

Silkworm powder containing manganese superoxide dismutase regulated the immunity and inhibited the growth of Hepatoma 22 cell in mice

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Received: 4 October 2007 / Accepted: 12 November 2007 / Published online: 22 November 2007
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Abstract The effects of SOD contained silkworm powder on immune regulation and inhibition against Hepatoma 22 tumor cells *in vivo* were investigated. The activity of natural killer cell (NK) and the ConA-stimulated spleen proliferation were measured. The results found that the SOD-contained silkworm powder caused an enhancement on NK cell activity, which implied this material modulated the immune system in mice *in vivo*. The NK cell activities of Hepatoma 22 tumor modeled mice treated with silkworm powder including SOD were increased significantly compared to a modeled control and silkworm powder without SOD, reaching 36.18%. In addition, the ConA-stimulated spleen proliferation of SOD treated mice was higher than that of the controls. The treatment of SOD contained silkworm powder presented 40.3% of average inhibition rate to Hepatoma 22 tumor, showing stronger inhibition against tumor. There were no significant differ-

ence in body weight between modeled control and SOD silkworm powder feeding in Hepatoma 22 tumor modeled mice, suggesting the SOD silkworm powder is safety as an inhibitor to tumor. In conclusion, these findings demonstrate that administration of silkworm powder containing SOD results in activation of NK cells and immunity, suggesting the silkworm powder containing SOD plays a positive role in tumor inhibition.

Keywords SOD-contained silkworm powder · Natural killer (NK) cells · Hepatoma 22 modeled mice · Immune regulation · Spleen proliferation

Introduction

Superoxide dismutase (SOD, EC 1.15.1.1) is a metalloenzyme, which catalyzes the conversion of the superoxide radicals into molecular O₂ and H₂O₂ and thus form a crucial part of the cellular antioxidant defense mechanism [1]. The amount of SOD present in cellular and extracellular environment is crucial for the prevention of disease linked to oxidative stress. Mutations in SOD account for approximately 20% of familial amyotrophic lateral sclerosis (ALS) cases [2].

Oxidation free radicals have caused more and more interests in medical. Researchers have found that there are close relationship between oxidation free radicals and tumor development. Antioxidants can cancel out the cell-damaging effects of free radicals [3], and people who eat fruits and vegetables rich in polyphenols and anthocyanins have a lower risk of cancer, heart disease and some neurological diseases [4]. This observation suggested that these compounds might prevent conditions such as macular degeneration [5], suppressed immunity due to poor

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nutrition [6], and neurodegeneration, which are due to oxidative stress [7].

While several trials have investigated supplements with high doses of antioxidants, the investigators found there was statistically significant effect of the antioxidants on overall survival, cancer, or heart disease, showing a 31% reduction in the risk of cancer in men [8].

In other hand, the silkworm bioreactor has showed its advantages such as high expression efficiency and low feeding cost, natural activity for its expressed products and safety for both environment and human [9]. Therefore, it is very promising to use the silkworm as vector for industrial large-scale mass production [10]. In our previous reports, we used a practical BmNPV bacmid system to express the Mn-SOD enzyme protein in silkworm larvae by the recombinant bacmid baculoviruses [11]. The availability of large quantities of SOD that the silkworm provides should greatly facilitate the future research and testing of this protein for potential application in medicine. We have also investigated the effects of silkworm powder containing SOD on the antioxidation and the immune system of mouse, focused on hemolysin response, hemagglutination against SRBC, PFC assay and the dose-dependent increase of phagocytic activity. All treated mice showed significant promotion in immunity [12, 13].

In this paper, we further investigated the effects of silkworm powder including SOD on NK cell activity and spleen proliferation of mice. The treatment of SOD contained silkworm powder presented 40.3% of average inhibition rate to Hepatoma 22 tumor cell growth in vivo, showing stronger inhibition against tumor. The results showed that silkworm powder containing SOD maybe have potential application against tumor cells.

Material and methods

Experimental animals

The experimental animals were supplied by Animal Experiment Center of Zhejiang Medical Institute, China. Half male and half female ICR mice of 6–8 weeks (weighing 20 ± 2 g) were used. The animals were housed in individual stainless steel cages in an air-conditioned room under a 12:12-h light: dark cycle. A commercial pellet diet and water were provided throughout the experiment. All procedures were conducted in accordance with the P.R. China legislation under No. 8910M047 on the use and care of laboratory animals and with the guidelines established by Institute for Experimental Animals of Zhejiang University.

Preparation of silkworm powder including superoxide dismutase (SOD)

The fifth instar silkworm larvae expressed SOD 96 h post-infection with recombinant virus (rBacmid/BmNPV/SOD) were collected [11], and dried with a vacuum dryer (Brocher CHRIST Beta-16, Germany) under low temperature of -56°C . The dried larvae were homogenized to powder and stored at -20°C up to use.

Animal experiments

The mice were randomly divided into groups with 15 animals in each group: normal healthy control group (Control), received silkworm powder without SOD; three treated groups, administrated through feeding with silkworm powder including SOD at the level of 100 mg/kg body weight/day (Low dosage group, Group-L), 200 mg/kg body weight/day (middle dosage group, Group-M) and 400 mg/kg body weight/day (high dosage group, Group-H), and a positive control was set as oral feeding of *Astragalus membranaceus* (Fisch.) Bunge (supplied by Yangzhi River Pharmaceutic Co. LTD, China) at level of 0.3 ml/day (Positive-Control); Cyclophosphamide group (CPA): 0.2 ml/10 g body weight was injected through abdominal cavity for successive 2 days.

The silkworm powder was made to suspension, and fed the mice with 0.5 ml per day. The immunity index was assayed 30 days after treatment.

Liver cancer Hepatoma 22 cell modeling

Thirty five male and 35 female ICR mice (5–7 weeks) weighing 18–22 g were used for liver cancer H22 modeling. The H22 cell line was supplied by Animal Experiment Center of Medical College, Zhejiang University, China. After three times of passage in abdominal cavity of mouse, 0.2 ml of 5×10^6 /ml H22 cells was inoculated through right armpit for successive 10 days.

Assessment of natural killer (NK) cell activity

Spleen of mice upon exposure to manganese SOD expressed in silkworm larvae was collected under aseptic conditions, in Hank's balanced salt solution (Sigma), was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension, The interface mononuclear cells were washed twice with Hank's solution, and the erythrocytes were lysed with NH_4Cl (0.8% (w/v)). After centrifugation (1,500g at 4°C

for 10 min), the pelleted cells were washed three times with phosphate buffered saline (PBS) and resuspended in RPMI 1640 complete medium (supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-sulfanylethanol, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% FCS). Cell numbers were counted with a hemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocytes were adjusted to 2 ± 10^5 cell/ml, and seeded into a 96-well flat-bottom microtiter plate with RPMI 1640 complete medium. NK activity was detected with the freshly isolated splenic mononuclear cells. Target cells for detection of NK cell cytotoxicity were YAC-1 cell line (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). The YAC-1 cells were maintained in continuous suspension culture in the complete culture medium at a concentration of about 8×10^5 cells/ml at 37°C in a humidified 5% CO₂ incubator for 24 h. Hundred µl of NK and YAC-1 cells were added in to a U- bottom microtiter plate hole, and 100 µl of 1% NP40 and YAC-1 cells were added into another hole for control. The plate was incubated in a humidified 5% CO₂ incubator for 4 h. The plate was centrifugated at 1,500g, 5 min, and take 100 µl into a 96-well flat-bottom microtiter plate, and add 100 µl of LDH substrate for 5 min of reaction. Thirty µl of 1 M HCl was added to stop the reaction. The absorbance at 492 nm was monitored using a spectrophotometer. The NK cell activity was calculated according to the formula:

$$\text{NK cell activity (\%)} = \frac{[(\text{NK} + \text{YAC} - 1)\text{OD} - (\text{Yac} - 1)\text{OD}]}{[(\text{Yac} - 1 + \text{NP40})\text{OD} - (\text{Yac} - 1)\text{OD}]}$$

In vivo spleen proliferation assay

Spleen of mice exposure to manganese SOD expressed in silkworm larvae was collected under aseptic conditions, in Hank's balanced salt solution (Sigma), was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension, and the erythrocytes were lysed with NH₄Cl (0.8% (w/v)). After centrifugation (1,500g at 4°C for 10 min), the pelleted cells were washed

three times with phosphate buffer saline (PBS) and resuspended in RPMI 1640 complete medium (supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-sulfanylethanol, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% FCS). Cell numbers were counted with a hemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described by Pan et al. [14]. Briefly, splenocytes were seeded into a 96-well flat-bottom microtiter plate at 1×10^6 cell/ml in 100 µl of complete medium, and then the Con A (final concentration 5 µg/ml), RPMI 1640 medium was added to give a final volume of 200 µl (tetraplicate wells). The plate was incubated at 37°C in a humidified atmosphere with 5% CO₂. After 44 h, 50 µl of MTT solution (5 µg/ml) was added to each well and incubated for further 4 h. The plates were centrifuged (1,400g, 5 min) and the untransformed MTT was removed carefully by pipetting. Two hundred µl of acidic isopropanol solution (192 µl of isopropanol with 8 µl of 1 N HCl) was added to each well, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630-nm reference after 15 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.

Statistical analysis

The data were expressed as mean \pm S.D, and compared statistically by *t*-test, *P* < 0.05 being considered significant.

Results

Effects of SOD-contained silkworm larvae powder on growth of organs and tumor in Hepatoma 22 modeled mice

Table 1 showed the inoculation of Hepatoma 22 cell caused severe effect on spleen growth, showing 3–5 times increases in splenocytes/body weight (mg/g) in modeled

Table 1 Effects of SOD-contained silkworm larvae powder on growth of organs and tumor in Hepatoma 22 modeled mice

Treatments	Animal No.	Splenocytes/body weight (mg/g)	Thoracic gland/body weight (mg/g)	Tumor weight (g)	Inhibition rate (%)
Normal mice	10	3.77 \pm 0.82	2.29 \pm 0.38		
Modeled control	10	11.91 \pm 1.98**	2.24 \pm 0.29	2.86 \pm 0.66	
SOD silkworm powder (400 mg/kg)	10	13.30 \pm 2.79**	3.09 \pm 0.73	2.13 \pm 0.31	25.52
SOD silkworm powder (200 mg/kg)	10	16.13 \pm 0.85**	1.91 \pm 0.18	1.75 \pm 0.42*	38.81*
SOD silkworm powder (100 mg/kg)	10	12.27 \pm 0.52**	1.94 \pm 0.13	2.16 \pm 0.30	24.48
Contro (400 mg/kg)	10	12.31 \pm 2.47**	2.01 \pm 0.38	2.67 \pm 0.62	6.62

Values are shown as the mean \pm SD. * *P* < 0.05, ** *P* < 0.01, *n* = 10

control and SOD silkworm powder treatment compared to normal mice. In contrary, the Thoracic gland/body weight (mg/g) has no significantly difference among treatments. The results suggested the SOD silkworm powder affects mainly on the spleen growth, not thoracic gland and caused the inhibition of tumor.

The tumor weight was reduced after treated with SOD silkworm powder compared to modeled control, ranging from 24.48% to 38.81% in inhibition rate. Among them, the treatment of 200 mg SOD silkworm powder/kg mouse body presented statistically significant data in tumor weight and inhibition rate.

The data also showed there are direct relationship between splenocytes/body weight and tumor weight and inhibition rate, suggesting the SOD silkworm powder enhanced anti-tumor capability through regulation of the spleen growth.

Table 2 showed the body weight has no significant difference among treatments, but the spleen weight of mice was statistically significantly increased after Hepatoma 22 modeled and treatment with SOD-contained silkworm powder in Hepatoma 22 modeled mice. The Tumor/body weight was reduced in mice treated with SOD-contained silkworm larvae powder, showing this material has positive effect against tumor cells in vivo.

Sub-effects of SOD silkworm powder on the growth of organs and tumor inhibition in vivo

As results in Table 3, the treatment of SOD contained silkworm powder presented 40.3% of average inhibition rate to Hepatoma 22 tumor, showing stronger inhibition than that of positive control (oral feeding of *Astragalus membranaceus* (Fisch.) Bunge).

In addition, there were no significant difference in body weight between modeled control and SOD silkworm powder feeding in Hepatoma 22 tumor modeled mice, suggesting the SOD silkworm powder is safety as an inhibitor to tumor.

Table 3 Effects of SOD contained silkworm larvae powder on Hepatoma 22 tumor inhibition in modeled mice

Treatments	Tumor weight (g)	Body weight (g)	Inhibition rate (IR,%)
Modeled control	3.25 ± 0.76	30.54	
SOD silkworm powder (400mg/kg)	1.94 ± 0.60**	30.5	40.33**
Control (400mg/kg)	2.11 ± 0.42	30.61	36
Positive-Control	2.43 ± 0.32	29.77	25.14
Cyclophosphamide control (CPA)	0.84 ± 0.16**	18.44**	74.23**

Values are shown as the mean ± SD. ** $P < 0.01$, $n = 10$

The cyclophosphamide is an effective tumor curing medicine. In cyclophosphamide control (CPA), the inhibition rate was highest, but this chemical showed strong side-effect, presenting reduced body weight (Table 3).

Effects of silkworm powder including SOD on splenocyte proliferation (SI) and NK cell activity in Hepatoma 22 tumor modeled mice

In order to realize the function of SOD contained silkworm powder against tumor growth in vivo, we investigated the effects of silkworm powder including SOD on splenocyte proliferation (SI) and NK cell activity in Hepatoma 22 tumor modeled mice. Table 4 showed the NK cell activities of Hepatoma 22 tumor modeled mice treated with silkworm powder including SOD were enhanced significantly compare to a modeled control and silkworm powder without SOD control (400 mg/kg), reaching 36.18%.

In addition, the ConA-stimulated splenocyte proliferation of SOD treated mice was higher than that of the controls. In the cyclophosphamide treatment, although the

Table 2 Effects of SOD-contained silkworm larvae powder on growth of body and ratio of Tumor/body weight in Hepatoma 22 modeled mice

Treatments	Animal No.	Start body weight (g)	Final body weight (g)	Spleen weight (g)	Tumor/body weight (g/g)
Normal mice	10	20.81 ± 0.43	34.70 ± 2.19	0.13 ± 0.02	
Modeled control	10	20.74 ± 0.52	35.19 ± 3.16	0.44 ± 0.07**	0.0813
SOD silkworm powder (400 mg/kg)	10	20.56 ± 0.18	33.78 ± 1.88	0.45 ± 0.11**	0.0631
SOD silkworm powder (200 mg/kg)	10	20.87 ± 0.45	33.45 ± 2.12	0.53 ± 0.05**	0.0523**
SOD silkworm powder (100 mg/kg)	10	20.45 ± 0.71	35.05 ± 2.43	0.42 ± 0.02**	0.0616
Control (400 mg/kg)	10	20.45 ± 0.62	33.98 ± 2.15	0.41 ± 0.09**	0.0550*

Values are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, $n = 10$

Table 4 Effects of silkworm powder including SOD on splenocyte proliferation (SI) and NK cell activity of Hepatoma 22 tumor modeled mice

Treatments	Splenocyte proliferation (SI)	NK cell activity (%)
Modeled control	0.973 ± 0.076	23.06 ± 11.24
SOD silkworm powder (400 mg/kg)	1.011 ± 0.096*	36.18 ± 10.64*
Control (400 mg/kg)	0.944 ± 0.058	24.97 ± 7.67
Positive-Control	0.951 ± 0.073	33.42 ± 10.16*
Cyclophosphamide control (CPA)	0.899 ± 0.049	43.53 ± 14.20**

Values are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, $n = 10$

NK cell activity was strong enhanced, but splenocyte proliferation (SI) was reduced compared to controls.

Discussion

Hepatomas are the most common type of cancer originating in the liver. In certain areas of Africa and Southeast Asia, hepatomas are even more common than metastatic liver cancer, and they are a prominent cause of death [15]. Usually, the survival rate for people with a hepatoma is poor because the tumor is detected at a late stage. At present, hepatoma was mainly treated with physical therapy (i.e. Radiofrequency ablation (RFA), Intra-arterial iodine-131-lipiodol administration, Combined PEI and TACE, High intensity focused ultrasound) and chemotherapy [16].

Chemotherapy drugs can be injected into a vein or into the hepatic artery, which then delivers a high concentration of the drugs directly to the cancer cells in the liver [17]. Although chemotherapy drugs can temporarily slow the growth of the tumor, they do not cure the cancer. Furthermore, almost all of chemical drugs kill both of cancer cells and normal cells at same time with treatment. This limited the dosage of chemotherapy drugs, and reduced the patients' immune function against tumor cells. Therefore, the anti-oxidant drugs were put more and more importance recently. These drugs are useful to reduce the clinical symptoms, enhance the life quality, inhibit the cancer cell transferring, and assist the radio and chemotherapy through the cellular antioxidant defense and immunity promotion.

In our previous reports, we used a practical BmNPV bacmid system to express the Mn-SOD enzyme protein in silkworm larvae by the recombinant bacmid baculoviruses. The expression level was about 20 mg per larval [18]. The time course of the expressed Mn-SOD activity in larvae infected with the recombinant virus (rBacmid/BmNPV/SOD) was determined [11]. The availability of large

quantities of SOD that the silkworm provides should greatly facilitate the future research and testing of this protein for potential application in medicine. We have also investigated the effects of silkworm larvae powder containing SOD on the antioxidation and the immune system of mouse, focused on hemolysin response, hemagglutination against SRBC, the activity of natural killer (NK) cells, the ConA-stimulated splenocytes proliferation, PFC assay and the dose-dependent increase of phagocytic activity. All treated mice showed significant promotion in immunity [12].

Liu et al. studied the effect of injury of SOD on lipid peroxidation of tumor cell, telomerase activities in tumor tissues, expression of protooncogene. It has been found that content of MDA increased in S180 sarcom group and Lewis lung cancer group in situation of SOD injury while the content of GSH, GPX and activity of T-SOD decreased. It has also been found that SOD can reduce the telomerase activities in S180 and H22 and expression of protooncogene in S180 sarcom, Lewis lung cancer tumor tissues. The radiosensitivity of SOD may have some relationship with the effect on expression of protooncogene and telomerase activities, and metabolism of SOD in different kinds of tumor [19].

For silkworm larvae powder containing SOD as potential application in medicine, the safety assessment is very necessary. Our previous data indicated the feeding treatment was safe with 360 folds of recommended human dosage in acute toxic test. In long-term test, there were no effects of silkworm larvae powder containing SOD on treated mice's growth and inside organs as long as 90 days. Further the electronic microscope investigation showed the intestine, liver, splenocytes and stomach in mice were no obvious changes both in organs and sub-organs such as nucleus, endoplasmic reticulum, mitochondrion, Golgi and peroxisomes after treated for as long as 90 days [18].

Natural killer cell is known as a major immune system in body through mediating cell death via several possible pathways [20]. NK cells are one of three subpopulations of lymphocytes functioning as scavenger of tumor, virus infected cells etc. Our present results found the NK cell activities of Hepatoma 22 tumor modeled mice treated with silkworm powder including SOD were enhanced significantly compared to a modeled control and silkworm powder without SOD control, reaching 36.18%, which implied this material modulated the immune system in the Hepatoma tumor modeled mice in vivo. The spleen is an important immunological organ that contains mainly B-lymphocytes. Hence, the spleen can indirectly reflect humoral immunity. Our data on the effects of continuous treatment with SOD-containing silkworm powder showed the ConA-stimulated spleen proliferation of SOD treated mice was higher than that of the controls. As results in

Table 3, the treatment of SOD contained silkworm powder presented 40.3% of average inhibition rate to Hepatoma 22 tumor, and there were no significant difference in body weight between modeled control and SOD silkworm powder feeding in Hepatoma 22 tumor modeled mice, suggesting the SOD silkworm powder is safety as an inhibitor to tumor.

The cyclophosphamide is an effective tumor curing medicine. In CPA, the inhibition rate was highest, but this chemical showed strong side-effect, presenting reduced body weight in treated mice.

In conclusion, these findings demonstrate that administration of silkworm powder containing SOD results in activation of NK cells and immune system, suggesting the silkworm powder containing SOD plays a positive role in tumor inhibition. The results also suggested the SOD expressed in silkworm maybe have potential application in medicine.

Acknowledgements The works were supported by the Hi-Tech Research and Development Program of China (No. 2006AA10A119) and a key project of Zhejiang Government (No.2005C22042).

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