

Selenium-*Bifidobacterium longum* as a delivery system of endostatin for inhibition of pathogenic bacteria and selective regression of solid tumor

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Abstract. *Bifidobacterium longum* (*B. longum*) as a delivery system for endostatin was shown to have definite antitumor effects. Moreover, it was found that the enrichment of selenium was able to enhance the immunity of mice. In order to further evaluate the safety and efficacy of *B. longum* carrying pBV22210-endostatin (*B. longum*-En) enriched with selenium (Se-*B. longum*-En), we determined the biochemical characteristics of Se-*B. longum*-En. We then investigated its effect on macrophage activity, as well as its inhibitory effect on the multiplication of pathogenic bacteria *in vitro* and the antitumor effects on murine hepatic (H22) tumor-bearing mice. The results showed that Se-*B. longum*-En exhibited similar biochemical characteristics to that of wild-type *B. longum*, i.e., Se-*B. longum*-En strongly enhanced macrophage phagocytosis in rats and inhibited the growth of pathogenic bacteria. In addition, Se-*B. longum*-En showed a definite inhibitory effect of tumor growth when H22 tumor-bearing mice were fed through oral or tail vein delivery. These results suggested that Se-*B. longum* is able to retain the advantages of wild-type *B. longum* and be used as a novel gene delivery system for liver cancer gene therapy.

Introduction

Angiogenesis is required in the growth and metastasis of solid tumors. Extensive studies have been conducted on various angiogenic inhibitors. O'Reilly *et al* reported on such an angiogenic inhibitor, endostatin, which has a broad antitumor spectrum and low toxicity (1). Mammalian studies indicated that endostatin had few side effects and induced no drug resistance after prolonged use (2). These properties make endostatin a promising candidate as an antitumor agent.

A primary problem for cancer gene therapy is the lack of specificity in current delivery systems. Many solid tumors in rodents exhibit similar features of hypoxic regions as those in humans. Therefore, anaerobic non-pathogenic bacteria such as *Bifidobacterium* and *Lactobacillus* are regarded as favorable media for the delivery of specific tumor-inhibiting genes (3-6). Previous studies have shown that the role of *Bifidobacterium longum* (*B. longum*) as a delivery system for endostatin is tumor-specific with no toxicity (7,8). Selenium (Se), essential micronutrient in the daily diet, was shown to be beneficial to human health, especially in cancer chemoprevention. Some studies demonstrated that a Se supplement reduces carcinogenic risk (9,10). Se is speculated to play a role by entering the protein as an amino acid selenocysteine (Sec), coded by UGA. Selenoproteins are associated with the deletion of carcinogen-initiated cells and the suppression of transformed cell expansion.

In the present study, Se was enriched to transformed *B. longum* carrying the shuttle vector pBV22210-endostatin (*B. longum*-En). Its biochemical characteristics and effect on pathogenic bacteria, as well as its antitumor effects on Se-*B. longum*-En were determined on murine hepatic (H22) tumor-bearing mice through oral and intravenous (i.v.) delivery. The results showed that Se-*B. longum*-En maintained its biochemical characteristics and exhibited inhibitory activity on tumor growth.

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Materials and methods

Plasmids, bacterial strains and reagents. A shuttle vector pBV22210 coding endostatin was constructed and transfected into *B. longum*-En (Xu *et al.* of our laboratory) (8). Wild-type (WT) *B. longum*, *shigella flexneri*51571, *salmonella typhimurium*50115, *escherichia coli*44155 and *pseudomonas aeruginosa*03 were obtained from the Inner Mongolia Shuangqi Medical Industry Corporation (Inner Mongolia, P.R. China). Chicken blood was obtained from a vein under the wing from a 2 kg hen. Chicken red blood cells (RBC) were separated following centrifugation at 2000 g for 5 min. Cyclophosphamide (CTX) was purchased from Shanghai Lianhua Pharmaceutical Co. Ltd. China (Shanghai, P.R. China). Ketamine was purchased from Shanghai First Biochemical Pharmaceutical Co. Ltd. China (Shanghai, P.R. China).

Animals and tumor cells. Male Kunming mice, aged 6-8 weeks (20±1 g), were purchased from the animal center of Nanjing Medical University (Nanjing, P.R. China). Mice were fed with standard rodent diet and water *ad libitum* and kept in an animal facility, maintained at 21±2°C on a 12-h light/dark cycle. H22 cells were supplied by the Shanghai Academy of Medical Industry (Shanghai, P.R. China). A liver tumor model was established by the subcutaneous injection of H22 tumor cells (1×10⁶/0.2 ml) into the flank of each mouse.

Selenium enrichment to *B. longum*-En and selenium quantification. Selenium enrichment to *B. longum*-En was performed according to the previously established protocol (7). Briefly, sodium selenite was weighed and dissolved in 200 ml TPY medium at a concentration of 5, 10, 15, 20 and 25 µg/ml, respectively. Overnight cultivated *B. longum*-En in TPY medium was washed three times with phosphate-buffered saline (PBS). The number of viable bacteria for Se-*B. longum*-En was measured according to the Manufacturing and Determining Regulations of Oral Live Bifidobacterium Preparatum in Regulations for Biological Products of People's Republic of China (11). The total selenium content in *B. longum*-En was determined according to the Determination of Selenium in foods (12). The inorganic and organic form of selenium content in *B. longum*-En was measured by atomic absorption spectrometry according to the appendix VIII D of Chinese Pharmacopeia (13).

Analysis of Se-*B. longum*-En. The identification of Se-*B. longum*-En was carried out by the Inner Mongolia Shuangqi Medical Industry Corporation (Inner Mongolia). Aerobic, dynamic, spore, catalase and gelatin liquefaction assays, as well as deoxidize by nitrate and carbohydrate fermentation were performed according to previously published protocols (14). *B. longum*-En and WT *B. longum* were used as controls.

Effect of measurements of Se-*B. longum*-En on macrophage phagocytotic activity. In order to determine the effect of Se-*B. longum*-En on immunity, we measured the macrophage phagocytotic activity in rats. Twenty-four Wistar rats (male, weighing 220±15 g) were divided into three groups. Each group received dextrose-saline solution intraperitoneally (i.p.)

on days 1-3, WT *B. longum* cells (i.p., days 1-3) and Se-*B. longum*-En cells (i.p., days 1-3), respectively. The rats in two of the treated groups were allergized using the method: 1 ml liquid bacterium [10⁸ colony-forming unit (CFU)] dissolved in dextrose-saline solution was injected into each mouse on day 1, 2 ml (10×10⁸ CFU) on day 2, and 4 ml (20×10⁸ CFU) on day 3. Rats in the dextrose-saline solution group were used as a negative control. On day 9, the peritoneal fluids of each group were collected for the preparation of macrophages. Briefly, 20 ml of PBS (pH 7.0) were injected into rat abdominal cavities. The rats were anesthetized with ketamine (120 mg/kg). Ascites (15 ml) were removed from each rat after 5 min cheirapsis. The ascites were washed three times with PBS before being adjusted to a concentration of 3×10⁶ cell/ml. Macrophage suspension was then obtained. RBC suspension (1 ml; containing 2% chicken RBC) was added to 1 ml macrophage suspension and the mixture was centrifuged at 1000 g for 8 min after incubation at 37°C for 30 min. The supernatant was discarded and precipitation was re-suspended before being plated onto the slides. The cells were stained and counted using the high microscope objective. The phagocytotic rate and index were calculated by the formula:

$$\text{Rate of phagocytosis} = \frac{\text{no. of macrophage cells in 200 cells}}{200} \times 100\%$$

$$\text{Phagocytosis index} = \frac{\text{no. of chicken RBC phagocytized by 200 macrophage cells}}{200} \times 100\%$$

Co-cultivation of Se-*B. longum*-En and pathogenic bacteria. The *shigella flexneri*51571, *salmonella typhimurium*50115, *escherichia coli*44155 and *pseudomonas aeruginosa*03 were co-incubated with WT *B. longum* or Se-*B. longum*-En under aerobic conditions. The co-cultures were incubated overnight at 37°C. Colonies on agar plates with or without 5 µg/ml chloramphenicol were counted at 0, 12, 24, 48, 72 and 96 h, respectively. Each pathogenic bacterium was cultured alone as a control under the same conditions.

Suppression of H22 tumor growth by Se-*B. longum*-En through oral delivery. The tumor-bearing mice were divided into five groups. Animals were treated with Se-*B. longum*-En [0.8 ml, intragastrically (i.g.), days 1-30], *B. longum*-En (0.8 ml, i.g., days 1-30) and WT *B. longum* (0.8 ml, i.g., days 1-30), respectively. However, the positive control group was injected with CTX (30 mg/kg, i.p., days 1-7) and another group received 13% fat-free milk (0.8 ml, i.g., days 1-30) as a negative control. Prior to injection, *B. longums* were washed three times with dextrose-saline solution and re-suspended in 13% fat-free milk to 7.5×10⁸ CFU/ml. Seventy-two hours after the last administration the animals were sacrificed and tumors were excised and weighed. The inhibition rate (IR) on the tumor growth was determined by the formula:

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

Suppression of H22 tumor growth by Se-*B. longum*-En through tail vein delivery. The H22 tumor-bearing mice were randomly divided into five groups. Se-*B. longum*-En, *B.*

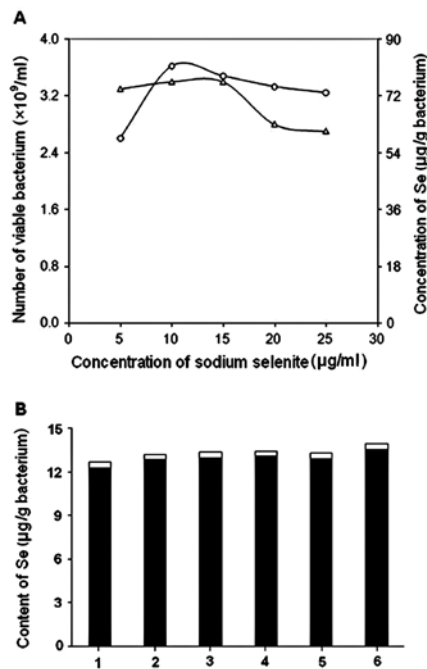


Figure 1. Selenium content in *Se-B. longum-En*. (A) The growth rates for *Se-B. longum-En* and selenium content changed by different concentrations of sodium selenite. Open triangles show the numbers of viable *Se-B. longum-En* and open circles show selenium content. (B) Selenium content in six samples of *Se-B. longum-En*. Six random samples (1-6) are shown. The white and black parts of the bars represent the inorganic and organic form of selenium in the samples, respectively.

longum-En, and WT *B. longum* were washed as mentioned above. These three groups were then re-suspended in dextrose-saline solution at a concentration of 2.5×10^8 cells/ml before injection. Mice were injected with *Se-B. longum-En* (0.4 ml, i.v., days 1-7), *B. longum-En* (0.4 ml, i.v., days 1-7), WT *B. longum* (0.4 ml, i.v., days 1-7), CTX (30 mg/kg, i.p., days 1-7) and dextrose-saline solution (0.4 ml/mice i.v., days 1-7), respectively. The animals were kept for an additional three days following the last injection, and were sacrificed seven days later. The weight of the excised tumors and IR of tumor growth were determined as described above.

Statistical analysis. The data were statistically analyzed using Student's t-test in both groups and the ANOVA test in multiple groups. Comparisons among the multiple groups were

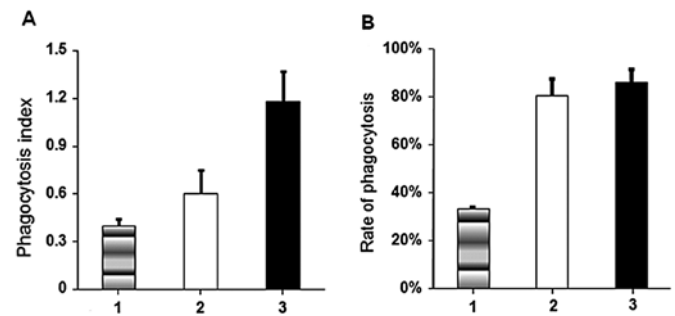


Figure 2. Enhancement of phagocytotic activity of macrophage by *Se-B. longum-En*. (A) Bars 1-3 show the rate of phagocytosis of rat macrophages in the negative control, WT *B. longum* and *Se-B. longum-En* groups, respectively. (B) Bars 1-3 show the phagocytotic indices of rat macrophages in the negative control, WT *B. longum* and *Se-B. longum-En* groups, respectively.

performed using the Student-Newman-Keuls Q-test. $P < 0.05$ was considered to be significant.

Results

Selenium content in *Se-B. longum-En*. The growth curve and selenium content of *Se-B. longum-En* were measured. As shown in Fig. 1A, the growth curve of *Se-B. longum-En* and selenium content did not increase with the increasing concentration of sodium selenite. Instead, both showed their peak values when the concentration of sodium selenite reached 10 $\mu\text{g/ml}$, suggesting that 10 $\mu\text{g/ml}$ was the most suitable concentration to enrich Se by *B. longum-En*. Measurement of the selenium content in inorganic and organic forms was also performed. Fig. 1B shows that the percentage of organic selenium was >96% of the total selenium content.

Biochemical characteristics of *Se-B. longum-En*. *B. longum* bacterium colonies were ivory white round scabrosities with a smooth surface. The diameter of these colonies ranged from 0.6 to 1.8 mm. *Se-B. longum-En* exhibited the same biochemical characteristics as those of *B. longum-En*. In our study, both showed minor differences in carbohydrate fermentation to the WT *B. longum*. *Se-B. longum-En* and *B. longum-En* were not able to ferment arabinose and mannose, both of which were fermented in WT *B. longum* cells. These results

Table I. The main biochemical characteristics of (A) *Se-B. longum-En*, (B) *B. longum-En* and (C) WT *B. longum*.

		Carbohydrate fermentation																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A	G ⁺	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-
B	G ⁺	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-
C	G ⁺	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-

1, Gram staining; 2, aerobic assay; 3, anaerobic assay; 4, spore assay; 5, dynamic assay; 6, catalase assay; 7, gelatin liquefaction assay; 8, deoxidize by nitrate; 9, glucose; 10, arabinose; 11, lactose; 12, cellobiose; 13, starch; 14, esculin; 15, mannose; 16, melibiose; 17, trehalose and 18, melezitose. (-), Negative reaction; (+), positive reaction.

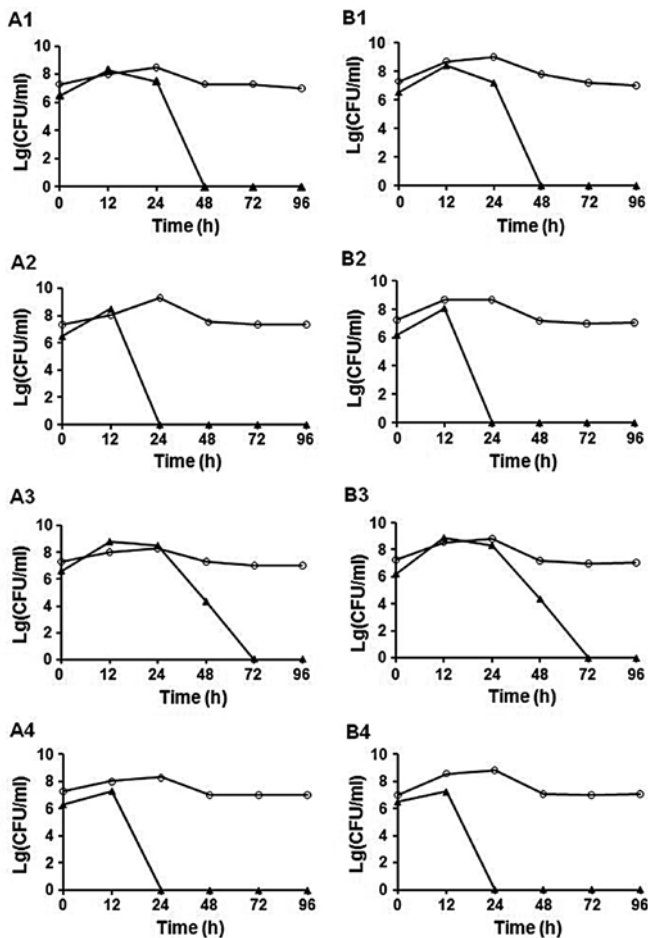


Figure 3. Growth curves of four pathogenic strains co-cultured with WT *B. longum* and Se-*B. longum*-En, respectively, are shown. A1-4 show the growth curve of *shigella flexneri*51571, *salmonella typhimurium*50115, *escherichia coli*44155 and *pseudomonas aeruginosa*03 cultured alone (open circle) or co-cultured with WT *B. longum* (filled triangle), respectively. B1-4 show the growth curves of *shigella flexneri*51571, *salmonella typhimurium*50115, *escherichia coli*44155 and *pseudomonas aeruginosa*03 cultured alone (open circle) or co-cultured with Se-*B. longum*-En (filled triangle), respectively.

suggested that gene transfection and selenium enrichment had little influence on the natural characteristics of *B. longum*. The results are shown in Table I.

Enhancement in phagocytotic activity of macrophage by Se-*B. longum*-En. Fig. 2 shows that the rates of phagocytosis in the transformed *B. longum* (Se-*B. longum*-En and *B. longum*-En) group were 46.72 and 52.40%, respectively. These rates were higher than those in WT *B. longum*. Selenium enrichment to *B. longum*-En markedly enhanced the phagocytotic activity of rat macrophages compared with *B. longum*-En and WT *B. longum*, as is shown by the phagocytotic indices of 1.18, 0.60 and 0.40, respectively.

Inhibition of pathogenic bacteria by Se-*B. longum*-En in vitro. Four pathogenic bacteria strains were strongly inhibited by co-cultivation with either WT *B. longum* or Se-*B. longum*-En (Fig. 3). *Salmonella typhimurium*50115 and *pseudomonas aeruginosa*03 were not detected 24 h after co-cultivation with WT *B. longum* or Se-*B. longum*-En. No colony of *shigella*

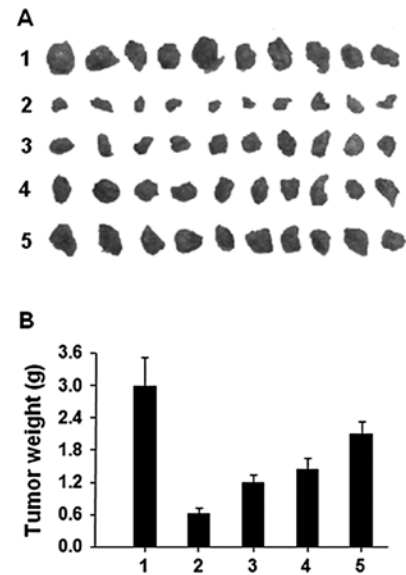


Figure 4. Inhibition of H22 tumor growth with Se-*B. longum*-En by intra-gastric administration. (A) Rows 1-5 show tumors excised from mice treated with 13% fat-free milk, CTX, *B. longum*-En, Se-*B. longum*-En and WT *B. longum*, respectively. (B) The average tumor weight of each group is shown. Bars 1-5 are the average weight of tumors in Rows 1-5.

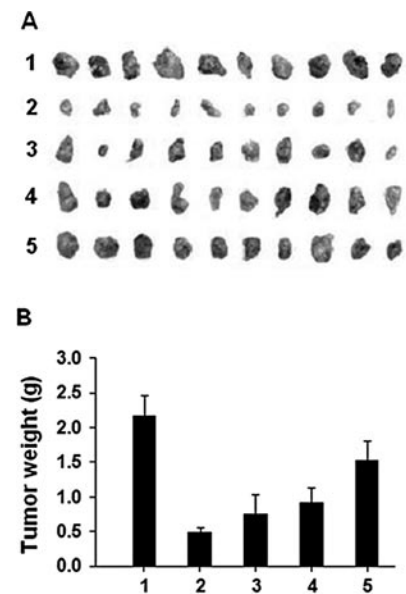


Figure 5. Inhibition of H22 tumor growth with Se-*B. longum*-En by i.v. administration. (A) Rows 1-5 show tumors excised from mice treated with dextrose-saline solution, CTX, *B. longum*-En, Se-*B. longum*-En and WT *B. longum*, respectively. (B) The average tumor weight of each group is shown. Bars 1-5 show the average weight of tumors in Rows 1-5.

*flexneri*51571 was found 48 h after co-cultivation, nor was any colony of *escherichia coli*44155 observed 72 h after co-cultivation. No significant difference was noted between the WT *B. longum* and Se-*B. longum*-En groups.

Suppression of H22 tumor growth with Se-*B. longum*-En by intra-gastric administration. Tumors excised from the mice are shown in Fig. 4A. Fig. 4B shows that mice treated with transformed *B. longum* grew more slowly than those treated with 13% fat-free milk. Compared with the negative control

group, tumor weights were significantly reduced by 51.8% ($P<0.00001$) in the *B. longum*-En and by 60.1% ($P<0.00001$) in Se-*B. longum*-En groups, respectively. Selenium enrichment to *B. longum*-En resulted in ~10% ($P=0.0087$) enhancement of the antitumor effect of *B. longum*-En. Fat-free milk showed partial tumor suppression with an IR of 29.7% ($P=0.00057$).

Suppression of H22 tumor growth by Se-*B. longum*-En through i.v. administration. The inhibition of H22 tumor growth was determined by treating the H22 tumor mice with Se-*B. longum*-En. Tumors excised from the mice and tumor weights of each group are shown in Fig. 5. Tumor growth, measured in weight, was inhibited by 65.16 ($P<0.00001$), 57.21 ($P<0.00001$) and 29.85% ($P=0.00013$) in the Se-*B. longum*-En, *B. longum*-En and WT *B. longum* groups, respectively, as compared with the dextrose-saline solution group. The positive control group inhibited tumor growth by 77.59% ($P<0.00001$). The results suggested that transformed *B. longum* cells significantly inhibited growth of the H22 tumor. No statistical difference was noted between the *B. longum*-En and Se-*B. longum*-En groups ($P=0.143$) though the tumor IR was 8% higher in the Se-*B. longum*-En group.

Discussion

As a non-toxic angiogenic inhibitor with no known side effects or drug resistance, endostatin was considered to be a novel candidate for cancer therapy (15). It can activate the downstream Wnt/catenin pathway and induce reorganization in endothelial cells by binding to several receptors on endothelial cells including $\alpha 5\beta 1$, $\alpha 5\beta 3$ and $\alpha 5\beta 5$ integrins, heparin sulfate, tropomyosin, caveolin-1, vascular endothelial growth factor receptor-1 (VEGFR-1; Flt-1) and VEGFR-2 (Flk-1). The Wnt/catenin pathway and reorganization in endothelial cells were considered to play a role in inhibiting the proliferation and metastasis of endothelial cells, thereby inducing tumor apoptosis (16). Several studies have demonstrated that endostatin has an inhibitory effect on vessel generation in tumors in a dose-dependent manner without apparent side effects or drug resistance (17-19). Endostatin efficacy on many tumor cells, such as renal carcinoma, bladder cancer, Hep3 liver cancer, C6 Glioma, K1735 melanoma and C666-1 nasopharyngeal carcinoma has been noted in gene therapy studies (14,20-25). However, endostatin failed to show a similar effect on acute lymphocytic leukemia and neuroblastoma (26-31).

The greatest obstacle of gene therapy is the lack of an efficient delivery system. As probiotics, bifidobacteria have been widely used in the prevention and treatment of gastrointestinal disorders. In previous years, the anaerobic property of bifidobacteria was explored for cancer gene therapy (32-36). Yazawa *et al* observed that *B. longum* was not detected in normal tissues but selectively germinated in tumors after i.v. administration. Further studies showed that *B. longum* was able to be used as a specific gene therapy delivery system for breast tumors (5,36). Fu *et al* used transformed *B. longum* carrying shuttle vector pBV220 (Amp^r) encoding human endostatin as an oral delivery system. The results of Fu *et al* showed that *B. longum*-pBV220-En strongly inhibited the growth of solid liver tumor in a HepG2 liver tumor xenograft model and prolonged survival in experimental animals (7). Xu *et al*

developed a more stable *B. longum*-pBV22210-En constructor that showed a stronger suppression of tumor growth in xenograft models (8).

Selenium was shown to benefit patients subjected to cancer chemoprevention (37). Furthermore, selenium supplementation enhanced lymphocyte activities and reduced the incidence of lung cancer (38-40). Studies have demonstrated that selenocysteine is responsible for the majority of Se biological effects (41). Presumably, selenoproteins in the form of selenocysteine or the S-Se-S compound were involved in the reversible cysteine/disulfide transformation which occurred in a number of redox-regulated proteins. The monomethylated form of Se was associated with the apoptosis-inducing process in transformed cells (10). In our previous study, Fu *et al* observed that the antitumor effect was enhanced when *B. longum*-pBV220-En was enriched with Se. In addition, selenium enrichment to *B. longum*-pBV220-En improved natural killing cell activity, stimulated T-cell proliferation and promoted the activity of IL-2 and TNF- α in BALB/c mice (7). It has been known that Se can only be utilized in its organic form *in vivo*. We determined the most suitable concentration of sodium selenite for enriched and quantified selenium in its organic form. We also observed that Se-*B. longum*-En markedly enhanced the phagocytotic activity of rat macrophages with a phagocytotic index of 1.18, which was significantly higher than that of *B. longum*-En (0.60) and WT *B. longum* (0.40).

Maintaining the inherent characteristics of *B. longum* is important for retaining the physiological role of *B. longum*, which contributes to the advantages of *B. longum* as a gene delivery system, including the enhancement of immunity and selective location in tumors. Carbohydrate fermentation is considered to be of great significance in identifying the bacterium strain. The results showed that *B. longum*-En and Se-*B. longum*-En differed from WT *B. longum* in carbohydrate fermentation in arabinose and mannose (Table I). This difference indicated that *B. longum*-En and Se-*B. longum*-En adopted similar morphological and biochemical characteristics as those of WT *B. longum*. It was reported that the fermentation of L-arabinose, gluconate, inulin, lactose, D-mannose, methyl α -D-glucoside, ribose, salicin, trehalose or xylose was variable in *B. longum* (42). Results of additional indices, including the aerobic, dynamic and spore assays, showed that there was no difference between *B. longum*-En, Se-*B. longum*-En and WT *B. longum*. In addition, we examined the *in vitro* activity of transformed *B. longum* in the inhibition of pathogenic bacteria and the results showed that both *B. longum*-En and Se-*B. longum*-En markedly enhance phagocytotic activity when compared with WT *B. longum*. However, no difference to WT *B. longum* was noted in the inhibition of pathogenic bacterium, suggesting that selenium enrichment was not able to enhance the regressive effect on the pathogenic bacteria of *B. longum*-En *in vitro*. Our results indicated that the gene transfection of endostatin and selenium enrichment had little influence on the characteristics of *B. longum*. Thus, it is important to further evaluate the safety and availability of Se-*B. longum*-En.

The present study showed that Se-*B. longum*-En resulted in an approximately 10% higher tumor IR than that of *B. longum*-En on tumor-bearing mice through intra-gastric ($P=0.0087$) and i.v. ($P=0.143$) administration. The higher

IR suggested that the enhancement of immunity and down-regulation of related gene expression by the transfection of endostatin and selenium enrichment is a possible mechanism of improvement on tumor inhibition.

In conclusion, selenium enrichment to *B. longum*-En not only retained the biochemical properties of *B. longum* but also enhanced the *B. longum* immune stimulatory activities, and increased the antitumor effect of *B. longum*-En. These results suggested that Se-*B. longum* be used as an effective delivery system of endostatin for the inhibition of liver tumors.

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