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Antitumor and immunostimulatory activity of a polysaccharide–protein complex from *Scolopendra subspinipes mutilans L. Koch* in tumor-bearing mice

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ABSTRACT

Scolopendra subspinipes mutilans L. Koch has been used for cancer treatment in traditional Chinese medicine for hundreds of years. In this study, the effects of a polysaccharide–protein complex from *Scolopendra subspinipes mutilans L. Koch* (SPPC) on the tumor growth and immune function were assessed in sarcoma S180 and hepatoma H22 bearing mice. Results showed that SPPC significantly inhibited the growth of S180 transplanted in mice and prolonged the survival time of H22- bearing mice. In S180-bearing mice, it promoted specific and nonspecific immune response as evidenced by enhancing the activities of natural killer (NK) cells, cytotoxic T lymphocytes (CTL) and the ratio of Th1/Th2 cytokines, and increasing the percentages of $CD4^+$ T cells, B cells and NK cells. Furthermore, SPPC not only significantly inhibited mRNA expression and production of the immunosuppressive cytokines (IL-10 and TGF-β), but also diminished arachidonic acid (AA)-metabolizing enzymes (COX-2 and CYP4A) and their products (PGE_2 and 20-HETE) in tumor-associated macrophages (TAMs). Taken together, our results indicate that SPPC inhibits tumor growth *in vivo* by improving antitumor immune responses at least partly *via* downregulating AA-metabolic pathways in TAMs, and could act as an anti-tumor agent with immunomodulatory activity.

1. Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. Although standard therapeutic protocols for cancer, including surgery, radiotherapy, chemotherapy, cancer vaccine and antibody therapy, have been improved during the last three decades, no particular regimen has provided a significant improvement in outcomes (Drake and Antonarakis, 2010; Chen et al., 2012). Therefore, it is very imperative to investigate novel antitumor strategies for eliminating the cancer cells with little toxicity to normal cells.

Immune suppression is clearly observed in cancer patients and tumor-bearing animals (Vasievich and Huang, 2011). It has been suggested that tumor cells impair the function of immune system through various mechanisms, including production of immunosuppression cytokines, inhibition of the function of immune cells, protection against lytic activity of immune effectors, reduction of the Th1/Th2 cytokine ratio, and activation of tumor-associated macrophages (TAMs) (Agarwal et al., 2010; Russo, 2011; Zamarron and Chen, 2011). Recently, it has been reported that aberrant metabolism of arachidonic acid (AA) results in increased production of eicosanoids such as prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and hydroxyeicosatetraenoic acid (HETE), all of which can lead to an immunosuppressive tumor microenvironment (Greene et al., 2011; Zhang and Dai, 2011). Therefore, therapeutic approaches directed toward the microenvironment *via* downregulating the AA metabolism could represent a novel strategy to counteract advanced tumor (Daurkin et al., 2011).

Many polysaccharide–protein complexes with immunomodulatory and antitumor activities have been isolated from *Lycium barbarum*, *Pleurotus geesteranus*, *Gekko*, *Grifola frondosa* (Gan et al., 2004; Chen et al., 2010; Chan et al., 2011; Zhang et al., 2011), and could be ideal candidates for therapeutics for tumor due to little toxicity to host (Ooi and Liu, 2000). The dried whole body of *Scolopendra subspinipes mutilans L. Koch* (class Chilopoda, phylum Arthropoda) has been used for cancer treatment in traditional Chinese medicine for hundreds of years. The water extracts of the organism were reported to possess antitumor and immunopotentiating activities (Cohen and Quistad, 1998; Xu et al., 2010; Zhou et al., 2011). However, to our knowledge, the active constituents of this drug and its mechanism of action remain largely unexplored. In the present study, we focused on a novel polysaccharide–protein complex from *Scolopendra subspinipes mutilans*

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L. Koch (SPPC) for its antitumor and immunostimulatory activity in tumor-bearing mice. The results will be helpful to develop novel drugs and functional foods.

2. Materials and methods

2.1. Drug and reagents

The dried whole bodies of *Scolopendra subspinipes mutilans L. Koch* were obtained from a centipede farm, Dangyang city, Hubei province and authenticated by our experts at the School of Pharmaceutical Sciences, Wuhan University, Wuhan, China. A voucher specimen (No. 20060209) was deposited in the Center for Drug Nonclinical Safety Studies of Wuhan University.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS) and AA were purchased from Sigma, Chemical Co., Saint Louis, Missouri, USA; RPMI-1640 medium and fetal calf serum (FCS) were purchased from Gibco, Grand Island, NY, USA; phycoerythrin (PE) anti-mouse CD4, fluorescein isothiocyanate (FITC) anti-mouse CD8, phycoerythrocyanin 5 (PECy5) anti-mouse CD19, PE anti-mouse CD49b were procured commercially (e-Bioscience or BD Biosciences); Two-site sandwich enzyme-linked immunosorbent assays (ELISA) for mouse interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4) and interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) were purchased from Pierce Biotechnology, Rockford, IL, USA; Enzyme immunoassay (EIA) kits for mouse PGE_2 and LTB_4 were purchased from Cayman Chemical, Ann Arbor, MI, USA; Trizol and M-MLV Reverse Transcriptase were purchased from Invitrogen Corp; SYBR Green PCR Master mix was purchased from Applied Biosystems. All other chemicals used were of analytical grade.

2.2. Isolation, purification and identification of SPPC

Isolation, purification and identification of SPPC were based on our previous published work (Yang et al., 2010). Briefly, whole animals (500.0 g) were ground into powder and extracted three times with hot distilled water (5, 4, and 2 l) at 90 °C for 6, 4, 2 h after being defatted twice for 3 h with methanol (4 and 2 l). Then the water solutions were filtered and concentrated in a rotary evaporator under reduced pressure. The concentrated product was precipitated by adding four volumes of ethanol (95%) at 4 °C for 24 h, followed by centrifugation for 10 min at 400*g*. The precipitate was dissolved in distilled water (250 ml). Solid ammonium sulfate was slowly added to the solution with stirring until its concentration got to 40–60% at 4 °C for 4 h. Sixty percentage saturation sediment was redissolved in distilled water (100 ml), and dialyzed against distilled water (cut-off Mw 5 kD). The retentate portion was concentrated, and lyophilized to afford crude SPPC (25.60 g). The crude SPPC was subjected to the DEAE Sephadex A-50 column chromatography (40 × 2.6 cm), and eluted with NaCl (0.1–2 mol/L) gradients. Acidic fractions were collected between 0.2 and 0.25 mol/L ionic strength and dialyzed, concentrated under a reduced pressure to an appropriate volume. The concentrated product was further chromatographied on a column of Sephadex G-200 gel filtration column (60 × 2.6 cm). The fraction with molecular weight between 12 and 13 kD was collected and lyophilized to yield purified SPPC (19.90 g). The percentage yield of the SPPC was 3.98% (w/w) and its structure was detected by ultraviolet, infrared spectroscopy, ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-nuclear magnetic resonance (^{13}C-NMR). The SPPC sample was composed of 91.8% polysaccharides (60.3% galactose, 19.0% fructose, 5.4% arabinose, 2.6% rhamnose, and 2.4% Mannose) and 8.2% proteins (18 different amino acids, mostly aspartic acid and glutamic acid). It contained α-glucose linkage (infrared spectroscopy: 851 cm^{-1}) and the linkage between glycan and protein was through glycan-O-serine by β-elimination method. It has a molecular weight of 128 kD, analyzed by laser light scattering (LLS), and was endotoxin-free demonstrated by Limulus amebocyte lysate test.

2.3. Cell lines

Mouse sarcoma S180 cell lines, mouse hepatoma H22 cell lines, EL-4, a murine T-cell lymphoma cell line (H-2b) and Yac-1 selected for resistance to NK cell lines were obtained from Chinese Centre for Type Cultures Collections, Wuhan University, Wuhan, China. They were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with 2 mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS at 37 °C under humidified air with 5% CO_2.

2.4. Experimental animals

Specific pathogen-free (SPF) BALB/c mice aged 5 weeks were obtained from Experimental Animal Center (Certificate No. 2006005), Wuhan University, Wuhan, China. Half of them were male and the others were female. The mice were acclimatized for at least 1 week before the study. Rodent laboratory chow and tap water were provided *ad libitum* and maintained in an air-conditioned SPF room with a temperature of 24 ± 1 °C, humidity of 50 ± 10%, and a 12/12 h light/dark cycle. All animal studies maintained in accordance with the standards established by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and were approved by the university committee for animal experiments.

2.5. Treatment and drug administration

Ascetic fluid was drawn out from tumor bearing mice under aseptic conditions and then diluted 4-fold with aseptic saline. The diluted tumor cell suspension was subcutaneously inoculated (0.2 ml, 2×10^6 cells/mouse) into the right armpits of BALB/c mice at day 0. The mice were randomly divided into five groups (10 mice in each group), three SPPC (5.0, 10.0, 20.0 mg/kg)-treated groups, S180-bearing group (model control group), and normal control group. SPPC was dissolved in normal saline, and then administered intragastrically once a day for 10 days, starting 24 h after tumor inoculation. A normal control group without tumor inoculation and drug administration was also used in this experiment. The mice in model control group and normal control group received an equal volume of normal saline (0.2 ml/10 g body weight). The mice were examined for localized tumor, and the tumor size was measured once every two days (beginning 1 day after injection) with micro calipers. Tumor volume was measured with a digital caliper and calculated using the formula $0.52 \times a \times b^2$, wherein *a* and *b* are the largest and smallest diameters as described previously (Cai et al., 2008). Twenty four hours after last tested administration, all the tumor-bearing mice were weighed and sacrificed by cervical dislocation on day 11. The spleen, thymus and tumor were immediately dissected and weighed. The spleen and thymus indices were calculated according to the following formula: spleen and thymus index (mg/10 g) = (weight of spleen or thymus/body weight). Blood samples with and without the anticoagulant were collected in polystyrene tubes. Serum samples were separated by centrifugation at 3000 rpm at room temperature for 10 min and stored at −80 °C until assayed.

For survival studies, H22 cells (0.2 ml, 2×10^6 cells/mouse) were implanted (right armpit) so as to observe the mice for longer time period. All experiments were performed in triplicate.

2.6. Splenocyte proliferation assay

The spleens collected from the above treated groups under aseptic conditions were immediately minced into small pieces and passed through sterilized stainless sieves (400 mesh) to obtain homogeneous cell suspensions. Recoverd spleen cells were resuspended in lysis buffer (0.15 M NH_4Cl, pH 7.2) for 5 min to remove erythrocytes. Cell viability (over 95%) was assessed microscopically using trypan blue dye exclusion technique. Splenocytes were seeded in 96-well plates (1×10^7 cells/well), thereafter Con A (2.5 μg/ml), or LPS (5.0 μg/ml) were added. After the cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 for 44 h, 50 μl of MTT solution (2.0 mg/ml) were added to each well and incubated for another 4 h. The medium was discarded and 100 μl dimethylsulfoxide (DMSO) was then added. The absorbance was evaluated in a microplate reader at 570 nm with a 630 nm reference after 30 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.7. Assay for the activities of natural killer (NK) cells and cytotoxic T lymphocytes (CTL)

2.7.1. NK activity assay

NK cells in splenocytes from mice of each treatment group were purified by magnetic-activated cell sorting (MACS) using a negative selection kit (NK cell isolation Kit mouse, Miltenyi Biotec) that included a mixture of antibodies against CD4, CD5, CD8a, CD19, Ly-6G, and Ter-119 and depletes the non-NK cells according to the manufacture's instructions. The purity of sorted cells was established and always greater than 93% by flow cytometry analysis.

The activity of NK cell was measured using the standard ^{51}Cr-release assay as previously described with minor modification (Zeytin et al., 2008). Briefly, Yac-1 cells as the target cells were maintained in RPMI 1640 containing 10% FCS and antibiotics in a 5% CO_2 incubator at 37 °C, and cultured to log phase growth. These target cells (1×10^6) were labeled with 250 μCi of $Na_2[^{51}Cr]O_4$ (Institute of High Energy Physics Chinese Academy of Sciences) for 30 min at 37 °C, washed, and resuspended at a concentration of 1×10^5 cells/ml. The NK cells (100 μl) of control or SPPC-treated groups were cocultured with $Na_2[^{51}Cr]O_4$ labelled Yac-1 cells (100 μl) at an effector to target (E:T) ratio of 50:1 in triplicate cultures in 96-well round-bottom plates for a total volume of 200 μl/well. After 5 h incubation at 37 °C in 5% CO_2 atmosphere, the cell-free supernatants were harvested and the radioactivity was counted by a Beckman 5500 gamma counter (Beckman Scientific Instruments, Irvine, CA). Specific cellular cytotoxicity was determined by the following formula: percentage of specific lysis = (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100. Maximum cpm was obtained by adding Triton X-100 (0.25%) (Sigma, St. Louis, MO) to the target cells in the absence of effector cells. Spontaneous cpm was the radioactivity from wells with target cells alone. EL-4 cells were used as negative control target cells. All assays were done in triplicate.