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Original articles

Antitumor activities and tumor necrosis factor producibility of traditional Chinese medicines and crude drugs*

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Summary. The antitumor activities and capacity for tumor necrosis factor (TNF) production of traditional Chinese herbal preparations (Zhu-ling-tang, Xiao-chai-hu-tang), crude drugs (Polyporus, Hoelen, Bupleuri radix, Angelica radix, Cnidii rhizoma, Cinnamomum cortex), and Krestin (PSK) were investigated. These drugs were given to DDY mice in the drinking water before and after transplantation of Ehrlich tumors, and the development of the intradermally transplanted Ehrlich tumors and survival rate were observed. A good survival rate and sometimes a complete cure were found in the groups administered Bupleuri radix, Xiao-chai-hu-tang, Angelica radix, or Cinnamomum cortex, while the group given Hoelen showed poor results. To examine the capacity for TNF production these drugs were given to DDY mice PO as initial stimulating agents, to stimulate the reticuloendothelial system (RES) prior to lipopolysaccharide injection. The TNF activity was tested from the cytotoxicity against L cells. Significant differences in capacity for TNF production were observed among the drugs. Relatively high levels of TNF activity were noted in the groups given Angelica radix, Bupleuri radix, Cnidii rhizoma, or Cinnamomum cortex, very low activities in the groups given Xiao-chai-hu-tang, Zhu-ling-tang, or Krestin, and no TNF activities in the groups given Polyporus or Hoelen. The TNF capacity for production broadly paralleled the survival rate of the mice transplanted to Ehrlich tumors. Our findings suggest that one mechanism underlying the antitumor activities of these drugs is based on stimulation of the RES and is closely related of TNF production.

Introduction

The antitumor activities of traditional Chinese herbal preparations (Zhu-ling-tang, Xiao-chai-hu-tang) and crude drugs (Polyporus, Hoelen, Bupleuri radix, Angelica radix, Cnidii rhizoma, Cinnamomum cortex), which are components of traditional Chinese preparations, have been reported previously [2]. It is considered that polysaccharide separated from these drugs partially reveals their antitumor activities through an increase in the immune response with macrophage involvement [3, 14, 16, 17].

Tumor necrosis factor (TNF) was discovered in 1975 and is an active component exhibiting antitumor activity [1]. TNF causes hemorrhagic necrosis of tumors in vivo [6], and displays cytotoxicity against cancer cells in vitro [5]. It has been reported that priming agents which stimulate the reticuloendothelial system (RES) are good substances for the production of TNF [4, 8]. In the present study the antitumor effects of the above traditional Chinese preparations and crude drugs and the capacity of these drugs for TNF production as priming agents with subsequent lipopolysaccharide (LPS) administration were investigated.

Materials and methods

Animals. DDY strain mice (Nihon Clea Inc., Tokyo, Japan) were used for the production of TNF and for tumor transplantation.

Drugs and mode of administration. Various Chinese medicines and Krestin (PSK, *Coriolus versicolor* Quél CM-101 strain; Kreha Chemical Ind., Tokyo, Japan), which consists of polysaccharides separated from *Coriolus*, were used. All the traditional Chinese prescriptions and crude drugs were obtained from Tsumura Juntendo Inc., Tokyo, Japan. Zhu-ling-tang 0.7 g contains spray-dried extracts of 3.0 g Polyporus, Hoelen, Alismatis rhizoma, glutinum, and kaolinum. Xiao-chai-hu-tang 2.0 g contains extracts of 7.0 g Bupleuri radix, 5.0 g Pinelliae tuber, 3.0 g Suxtallariae radix, 3.0 g of Zizyphi fructus, 3.0 g Ginseng radix, 2.0 g Glycyrrhizae radix and 1.0 g Zingiberis Recens rhizoma. Spray-dried aqueous extracts of Polyporus, Hoelen, Bupleuri radix, Angelica radix, Cnidii rhizoma, and Cinnamomum cortex were obtained from Tsumura Juntendo Inc. LPS obtained from *Escherichia coli* O111: B4*w* (Difco Lab., Mich, USA) was used for TNF production.

Spray-dried aqueous extracts of 560 mg/kg Zhu-ling-tang, 1600 mg/kg Xiao-chai-hu-tang, 400 mg/kg Polyporus, 400 mg/kg Hoelen, 640 mg/kg Bupleuri radix, 640 mg/kg Angelica radix, 560 mg/kg Cnidii rhizoma, 560 mg/kg Cinnamomum cortex, or 500 mg/kg Krestin were administered to mice in their drinking water 2 weeks before LPS injection. LPS 20 μg/mouse was injected IV, and 2 h later blood was collected. Each group consisted of six or more mice. The spleen and liver weights were measured as indicators of RES stimulation.

TNF assay in vitro. L(S) cells were cultured in Eagle's MEM with heat-inactivated FBS (10%), penicillin, strep-

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Table 1. TNF producibility, spleen and liver weights in mice treated with various drugs for 2 weeks prior to LPS injection[a]

Group (*n*)	Spleen wt (mg)	Liver wt (g)	TNF activity (DF)[b]	% TNF positive (High titer)	Meth A[c]
Control (5)	109.9 ± 16.9[d]	1.34 ± 0.07	0	0	–
Zhu-ling-tang (8)	157.7 ± 25.4	1.58 ± 0.17	16.5 ± 43.6	12.5 (131.9)	–
Xiao-chai-hu-tang (8)	258.4 ± 86.8	1.56 ± 0.19	44.1 ± 116.6	12.5 (352.6)	–
Polyporus (7)	200.9 ± 21.3	1.63 ± 0.04	5.0 ± 12.1	14.3 (34.7)	–
Hoelen (6)	287.3 ± 107.8	1.82 ± 0.11	1.2 ± 2.6	16.7 (7.1)	–
Bupleuri radix (8)	198.8 ± 75.6	1.28 ± 0.21	613.2 ± 803.6	62.5 (1977.4, 1769.0, 1062.8)	+
Angelica radix (13)	302.2 ± 100.8	1.40 ± 0.15	350.7 ± 361.9	76.0 (1737.4, 976.6, 171.6, 117.3)	+
Cnidii rhizoma (8)	267.3 ± 109.2	1.46 ± 0.20	425.6 ± 594.6	50.0 (1445.9, 788.7, 431.1, 215.4)	+
Cinnamomum cortex (9)	194.2 ± 63.8	1.14 ± 0.23	803.7 ± 1510.7	44.4 (4490.1, 2479.4, 284.7)	+
Krestin (8)	186.1 ± 57.8	1.35 ± 0.20	62.7 ± 127.4	25.0 (384.1, 117.2)	–

[a] LPS 20 μg/mouse was injected IV and 2 h later blood was collected
[b] TNF activity was assessed from the DF (dilution factor) which revealed 50% L cell cytotoxicity, individually
[c] Tumor necrosis at 24 h after administration of pooled TNF-positive serum
[d] Values are means ± SD

tomycin, and HEPES buffer (10 m*M*). Serially diluted sera were incubated with 2×10^5/ml L cells for 48 h in 5% CO_2 in air at 37 °C. The dilution of the serum having 50% cytotoxicity was expressed as the dilution factor (DF) with the dye exclusion method: that is, after being drawn out of the medium the cells were fixed with methanol and stained with 0.05% methylene blue for 5 min, the dye was extracted from the cells with 3% HCl, and the optical density at 665 nm was measured.

TNF assay in vivo. Five million Meth A sarcoma cells were injected intradermally into the flank of BALB/C mice. Seven days after the transplantation, 0.5 ml of a test sample (mixed sera from the group) was injected IV and the degree of tumor necrosis was assessed 24 h later [7].

Antitumor activity of various drugs against Ehrlich tumors. Drugs were given PO to DDY mice 2 weeks before and after Ehrlich tumor transplantation at the same doses as described above. Ehrlich tumor cells (1×10^6/mouse) were inoculated intradermally into the flank of DDY mice. Control mice were given sterilized water. The tumor size and the extent of necrosis were measured (length and width) with vernier calipers every other day for 1 month. The tumor weight (w) in milligrams was estimated from the linear dimensions using the formula:

$$w = \frac{\pi}{6} X \left(\frac{A+B}{2}\right)^3 - \frac{\pi}{6} X \left(\frac{nA+nB}{2}\right)^3,$$

where A represents the tumor width in millimeters, B the tumor length, nA the width of necrosis, and nB the length of necrosis. To standardize the variability in tumor weights among different test groups, the value of T/C (tumor weights of treated group/tumor weights of control group) was estimated. The ratio of the tumor-doubling time (T_D) of the treated group to that of the control group was calculated, where

$$T_D = \frac{\ln 2}{(\ln W_{14} - \ln W_7)/7},$$

W_{14} and W_7 being the tumor weights at 14 and 7 days after transplantation, respectively. The survival rate and the percentage of completely cured mice after the transplantation of Ehrlich tumors were estimated. The mean survival times (excluding completely cured mice) were also estimated.

Results

TNF production capacity of various drugs

Table 1 shows the spleen and liver weights and the TNF activities after LPS injection in the groups to which various drugs were administered for 2 weeks. Splenomegaly was observed in all groups that received the drugs. Relatively high TNF activities were observed in the Cinnamomum cortex, Bupleuri radix, Cnidii rhizoma and Angelica

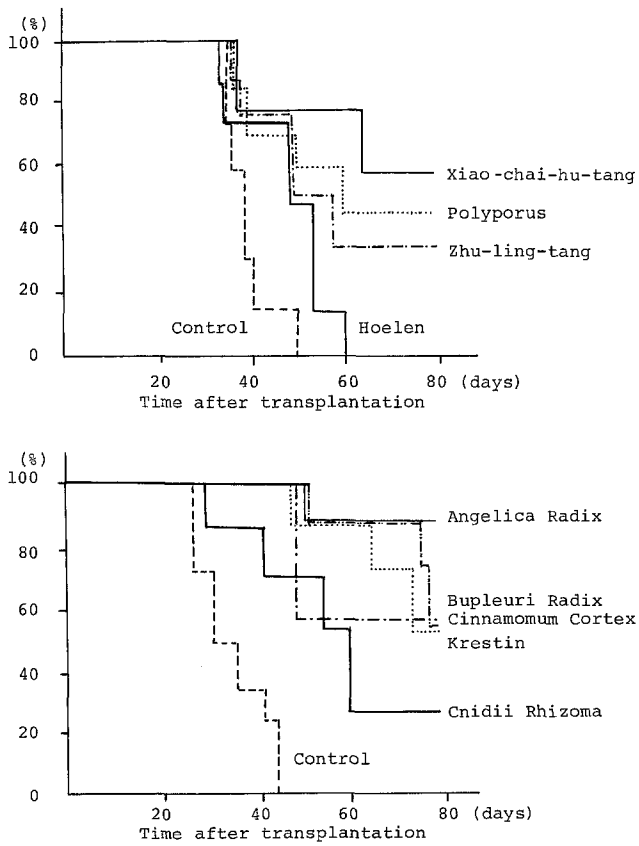

Fig. 1. Survival curves of mice following intradermal transplantation of Ehrlich tumor cells. Various drugs were administered 2 weeks before and after the transplantation and the mice were not given LPS

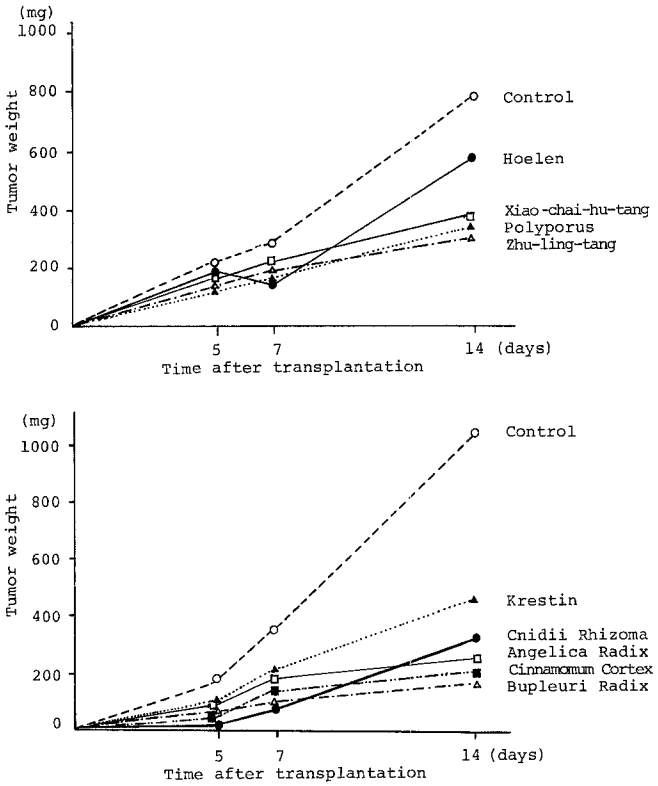

Fig. 2. Changes of transplanted Ehrlich tumor weight in mice. Various drugs were administered 2 weeks before and after the transplantation without subsequent LPS administration. Tumor weights in milligrams were estimated from the linear dimensions

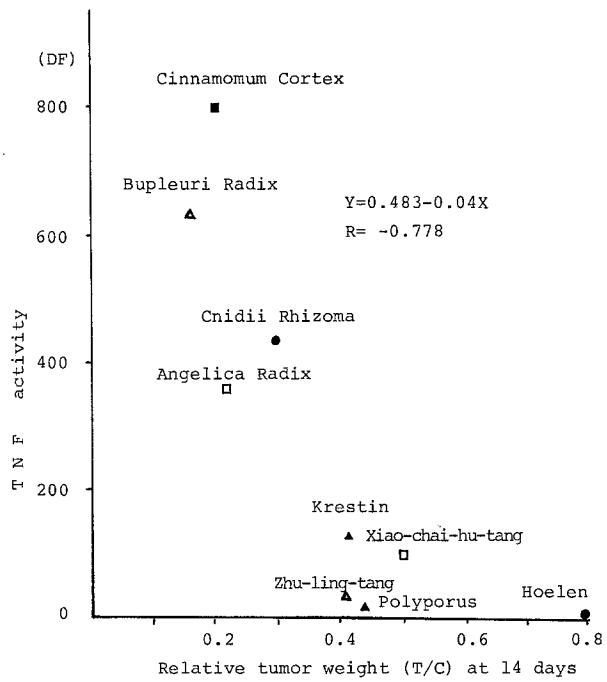

Fig. 3. Correlation between TNF activity and relative tumor weight. The TNF producibility is plotted against relative tumor weight of Ehrlich tumors at 14 days after transplantion

radix groups, while very low activities were noted in the Xiao-chai-hu-tang, Zhu-ling-tang and Krestin groups, and none in the Polyporus and Hoelen groups.

Antitumor activity of various drugs and Krestin against Ehrlich tumors

Figure 1 shows the survival curves for tumor-bearing mice treated with various drugs and Krestin. High survival and complete cure rates were observed in the groups that received Angelica radix, Cinnamomum cortex, Bupleuri radix, Xiao-chai-hu-tang, and Krestin, while relatively high survival rates were noted in the groups that received Polyporus and Zhu-ling-tang, but low survival and complete cure rates were seen in the groups treated with Hoelen and Cnidii rhizoma compared with the control group.

Figure 2 illustrates the changes in transplanted Ehrlich tumor weights. Development of the transplanted tumors tended to be inhibited in all groups compared with the control groups. The development of transplanted tumors was inhibited best in the Bupleuri radix, Angelica radix and Cinnamomum cortex groups, followed by the Cnidii rhizoma and Krestin groups and then the Zhu-ling-tang, Polyporus, Xaio-chai-hu-tang and Hoelen groups.

Table 2. Antitumor activity against Ehrlich tumors[a]

Drugs	(*n*)	Tumor weight (T/C)[b]			T_D (T/C)[c]	Complete cure (%)	Survival days[d] (*n*)
		5 days	7 days	14 days			
Control	(8)	1.00	1.00	1.00	4.62	0	35.8 ± 5.8[e] (8)
Zhu-ling-tang	(6)	0.57	0.72	0.39	11.80/4.71 = 2.51	33.3	44.5 ± 9.0 (4)
Xiao-chai-hu-tang	(12)	0.56	0.71	0.47	7.82/4.71 = 1.66	58.3	46.6 ± 15.0 (5)
Polyporus	(7)	0.84	0.63	0.42	7.95/4.71 = 1.69	42.9	44.8 ± 10.9 (4)
Hoelen	(7)	0.72	0.63	0.42	4.08/4.71 = 1.69	0	47.7 ± 10.4 (7)
Bupleuri radix	(6)	0.33	0.27	0.15	10.30/4.62 = 2.23	66.7	51.0 ± 0 (2)
Angelica radix	(9)	0.40	0.52	0.23	10.78/4.62 = 2.33	88.9	51.0 ± (1)
Cnidii rhizoma	(7)	0.17	0.24	0.31	3.76/4.62 = 0.81	28.6	49.8 ± 10.7 (5)
Cinnamomum cortex	(9)	0.20	0.42	0.21	14.70/4.62 = 3.18	66.7	70.3 ± 11.6 (3)
Krestin	(7)	0.49	0.58	0.40	7.13/4.62 = 1.54	57.1	64.0 ± 13.5 (3)

[a] Drugs were given PO to DDY mice 2 weeks before and after Ehrlich tumor transplantation without subsequent LPS administration
[b] Tumor weights (mg) were estimated from the linear dimensions
[c] T/C = tumor weights of treated group/tumor weights of control group
[d] The tumor-doubling time (T_D) between 7 and 14 days after tumor transplantation was calculated
[e] Survival days excluding completely cured mice
[f] Values are means ± SD

The data obtained are summarized in Table 2. Judging from the lowest T/C values and the ratio of the tumor-doubling times, administration of these drugs was effective against Ehrlich tumors.

We investigated the toxicity of the drugs at the same doses in tumor-free controls. The survival rates were 100%.

Correlation between capacity for TNF production and antitumor activities

The capacity for TNF production broadly paralleled the survival rate of the mice that received transplants of Ehrlich tumors. The correlation index between the TNF activity and relative tumor weight of Ehrlich tumors at 14 days after transplantation (R) was −0.778, as shown in Fig. 3.

Discussion

In therapy with Chinese traditional medicines, the basic principle is to regulate the homeostasis of the whole body through the compound actions of original prescriptions and to restore an abnormal state to a normal state, rather than to make a selective attack on the target region involved in the ailment. It has been reported that Xiao-chai-hu-tang and Zhu-ling-tang may increase the immune response through macrophage involvement in vivo and in vitro, and antitumor activities against transplanted Ehrlich carcinoma were observed [11]. Ikemoto et al. examined the effects of Xiao-chai-hu-tang on antibody response in vitro. Their results suggested that Xiao-chai-hu-tang had a enhancing effect on antibody response and that this might be due in part to macrophage activation [10].

Polyporus and Hoelen are components of Zhu-ling-tang, and it has been reported that glucans prepared from Polyporus and Hoelen exert antitumor activities [3, 16, 17].

Bupleuri radix is one of the components of Xaio-chai-hu-tang, and the anti-inflammatory effect of Bupleuri radix is the most common. The possible immunological involvement of Bupleuri radix through activation of macrophages has been discussed [13].

Angelica radix, Cnidii rhizoma, and Cinnamomum cortex are also important components of traditional Chinese preparations which are widely used clinically in Japan and China. It has been reported that polysaccharides separated from Angelica radix can stimulate immunocomponent cells and activate macrophages derived from peritoneal cells [11, 14]. These polysaccharides therefore revealed antitumor activities against leukemia cells and Ehrlich carcinoma. Cnidii rhizoma is reported to have a tumor-sensitizing activity through enhancement of antibody-dependent cytolysis of Raji cells [18].

Old and his coworkers demonstrated that hemorrhagic necrosis of tumors could be transferred by the serum of endotoxin-treated mice which had been presensitized with BCG or *Corynebacterium parvum* [1, 4]. The mediator molecule was termed tumor necrosis factor (TNF), and TNF is known to be a product of macrophages [15, 19, 21]. Purification and physicochemical characterization of murine TNF have been undertaken by Old and our group [20]. We recently reported the cytotoxic activity of murine TNF against murine cancer cells and human cancer cells in vivo [6] and in vitro [5].

The role of initial stimulating agents in the production of TNF has also been investigated by our group [8]. Wide differences in capacity for TNF production are observed among different priming agents. In a previous report we substantiated the involvement of macrophages in TNF production [21]. Hepatosplenomegaly and macrophage hyperplasia in the spleen and liver were observed after administration of the priming agents, and there were good positive correlations between the TNF producibility and the weights of the spleen and liver [7]. For TNF production, it appears that there are two important steps: stimulation of the RES, and release of TNF from the activated site with the aid of LPS [7, 21].

In the present study, traditional Chinese medicines and crude drugs were used as RES-stimulating agents because of their low toxicity and easy administration PO. Although the clinical effectiveness of these drugs has been demonstrated their detailed mechanisms of action remain unknown. To clarify the mechanisms of antitumor activity displayed by these drugs we examined their effectiveness against Ehrlich tumors and the correlation with the capacity for TNF production shown on subsequent LPS administration.

The present findings indicated a good survival rate and sometimes a complete cure for transplanted Ehrlich tumors in the groups given Bupleuri radix, Xiao-chai-hu-tang, Angelica radix, or Cinnamomum cortex. In these groups, the capacity for TNF production was also high, except in the case of Xiao-chai-hu-tang. The group given Hoelen revealed poor results for both antitumor activity and capacity for TNF production. Krestin has been found to exert antitumor activity against several experimental tumors [22, 23]. The group given Krestin had a relatively good survival rate and a complete cure rate of over 50%, though the capacity for TNF production was not so high. The reason for this discrepancy may be the existance of an action regulating the homeostasis of the whole body, that is not only the macrophage involvement but also lymphocyte activation, etc. There was also a discrepancy in the group given Cnidii rhizoma. An aqueous extract of Cnidii rhizoma following precipitation with ammonium sulfate at 100% saturations has been reported to have a tumor-sensitizing activity [18]. The aqueous extract of Cnidii rhizoma is known to contain ligustilide, butilidenephthalide, ferelic acid, and many kinds of unknown substances, which means it has complicated pharmacological actions. The long-term toxicity of this drug has been reported to be the most severe of any of the various drugs. When large doses of the aqueous extract were used (more than twice the ED_{50}), anti-inflammatory, antimicrobial, and antiulcerative actions were suppressed [12]. Cnidii rhizoma had low complete cure and survival rates and a low T_D value in spite of a high capacity for TNF production. In this experiment we used five times the ED_{50}, so that the immunosuppressive effect against tumor-bearing individuals after long-term administration of this dose was considerable.

Following administration of the above drugs to tumor-bearing mice central necrosis was observed, which was very similar to that induced by TNF administration. It is known that tumor-bearing animals are very sensitive to LPS, and we speculated therefore that very low quantities of LPS, for example that of gastrointestinal origin, could be replaced as eliciting agents. Tumor necrosis was observed following administration of TNF-positive sera, which were derived from post-endotoxin mice. However, such sera contain only nanogram quantities of LPS. In the

in vivo Meth A assay system tumor necrosis is induced by LPS at amounts over 10 μg. The tumor-necrotizing activities of TNF and LPS are different in some respects [9]. The antitumor activity against Ehrlich tumors was examined without giving LPS. We speculate that a very low titer of a host-mediated factor like TNF may be induced, and the antitumor activity may be enhanced by this molecule in addition to stimulation of the RES.

The capacity for TNF production broadly paralleled the survival rate in the mice that received transplants of Ehrlich tumors. Large differences in the effects of the various drugs were found among individuals in spite of the administration of similar doses to those used in Western medicine. Such findings are very common in clinical applications and may reflect the complicated mixed functions of Oriental medicines involving unknown mechanisms.

It is suggested that the antitumor activities and capacity for TNF production of the present drugs are probably due partly to stimulation of the RES, including macrophages, and the induction of a host-mediated antitumor substance like TNF.

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