Abstract. Sarcoma is one of the most prevalent pediatric tumors and the therapeutic role of chemotherapy has yet to be elucidated. It has been reported that extracts of Longyanshen (Yulangsan) may enhance the sensitivity of drug-resistant cancer cell lines, and improve the immune dysfunction induced by cyclophosphamide (CTX) in mice. The present in vivo study investigated the antitumor effects of Yulangsan polysaccharides (YLSPS) and their interaction with CTX in murine sarcoma 180 (S180)-bearing mice. Immunohistochemistry was used to detect the expression of apoptosis-related proteins. The ultrastructure of sarcoma cells was examined by transmission electron microscopy and the tumor growth rate was determined by measuring the tumor weight. A dose-dependent inhibition of sarcoma growth was observed in S180-bearing mice following administration of YLSPS. In combination with CTX, an additive antitumor effect was obtained, which was accompanied by amelioration of immune function. YLSPS also potentiated the tumor suppression effect of CTX while avoiding cytotoxicity against immune cells. YLSPS inhibited sarcoma growth in S180-bearing mice through the induction of apoptosis in S180 sarcoma cells. YLSPS also attenuated CTX-induced cytotoxicity to the immune system while potentiating the tumor suppression effect. These results provide additional information regarding combination therapy with YLSPS and chemotherapy for the treatment of sarcoma.

Introduction

Sarcomas are a heterogeneous group of tumors that are mesenchymal in origin and account for 1% of adult tumors and 15% of pediatric tumors (1,2). Approximately 80% of sarcomas originate from soft tissue and 20% from bone. Similar to other types of solid tumors, conventional therapy includes surgery, radiotherapy and chemotherapy. The role of adjuvant or neoadjuvant chemotherapy for sarcoma has not yet been fully elucidated, as large prospective analyses have not demonstrated their unequivocal benefit (3,4); however, a previous meta-analysis suggested that adjuvant chemotherapy is associated with a moderate overall survival advantage (5). A number of agents are currently used for sarcoma therapy, including doxorubicin, ifosfamide, dacarbazine, cisplatin, vincristine, cyclophosphamide (CTX) and etoposide. Under most circumstances, the combination of ≥2 agents is commonly used in patients with sarcoma, as combination chemotherapy appears to increase response rates; however, it is associated with greater toxicity, with immunosuppression being one of the most serious side effects, with no overall survival advantage (6).

Yulangsan, also referred to as Longyanshen, is a commonly used herb in the indigenous Zhuang community in Guangxi, China, and is derived from the dried roots of *Millettia pulchra Kurz var-laxior* (Dunn) Z. We. Locally, this herb is used for patients recuperating after illness and those concerned about their general health. In the published literature, it has been reported that Yulangsan may enhance the function of the immune system (7). Kong et al found that extracts of Longyanshen (mainly the polysaccharide component) enhance sensitivity to chemotherapy in drug-resistant cancer cell lines (8), and improve CTX-induced immune dysfunction in mice (9).

The present study was designed to investigate the antitumor activity of YLSPS in mice bearing S180 sarcoma tumors. In order to demonstrate the antitumor effect of YLSPS, as well as its effects on the immunological organs, paralleled and
combined studies with CTX were simultaneously conducted under the same experimental conditions.

Materials and methods

Chemicals. YLSPS was prepared as previously described (10). The root of *Millettia pulchra* Kurz var. laxior (Dunn) Z. Wei was dried, and extracted three times with boiling water. The polysaccharide in the filtrate was precipitated fractionally with alcohol. The protein in the product was removed and further purified using diethylaminoethyl (DEAE) ion exchange cellulose (DEAE-52). Gas chromatography and thin-layer chromatography analysis demonstrated that YLSPS was composed of D-glucose and D-arabinose in a molar ratio of 90.79 and 9.21%, respectively, with an average molecular weight of ~14,301 Da. CTX injection was purchased from Shaxi Pude Pharmaceutical Co., Ltd. (Datong, China).

Animals and sarcoma model. A total of 80 male and 80 female BALB/c mice (aged 4-6 weeks and weighing 20±2 g) were purchased from the Institute of Animal Care Center of Guangxi Medical University. The mice were acclimatized for 1 week prior to being used in the study. All animals were cared for in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. The experimental protocols of the study were approved by the Animal Care Committee at Guangxi Medical University (certificate no. SYKG 2013-0005).

Murine sarcoma 180 (S180) cells were injected into the peritoneal cavity of the mice and proliferated to produce ascites. The cell colonies were maintained by weekly transplantation of the tumor cells from the ascitic fluid into the peritoneal cavity of another mouse. S180 cells were isolated from the ascitic fluid and suspended in saline at a density of 1x10⁶ cells/l. Subsequently, 2x10⁶ cells (in 200 µl saline) were injected into the axillary fossa of the right foreleg to prepare the tumor-bearing mice. Two days after S180 cell implantation, the mice were randomly divided into five groups (10 mice per group), as follows: i) CTX group [0.02 g/kg, intraperitoneal (i.p.) injection on days 1, 4, 7 and 10]; ii) high-dose YLSPS group [0.6 g/kg/day, intragastric (i.g.) administration]; iii) intermediate-dose YLSPS group [0.3 g/kg/day, i.g.]; iv) low-dose YLSPS group [0.15 g/kg/day, i.g.]; and v) control group (saline, 0.2 ml/10 g/day, i.g.). The animals were treated for 10 days and then sacrificed by cervical dislocation under ethyl ether anesthesia on day 12.

Calculation of tumor inhibition, spleen index and thymus index. The tumor inhibition rate was calculated as follows: Inhibition (%)=\((C-T)/C\times100\)%, where C and T represent the tumor weights (in mg) of control and treated mice, respectively. Based on the correlation between immune activity and the weights of the spleen and thymus, the relative weights of the spleen and thymus (in mg) with regards to the mouse body weight (10 g) were used to obtain the spleen index (SI) and thymus index (TI), as previously described (11,12).

Immunohistochemistry (IHC). B-cell lymphoma 2 (Bcl-2), p53 and Bcl-2-associated X (BAX) proteins were detected by IHC, as previously described (13). Briefly, all specimens were fixed in formalin, embedded in paraffin and cut into 4-µm sections for IHC. Bcl-2, p53 and BAX were detected by rabbit anti-mouse polyclonal antibodies (N-20; dilution, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and IHC was performed using the immunoperoxidase method as follows: Antigen retrieval was performed using cell conditioning 1 antigen retrieval buffer (pH 7.4) with 1% bovine serum albumin and stained on a BenchMark XT automated slide stainer using a diaminobenzidine detection kit (ultraView Universal DAB detection kit; Ventana Medical Systems). The positive reaction, shown by brown color, was evaluated under a light microscope and scored by two pathologists who were blinded to the origins of the sections. The staining was scored on semi-quantitative scales as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining.

Spleen lymphocyte preparation and cell proliferation assessment. Spleens from S180 sarcoma-bearing mice treated with CTX and/or YLSPS were aseptically removed for the preparation of spleen lymphocytes. The removed spleens were homogenized in sterile Hank’s balanced salt solution (HBSS) and the homogenized spleen tissue was passed through a mesh (200 mesh sieve) and washed twice with HBSS. The erythrocytes were lysed with red blood cell lysis buffer, while the remaining cells were suspended in RPMI-1640 medium with 10% fetal calf serum. Cells were counted with a hemocytometer using the trypan blue exclusion technique (14). Cell viability exceeded 95% and cells were resuspended with complete medium at a density of 1x10⁵ cells/l. ConA-stimulated cell proliferation was detected with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, cell suspensions (100 µl/well) were added to 96-well culture plates followed by the addition of 100 µl ConA (5 mg/l). The cells were then incubated at 37°C in a humidified, 5% CO₂ atmosphere for 68 h. Subsequently, 10 µl MTT (5 mg/ml) was added into each well followed by a 4-h additional incubation. The cultures were centrifuged at 444 x g for 5 min and the supernatant was carefully removed. After adding 200 µl dimethyl sulfoxide, absorbance was assessed with a microplate reader at a wavelength of 570 nm (A570; Jupiter ASYS, Montreal Biotech, Kirkland, PQ, Canada).

Transmission electron microscopy. Mouse sarcoma samples were fixed with 4% paraformaldehyde and 1% glutaraldehyde. The fixed tissues were washed with phosphate buffer (pH 7.4) and post-fixed with osmium tetroxide. These tissue samples were dehydrated through a series of graded ethanol solutions and propylene oxide, and then embedded in epoxy resin. Ultrathin sections (0.5 µm) of each sample were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM; JEM-1200EX, JEOL, Tokyo, Japan) with an accelerating voltage of 80 kV.

Statistical analysis. Numerical data were expressed as mean ± standard error. Statistical differences between the means for the different groups were evaluated with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) using Student’s t-test, with the level of significance set at P<0.05.
Results

Antitumor effect of YLSPS in mice bearing SI80 solid sarcoma tumors. The tumor growth was significantly inhibited by YLSPS administration at a low dose (39.7%), intermediate dose (41.3%) and high dose (52.6%), compared with control mice. The dose-dependent pattern of YLSPS-induced inhibition indicated the therapeutic effect of YLSPS on SI80 sarcoma tumors. To provide an antitumor effect comparable with commonly used anticancer drugs, CTX was also used at a standard dose (0.02 g/kg) in separate positive-control mice, resulting in a tumor inhibition rate of 71.1%.

YLSPS- and CTX-induced changes of the SI and TI in SI80 sarcoma-bearing mice. Immunosuppression is considered to be a major side effect of anticancer agents. As such, the changes in the SI and TI induced by YLSPS and CTX treatment were compared. YLSPS increased the SI as well as the TI at all three dose levels. With increasing YLSPS dose (0.15, 0.30 and 0.60 g/kg/day), the SI values were 90.89±16.13, 92.93±7.26 and 104.52±8.22 mg/10 g, respectively, with related TI values of 29.49±7.11, 33.81±6.02 and 38.39±5.50 mg/10 g, respectively. The SI and TI values were 81.90±6.95 and 26.90±4.94 mg/10 g in the control group. By contrast, CTX significantly decreased the SI and TI compared with the YLSPS and control groups (28.45±7.65 and 8.76±2.59 mg/10 g, respectively).

Expression of apoptosis-related proteins in the sarcoma tissue of SI80-bearing mice. The changes in p53, Bcl-2 and BAX expression following administration of YLSPS or CTX are summarized in Fig. 1. The accumulated scores represent the relative expression of the studied proteins in the sarcoma tissues of SI80-bearing mice.

Ultrastructural changes of sarcoma cells. The majority of the cells in the sarcoma tissue were ceroid-shaped, with increased internuclear distance and atypical nuclei (Fig. 2A). SI80 cells exhibited nuclear membrane integrity, typical organelles and mitotic activity (Fig. 2B). Following treatment with a low dose of YLSPS, the cell size decreased, with formation of mitochondrial vacuoles (Fig. 2C). SI80-bearing mice treated with an intermediate dose of YLSPS exhibited an increased number of apoptotic cells with overt karyopyknosis and disordered cell structure (Fig. 2D). After treatment with a high dose of YLSPS (Fig. 2E), there was an increase in cell debris in the intercellular space accompanied by nuclear fragmentation and the formation of apoptotic bodies. Furthermore, in addition to organelle fragmentation and apoptotic body formation, the cell membrane structure completely dissolved following treatment with CTX (Fig. 2F).

Effects of combined YLSPS and CTX treatment on tumor growth and immune function. The additive effect of YLSPS and CTX is shown by the q-value: q=EAB/[EA+(1-EA) EB], where EA and EB are the tumor inhibition rates of drug A and drug B, respectively, and EAB is the tumor inhibition rate of drugs A and B used in combination. In this case, q=0.85-1.25 indicates an additive effect of drugs A and B, q>1.25 indicates enhancement (or a synergistic effect) of the two drugs, and q<0.85 indicates that the two drugs are antagonistic (15).

The tumor inhibition rate was 64.8% for the CTX treatment group, whereas for the high, medium and low doses of YLPS together with CTX, the tumor inhibition rates were 74.8, 76.1 and 67.4%, respectively, with related q-values of 0.905, 0.984 and 0.887, respectively. All the q-values were in the range of 0.85 to 1.25, which indicated that different doses of YLSPS may enhance the CTX antitumor effect on mouse SI80 tumors in an additive manner.

To investigate the mechanisms through which YLSPS potentiates the antitumor effect of CTX, the SI, TI and peripheral blood leukocyte count were assessed following administration of various drug combinations. As shown in Table I, the administration of CTX significantly inhibited tumor growth, but its antitumor activity was accompanied by a decrease in SI, TI and peripheral blood leukocytes. However, the combination of CTX with YLSPS at the three tested doses achieved further tumor growth inhibition, but improved the SI, TI and peripheral blood leukocyte count compared with CTX alone. The results of spleen lymphocyte proliferation and tumor necrosis factor α (TNF-α) production analyses in SI80-bearing mice were in accordance with the YLSPS-induced SI and TI changes following CTX treatment (Table II).

Discussion

Sarcomas are tumors of mesenchymal origin that comprise ~1% of human cancers. Soft tissue sarcomas are a relatively rare and heterogeneous group of malignancies that are characterized by mesodermal differentiation (16). The international incidence is estimated to be ~1.8-5 per 100,000 people annually (17). There are at least 50 different subtypes of soft tissue sarcoma, with new ones described at an ever increasing frequency (2). Sarcomas present a challenge with regards to their treatment due to their rarity, biological heterogeneity and the need for multimodality therapy. Current conventional cancer therapies (surgery, chemotherapy and radiotherapy) are, to a significant extent, symptomatic and passive in nature. The majority of cancer patients (including those with sarcoma) succumb to recurrence, metastasis, or therapy-related life-threatening complications (18,19), in which chemotherapy-induced immunosuppression plays a major role. Certain polysaccharides extracted from various
Chinese herbs have been demonstrated to possess antitumor properties in recent studies, including the polysaccharides from *Ganoderma lucidum* (20), *Ganoderma atrum* (21) and ginseng neutral (22). One of the most obvious advantages of herbal-extracted polysaccharides is their low incidence of side effects and potent anticancer activity. In the present study, the
antitumor effect of YLSPS was investigated in combination with CTX, using a model of S180 sarcoma-bearing mice.

YLSPS significantly inhibited the growth of S180 tumors at three dosages; however, the rate of inhibition with YLSPS was lower compared with that of CTX. However, CTX exerted obvious damage to the immune organs. The changes in the apoptosis-related protein expression levels, i.e., the decrease in Bcl-2 and the increase in BAX, suggested the involvement of S180 cell apoptosis induced by administration of YLSPS (Fig. 1). This hypothesis was supported by the apoptotic appearance of cells observed under TEM, i.e., condensation of the chromatin at the margins of the nuclei, and disintegration of the nucleus and the cytoplasmic vacuoles (Fig. 2). An additive antitumor effect was obtained when YLSPS was combined with CTX in S180 sarcoma-bearing mice. Interestingly, in this combined therapy, YLSPS ameliorated CTX-induced changes in the SI, TI and peripheral leukocyte count (Table I). This additive effect of YLSPS on CTX was confirmed by consideration of the q-values.

The beneficial effect of YLSPS on CTX with regards to the treatment of sarcoma in S180-bearing mice may be considered to have two aspects. First, YLSPS itself exerted an antitumor action, most likely through the induction of S180 cell apoptosis, although this activity was weaker in comparison to that of CTX alone. Second, YLSPS attenuated CTX-induced toxicity in the immune system, which was shown by the changes in the SI and TI (Table I), as well as the CoA-induced proliferation of splenic lymphocytes and TNF-α production in S180-bearing mice (Table II).

In conclusion, YLSPS inhibited sarcoma growth in S180-bearing mice. The antitumor effect of YLSPS likely resulted through the induction of apoptosis in S180 sarcoma cells. YLSPS also attenuated CTX-induced immune system cytotoxicity when used in combination, thereby potentiating the tumor suppression effect of CTX, while limiting side effects. These results provide additional information on combination therapy for the treatment of sarcoma.

Acknowledgements

The authors are grateful to James Dai for his assistance in preparing the manuscript. The present study was supported by the Guangxi Science and Technology Development Project (grant no. 14124003-8), and the Guangxi Natural Science Foundation (grant nos. 2013GXNSFAA019253 and 2015GXNSFAA139163).

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