Preventive Effects of a Traditional Chinese Medicine (Sho-saiko-to) on Endotoxin-Induced Cytotoxicity and Tumor Necrosis Factor-α Production in J774A.1 Cells

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Sho-saiko-to, one of the most frequently prescribed Kampo medicines, is used clinically to treat chronic hepaticitis and has shown confirmed clinical efficacy. The present study investigated whether Sho-saiko-to can suppress cytotoxicity and tumor necrosis factor (TNF)-α production in endotoxin-treated J774A.1 cells. Sho-saiko-to (10—20 μg/ml) did not affect the proliferation of J774A.1 cells, while a high concentration (50 μg/ml) of Sho-saiko-to induced a slight reduction in cell viability. Treatment with Sho-saiko-to (10—50 μg/ml) significantly inhibited endotoxin (10 μg/ml)-induced cytotoxicity in J774A.1 cells. In addition, Sho-saiko-to (20 μg/ml) suppressed TNF-α production by endotoxin (1 μg/ml)-activated J774A.1 cells. These findings suggest that the Kampo prescription Sho-saiko-to suppresses cytotoxicity or TNF-α production in macrophages treated with endotoxin and that it may be useful in improving septic shock symptoms. Sho-saiko-to may therefore protect against some of the various disturbances caused by endotoxins through its ability to inhibit TNF-α production in septic shock.

Key words: Kampo medicine; Sho-saiko-to; endotoxin; cytotoxicity; tumor necrosis factor (TNF)-α production; preventive effect

Sepsis is defined as the systemic response to infection, with the most common cause being the contamination of the blood with bacteria. Endotoxins of gram-negative bacteria exhibit numerous biological activities. Therefore endotoxins induce shock in both humans and animals characterized by fever, hypotension, intravascular coagulation, and finally multiorgan failure. Endotoxins are believed to be initially detoxified in the reticuloendothelial system (RES), particularly in liver Kupffer cells. These cells are among the main targets in poisoned animals. Macrophages stimulated by microorganisms or their toxins induce a variety of biologically active mediators known as cytokines, and tumor necrosis factor (TNF)-α is recognized as an important mediator in the development of endotoxicity.1,2) TNF-α is considered to be a major early mediator in the systemic inflammatory response syndrome observed during gram-negative sepsis.3)

Kampo medicines involve a system of drug therapy developed from clinical experience accumulated over some thousands of years in China. Sho-saiko-to has been used to treat various inflammatory diseases including hepatitis and is currently one of the most important prescriptions in Kampo medicine in Japan.4) We previously reported5—7) that Sho-saiko-to decreases recombinant human TNF-α (rhTNF-α)-induced lethality in galactosamine (GalN)-hypersensitized mice and protects mice against oxygen toxicity, Ca2+ overload in the cytoplasm or mitochondria, and carbohydrate metabolic disorders during endotoxia. In addition, we suggested in previous studies8—9) that Sho-saiko-to suppresses nitric oxide (NO) production in macrophage J774A.1 cells stimulated with endotoxin or rhTNF-α, and that it may be useful in improving endotoxin-shock symptoms. Therefore the present study investigated whether Sho-saiko-to can suppress cytotoxicity and TNF-α production by endotoxin-treated J774A.1 cells to study the preventive mechanism of Sho-saiko-to against endotoxia.

MATERIALS AND METHODS

Materials: Salmonella typhimurium lipopolysaccharide (endotoxin, Westphal obtained from Difco Laboratories, Detroit, MI, U.S.A.) was used throughout this study. The traditional Chinese preparation Sho-saiko-to was obtained from Tsumura Co. (Tokyo, Japan). Tsumura Sho-saiko-to (crude powder extract, TJ-9) contains spray-dried aqueous extracts of seven crude drugs in the following amounts: Bupleuri Radix 7.0 g, Pinelliae Tuber 5.0 g, Scutellariae Radix 3.0 g, Zizyphi Fructus 3.0 g, Ginseng Radix, 3.0 g Glyrrhizae Radix 2.0 g, and Zingiberis Rhizoma 1.0 g.

Cell Line and Culture: The murine monocyte-macrophage cell line J774A.1 was obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). The cells were maintained continuously in 75-cm² plastic culture flasks (Becton Dickinson Co., Franklin Lakes, NJ, U.S.A.) in RPMI-1640 medium supplemented with penicillin (100 U/ml, Meiji Seika Co., Ltd., Tokyo, Japan), streptomycin (100 μg/ml, Meiji Seika Co., Ltd., Tokyo, Japan), and 10% fetal bovine serum (FBS, JRH Biosciences Inc., Lenexa, KS, U.S.A.). The cells were incubated at 37°C in a 5% CO2/air environment.

Cytotoxicity Assay: Cell number was determined by trypan blue dye staining or counting using a Coulter model ZI (Coulter Inc., Hialeah, FL, U.S.A.). Cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, U.S.A.)-based colorimetric assay.10) Cells were plated at 5×10⁴ cells/ml in 96-well plates (Corning Co., NY, U.S.A.). To study the cytotoxic effects of endotoxin alone (10 μg/ml) and the combined effects of endotoxin with Sho-saiko-to (10—50 μg/ml), the cells were exposed to different concentrations of drugs for 48 h at 37°C in an atmosphere of 5% CO2 in air. After completion of drug treatment, MTT 10 μl (5 mg/ml) solution was added to each well followed by
100 μl of complete medium. After incubation for 4 h at 37°C, the MTT solution was removed. The incorporated formazan crystals in viable cells were solubilized with dimethyl sulfoxide 100 μl (Nacalai Tesque, Inc., Kyoto, Japan). Cells for each experimental time point were plated into at least eight wells, and the absorbance of each well was read at 540 nm using an ELISA analyzer. The surviving fraction was determined by dividing the absorbance of treated wells by that of control wells.

**TNF-α Assay**  
The J774A.1 macrophages were cultured in 96-well flat-bottomed plates with endotoxin (1 μg/ml). TNF-α production in J774A.1 cells was measured after 2 h. To assess the effects of Sho-saiko-to, the agent (20 μg/ml) was added to cells (1 × 10⁵ cells/ml) 18 h before treatment with endotoxin. TNF-α production in the culture supernatant was measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cytoscreen Mouse TNF-α, Biosource International Inc., Camarillo, CA, U.S.A.). Optical density at 450 nm was measured with an Immuno-Mini NJ-2300 ELISA analyzer (InterMed Inc., Tokyo, Japan). The TNF-α concentrations were calculated by comparison with a standard solution of TNF-α.

**Statistical Analysis**  
Data are expressed as the mean ± S.E. Statistical significance was evaluated using Student’s t-test. Differences with p values of less than 0.05 were regarded as significant.

**RESULTS**

**Preventive Effects of Sho-saiko-to on Cytotoxicity in Endotoxin-Treated J774A.1 Cells**  
Cell death induced by free radicals or NO radicals has been described in various cell lines.1) We previously observed2,8,9) that Sho-saiko-to protected against oxygen toxicity during endotoxemia and NO production from endotoxin or TNF-α-activated macrophages. Therefore we designed this experiment to investigate whether Sho-saiko-to can suppress cytotoxicity in endotoxin-treated J774A.1 cells. Figure 1 shows the preventive effect of Sho-saiko-to on cytotoxicity in endotoxin-treated cells. J774A.1 cells were incubated with endotoxin (10 μg/ml) and Sho-saiko-to (10—50 μg/ml) at 37°C for up to 48 h. Cell viability in the presence of Sho-saiko-to (10—20 μg/ml) was indicated to be similar to that of the untreated control cells, while the addition of Sho-saiko-to 50 μg/ml resulted in 87% cell viability. On the other hand, J774A.1 cells in the presence of endotoxin alone (10 μg/ml) exhibited a high level of cytotoxicity (42% cell viability). In contrast, treatment with Sho-saiko-to (10—50 μg/ml) showed a significant inhibitory effect on endotoxin-induced cytotoxicity in J774A.1 cells.

**Influence of Sho-saiko-to on TNF-α Production in Endotoxin-Activated J774A.1 Cells**  
The results of the above experiments suggested that Sho-saiko-to may protect against some of the various metabolic disorders caused by endotoxin challenge. On the other hand, the cytokine TNF-α has frequently been reported to cause a shock syndrome similar to endotoxin shock and it has been suggested to be the major mediator of this shock.1,2) Thus antibodies against TNF-α have been shown in different experimental models to protect mice against endotoxin lethality. Therefore we examined the effects of Sho-saiko-to on TNF-α production by endotoxin-activated J774A.1 cells. The cells were incubated with endotoxin (1 μg/ml) and Sho-saiko-to (20 μg/ml). TNF-α levels in cells were analyzed after 2-h treatment using a standard ELISA system. As shown in Fig. 2, when cells were treated with endotoxin, the TNF-α level was markedly increased, and treatment with Sho-saiko-to (20 μg/ml) showed a significant inhibitory effect (44%) on endotoxin-activated TNF-α production in J774A.1 cells. This result suggests that the preventive effects of Sho-saiko-to on endotoxin-induced shock syndrome are due to inhibition of TNF-α production in macrophages. Sho-saiko-to may therefore prove to be important in the treatment of gram-negative bacteria-induced shock.

**DISCUSSION**

Despite the remarkable progress in clinical medicine, sepsis and shock continue to be major clinical problems in intensive care units. Sepsis is the leading cause of death in critically ill patients in the U.S.A. Sepsis develops in 750000...
TNF-α affects of Sho-saiko-to on endotoxin-induced cytotoxicity and macrophage J774A.1 cells, we described the preventive effects of Sho-saiko-to on endotoxin-induced cytotoxicity and TNF-α production in macrophage cells.

NO contributes to the cytotoxic or cytostatic actions of macrophages activated by various immunologic stimuli. Previously, we suggested that Sho-saiko-to confers protection against oxygen toxicity, liver plasma membrane damage during endotoxemia, and NO production by endotoxin-activated J774A.1 cells. Asahi et al. reported that glutathione peroxidase is inactivated by endogenous NO and that this mechanism may at least in part explain the cytotoxic effects of NO on cells and NO-induced apoptotic cell death. In this experiments, endotoxin (10 μg/ml)/Sho-saiko-to (10—50 μg/ml)-treated J774A.1 cells exhibited less cytotoxicity, suggesting that macrophage cell lines are stabilized by treatment with Sho-saiko-to (Fig. 1). It appears that Sho-saiko-to has a protective effect against endotoxin-induced cytotoxicity through its ability to inhibit NO production and superoxide anion generation. In support of our finding, a previous study indicated the protective effect of lactate dehydrogenase-5 isozyme leakage in the serum of endotoxin-poisoned mice after Sho-saiko-to administration. It has been suggested that Sho-saiko-to might be associated with increased stability of hepatic lysosomal particles. Therefore we assumed that Sho-saiko-to enhances the host defense mechanism by changing the RES function. Oghihara’s coworkers have already shown that some Kampo-hozai prescriptions modulate immune responses. Sho-saiko-to, one such Kampo-hozai prescription, is known to facilitate the development of immunity by stimulating macrophages. Nagatsu et al. suggested that Sho-saiko-to enhances the immune response through at least two different routes: by eliminating the inhibition of lymphocyte functions by prostaglandin E2 and by presenting antigen more efficiently. In the present study, judging from the protective effect against endotoxin-induced cytotoxicity in J774A.1 cells after Sho-saiko-to treatment, we assumed that Sho-saiko-to is primarily associated with increased stability of macrophage cell lines.

On the other hand, TNF-α, a macrophage-derived cytokine inducible by endotoxins, has frequently been reported to cause a shock syndrome similar to that caused by endotoxins and has been suggested to be one of the major mediators of shock. This mediator is responsible, at least in part, for a number of pathophysiologic responses in the liver, including the acute-phase response, hyperlipidemia, free oxygen radical formation, fibrogenesis, and cholestasis. The present results demonstrated a clear inhibitory effect of Sho-saiko-to (20 μg/ml) on the production of TNF-α from endotoxin (1 μg/ml)-activated J774A.1 cells (Fig. 2). Previously, we reported that Sho-saiko-to protects against rhTNF-α-induced lethality in GalN-hypersensitized mice. In addition, our previous report suggested that Sho-saiko-to may protect mice from severe shock syndrome due to rhTNF-α. Endothelium-derived NO plays a role in the physiologic regulation of vascular tone and blood pressure. In addition, NO exerts protective effects against endotoxin- or TNF-α-induced shock symptoms through its ability to inhibit NO production, as shown in our previous studies. From the findings described above, the suppression of TNF-α production by endotoxin-activated macrophages by Sho-saiko-to may also prove useful in improving endotoxin-induced shock symptoms. Sho-saiko-to may therefore prove to be an important defense factor in septic shock in mice.

REFERENCES