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Inhibition by Shi-Quan-Da-Bu-Tang (TJ-48) of Experimental Hepatocarcinogenesis Induced by *N*-Nitrosomorpholine in Sprague–Dawley Rats

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The effect of Shi-Quan-Da-Bu-Tang (TJ-48) on hepatocarcinogenesis induced by *N*-nitrosomorpholine (NNM) was investigated in male Sprague–Dawley rats. Rats were given drinking water containing NNM for 8 weeks, and also from the start of the experiment, regular chow pellets containing 2.0 or 4.0% TJ-48 until the end of the experiment. Preneoplastic and neoplastic lesions staining for the placental type of glutathione-S-transferase (GST-P) or gamma-glutamyl transpeptidase (GGT) were examined histochemically. In week 15, quantitative histological analysis showed that prolonged administration of either 2.0 or 4.0% TJ-48 in the diet significantly reduced the size, volume and/or number of GST-P-positive and GGT-positive hepatic lesions. This treatment also caused a significant increase in the proportion of interleukin-2 receptor-positive lymphocytes among the lymphocytes infiltrating the tumours as well as a significant decrease in the labelling index of preneoplastic lesions. These findings indicate that TJ-48 inhibits the growth of hepatic enzyme-altered lesions, and suggest that its effect may be in part due to activation of the immune system.
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INTRODUCTION

SHIN-QUAN-DA-BU-TANG (TJ-48) is a traditional Chinese herbal medicine composed of a mixture of 10 medicinal plants [1]. Pharmacologically, TJ-48 has anti-inflammatory and anti-allergic activities. TJ-48 also affects the immune system; it significantly enhances anti-sheep red blood cell (SRBC) response, phagocytosis and mitogenic activity against spleen cells in mice [2].

TJ-48 has been found to improve the general condition of cancer patients receiving chemotherapy and/or radiation therapy [3, 4]. In addition, TJ-48 induces various anti-tumour factors such as tumour necrosis factor (TNF) [5] and interferon [6]. Haranaka *et al.* [7] found that in two-step carcinogenesis experiments involving treatment with dimethylbenzanthracene and 12-0-tetradecanoyl phorbol-β-acetate to induce skin papillomas. TJ-48 appeared to be effective in inhibiting carcinogenesis. These findings suggest that treatment with TJ-48 might inhibit hepatocarcinogenesis. To test this possibility, we have now investigated the effect of administration of TJ-48 to rats on the development of enzyme-altered lesions of the liver induced by *N*-nitrosomorpholine (NNM).

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MATERIALS AND METHODS

Animals

Sixty young (initially, 6-week-old) male Sprague–Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in suspended cages with a wire mesh bottom in a room at 21 ± 1°C with 40 ± 10% humidity and a 12:12 h light/darkness cycle.

Treatments

The rats were randomly divided into three groups of 20 rats each, and were given drinking water containing 175 mg/l of NNM (Sigma, St. Louis, Missouri, U.S.A.) for 8 weeks. NNM was dissolved in distilled water at a concentration of 50 g/l, and stored in a cool place. This stock solution was diluted to 175 mg/l with tap water just before use, supplied to rats from bottles, and renewed every other day. From week 9 onwards, rats were given normal tap water only until the end of the experiment.

From the start of administration of the carcinogen, the animals were also given regular chow pellets (Oriental Yeast, Tokyo, Japan) (Group 1) or chow pellets containing 2.0% (Group 2) or 4.0% (Group 3) TJ-48 (Tsumura, Tokyo, Japan) throughout the experiment.

Two grams of TJ-48 preparation contained spray-dried aqueous extracts of 3.0 g of *Astragali radix*, 3.0 g of *Cinnamomi cortex*, 3.0 g of *Rehmannia radix*, 3.0 g of *Paeoniae radix*, 3.0 g of *Cnidii rhizoma*, 3.0 g of *Atractylodis lanceae rhizoma*, 3.0 g of *Angelicae radix*, 3.0 g of *Ginseng radix*, 3.0 g of *Hoelen* and 1.5 g of *Glycyrrhizae radix*. Chemically, TJ-48 contained at least two types of substance: a protein or glycoprotein molecule insoluble

in methanol and water and a polymer containing acidic pectic polysaccharide [8]. The TJ-48 preparation was standardised according to Regulations for Manufacturing Control and Quality Control of Ethical Extract Products in Kampo Medicine (Oriental Medicine) Formulation, Japan [9].

Histological and histochemical observations of hepatic lesions

In week 15, all surviving rats (not starved) were killed by ether anaesthesia. The liver was promptly excised and sections of 2–3 mm thickness obtained from the left and middle lobes were fixed in cold acetone (0–4°C) for 6 h, and embedded in paraffin. Serial sections of 3 μm thickness were stained with haematoxylin and eosin, for examination of gamma-glutamyl transpeptidase (GGT) activity as described by Ruttenberg *et al.* [10], and for examination of placental type of glutathione-S-transferase (GST-P) by an immunohistochemical PAP method [11] using anti-rat GST-P rabbit serum (Bio Prep Medlabs, Stillogan, CD).

Carcinogen-induced hepatocellular lesions in rats were classified histologically according to the report of the Rat Liver Tumor Workshop [12]. The hepatocellular carcinomas compress or extend into the surrounding parenchyma. The tumour cells may resemble normal hepatocytes, or they may be enlarged or anaplastic. They are in broad sheets in plates one to several cells in thickness.

Volumetric analysis

Serial sections were scored for GGT-positive lesions and GST-P-positive lesions without knowledge of their group of origin. Only preneoplastic or neoplastic lesions of 0.2 mm or more in longest diameter in the plane of section were counted, because reproducible evaluation of lesions of less than 0.2 mm in diameter was impossible. The transectional area of the lesions in the plane of the tissue section and the area of the entire liver section were measured with an LA-500 Personal Image Analyzer System (Pias, Tokyo, Japan). From the measured areas of transected lesions, the number of lesions per unit volume was estimated by the method of Pugh *et al.* [13], and the mean volume of the lesions per unit liver volume was calculated by the method of Campbell *et al.* [14].

Immunological examination

The localisation of interleukin-2 receptor (IL-2R)-positive lymphocytes was determined by an indirect immunoperoxidase technique with monoclonal antibody OX-39 in cryostat sections of the liver [15] in week 15. For this, tissues were removed from the left lobe of the liver of each group, promptly embedded in OCT embedding compound (Raymond A. Lamb, London, U.K.), and stored at −70°C. Cryostat sections of 7 μm thickness were air-dried for 1 h, fixed in absolute ethanol at 0–4°C for 10 min and washed with phosphate buffered saline. Mouse anti-rat IL-2R monoclonal antibody OX-39 (Chemicon, California, U.S.A.) was layered over the sections for 2 h at 4–6°C. The sections were then stained by the avidin–biotin–peroxidase complex method [16] using a Vectastain ABC kit (Vector Laboratories, Burlingam, California, U.S.A.). The percentage of IL-2R-positive lymphocytes among the lymphocytes infiltrating the tumours was determined by counting the labelled cells among lymphocytes in five areas in or surrounding the preneoplastic or neoplastic lesions.

Labelling indices of enzyme-altered lesions and surrounding liver

The labelling indices of the enzyme-altered lesions and the surrounding liver were examined in week 15. The labelling index was measured with an immunohistochemical analysis kit (Becton-Dickinson, Mountain View, California, U.S.A.) for assaying bromodeoxyuridine (BrdU) incorporation [17, 18]. For this purpose, five unstarved rats in each group received an intraperitoneal (i.p.) injection of 20 mg/kg of BrdU, and 1 h later they were killed with ether. Sections obtained from the left liver lobe were immediately mounted on brass chucks using OTC compound, frozen in dry ice-acetone (−86°C), and stored at −70°C. Serial cryostat sections of 6 μm thickness obtained from the frozen slices were fixed in 70% ethanol (0–4°C) for 10 min. After washing, these sections were immersed in 2 N HCl solution for 30 min at room temperature and then in 0.1 mol/l $Na_2B_4O_7$ to neutralise the acid. The sections were then stained with anti-BrdU monoclonal antibody (diluted 1:25) for 2 h at room temperature; they were washed, stained with biotin-conjugated horse anti-mouse antibody (diluted 1:200) for 30 min, and stained with avidin–biotin–peroxidase complex for 30 min. The reaction product was located with 3,3′-diaminobenzidine tetrahydrochloride and cells containing BrdU were identified by the presence of dark pigment over their nuclei. For determination of the labelling index, then numbers of BrdU-labelled cells were determined from a total cell count of 500 cells in either the surrounding liver or in enzyme-altered lesions of 0.7–1.2 mm longest diameter. The labelling index was expressed as the percentage of labelled cells among the cells examined.

Statistical analyses

Results were analysed by the χ^2 test or by one-way analysis of variance with Dunn's multiple comparison [19–21]. Data are shown as means ± S.E. "Significant" indicates a calculated P value of less than 0.05.

RESULTS

Body and liver weights

The body and liver weights of the NNM-treated rats are summarised in Table 1. In week 15, the rats treated with TJ-48 at either dosage had slightly, but not significantly lower body weights than the controls. Treatment with 4.0% TJ-48 resulted in significantly reduced liver weight, but not liver weights per 100 g body weight.

Number, size and volume of enzyme-altered lesions in the liver

Table 2 summarises the numbers, sizes, volumes and relative volumes of GST-P-positive lesions and GGT-positive lesions including hepatocellular carcinoma. Two-dimensional data showed that GST-P-positive lesions and GGT-positive lesions were significantly smaller in groups 2 (2.0% TJ-48) and 3 (4.0% TJ-48) than in group 1 (control). Statistical analysis of the calculated volumetric data showed that the mean volume and the volume as a percentage of parenchyma of GST-P-positive and GGT-positive lesions were significantly less in groups 2 and 3 than in group 1.

Incidence, number, size and volume of hepatocellular carcinomas

Table 3 summarises the incidences, numbers, sizes, volumes and relative volumes of hepatocellular carcinomas in NNM-treated rats. Hepatocellular carcinomas were found in 11 (55%) of the 20 control rats examined. The incidences of hepatocellular carcinomas were significantly less in groups 2 (2.0% TJ-48) and 3 (4.0% TJ-48) than in group 1 (control). The mean area and volume of hepatocellular carcinoma were significantly less in groups 2 and 3 than in group 1.

Table 1. Body weight and liver weight of NNM-treated rats

Group no.	Treatment*	Body weight (g) Initial	Body weight (g) Week 15	Effective no. of rats	Liver weight (g)	Liver weight /100 g body weight
1	None	156 ± 8	410 ± 9	20	18.6 ± 0.6	4.6 ± 0.1
2	2.0% TJ-48	156 ± 6	394 ± 9	20	17.0 ± 0.5	4.5 ± 0.2
3	4.0% TJ-48	158 ± 7	385 ± 6	19	16.4 ± 0.4†	4.3 ± 0.1

*Treatment: rats were given drinking water containing 175 mg/l of NNM for 8 weeks, and fed regular chow pellets (Group 1) or chow pellets containing 2.0% (Group 2) or 4.0% (Group 3) TJ-48. †Significantly different from the value for Group 1 at $P < 0.01$.

Table 2. Number and size of GST-P-positive lesions and GGT-positive lesions of the liver in NNM-treated rats

Enzyme-altered lesions	GST-P-positive lesions			GGT-positive lesions		
Group no.	1	2	3	1	2	3
Treatment*	None	2.0% TJ-48	4.0% TJ-48	None	2.0% TJ-48	4.0% TJ-48
Observed transectional data on lesions						
No./cm²	66 ± 4	59 ± 4	56 ± 4	38 ± 3	22 ± 2†	24 ± 3†
Mean area (mm²)	0.57 ± 0.05	0.29 ± 0.04‡	0.27 ± 0.02‡	0.55 ± 0.07	0.30 ± 0.04†	0.22 ± 0.03‡
Calculated volumetric data on lesions						
No./cm³	1084 ± 84	1369 ± 196	1209 ± 75	611 ± 59	491 ± 59	547 ± 70
Mean volume (mm³)	0.39 ± 0.05	0.16 ± 0.03‡	0.14 ± 0.02‡	0.39 ± 0.07	0.18 ± 0.04‡	0.11 ± 0.02‡
Volume as % of parenchyma	36.7 ± 3.4	17.6 ± 2.5‡	16.1 ± 2.3‡	20.7 ± 2.4	7.2 ± 1.3‡	5.9 ± 1.1‡

*For explanation of treatments, see Table 1. †‡Significantly different from the value for Group 1 at: †$P < 0.01$, ‡$P < 0.001$.

Labelling index of enzyme-altered lesions and % IL-2R-positive lymphocytes

Table 4 summarises date on the labelling indices of enzyme-altered lesions and adjacent normal liver and the percentage of IL-2R-positive lymphocytes. Treatment with 2.0% (group 2) or 4.0% (group 3) TJ-48 significantly decreased the labelling indices of preneoplastic lesions and adjacent normal liver. Neither dosage increased the number of tumour-infiltrating lymphocytes, but both dosages significantly increased the proportion of IL-2R-positive lymphocytes among the lymphocytes infiltrating tumours.

Table 3. Number, size and volume of hepatocellular carcinomas in tumour-bearing rats

	Group no. 1	2	3
Treatment*	None	2.0% TJ-48	4.0% TJ-48
Effective no. of rats	20	20	19
No. of rats with hepatocellular carcinoma (%)	11 (55)	2 (10)‡	3 (16)†
Observed transectional data on lesions			
No./cm²	2.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.0
Mean area (mm²)	8.03 ± 1.17	0.75 ± 0.25†	0.87 ± 0.13†
Calculated volumetric data on lesions			
No./cm³	14.5 ± 3.8	4.0 ± 3.0	2.9 ± 0.6
Mean volume (mm³)	6.49 ± 0.71	0.70 ± 0.10‡	0.30 ± 0.15‡
Volume as % of parenchyma	5.3 ± 1.7	0.8 ± 0.3	0.3 ± 0.1

*For explanation of treatment, see Table 1. †‡Significantly different from the value for Group 1 at: †$P < 0.05$, ‡$P < 0.01$.

DISCUSSION

In the present study, oral treatment with TJ-48 resulted in significant inhibition of the growth of GST-P-positive and GGT-positive lesions in the liver. The exact mechanism(s) of this effect is unknown, but at least two possible explanations can be considered. The first possibility is production of TNF induced by TJ-48. TNF was discovered in 1975 as a compound exhibiting antitumour activity [22]. TNF causes haemorrhagic necrosis of tumours *in vivo* [23], and has cytotoxic effect on cancer cells *in vitro* [24]. Haranaka *et al.* [5] examined the antitumour activities and capacities to induce TNF production of traditional Chinese herbal preparations and crude drugs from TJ-48 (*Hoelen*, *Angelicae radix*, *Cnidii rhizoma* and *Cinnamomi cortex*) in DDY mice transplanted with Ehrlich tumours. They observed good survival and sometimes complete cure of transplanted tumours and a high capacity to produce TNF in the groups given *Angelicae radix* or *Cinnamomi cortex*. Moreover, recently, Haranka *et al.* [7] examined the effects of combination therapy with TJ-48 and OK 432 or mitomycin C on the antitumour activities and TNF production of mice with transplanted Ehrlich or Meth A tumour cells. They found that development of transplanted tumours was strongly inhibited by this treatment, and that TNF production

Table 4. Labelling indices of the enzyme-altered lesions and adjacent normal liver, and % IL-2R-positive lymphocytes

Group no.	Treatment*	Labelling index (%)		IL-2R-positive lymphocytes	
		Enzyme-altered lesions	Adjacent liver	No. of tumour-infiltrating lymphocytes/mm²	% IL-2R-positive lymphocytes
1	None	5.2 ± 0.3	1.2 ± 0.1	121 ± 11	8.0 ± 0.8
2	2.0% TJ-48	2.5 ± 0.3‡	0.4 ± 0.0‡	142 ± 23	15.6 ± 1.4‡
3	4.0% TJ-48	2.4 ± 0.3‡	0.7 ± 0.2†	114 ± 12	16.0 ± 0.2‡

*For explanation of treatments, see Table 1. †‡Significantly different from the value for Group 1 at: †$P < 0.05$, ‡$P < 0.001$.

was increased by treatment with the combination of TJ-48 and OK 432. They also observed marked lymphocytosis, hyperplasia and hypertrophy of Kupffer cells in the liver of tumour-bearing mice receiving TJ-48 or OK 432.

A second possibility is an effect of TJ-48 on the immune system. Phagocytes such as macrophages and polymorphonuclear leucocytes are involved in immunological function, autoimmune disease, inflammation, infection, tumour necrosis and other biological responses. They are also known to release many cytokines that are important in maintaining homeostasis. Maruyama *et al.* [25] found that i.p. or oral treatment with TJ-48 enhanced the phagocytic activity or peritoneal exudate cells. Ito and Shimura [26] examined the effect of aqueous extracts of TJ-48 on implanted tumour cells in mice, and concluded that inhibition of tumour growth by TJ-48 may be due to activation of complement C3 and stimulation of phagocytic activity. Kawamura *et al.* [27] examined the effects of TJ-48 on the anti-sheep red blood cell response in mice, and found that oral administration of TJ-48 augmented this response both *in vivo* and *in vitro* and enhanced cytotoxic lymphocyte induction *in vitro* and delayed type hypersensitivity *in vivo*. Sakagami *et al.* [6] examined the effect of TJ-48 on IL-2 production in mice, finding that the production of IL-2 from the PHA-stimulated peripheral blood mononuclear cells was significantly enhanced by TJ-48. They concluded that the antitumour activity of TJ-48 may be mediated, at least partially, by IL-2 production. In the present work, we found that TJ-48 at both dosages caused a significant increase in the production of IL 2R-positive lymphocytes among the lymphocytes infiltrating tumours. This increase in the proportion of IL-2R-positive lymphocytes is indirect evidence that TJ-48 activates T cells in rats.

Our results show that TJ-48 inhibits the development of enzyme-altered foci induced by NNM. Recently, we started a controlled clinical prospective study on the potential ability of TJ-48 to prevent hepatocellular carcinoma. The actions of TJ-48 are not as well understood as some of those of Western drugs because it comprises a complex mixture of natural products, many of which have yet to be evaluated.

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Relation of Early Photofrin® Uptake to Photodynamically Induced Phototoxicity and Changes of Cell Volume in Different Cell Lines

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For efficacy of photodynamic therapy, selective uptake and retention of photoactive substances has been postulated. Therefore, measurements were performed to find out whether the photosensitiser Photofrin® is taken up differently in malignant and non-malignant cells *in vitro*. In addition, the sensitivity of malignant cells and non-malignant cells to photodynamic exposure was investigated, by quantifying viability and volume alterations of the cells. Bovine aortic endothelial cells, mouse fibroblasts and amelanotic hamster melanoma cells were suspended in a specially designed incubation chamber under controlled conditions (e.g. pH, pO_2, pCO_2 and temperature). After establishing constant baseline conditions, the cellular fluorescence intensity per cell volume, indicative of the uptake of Photofrin®, and cell volume were assessed by flow cytometry, and cell viability was quantified by the trypan blue exclusion test. Photodynamic exposure of cells was performed using an argon-pumped dye laser system via a 600 μm optical fibre at energy density of 4 Joules at the cell surface (40 mW/cm², 100 s). In comparison to endothelial and fibroblast cells, the melanoma cells exhibited no increased uptake of Photofrin®, and no enhanced sensitivity to photodynamic therapy (PDT). However, the fluorescence intensity/volume of endothelial cells was two to three times higher at each concentration of the photosensitiser. Following PDT, reduction in cell viability was dependent on the concentration of Photofrin®, and directly correlated with fluorescence intensity per cell volume. In addition, the cells of all three lines, treated by PDT, revealed dose-dependent changes in cell volume. Melanoma cells exhibited the most excessive increase. It is suggested that selective uptake of photosensitiser *in vitro* is not characteristic for tumour cells. The high uptake of Photofrin® by endothelial cells may indicate that the vascular endothelium is a major target for PDT, leading to cessation of tumour blood flow and subsequent destruction of tumour tissue. In addition, PDT-induced swelling of tumour cells might represent and effect synergistically impairing tumour perfusion, and thereby promoting tumour death.
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INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) is a promising treatment modality in the management of malignant tumours. Therapy using haematoporphyrin derivative (HPD) or Photofrin® is based on preferential labelling of neoplastic tissue by these drugs, producing transiently toxic oxygen species under laser irradiation [1–3]. The mechanisms of tumour destruction in response to PDT are the subject of intensive investigations. In some studies, malignant cells have been shown to take up greater amounts of porphyrin or to exhibit a higher sensitivity to PDT