and A549 (alveolar epithelial cell line) cells were cultured in a medium with SA4 (aqueous fraction of SA) (dotted line) or *kakkon-to* (KA; assay control) (straight line). (A) Viability of cultured cells was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium assay. Cells of A549 and MS1 were plated at a density of 2×10^4 /mL and cultured for 3 days in the following conditions: (i) culture medium alone (control); (ii) culture medium plus SA (5–10 µg/mL); (iii) culture medium plus KA (5–10 µg/mL). SA4 significantly suppressed proliferation of MS1 cells but not A549 cells in a time-dependent manner. (—) KA; (---) SA. (B) Similarly, SA4 significantly inhibited the growth of MS1 in a dose-dependent manner. (■) KA; (□) SA. (C) MS1 (endothelial cell line) cells were incubated in normal culture medium (CTR) or medium supplemented with SA4 (10 mg/mL) (SA4) or KA (10 mg/mL). After 24 h, cultured cells were stained with a combination of annexin V and propidium iodide (PI) and evaluated by flow cytometry. (D) Ratio of apoptotic cells (annexin V (+) and PI (-)) to total cells was significantly higher when cultured with SA4. * $P < 0.05$ versus control.

infiltration along the tracheal walls, but the trachea allograft in SA4-injected mice showed luminal fibrosis at a significantly higher degree (Fig. 6) than control.

Interindividual differences exist in monocytes reactivity to *Sauropus androgynus* solution

Although SA might induce monocyte/macrophage activation, it did not always induce bronchiolitis in those who consumed SA. We speculated that there might be individual variation in the reactivity of macrophage/monocyte to SA. Thus, we investigated TNF- α production by peripheral monocytes from 10 healthy volunteers (five males and five females) under SA4 stimulation. Intriguingly, there were obvious individual differences in TNF- α production induced by SA4. These experiments were repeated at least two different times per person, and the amounts of TNF- α in each experiment were compatible in the same indi-

vidual. Furthermore, peripheral monocytes from two SA-BO patients showed a rather high level of TNF- α production when stimulated with SA4 (Fig. 7). We speculate that the individual difference in susceptibility to SA could be one reason why not all people who ingested SA developed BO.

DISCUSSION

In the present study, we showed that an aqueous extract of SA could significantly induce production of TNF- α and other pro-inflammatory chemokines from macrophages or monocytes. In the inflammation of small airways of SA-BO patients, macrophages were thought to play various important roles in the disease progression.⁴ Together with the fact that serum concentration of TNF- α was reported to be higher in the SA-BO patients than in healthy controls,³ our results

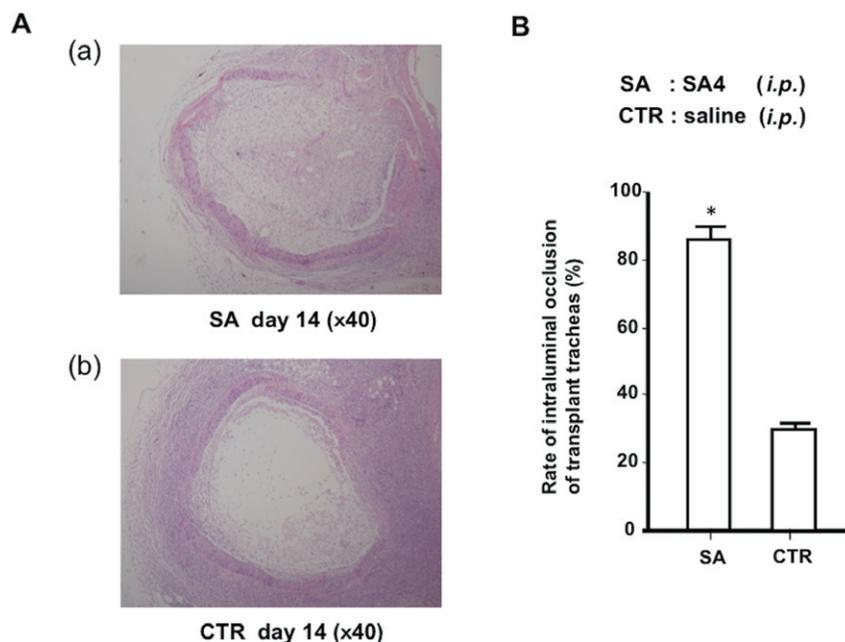


Figure 6 Aqueous fraction of *Sauropus androgynus* (SA) enhances obstructive bronchiolitis in a murine bronchiolitis obliterans syndrome (BOS) model. Pathological findings of murine trachea allograft heterotopically transplanted into allogeneic mice (BOS model) with administration of SA4 or saline. Two weeks after transplantation, trachea allograft was removed and analysed. (A) Representative microphotograph lumen of trachea allograft from mice injected with SA4 (a) or saline (b) (haematoxylin and eosin stain, original magnification $\times 40$). (B) Quantification of intraluminal fibrotic occlusion was estimated by percent area analysis. Data are represented as mean \pm standard error of the mean of four independent experiments. * $P < 0.05$ versus control. *i.p.*, intraperitoneally.

indicate that TNF- α and other pro-inflammatory chemokines induced by the aqueous part of SA are important in the pathogenesis of SA-BO. Further, we demonstrated that the aqueous part of SA could induce enhanced apoptosis of endothelial cells. These findings might be related to the obliterative arteriopathy (fibromuscular intimal sclerosis) that was also observed in SA-BO pathology.^{4,19}

Previous investigators have reported that the leaves of SA contain a high level of the alkaloid papaverine that is thought to be responsible for the development of SA-BO.^{2,3,20} An animal model of BO induced by intratracheal papaverine was also reported.² However, in our experimental analyses, we could not detect the presence of papaverine in aqueous or methanol-soluble fractions of the plant material SA (data not shown). From our analysis of the SA powder, which had been actually ingested by our SA-BO patients, our study clearly showed that papaverine is not the cause of BO in our patients.

To our knowledge, there has been no report of successful induction of BO by ingestion of SA in an animal model.²¹ In our study, we demonstrated that the aqueous part of SA could induce enhanced bronchiolar obstruction in a murine BOS model. We also showed significant individual differences in TNF- α production from peripheral monocytes stimulated with SA. Based on these findings, we speculate that the SA-BO may require some additional factors, such as chronic inflammatory background, genetic background or gender. Most reported cases of SA-BO were women, who ingested SA for the purpose of weight

control. Further investigation would be needed to elucidate the precise mechanism of individual differences in the response to SA.

There are some limiting factors in our study. First, our analyses could not detect the substance responsible for BO development. Although our study clearly showed that the aqueous fraction of SA is the most responsible fraction, the substance causing SA-BO is still unknown. This fraction could contain heavy metal and its salt. The SA powder we used in the study is relatively rich in cadmium (unpubl. data). Further investigation would be needed to clarify whether cadmium is related to SA-BO development.

Second, we could not demonstrate a causative mechanism as to how SA-induced pro-inflammatory factors lead to the bronchiolar obstruction *in vivo*. Alho *et al.* reported inhibition of TNF- α -reduced inflammation, rate of epithelial loss and bronchiolar obliteration in a BOS model after transplantation.²² Recent evidences showed that inflammatory cytokines not only TNF- α but also interferon-gamma may be essential for BO development.²²⁻²⁴ In a future study, we are planning to explore the roles of pro-inflammatory factors in our murine model and human post-transplantation BOS.

In conclusion, an aqueous fraction of SA has the potential to induce inflammation of macrophage, apoptosis of endothelial cells and enhancement of obliterative bronchiolitis in a murine BOS model. This fraction appears to induce human BO together with some unknown factors.

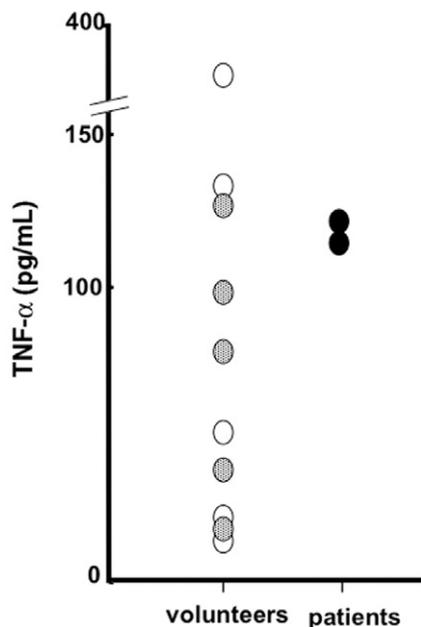


Figure 7 Individual differences in peripheral mononuclear cell reactivity to *Sauropus androgynus* (SA). Peripheral blood mononuclear cells from 10 healthy volunteers (five males and five females) and two SA-induced bronchiolitis obliterans (BO) patients were cultured under the SA4 stimulation. Tumour necrosis factor α (TNF- α) concentration in the each culture medium was measured. Experiments were repeated at least two different times, and the TNF- α amount in each experiment was compatible in the same individual. Intriguingly, peripheral monocytes from two SA-BO patients showed high TNF- α production when stimulated with SA4. (○) female (healthy volunteers); (◐) male (healthy volunteers); (●) SA-BO patients

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