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Effective inhibition of a *Strongylocentrotus nudus* eggs polysaccharide against hepatocellular carcinoma is mediated via immunoregulation *in vivo*

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ABSTRACT

This study was aimed at evaluating the inhibitory effect of a polysaccharide that was isolated from *Strongylocentrotus nudus* eggs (SEP) against hepatocellular carcinoma in H22-bearing mice and elucidating its immunological mechanisms by determining its effects on the growth of transplanted tumors and immune response in H22-bearing mice. ICR mice inoculated with mouse hepatoma carcinoma cell lines H22 were treated with SEP at doses of 4, 8, 16 mg/kg/d for 12 days. The effects of SEP were measured via the growth of the transplanted tumors, splenocyte proliferation, T lymphocytes counts, CTL activity, the production of cytokines from splenocytes and the levels of serum Ig in tumor-bearing mice. In addition, the effects of SEP on Erk phosphorylation in mouse splenocytes and on the transcriptional activity of NFAT in Jurkat T cells were also investigated. Our results showed that SEP significantly inhibited the growth of transplanted tumors in mice. SEP could not only remarkably enhance splenocyte proliferation, $CD4^+$ and $CD8^+$ T cell numbers as well as CTL activity, but it also elevated IL-2 and TNF-α secretion as well as IgA, IgM and IgG levels in the serum. Furthermore, the activation of Erk phosphorylation and the NFAT promoter by SEP promoted the transcription and expression of downstream gene IL-2. In conclusion, our study demonstrates that SEP effectively inhibits hepatocellular carcinoma *in vivo* via enhancement of host immune system function, and it could be a potential therapeutic drug for hepatocarcinoma.

1. Introduction

Malignancy is one of the most serious diseases in humans and accounts for 12% of all deaths worldwide according to estimates by the World Health Organization (WHO) [1]. Hepatocarcinoma is a kind of tumor with both a high incidence and lethality rate. Although administration of chemotherapeutic agents is the primary approach to cancer treatment, severe adverse effects, such as immune system damage, limit their use and development. Moreover, immunomodulatory agents, including either natural or synthetic products, may be a possible means to inhibit tumor growth without harming the host [2,3]. Therefore, it is very important to investigate effective antitumor substances with improved immunity potential.

T cells, having a central role in the generation and regulation of the immune response to tumor antigens, are divided into either $CD4^+$ or $CD8^+$ subsets. CD4 T helper cells (Th) are important regulators of the immune system [4,5], and they secrete a series of cytokines, such as IL-2, IFN-γ and TNF-α, to initiate antibody production in B cells and enhance the production of CD8 cytotoxic T cells [6]. CD8 cytotoxic T lymphocytes (CTL) are major effectors in protection against cancer and against many infectious diseases [7]. IL-2 is associated with many immunopotentiating effects, such as the proliferation of T cells, B cells, NK cells and monocytes; augmentation of the cytotoxicities for T and NK cells; and *in vivo* generation of lymphokine-activated killer (LAK) cells, which exhibit high cytolytic activities against autologous tumor cells [8]. Cytokine gene transcription is initiated via the participation of transcription factors such as NFAT, AP-1, and NF-κB. NFAT was initially identified as an inducible nuclear factor that could bind the IL-2 promoter in activated T cells [9].

Sea urchins belong to echinoderm [10], and their roes or eggs are a favorite seafood in China, where they are valued for their pleasurable taste and high nutritional value. According to traditional Chinese medicine, sea urchins have broad pharmacological functions, such as curing precordial pain and enhancing immunity [11]. Recently, many polysaccharides that were isolated from biological organisms have attracted a great amount of attention due to their effective treatment, low toxicity and limited side effects [12]. In our previous study, *Strongylocentrotus nudus* eggs polysaccharide (SEP), which was found to be a D-glucan containing an α-(1 → 4)-linked backbone (branched α-(1 → 6)-linkage) (Fig. 1), was proven to inhibit the growth of an S180 tumor *in vivo* [13], and induce

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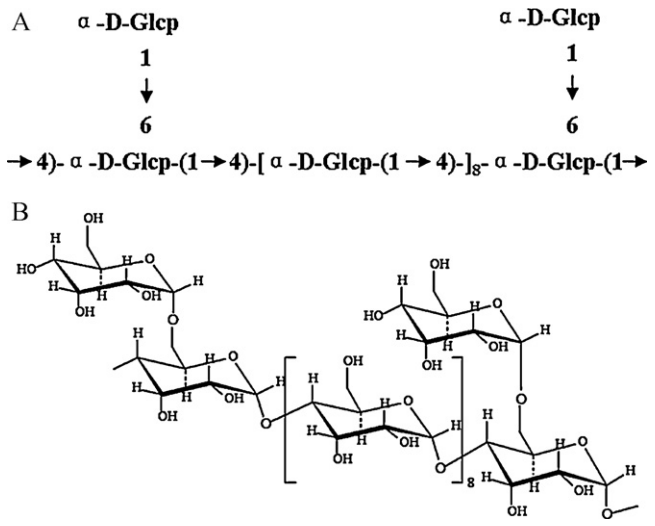

Fig. 1. Structure of SEP. (A) Predicted structure of SEP. (B) Molecular structure of SEP.

spleen proliferation and IL-2 production via activation of TLR2 and TLR4 [14]. However, its efficacy on hepatocarcinoma has not been investigated.

In the present study, we investigated for the first time the antitumor and immunomodulatory activity of SEP in H22-bearing mice via analyzing *in vivo* tumor growth, splenocyte proliferation, splenocyte cytokine expression, T lymphocytes counts, CTL cytotoxicity and Ig levels. Our data showed that SEP could activate NFAT transcription factors, induce IL-2 gene transcription and protein production, and promote T cells to proliferate and differentiate into CTL, which protect against the growth of H22 carcinoma transplanted in mice.

2. Materials and methods

2.1. Preparation of Strongylocentrotus nudus eggs polysaccharide

Dalian purple sea urchins were collected from the Huanghai Sea, China. After removing the shell, spines and intestine, the eggs were immediately frozen at −20 °C for further use. SEP was isolated and purified from *Strongylocentrotus nudus* eggs as described previously [13]. Briefly, the crude, water-soluble polysaccharide from the eggs of sea urchins was separated and sequentially purified via Cellulose DE-52 and Sephacryl S-400 to yield the SEP polysaccharide. The main fraction (SEP) was collected for subsequent analysis. A sample solution of SEP (20 μl) was analyzed via a high performance liquid chromatography (HPLC) system that was equipped with a TSK gel 4000 PW_{XL} column and a Waters 2414 refractive index detector (Sigma–Aldrich, USA). Distilled water was the mobile phase at a flow rate of 0.8 ml/min, and the column temperature was maintained at 30 °C. The HPLC profile showed a single symmetrical, sharp peak, and the content of SEP was 98.0%. To remove endotoxin contamination, 1 ml of Affi-Prep Polymyxin Matrix was packed in a Bio-spin column; centrifuged at 200 × *g* for 2 min; and then 0.5 ml of either SEP (100 μg/ml), LPS (2 μg/ml) or a mixture of SEP (100 μg/ml) and LPS (2 μg/ml) was added. After incubating overnight at 4 °C, the effluent was collected and centrifuged under the same condition. The endotoxin level of SEP was determined using the E-TOXATE kit [15].

2.2. Reagents

The RPMI-1640 medium, fetal calf serum and antibiotics solution were provided by Gibco (Vienna, NY, USA). The injectable cyclophosphamide (Cy) was obtained from Hualian Pharmaceutical Co. Ltd. (Shanghai, China). The Ig kit was purchased from Sun Biological Products Co. Ltd. (Shanghai, China). Anti-CD3, anti-CD4 and anti-CD8 antibodies, as well as cytokine-detecting ELISA kits (IL-2 and TNF-α), were obtained from eBioscience (San Diego, CA, USA). A rabbit polyclonal antibody against phosphorylated Erk (P-Erk), as well as the Phototope-HRP Western Blot Detection System, which included an anti-mouse IgG horseradish peroxidase (HRP)-labeled secondary antibody, biotinylated protein ladder, 20× LumiGLO reagent and 20× peroxide, were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibodies against total Erk (T-Erk) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Affi-Prep Polymyxin Matrix and Bio-spin columns were obtained from Bio-Rad Laboratories (USA). The 3-(4, 5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS), and E-TOXATE kit were purchased from Sigma–Aldrich Biotechnology LP and Sigma–Aldrich Co. (St. Louis, MO, USA). The Jurkat T cell line was ordered from the American Type Culture Collection (ATCC, TIB-152), and the Luciferase reporter plasmid NFAT-luc was obtained from BD Biosciences (San Diego, CA, USA).

2.3. Animals and cell lines

Male ICR mice between 6 and 8 weeks of age (weight: 18–22 g) were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, Jiangsu, China) and acclimatized for 1 week before use. Animals were provided with continuous standard rodent chow and water, and they were housed in a rodent facility at 22 ± 1 °C with a 12-h light-dark cycle. All procedures involving animals and their care in this study were in strict accordance with protocols that were approved by the Ethics Committee of China Pharmaceutical University.

The mouse H22 hepatocarcinoma cell lines that were used in this study were preserved in the Department of Microbiology, School of Life Science and Technology, China Pharmaceutical University. H22 cells were cultured in RPMI-1640 medium that was supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 U/ml of streptomycin in a humidified, 5% CO_2 atmosphere at 37 °C.

2.4. In vivo tumor xenograft model and administration

Sixty male mice were randomly divided into six groups ($n = 10$). One group of healthy mice without medicine administration and tumor inoculation was used as a normal control (NS group). Seven-day-old H22 ascites (0.2 ml, 2×10^7 cells) were transplanted subcutaneously into the right axilla of each mouse in the other five groups. Twenty-four hours after tumor implantation, the mice were administered the following: the NS group was administered normal saline; the model group (tumor control) was administered normal saline; the three SEP groups were administered either 4, 8 or 16 mg/kg/d body weight SEP; and the positive control group was administered 20 mg/kg/d body weight Cy. All of the solutions were dissolved in saline, filtered through a 0.22-μm Millipore filter and administered daily via intraperitoneal injection (200 μl) for 12 days.

Twenty-four hours after the last drug administration, peripheral blood samples from mice of each group were collected and kept at 4 °C for measurement of serum immunoglobulin. Then all animals were weighed and sacrificed via cervical dislocation. The spleens were immediately removed and weighed, and were used freshly for the splenocyte proliferation assay, IL-2 expression, T-lymphocyte subsets determination and western blot assay. Samples of mice spleen and thymus in each group were kept in 10% formalin for histochemical examinations.