Effects of Antitumor Activity and Protection of Shock Symptoms by a Traditional Chinese Medicine (Sho-Saiko-To) in Recombinant Human Tumor Necrosis Factor Administered Mice

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The effects of a traditional Chinese medicine Sho-saiko-to (Kampo prescription) were investigated on the various metabolic disorders and antitumor activity of recombinant human tumor necrosis factor (rTNF) administered to mice. The oxygen level in liver of rTNF (5 x 10^6 units/mouse, i.v.)-injected mice was markedly lower at 4 h post-intoxication than that in the control, whereas the administration of rTNF to Sho-saiko-to (500 mg/kg/d, p.o.)-pretreated mice resulted in a greater level of glycogen than that in rTNF alone-pretreated mice. In mice pretreated with Sho-saiko-to, the level of fibrinogen 4 h after rTNF injection markedly increased as compared to that in mice treated with rTNF alone. We also estimated the NO2- in murine macrophage cell line J774A.1 using mice serum after administration of Sho-saiko-to. Our results clearly demonstrated that J774A.1 cells stimulated with endotoxin (1 µg/ml) and rTNF (1 x 10^4 units/ml) can effectively produce nitric oxide (NO), and ascertained the suppressive effect of Sho-saiko-to (500 mg/kg/d, p.o.)-pretreated serum on NO generation by endotoxin/rTNF-activated J774A.1 cells. When the cells were incubated with endotoxin, TNF and Sho-saiko-to pretreated serum (10-100 µl), the NO level was significantly lower than that in control serum incubated with endotoxin/rTNF alone. The effect of Sho-saiko-to (1 and 10 µg/ml) on in vitro cytotoxicity by rTNF in Meth-A Sarcoma cells was observed to be in a dose dependent fashion. In addition, there was a remarkable enhancement of antitumor activity of rTNF by Sho-saiko-to pretreatment in mice. These findings suggest that the Kampo prescription Sho-saiko-to may protect mice from severe shock syndrome by rTNF, and that it may enhance rTNF-induced antitumor activity.

Key words Kampo medicine; Sho-saiko-to; antitumor activity; tumor necrosis factor; preventive effect; shock symptom

Endotoxin from gram-negative bacteria induces shock symptoms in humans and animals, a state characterized by fever, hypotension, intravascular coagulation, and finally multiorgan failure. Therefore, investigators have recently confined their interest to the metabolic alterations which develop during gram-negative sepsis or endotoxin shock. Tumor necrosis factor (TNF) was discovered in 1975, in the serum of Bacillus Calmette-Guerin (BCG)-primed and endotoxin-treated animals, as a polypeptide that induces hemorrhagic necrosis of tumors in recipient animals, and has since then been thought to be useful for tumor therapy. TNF is a cytokine released from macrophage by endotoxin and has frequently been reported to cause symptoms similar to endotoxin shock; it has been suggested to be one of the major mediators of septic shock. Therefore, its use in tumor therapy requires investigations to guard against the shock symptoms it can induce.

Sho-saiko-to, one of the most frequently prescribed Kampo medicines, is applied clinically as a therapy for chronic hepatitis, and has been reported to show good therapeutic efficacy.2) Its in vivo or in vitro administration has been reported3) to have chemopreventive effects and the medicine functions as a biological response modifier. Animal studies on rats showed that this drug possesses cancer-preventive effects.5) We recently reported7) that Sho-saiko-to decreases the recombinant human TNF (rTNF)-induced lethality in galactosamine-hypersensitized mice, and protects mice against oxygen toxicity, Ca2+ overload in cytoplasm or mitochondria and carbohydrate metabolic disorders during endotoxemia. We further suggested10) that Sho-saiko-to shows a suppressive effect on nitric oxide (NO) generation in macrophages stimulated with endotoxin, and that it may be useful in improving endotoxin-shock symptoms. Therefore, the present study was carried out to observe the effects of Sho-saiko-to on rTNF-induced shock symptoms and antitumor activity of rTNF.

MATERIALS AND METHODS

Animals and Treatment Male ddY mice, 4 weeks old and weighing 18 to 20g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in the Tohoku College of Pharmacy Experimental Animal Center. Salmonella typhimurium lipopolysaccharide (endotoxin) (Westphal preparation obtained from Difco Laboratories, Detroit, Mich., U.S.A.) was used throughout this study. rTNF1) was generously provided by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Endotoxin content of this cytokine was less than 0.04 ng/mg protein by Limulus Test kit (Pyrodik, kit, Seikagaku Co., Tokyo). The traditional Chinese preparation, Sho-saiko-to, was obtained from Tsumura Co., Tokyo. Tsumura-Sho-saiko-to (crude power extract, TJ-9) contains spray-dried aqueous extracts of seven crude drugs in the following proportions: 7.0 g Bupleuri Radix, 3.0 g Zizyphi Fructus, 3.0 g Ginseng Radix, 2.0 g Glycyrrhizae Radix, and 1.0 g Zingiberis. TJ-9 was dissolved in distilled water at a dose of 500 mg/kg through a stomach catheter once per day for 5d. On the 6th d, rTNF was injected at a dose of 5 x 10^4 units/mouse i.v. into TJ-9 pretreated mice. In the experiment in Fig. 2, the serum was pooled from 10 mice administered with TJ-9. Then, the TJ-9-
pretreated serum obtained was subjected to an in vitro NO assay. Control mice were injected with 0.2 ml of saline alone.

**Measurements of Liver Glycogen and Plasma Fibrinogen**

Estimation of liver glycogen was carried out by a modification of the method of Wilder and Sword. Liver homogenate was heated at 100°C for 20 min in a 30% KOH solution. Glycogen was isolated by the addition of ethanol, and estimated colorimetrically by an anthrone reagent (Tokyo Kasei Kogyo Co., Ltd., Tokyo). The level of plasma fibrinogen was estimated by the Biopool Fibrinogen Assay Kit (Travenol Laboratories, Inc., Costa Mesa, Calif., U.S.A.).

**Measurement of Nitrite Production as an Assay of NO Release in J774A.1 Cells**

NO production by J774A.1 cells was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction. J774A.1 cells were removed from culture flasks by vigorous pipetting and centrifuged, then resuspended in the medium to a concentration of 1 x 10^6 cells/ml. Cells (1 x 10^5 cells/well) were plated in 24 well culture plates (Falcon) and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium or that containing endotoxin (1 µg/ml) and rhTNF (1 x 10^4 units/ml) in the presence or absence of TJ-9 pretreated serum, and was incubated at 37°C in 5% CO_2 in air for up to 24 h. Briefly, 300 µl of culture supernatant from control cells or cells stimulated with endotoxin/TNF were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethendiamine dihydrochloride and 2.5% H_3PO_4) at room temperature for 10 min.

**Cell Lines and Cultures**

The murine monocyte-macrophage cell line, J774A.1, was obtained from Japan Cancer Research Resources Bank (JCRB,Tokyo). Mouse Meth-A sarcoma cells were kindly supplied by the Cancer Cell Repository, Institute of Development Aging and Cancer, Tohoku University, Sendai, Japan. The cells were maintained continuously in 75 cm^2 plastic culture flasks (Falcon) in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% fetal calf serum. The cells were incubated at 37°C in a 5% CO_2/air environment.

**Cytotoxicity Assay**

Cytotoxicity was measured using a microculture tetrazolium (MTT) assay as described by Carmichael et al. Briefly, cell concentration and viability were determined by hemocytometer counts of cells excluding 0.1% trypan blue dye. Cells (1 x 10^5 cells/well) were plated in 96-well round bottomed plates (Corning Co., NY., U.S.A.), and treated with different concentrations of rhTNF in the presence or absence of TJ-9 (1 and 10 µg/ml) for 48 h at 37°C in 5% CO_2/air. After completion of drug treatment, 10 µl of MTT (5 mg/ml) solution was added to each well followed by 100 µl of complete medium. After incubation for 4 h at 37°C, MTT solution in the medium was removed. The formazan crystals in viable cells were solubilized with 100 µl of dimethylsulfoxide. Cells for each experimental point were plated into at least eight wells, then the absorbance of each well was read at 540 nm, using an ELISA analyzer (Model ETY-96, Toyo Instruments Inc., Japan). Surviving fractions were determined by dividing the absorbance of treated wells by the absorbance of control wells.

**Antitumor Activity**

Meth-A sarcoma cells maintained in BALB/C mice (Japan SLC Inc., Hamamatsu, Japan) by weekly passage in our laboratory were used. Meth-A sarcoma (1 x 10^5 cells) was inoculated to the right thigh of male BALB/C mice (5-weeks-old) on day 0. On days 11, 12, 13, 14 and 15, mice were given p.o. TJ-9 (500 mg/kg/d), and on day 15, they were injected i.v. with rhTNF (1 x 10^4 units/mouse). Antitumor activity of drugs was estimated by comparison with the control by weighting the solid tumor on day 30 after cell inoculation. The antitumor activity was evaluated in terms of the mean weight of tumors in each group of mice as a percentage of the mean weight to control mice.

**Statistical Analysis**

Student's t-test was used to evaluate statistical significance of differences between groups.

**RESULTS**

**Effects of TJ-9 Administration on the Plasma Fibrinogen and Liver Glycogen Levels in Mice Given rhTNF**

In this study, we examined whether or not Sho-saiko-to prevents TNF-induced shock symptoms. As shown in Fig. 1a, the fibrinogen level in plasma of rhTNF (5 x 10^4 units/mouse, i.v.)-treated mice was lower than that in control at 4 h post-intoxication, while a more significant increase was found in the plasma fibrinogen of TNF-TJ-9-treated mice as compared to that in mice treated with rhTNF. Figure 1b shows the effect of TJ-9 pretreatment on glycogen level in mouse liver 4 h after rhTNF injection. The level of liver glycogen in mice given rhTNF alone was markedly lower than that in the control mice, while the level in mice treated with rhTNF plus TJ-9 was higher than that in the animals given rhTNF alone. These results suggest that TJ-9 may protect mice from shock symptoms induced by rhTNF.

**Effect of TJ-9-Pretreated Serum on NO Production in Endotoxin and rhTNF-Activated J774A.1 Cells**

It is believed that excessive NO production mediates the hypotensive effect of septic or cytokine-induced shock. Therefore, we examined the effect of TJ-9-pretreated serum (10—100 µl) on NO production of endotoxin (1 µg/ml) and rhTNF (1 x 10^4 units/ml)-activated J774A.1 cells. As seen in Fig. 2, the unstimulated or rhTNF alone-activated J774A.1 cells did not produce detectable amounts of NO_2 in the absence of endotoxin. Treatment of cultured J774A.1 cells with endotoxin led to high levels of NO production. Further, the treatment with endotoxin and rhTNF showed a significant enhancement effect on NO production in J774A.1 cells. With the addition of TJ-9 non-treated serum (control) in a range of 10—100 µl, the NO_2 level following the incubation of cells in the presence of endotoxin/rhTNF was higher than that in cells treated with endotoxin/rhTNF alone, and the production was dependent on the dose of serum (10—100 µl). This result is believed due to the effect of cytokines contained in normal serum. However, addition of serum (10—100 µl) of mice given TJ-9 showed a significant inhibitory effect on NO production in J774A.1 cells by endotoxin/rhTNF-stimulation.
Fig. 1. Changes in Plasma Fibrinogen (a) and Liver Glycogen (b) Levels of TJ-9-Pretreated Mice 4h after rhTNF Administration

Each value represents the mean±S.E. of 8 mice. * Significant difference from the value of rhTNF-injected mice at p<0.05. Control, saline; rhTNF, recombinant human tumor necrosis factor (5×10⁴ units/mouse, i.v.); TJ-9, Sho-saiko-to (500 mg/kg/d, p.o.). Mice were pretreated with TJ-9 as described in Materials and Methods.

Fig. 2. Effect of TJ-9-Pretreated Serum on Production of NO in J774A.1 Cells Stimulated with Endotoxin/rhTNF

Mice were pretreated with TJ-9 (500 mg/kg/d, p.o.) as described in Materials and Methods. The pooled serum was obtained from 10 mice pretreated with TJ-9. Control serum was obtained from TJ-9-non-treated 10 mice. Cells (1×10⁶ cells/well) were incubated with combinations of endotoxin/rhTNF and TJ-9-pretreated serum (10–100 µl). Nitric oxide production in the culture medium was determined as described in Materials and Methods. Each bar represents the mean±S.E. of 3 experiments. None, unstimulated J774A.1 cells; LPS, endotoxin (1 µg/ml); rhTNF, recombinant human tumor necrosis factor (1×10⁵ units/ml); Control, TJ-9-non-treated serum; TJ-9, Sho-saiko-to (500 mg/kg/d, p.o.)-pretreated serum. a) p<0.05, compared with LPS alone-treated group. b) p<0.05, compared with rhTNF/LPS-treated group. c) p<0.05, compared with group treated with rhTNF/LPS plus control serum.

Effect of TJ-9 on rhTNF Cytotoxicity to Meth-A Sarcoma Cells

As can be seen in results of the MTT assay in Fig. 3, IC₅₀ of rhTNF was 0.098 unit/ml. Treatment with TJ-9 alone, however, was ineffective until 100 µg/ml on rhTNF cytotoxicity against Meth-A sarcoma cells (data not shown). The cell cytotoxicity of rhTNF in the presence of TJ-9 (1 µg/ml) was 0.029 units/ml as IC₅₀, whereas the addition of 10 µg/ml of TJ-9 showed 0.015 units/ml. The enhancing effect of TJ-9 on cytotoxicity of rhTNF in vitro was observed to be in a dose dependent fashion.

Enhancement of Antitumor Activity of rhTNF by TJ-9 Pretreatment

The following experiments were conducted to observe the antitumor activity of rhTNF by TJ-9-pretreatment in vivo. Table 1 shows tumor growth inhibition on TJ-9-pretreated mice after rhTNF administration. The solid tumor weight (mean±S.E.) in control mice on day 30 after the implantation was 7.44±0.59 g. The rhTNF and TJ-9 induced 13.4% and 5.6% reduction of tumor weight, respectively. When TJ-9-pretreated mice (500 mg/kg/d, p.o.) were injected once with rhTNF (1×10⁶ units/mouse, i.v.), however, the tumor growth was significantly suppressed, inducing 42.8% reduction of tumor weight by TJ-9-pretreatment. From Fig. 3 and
Fig. 3. rhTNF Cytotoxicity in Meth-A Sarcoma Cells by TJ-9

Meth-A sarcoma cells (1 x 10^5 cells) were incubated for 48 h with various concentrations of rhTNF in the presence or absence of TJ-9 (1 and 10 μg/ml). Then, drug effects on cell proliferation were determined using MTT assay as described in Materials and Methods. TNF cytotoxicity is expressed as IC_{50}, i.e., TNF concentration providing a 50% reduction in cell numbers as compared to controls cultured in parallel without drug. Each value represents the mean of quadruplicate determinations varying less than 10%.

Table 1. Enhanced Antitumor Activity of rhTNF by TJ-9 Injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor weight (g) ± S.E.</th>
<th>Inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7.44 ± 0.59</td>
<td></td>
</tr>
<tr>
<td>TJ-9 900 mg/kg (p.o.)</td>
<td>7.03 ± 0.91</td>
<td>5.6</td>
</tr>
<tr>
<td>rhTNF 1 x 10^6 units/mouse (i.v.)</td>
<td>6.44 ± 0.72</td>
<td>13.4</td>
</tr>
<tr>
<td>TJ-9 + rhTNF</td>
<td>4.26 ± 0.56*</td>
<td>52.8</td>
</tr>
</tbody>
</table>

Male BALB/c mice were inoculated i.m. with 1 x 10^5 Meth-A sarcoma on day 0. On days 11, 12, 13, 14 and 15, they were given p.o. injection of TJ-9 (500 mg/kg), and on day 15 an i.v. injection of rhTNF (1 x 10^6 units/mouse). Each group consisted of ten mice. The antitumor activity was evaluated by weighing the solid tumor on the 30th day after Meth-A sarcoma inoculation. * p < 0.05, significantly different from the rhTNF alone-treated group.

Table 1, in vitro or in vivo, these results suggest that Sho-saiko-to may enhance rhTNF-induced antitumor activity.

DISCUSSION

Septic shock may be associated with a toxic state initiated by stimulation of monocytes by a bacterial toxin, such as endotoxin, which is released into the bloodstream. It seems that endotoxin exhibits its toxic effects by stimulating host cells, especially macrophages, to release various proinflammatory mediators which act as secondary messengers. Beutler and Cerami reported that TNF may be identical to cachectin. TNF/cachectin is a factor which has been found to play a pivotal role in the development of shock and tissue injury during sepsis and cachexia, and TNF also possesses antitumor activity for some cancer cells. We recently reported that Sho-saiko-to improves endotoxic shock based on our series of studies on metabolic pharmacological effects. Therefore, we investigated whether or not Sho-saiko-to can protect mice from the severe shock symptoms induced by TNF, and can exhibit an enhancing effect of rhTNF on antitumor activity by TJ-9 administration.

In the present study, we found that fibrinogen was at a markedly lower level in plasma of mice 4 h after rhTNF (5 x 10^6 units/mouse, i.v.) injection than that in control. It was noted that the level showed a significant increase in plasma of mice treated with rhTNF plus TJ-9. Similarly, the glycogen level was significantly increased in liver of rhTNF-treated mice given TJ-9 than that in mice given rhTNF alone. TNF is known to have an enhancing effect on procoagulant activity and concomitant suppression of the protein C pathway. Thus, endothelial-directed actions of TNF may be relevant to intravascular coagulation and hemorrhagic tumor necrosis. Kubo et al. suggested that the compounds bicalin and baicalin can prevent the decrease of blood platelets and fibrinogen in disseminated intravascular coagulation of rats which is induced by endotoxin. It can be inferred, therefore, that the administration of TJ-9 protected mice from rhTNF toxicity. NO produced by activated macrophages has been shown to be involved in TNF-induced shock, hypotension and vasodilatation, and NO, a highly reactive free radical produced by activated macrophages, has emerged as another important mediator of inflammatory responses. Moreover, NO may react with superoxides resulting in the formation of peroxynitrite, which can lead to the iron-independent generation of hydroxyl radicals. We employed a seropropharmacological method to investigate the suppressive effect of TJ-9 (500 mg/kg/d, p.o.)-pretreated serum on NO production by endotoxin/TNF-activated J774A.1 cells. As shown in Fig. 2, since the serum contained a variety of cytokines, the effect of normal serum on NO production in J774A.1 cells was seen to be increased in a dose dependent fashion. In contrast, we noted that TJ-9 showed a significant inhibition on the NO production in J774A.1 cells stimulated by endotoxin/TNF in the normal serum. Endothelium-derived NO plays a role in the physiological regulation of vascular tone and blood pressure. It has also been implicated in the pathogenesis of vascular injury, hypotension and shock induced by endotoxin and TNF. Thus, the various symptoms in TNF-induced shock may result, at least in part, from NO radical generation during endotoxemia. It is possible that the preventive effects of TJ-9 on TNF-induced shock symptoms are caused, at least in part, by the inhibition of NO production as described above. Sho-saiko-to may, therefore, prove to be an important defense factor in septic shock of mice.

The Meth-A sarcoma cell is immunogenic and the system is so sensitive that not only hemorrhagic necrosis, but also the tumor can completely vanished with a single injection of a low, non-toxic concentration of TNF. It was originally a surprise, however, to find that Meth-A sarcoma cells in tissue culture were completely resistant to the action of TNF. Despite the in vitro findings as shown in Fig. 3, it was noted in this study that rhTNF-induced cytotoxicity of Meth-A sarcoma cells was en-
hanced in vitro in a dose dependent fashion by added TJ-9. We also observed in vivo the increase of antitumor activity of rhTNF by TJ-9 pretreatment (Table 1). It is of interest to note that rhTNF (1 x 10^4 units/mouse, i.v.) at a single injection had little effect on antitumor activity to Meth-A sarcoma cells, while the activity in rhTNF-TJ-9 (500 mg/kg/d, p.o.) treated mice was significantly increased compared with that in mice treated with rhTNF or TJ-9 alone. Two active ingredients of Sho-saiko-to, i.e., glycyrrhizin and baicalin, have been reported to suppress proliferation of a human hepatocellular carcinoma cell line (HuH-7) in vitro interfering with cell cycle at the G0/G1 phase and with DNA synthesis. Thus, the present in vitro or in vivo investigation clearly demonstrated a synergistic effect on cytotoxicity or antitumor activity in Meth-A sarcoma cells by rhTNF and TJ-9, since TJ-9 alone did not effectively show cytotoxicity in vitro. It is, therefore, of interest that TNF-induced shock symptoms are prevented by Sho-saiko-to which has an antitumor activity.

The systemic administration of TNF can mediate the regression of a number of established experimental murine tumors, but has no therapeutic effect when administered to humans, probably because humans can tolerate only 2% of the dose required for regressions in mice. TNF has been delivered via isolation perfusion of the limbs or by direct intravenous injection. Though it has been effective in mediating tumor regression in cancer patients, our findings suggest that antitumor effects after administration of TJ-9 are more probable if high TNF concentrations can be used at the local site. From the findings described above, the preventive effect of Sho-saiko-to from shock symptoms by rhTNF may make use of this tumor therapy beneficial. Further investigation on this point is required.

Acknowledgment We thank Daippon Pharmaceutica Co., Ltd. (Osaka, Japan) for generously providing the recombinant human TNF.

REFERENCES