

Full Length Research Paper

Antitumor efficacy of *Bifidobacterium longum* carrying endostatin gene enriched with selenium and the distribution of selenium

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As a domestic anaerobic bacterium in the human body, *Bifidobacterium longum* can selectively germinate and proliferate in the hypoxic regions of solid tumors. Selenium (Se) has been found to inhibit carcinogenesis and tumor growth. In our previous study, we demonstrate that *B. longum* carrying pBV22210-Endostatin (*B. longum*-En) inhibit tumor growth. In this study, we enriched Se to *B. longum*-En (Se-*B. longum*-En) to evaluate the antitumor efficacy when delivered orally or intravenously on H22 tumor-bearing mice and examined the distribution of Se *in vivo*. The results showed that Se-*B. longum*-En could significantly inhibit tumor growth as well as extend the median survival time of tumor-bearing mice. The concentration-time curve suggested that Se could be absorbed and disseminated rapidly *in vivo*. The results also showed that there was a dose-effect relationship between Se and tumor inhibition rate and the antitumor effect was more pronounced when Se-*B. longum*-En was delivered via vein than by oral route. Based on the results, *B. longum* carrying endostatin gene and enriched with Se may be a potential agent in cancer gene therapy.

Key words: *Bifidobacterium longum*, endostatin, selenium, tumor, gene therapy.

INTRODUCTION

Se is an essential trace element for animals and humans which has comprehensive biological effects. It has been recognized that Se is important for health and deprivation of Se is closely linked to many kinds of diseases (Rayman, 2000). The association between Se and tumors has become the focus of intensive studies and a number of studies have showed that Se had anticarcinogenic effect in many kinds of tumor models (Clement, 1998; Combs and Gray, 1998; Bjorkhem-Bergman et al., 2005;

Clement, 1984; Sundaram et al., 2000). Se is a constituent part of the enzyme glutathion peroxidase which catalyzes the conversion of hydrogen peroxide and organic hydroperoxides into water and corresponding alcohols. The antioxidant and pro-oxidant property of Se may be a possible mechanism for its antitumor activity. Live bacteria have been reported to be associated with tumor regression for more than a century. In the last few years, several species of live bacteria such as *Salmonella*, *Listeria*, *Bifidobacterium* and *Clostridium* have been explored either as direct antitumor agents, or delivery system of antitumor molecules (Fialho and Chakrabarty, 2010). *Bifidobacterium longum* has unique advantage among these bacteria because it can

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selectively localize and proliferate in the hypoxic regions of solid tumors without any toxicity (Yazawa et al., 2000, 2001; Sasaki et al., 2006).

Angiogenesis is an important process in tumor growth and metastasis. Endostatin, the COOH-terminal part of the collagen XVIII α 1-chain in the tumor cell, is an endogenous inhibitor of angiogenesis and has been demonstrated to inhibit tumor growth and considered as a potential therapeutic agent for a number of tumors (O'Reilly et al., 1997; Dejana, 1999; Yamagata et al., 2000; Read et al., 2001). In our previous studies, *B. longum* was transfected by electroporation with pBV22210-Endostatin, and we demonstrated that it could significantly inhibit tumor growth with no toxicity (Fu et al., 2005; Xu et al., 2007). In this study, H22 tumor-bearing mice were treated with different doses of Se-*B. longum-En* delivered through oral or tail vein route and the effects of Se-*B. longum-En* on tumor growth inhibition and survival time of tumor-bearing mice were observed. Besides, the dose-effect relationship between Se and antitumor efficacy as well as the content and distribution of Se in blood and organs were determined. The results showed that Se-*B. longum-En* had significant antitumor effects and Se could be absorbed adequately by tumor-bearing mice.

MATERIALS AND METHODS

Bacterial strains and reagents

WT *B. Longum* and Se-*B. longum-En* by intra-gastric administration ($100 \mu\text{g Se}/1 \times 10^{11}$ bacteria/g) were obtained from the Inner Mongolia Shuangqi Medical Industry Corporation (Inner Mongolia, China). *B. longum-En* by i.v. administration was preserved in our laboratory. Sodium selenite was purchased from Shanghai LuYuan Fine Chemical Factory (Shanghai, China). Selenium Yeast Tablet was purchased from Mudanjiang Lingtai Medicine Co, Ltd (Harbin, China). Germanium oxide was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cyclophosphamide (CTX) was purchased from Jiangsu Hengrui Medicine Co, Ltd (Lianyungang, China).

Animals and tumors

Beagle dogs were purchased from Beijing Laboratory Animal Center (Beijing, China). Male Kunming mice (20 ± 2 g) were obtained from Qinglongshan Animal Center (Nanjing, China). The animals were fed with standard food and water *ad libitum* and kept in an animal room that was maintained at 22°C with a 12 h light or dark cycle. Murine hepatoma cells (H22) were supplied by Shanghai Academy of Medical Industry (Shanghai, China). Tumor model was established by subcutaneous injection of H22 tumor cells (1×10^6 cells/0.2 ml) into the right flank of each mouse.

Enrichment of selenium to *B. longum-En* and growth assay of Se-*B. longum-En*

B. longum-En cultured overnight in Trypticase-Peptide-Yeast extract (TPY) medium was inoculated into fresh medium supplemented with sodium selenite at concentrations of 0, 5, 10, 15, 20 and 25 $\mu\text{g/ml}$, respectively. After 18 h of culture, the $\text{OD}_{600\text{nm}}$

value was then measured.

Suppression of H22 tumor growth with Se-*B. longum-En* by oral administration

The tumor-bearing mice were divided into six groups (ten per group). Mice in each group were administered with high dose of Se-*B. longum-En* (3×10^{10} bacteria/kg, 30 $\mu\text{g Se/kg}$, i.g., days 1 to 15), middle dose of Se-*B. longum-En* (1.5×10^{10} bacteria/kg, 15 $\mu\text{g Se/kg}$, i.g., days 1 to 15), low dose of Se-*B. longum-En* (0.75×10^{10} bacteria/kg, 7.5 $\mu\text{g Se/kg}$, i.g., days 1 to 15), WT *B. longum* (1.5×10^{10} bacteria/kg, i.g., days 1 to 15), Selenium Yeast Tablet (30 $\mu\text{g Se/kg}$ or 60 $\mu\text{g Se/kg}$, i.g., days 1 to 15), CTX (10 mg/kg, i.p., days 1 to 7) and 13% nonfat milk as a negative control group, respectively. Before administrated, all *B. longums* suspended in 13% nonfat milk. Twenty-four h after the last administration, the animals were sacrificed and tumors were excised and weighed. The inhibition rate of tumor growth was determined by the formula as follows:

$$\frac{\text{tumor weight of control group} - \text{tumor weight of treatment group}}{\text{tumor weight of control group}} \times 100\%$$

Distribution of selenium in beagle dogs with Se-*B. longum-En* administered orally

The beagle dogs were randomly divided into three group (six per group), and were respectively administered with a high dose of Se-*B. longum-En* (6.2×10^9 bacteria/kg), a middle dose of Se-*B. longum-En* (3.1×10^9 bacteria/kg) and a low dose of Se-*B. longum-En* (1.5×10^9 bacteria/kg) after 12 h fasting. Blood samples (5 ml) were collected into a heparin-containing tube at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h, respectively, and centrifuged at 3000 rpm for 10 min. The content of Se in the blood was determined.

Six beagle dogs were administered with a high dose of Se-*B. longum-En* (6.2×10^9 bacteria/kg). Two hours after the administration, the animals were sacrificed and the heart, liver, spleen, lung, kidney, brain, skin, muscle, stomach, small intestine, adrenal gland, uterus, ovary and sperm of them were excised, and the distribution of Se in the organs was analyzed.

Suppression of H22 tumor growth by Se-*B. longum-En* through tail vein delivery

B. longum-En was cultured in TPY medium supplemented with sodium selenite (5 $\mu\text{g/ml}$) to obtain Se-*B. longum-En* and was cultured in TPY medium supplemented with germanium (Ge) oxide (5 $\mu\text{g/ml}$) to obtain Ge-*B. longum-En* as control. The mice were weighted and divided into five groups randomly (ten per group) after subcutaneous inoculation of H22 cells. For negative control, mice were injected with 5% dextrose-saline solution. Four treated groups were injected with CTX (30 mg/kg, i.p., on days 2, 4, 6, 8, respectively), *B. longum-En* (0.4 ml/day, 5.0×10^9 bacteria/kg, i.v., on days 1 to 7), Ge-*B. longum-En* (0.4 ml/day, 5.0×10^9 bacteria/kg, i.v., on days 1 to 7) and Se-*B. longum-En* (0.4 ml/day, 5.0×10^9 bacteria/kg, i.v., on days 1 to 7), respectively. All *B. longum* were washed three times and re-suspended in dextrose-saline solution at a concentration of 2.5×10^8 cells/ml before injection. The mice were sacrificed and the tumors were excised and weighed on day 10. The tumor growth inhibition rate was determined by using the above-mentioned formula. Meanwhile, eye-pit blood, kidneys and tumors were removed from two mice of the dextrose-saline and Se-*B. longum-En-treated* group, and were analyzed for the Se content.

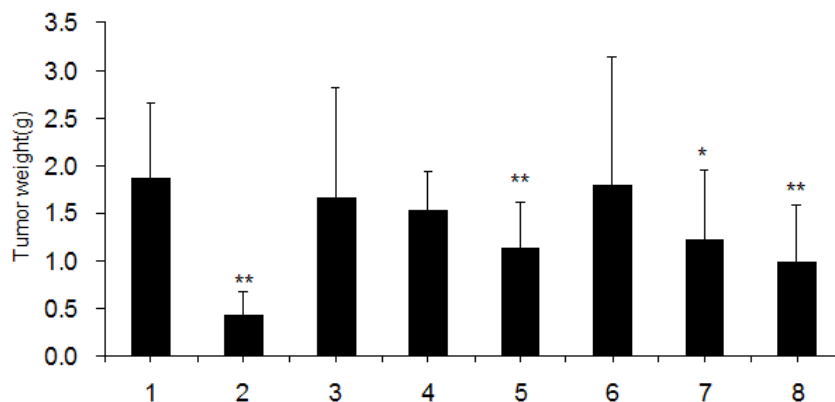


Figure 1. The average weight of tumors excised from tumor-bearing mice treated orally. Bar 1, 13% defatted milk group; Bar 2, CTX group; Bar 3, WT *B. longum* group; Bar 4 Selenium Yeast Tablet group (30 µg Se/kg); Bar 5 Selenium Yeast Tablet group (60 µg Se/kg); Bar 6, low dose of *Se-B. longum-En* group (7.5 µg Se/kg); Bar 7, middle dose of *Se-B. longum-En* group (15 µg Se/kg); Bar 8, high dose of *Se-B. longum-En* (30 µg Se/kg). The tumor weights were presented as mean ± S.D. (n = 10). * P < 0.05, ** P < 0.01.

Effect of *Se-B. longum-En* on survival time of H22 tumor-bearing mice

The mice were weighed and divided into five groups randomly (ten per group) as mentioned above. Mice in each group were injected with 5% dextrose-saline solution, CTX (30 mg/kg, i.p., on days 2, 4, 6, 8, respectively), *B. longum-En* (0.4 ml/day, i.v., on days 1 to 7), *Ge-B. longum-En* (0.4 ml/day, i.v., on days 1 to 7) and *Se-B. longum-En* (0.4 ml/day, i.v., on days 1 to 7), respectively. The dose of bacteria was the same as the above experiment. The mice were kept after the last administration and their survival time was recorded till day 60. All mice were sacrificed on day 61 and the survival data was analyzed.

Statistical analysis

The results were presented as mean ± SD value. The data were statistically analyzed using Student's t-test in both groups and analysis of variance (ANOVA) test in multiple groups. The comparisons among multiple groups were performed using Student-Newman-Keuls *q*-test. Survival analysis was performed using SPSS 15.0. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Growth characteristics of *B. longum-En* enriched with different concentration of Se

To study the effect of Se on the growth of *B. longum-En*, *B. longum-En* was enriched with different concentration of Se. The OD_{600nm} values of *B. longum-En* containing Se at concentrations of 0, 5, 10, 15, 20 and 25 µg/ml were 0.7515, 0.6625, 0.6445, 0.4845, 0.4125 and 0.4115, respectively. The result suggested that the growth of *B. longum-En* was little affected when the concentration of

Se at less than 10 µg/ml.

Effect of *Se-B. longum-En* on the growth of H22 tumor xenografts by oral delivery

To examine the antitumor effect of *Se-B. longum-En* delivered by oral route, a H22 tumor xenograft model was used. The average tumor weight of each group was shown in Figure 1. It can be seen from the figure that tumors grew slower in the treated groups. When compared with the control, animals treated with CTX, WT *B. longum*, Selenium yeast tablet (30 and 60 µg Se/kg) and *Se-B. longum-En* (7.5, 15 and 30 µg Se/kg) exhibited reduction in tumor weight by 76.9% (*P* < 0.001), 11.6% (*P* > 0.05), 18.2% (*P* > 0.05), 39.3% (*P* < 0.01), 3.91% (*P* > 0.05), 34.8% (*P* < 0.05) and 47.3% (*p* < 0.001), respectively. Compared with WT *B. longum* group, animals treated with middle and high doses of *Se-B. longum-En* exhibited significant tumor growth inhibition by 26.2% (*P* < 0.05) and 40.3% (*P* < 0.001) in tumor weight, respectively. The results suggested that both middle and high doses of *Se-B. longum-En* had higher tumor inhibition when delivered by oral route.

Distribution of orally delivered Se of *Se-B. longum-En* in beagle dogs

Blood samples (5 ml for each) were taken at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h, respectively, from beagle dogs administered with different dosage of *Se-B. longum-En* and the amount of Se in blood was determined. The concentration-time curves of three doses of *Se-B. longum-En* were shown in Figure 2. From

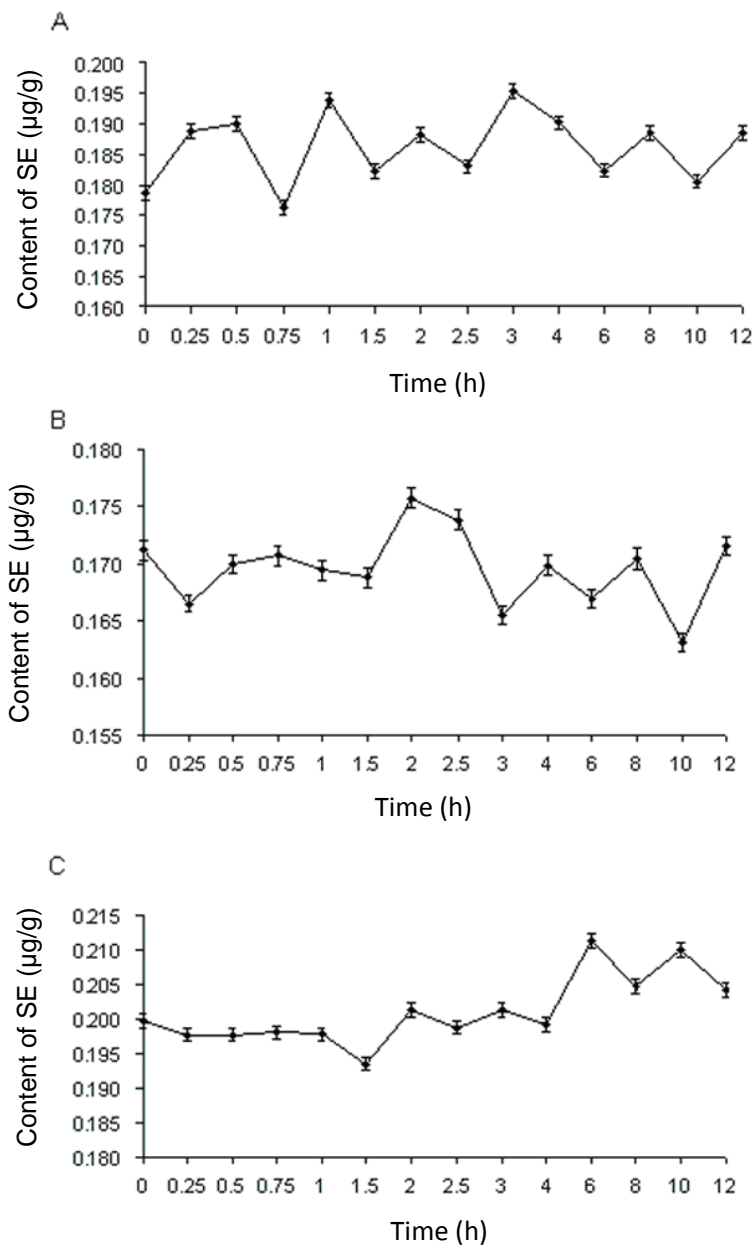


Figure 2. The serum drug concentration-time curves of *Se-B. longum-En* in different dose. (A, B and C) Content of Se in blood of beagle dogs treated with low, middle and high dose of *Se-B. longum-En* at different time points, respectively.

the curves, we determined that the serum level of Se in beagle dogs orally treated with low, middle and high doses of *Se-B. longum-En* was between 0.1763 to 0.1953, 0.1631 to 0.1758 and 0.1934 to 0.2117 µg/g, respectively. The data suggested that the content of Se in blood didn't changed obviously with the change of *Se-B. longum-En* in dose.

Beagle dogs administered with high dose of *Se-B. longum-En* were sacrificed 2 h after the drug administration. The amount of Se in different organs were

as follows: heart 0.1603 ± 0.0186 µg/g, liver 0.4117 ± 0.0441 µg/g, spleen 0.2544 ± 0.0247 µg/g, lung 0.2347 ± 0.0240 µg/g, kidney 0.7600 ± 0.0798 µg/g, brain 0.1746 ± 0.0142 µg/g, skin 0.1115 ± 0.0359 µg/g, muscle 0.0923 ± 0.028 µg/g, stomach 0.2102 ± 0.0827 µg/g, small intestine 0.1916 ± 0.0295 µg/g, adrenal gland 0.2535 ± 0.0511 µg/g, uterus 0.1603 ± 0.0040 µg/g, ovary 0.1538 ± 0.0308 µg/g, sperm 0.2486 ± 0.0536 µg/g. The result showed that the amount of Se was higher in kidney and liver.

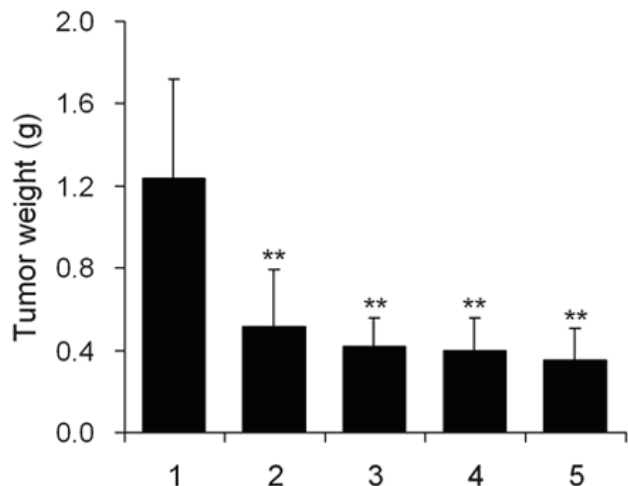


Figure 3. The average weight of tumors excised from tumor-bearing mice treated intravenously. Bar 1, dextrose-saline solution group; Bar 2, CTX group; Bar 3, *B. longum*-En group; Bar 4, *Ge-B. longum*-En group; Bar 5, *Se-B. longum*-En group. The tumor weights were presented as mean \pm S.D. (n = 10). ** P < 0.01.

Effect of tail vein-delivered *Se-B. longum*-En on growth of H22 tumor xenografts

Se-B. longum-En was delivered to H22 tumor-bearing mice intravenously to assess its efficacy. Figure 3 showed the average tumor weight of mice in each group. Compared with dextrose-saline solution, CTX, *B. longum*-En, *Ge-B. longum*-En and *Se-B. longum*-En inhibited tumor growth by 58.5 (P < 0.001), 66.2 (P < 0.001), 67.9 (P < 0.001) and 71.3% (P < 0.001), respectively. In addition, compared with *B. longum*-En, *Se-B. longum*-En suppressed tumor weight by 15.2%. Moreover, the content of Se in kidney, blood, and tumor was 0.1900 ± 0.0247 , 0.0560 ± 0.0071 and 0.1520 ± 0.0021 $\mu\text{g/g}$, respectively. These results suggested that when delivered via tail vein *Se-B. longum*-En could be delivered to tumors and exhibited significant inhibitory effect on tumor growth.

Effect of *Se-B. longum*-En on survival time of H22 tumor-bearing mice

To examine the effect on survival rate of tumor-bearing mice treated with *Se-B. longum*-En intravenously, a xenograft model using Kunming mice were established by injecting H22 cells subcutaneously. As shown in Figure 4, the median survival time of the mice treated with dextrose-saline solution, CTX, *B. longum*-En, *Ge-B. longum*-En and *Se-B. longum*-En was 19, 26, 27, 19 and 45 days, respectively. Compared with the dextrose-saline treatment, *Se-B. longum*-En significantly prolonged the survival time (P < 0.01), while CTX and *B. longum*-En

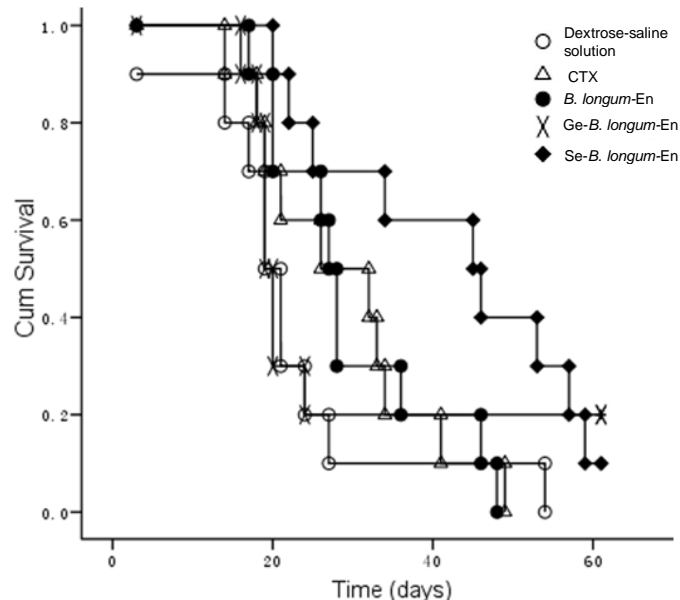


Figure 4. Kaplan-Meier curves of survival of tumor-bearing mice. Mice in each group received dextrose-saline solution, CTX, *B. longum*-En, *Ge-B. longum*-En, *Se-B. longum*-En, respectively.

prolonged to the survival time but with no statistical significance. In contrast, *Ge-B. longum*-En shortened the survival time of the mice.

DISCUSSION

Many studies have demonstrated that Se compounds could efficiently inhibit the growth of various tumors in animal models (Bjorkhem-Bergman et al., 2005; Clement, 1984). The mechanism of the inhibition has not been elucidated. However, there are a number of possible mechanisms which are shown as follows:

- (i) Regulation of thioredoxin reductase (TR) activity in cancer cells. Se can increase TR activity cells, thus leading to the reduction of thioredoxin, and affect the growth and transformed phenotype of cancer cells (Berggren et al., 1997; Gallegos et al., 1997).
- (ii) Alteration of the metabolism of the carcinogen. Se plays the roles by decreasing activation or increasing detoxification of carcinogens, inhibiting the DNA binding of carcinogens and the adduct formation, preventing carcinogens from reaching critical target sites in the cells through scavenging and stimulating the repair of DNA damage caused by carcinogens (Harbach and Swenberg, 1981; Shi et al., 1994; Lawson, 1989).
- (iii) Direct inhibition of tumor cells. Se can affect the regulation of the cell cycle, stimulate apoptosis and inhibit tumor cell migration and invasion (Zeng and Combs, 2008; Zeng et al., 2006; Webber et al., 1985; Zhu et al., 1995).

(iv) Enhancement of immune surveillance. Immunity is a series of processes that act together to protect organisms against attacks by pathogens and malignancy. Se supplements can augment the cellular immune response through an increased production of interferon and other cytokines, an earlier peak T cell proliferation, and an increase in T helper cells. However, humoral immune responses were unaffected (Petrie et al., 1989a, b; Arthur et al., 2003; Arvilommi et al., 1983). Enhancement of immune function may result in the decrease of cancer incidence.

A crucial obstacle in cancer gene therapy is the specific targeting of therapeutic agents directly to solid tumors. *B. longum* which can selectively germinate in the hypoxic regions of tumors has been demonstrated to be a tumor-specific and nontoxic delivery system for antitumor genes (Yazawa et al., 2000, 2001). Endostatin has been shown to be an inhibitor of angiogenesis and considered an attractive new strategy for cancer gene therapy. Our previous work has shown the potential antitumor activity of *B. longum*-En (Fu et al., 2005; Xu et al., 2007). We enriched Se to *B. longum*-En in the present study, and in this way it could more efficaciously inhibit tumor growth.

In the experiment, where different doses of *Se-B. longum*-En were used for an oral infection, a dose-dependent inhibitory activity could be shown. Middle dose and high dose of *Se-B. longum*-En inhibited the tumor growth significantly. The Se content of high dose of *Se-B. longum*-En (30 µg Se/kg) was the same as low dose of Selenium Yeast Tablet control (30 µg Se/kg), however, the tumor inhibitory rate of Selenium Yeast Tablet was only 18.2% while that of *Se-B. longum*-En was 47.3%. The results suggested that *B. longum* was an ideal live bacterium to enrich Se. In addition, in our previous studies, we had shown that the tumor inhibition rate of *Se-B. longum*-En was higher than that of *B. longum*-En (Fu et al., 2005). When *Se-B. longum*-En was delivered intravenously to the tumor-bearing mice. It could significantly inhibit tumor growth as well as prolonged the survival time of mice. From the growth curve of *B. longum*-En enriched with different concentration of Se, it is clear that low concentration of Se had little effect on the growth of *B. longum*-En, suggesting the safety of Se. Based on these results, we concluded that *Se-B. longum*-En was a safe and an effective antitumor agent. In addition, when tumor-bearing mice were treated with *Se-B. longum*-En orally at the dose of 0.75×10^{10} bacteria /kg for 15 days, there was no obvious inhibitory effect, and when the doses increased to 1.5×10^{10} bacteria /kg and 3×10^{10} bacteria /kg, the tumor inhibition was only 34.8 and 47.3%, respectively. However, when *Se-B. longum*-En was delivered intravenously at the dose of 5.0×10^9 bacteria /kg for 7 days, the tumor inhibition was 71.3%. The results suggested that tail vein delivery was more effective than oral delivery. Studies have proved that *Bifidobacterium* can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection (Yazawa et al., 2000; Kimura et al., 1980), which

may explain the observation that the tumor inhibition of *Se-B. longum*-En delivered by tail vein is higher than delivered by oral route.

The serum concentration-time curves of low, middle and high doses of *Se-B. longum*-En suggested that the Se level in blood was not affected obviously within the dosage and the parameters of drug metabolism could not be calculated. This is probably due to the fact that the treatment dosage of Se was very low and comparable to the intrinsic level *in vivo* and that Se was quickly absorbed and distributed without obvious excretory process. When high dose of *Se-B. longum*-En was administered orally, the highest Se level was detected in kidney and in liver, suggesting that Se was firstly delivered to liver and kidney and redistributed later. The level of Se in kidney, blood, and tumor of mice with intravenous treatment, suggested that *Se-B. longum*-En delivered to tumors by tail vein and played a role in inhibiting tumors.

Moreover, in order to further study the safety of *Se-B. longum*-En, some toxicity test was also done. The acute toxicity test of rats showed that the safe dose of *Se-B. longum*-En was 2.6×10^{12} bacteria/kg daily, while the safe dose was 5.4×10^{11} bacteria/kg daily in the long-term toxicity test of rats and was 0.9×10^{11} bacteria /kg daily in the acute toxicity test of dogs. In view of results above, the effective dose of *Se-B. longum*-En which was 1.0×10^{10} bacteria/kg was safe to animals, and the dose we used in the experiment was both effective and safe for tumor bearing animals.

In conclusion, the results suggested that Se in *Se-B. longum*-En could be absorbed and utilized sufficiently by organs and the enrichment of Se to *B. longum*-En significantly increased the antitumor efficacy, which can be used as a novel and safe strategy for cancer gene therapy.

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