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Comparison of antitumor effect of recombinant *L*-asparaginase with wild type one *in vitro* and *in vivo*

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ABSTRACT

AIM: To investigate the antitumor effect of recombinant *L*-asparaginase on the growth of several tumors such as P388, L1210, hepatocellular carcinoma (Heps), K562, P815, and sarcoma 180 (S_{180}). **METHODS:** Tumor cells (K562, L1210, and P815) were cultured *in vitro* and the morphology of those cells was observed with inverse microscope and transmission electron microscope. MTT assay was performed to measure the cell proliferation and inhibition rate. DNA content was assayed by flow cytometry. According to protocols of transplant tumor research, mice were transplanted with tumor cells L1210, P388, Heps, and S_{180}. The survival rate and weight of tumor were observed after the treatment of test drugs. **RESULTS:** The antitumor effects of *L*-asparaginase were observed *in vitro* with tumor cells K562, L1210, and P815 ($P<0.01$). *In vivo* experiments showed that ip administration of *L*-asparaginase significantly increased the survival rate and life span of mice with P388 or L1210 tumor cells ($P<0.01$). Tumor growth induced with Heps was also significantly suppressed. Furthermore, significant suppression of tumor growth was observed in mice induced with Heps and S_{180} by iv administration of *L*-asparaginase. **CONCLUSION:** Recombinant *L*-asparaginase markedly inhibited tumors tested in this study and the results strongly suggest that recombinant *L*-asparaginase has great potential for clinical treatment of these tumors.

INTRODUCTION

L-Asparaginase is an important natural product that possesses a broad spectrum of antitumor activity. It has been successfully applied to the treatment of several diseases such as lymphocyte sarcoma and leukemia[1-3]. Clinical studies have demonstrated higher recovery efficiency for leukemia patients treated with *L*-asparaginase than those with IL-2[4]. *L*-asparaginase is an important anti-cancer drug and was the focus of intensive investigations during 1970s that made large-scale industrial manufacture of this enzyme practical in both Japan and the United States from wild type *E coli* strains. However, no reports are available concerning the production of this important anti-cancer drug using recombinant DNA techniques. Currently, commercial *L*-asparaginase is produced by wild-type gene fermentation. Unfortunately, production of large quantities of *L*-asparaginase through this process is restricted by the extremely low efficiency of the technique. Consequently, *L*-asparaginase for most clinical applications in China is largely depended on imports from Japan and USA. The extremely high price of this drug poses the severe restriction on the clinical application of this po-

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tent drug to patients in China. Therefore, it is remarkably attractive to search for systems that display high expression levels of *L*-asparaginase and construct suitable genes for this enzyme through DNA recombinant techniques[5-7].

Department of Biochemistry of the School of Biopharmacy in China Pharmaceutical University has initiated a project of designing and constructing efficient expression systems for recombinant *L*-asparaginase (isozyme II) since 1995. The chromosome DNA of the wild type *E coli* strain AS-1357 was used as the template. After PCR amplification, the gene (AnsB) of *L*-asparaginase II was isolated and overexpressed in *E coli* (PKA/CPU 210009), therefore, providing an efficient technology for the large-scale manufacture of high-quality and low-cost *L*-asparaginase for clinical appli-cations.

This paper described the antitumor effect (*in vivo* and *in vitro*) of the recombinant *L*-asparaginase from a system (PKA/CPU 210009) that displayed extremely high expression efficiency for this enzyme. The results reported here validated the major function of *L*-asparaginase and provided experimental supports for the clinical application of this promising medicine.

MATERIALS AND METHODS

Chemicals and reagents Recombinant *L*-asparaginase (230 IU/mg) was provided as a kind gift from the Department of Biochemistry, School of Biopharmacy, China Pharmaceutical University. Wild type *L*-asparaginase (1000 IU/ampoule) was purchased from Zymosis Industries Inc, Japan. Cyclophosphamide (200 mg/ampoule) was purchased from Hualian Pharmaceutical Company Ltd, Shanghai. Culture media was prepared from RPMI-1640 (Gibco, USA) supplemented with 40 % bovine serum.

Animals Kunming mice (Grade II, Certificate № 9700047) were provided by the Department of Experimental Animals of China Pharmaceutical University. DBA/2 mice (Grade II, Certificate № 9704) were purchased from Center for Experimental Animals, Chinese Academy of Sciences, Shanghai, China.

Tumor cell lines Both S_{180} and Heps were from Jiangsu Institute of Antitumor Pharmaceuticals. All other tumor cells including L1210, P388, K562, and P815 were obtained from Cell Bank of the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China.

Morphology Tumor cells (K562, L1210, and P815) at the logarithmic growth phase with a cell density of 5×10^8/L were inoculated into 25 mL culture media containing either recombinant or imported *L*-asparaginase at a concentration of 30 mg/L. Cells were grown in 5 % CO_2 incubator at 37 ℃ for 24 h. The morphology of cells was monitored with an inverse microscope (Chongqing Optical Instruments, China). Cells were then isolated by centrifugation at 200×*g* for 10 min. After discarding the supernatant, cells were fixed with 4 % pentadialdehyde for 24 h followed by a thorough wash with double-distilled water. After dehydration with gradient alcohol washing, cells were embedded with EPON-812 resin. Dissection was accomplished using an LKB-V model microtome. Cell sections were stained with sodium acetate and lead citrate, and subjected to morphology investigation with an H-300 transmission electron microscope.

MTT assay Both single layer and suspension cultures were carried out with media containing either recombinant or imported *L*-asparaginase at concentrations of 2.5, 5.0, 10, 20, and 40 mg/L. The control culture was supplied with regular media only. MTT colorimetry was employed to investigate the proliferation of cells. Typically, cells (K562, L1210, and P815) at the logarithmic growth phase were suspended in solution to a cell density of 3×10^7/L. This suspension (100 μL per well) was then transferred to each aperture of a 40-aperture zymogram plate. Each aperture was further supplied with an equal volume (100 μL per well) of RPMI-1640 culture media. The plate was incubated in 5 % CO_2 incubator at 37 ℃ for 24 h. Stock MTT solution (5 g/L) 20 μL was then added to each aperture and the plate shaken for 1 min. Incubation was continued for another 4 h in the same incubator. Cells were then isolated by centrifugation and cell pellets were treated with isopropanol hydrochloric acid 0.04 mol/L (100 μL per well). Absorbance was measured at 570 nm after cells were completely dissolved.

DNA quantitation by flow cytometer Cells (K562, L1210, and P815) at the logarithmic growth phase were suspended in solution to a final cell density of 5×10^8/L. The suspension was inoculated into 25 mL culture media containing either recombinant or imported *L*-asparaginase at a final concentration of 30 mg/L. Cells were grown in 5 % CO_2 incubator at 37 ℃ for 24 h. Cells were then isolated by centrifugation at 200×*g* for 10 min and fixed with appropriate fixing solution. Cell DNA was stained with iodinated tincture and analyzed

with flow cytometer. About 5000 individual cells were consumed in a typical analysis.

Life span determination DBA/2 mice with body weight of 18-22 g were transplanted with L1210 (or P388) according to protocols of transplant tumor research[8]. Twenty-four hours after tumor transplantation, animals were randomly divided into 7 groups and each group consisted of 10 mice. Animals were then subjected to ip treatment with different lead materials with different dosages in each group for 8 consecutive days. The high dosage groups received a single dose of recombinant and imported *L*-asparaginase 5400 $IU \cdot kg^{-1} \cdot d^{-1}$ and the medium dosage groups were treated with 2700 $IU \cdot kg^{-1} \cdot d^{-1}$, respectively. The doxorubicin group was so identified as animals in this group treated with doxorubicin (2 $mg \cdot kg^{-1} \cdot d^{-1}$) instead of *L*-asparaginases. The negative control group received 0.9 % normal saline. The total number of mice survived and the overall survival rate during the 60-d observation period after termination of treatments were used to evaluate the efficiency of the test drugs. The survival rate was calculated only for animals that survived for 60 d or longer and that showed no symptoms of tumor development.

Tumor growth measurement Kunming mice with body weight of 18-22 g were transplanted with Heps (or S_{180}) according to protocols of transplant tumor research[8]. Twenty-four hours after 24 h of tumor transplantation, animals were randomly divided into 6 groups. The groups with *L*-asparaginase treatment received three dosages, respectively. The positive control group was treated with imported *L*-asparaginase. The other group was treated with cyclophosphamide. The negative control group received 0.9 % normal saline. All test drugs were given through injections 24 h after tumor transplantation (or inoculation). Treatments were done at a frequency of ip one dose per day for a total of 8 consecutive days and iv one dose per two day for a total of four treatments. All animals were killed 24 h after the treatments.

Statistical analysis All data were presented as mean±SD. Statistical significance was evaluated by *t*-test.

RESULTS

Effects of recombinant *L*-asparaginase on the morphology of tumor cells The treatment with either recombinant or imported *L*-asparaginase for 24 h significantly inhibited the growth rate of all tumor cells tested (K562, L1210, and P815) as manifested by the equivocal and elongated structure of cells from microscope observations. This inhibitory effect was further supported by the pycnosis of nucleus, increase in heterochromatin secretion, karyorrhexis, and apoptosis from transmission electron microscope observations (Fig 1).

Effects of recombinant *L*-asparaginase on proliferation rate of tumor cells As the concentration of *L*-asparaginases increased, the rate of cell proliferation decreased as a result of increased inhibition efficiency on cell growth. The growth of all three types of tumor cells was significantly inhibited by treatment with either recombinant or imported *L*-asparaginases compared with the control group (Fig 2).

Effects of recombinant *L*-asparaginase on DNA synthesis in tumor cells Results were processed with Cellfit software package. Compared with the control group, treatment with either recombinant or imported *L*-asparaginases significantly increased the number of cells (K562, L1210, and P815) at the G_0/G_1 stages. On the other hand, the number of cells at the S stage was dramatically decreased, indicative of reduction in DNA synthesis due to interception at the G_0/G_1 stages by *L*-asparaginase treatments. Meanwhile, the appearance of the sub-peak at the G_1 stage before the G_0/G_1 stages is due to apoptosis. It suggested that the accelerated tumor cell death was achieved through cell apoptosis after treatment with *L*-asparaginases (Tab 1).

Recombinant *L*-asparaginase increased life

Tab 1. The flow cytometry analysis of different phases of tumor cell proliferation after treatment of recombinant *L*-asparaginase (30 mg/L).

Group	P815	K562	L1210
Control			
G_0/G_1	34.6	52.6	21.0
S	59.9	47.4	67.5
G_2/M	5.5	–	11.5
Recombinant *L*-asparaginase			
G_0/G_1	59.0	65.1	40.3
S	19.6	34.9	57.0
G_2/M	21.4	–	2.7
Imported *L*-asparaginase			
G_0/G_1	60.6	69.3	38.0
S	13.8	30.9	60.1
G_2/M	25.6	–	1.9

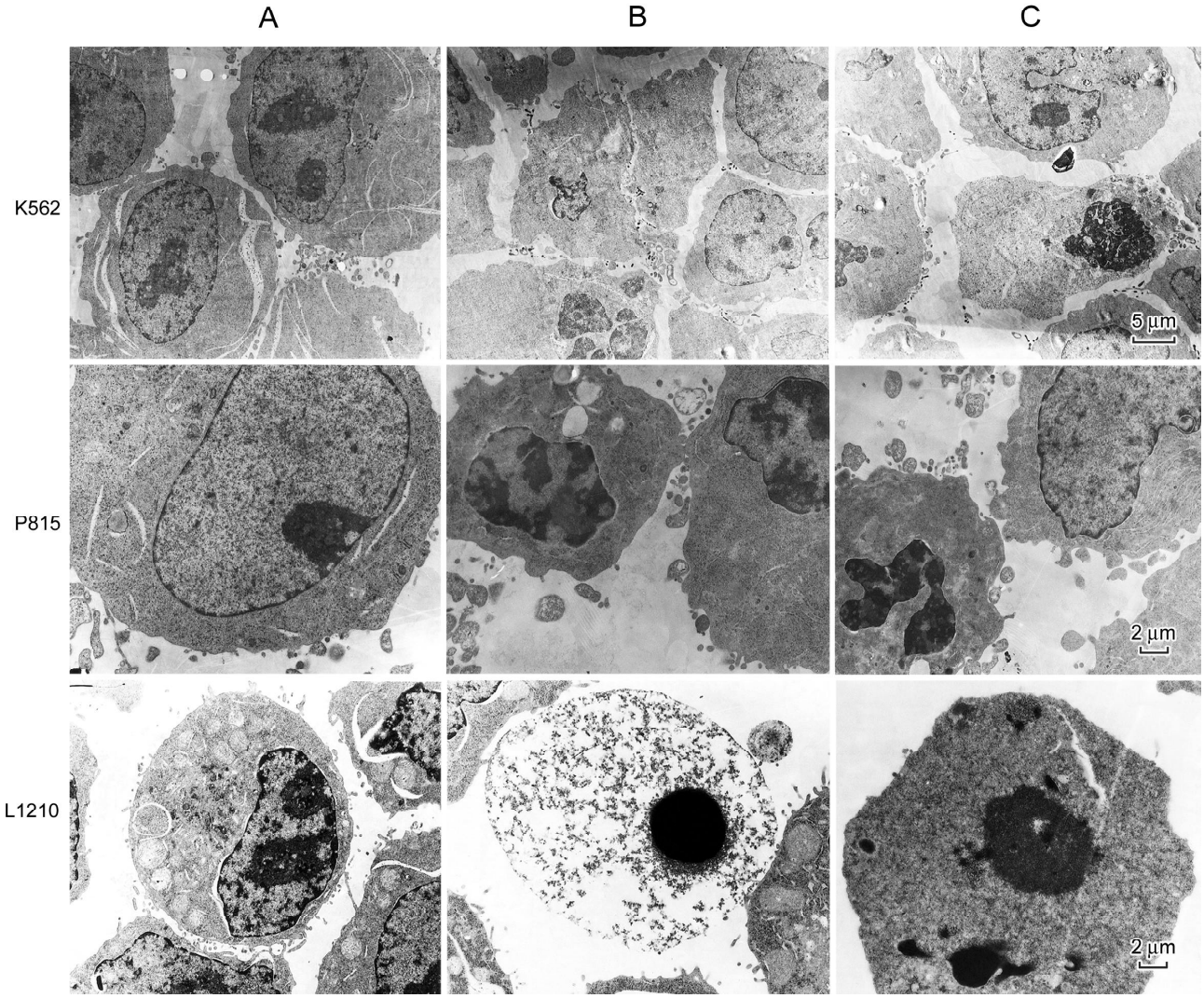

Fig 1. Morphological changes of tumor cell K562 (×3000), P815 (×5000), and L1210 (×5000) with transmission electron microscope. A: control; B: treated with imported *L*-asparaginase; C: treated with recombinant *L*-asparaginase.

span of mice with leukemia L1210 or P388 Obviously, compared with the control group, recombinant *L*-asparaginase (ip) significantly increased the life expectancy of mice inoculated with L1210 lymphocyte leukemia or P388 lymphocyte leukocyte (P<0.01, Tab 2).

Recombinant *L*-asparaginase inhibited tumor growth of Heps or S_{180} transplanted into mice In the treatment route of ip, recombinant *L*-asparaginase (5400 and 2700 IU·kg^{-1}·d^{-1}), imported *L*-asparaginase (5400 IU·kg^{-1}·d^{-1}), and cyclophosphamide (20 mg·kg^{-1}·d^{-1}) had significant inhibitory effect on the growth of inoculated Heps in mice (Tab 3). In all test drugs of iv administration, recombinant *L*-asparaginase (8000 and 4000 IU·kg^{-1}·d^{-1}), imported *L*-asparaginase (8000 IU·kg^{-1}·d^{-1}), and cyclophosphamide (30 mg·kg^{-1}·d^{-1}) have significant inhibitory effect on the growth of inoculated Heps in mice (Tab 4). In all test materials of iv administration of recombinant *L*-asparaginase (8000 and 4000 IU·kg^{-1}·d^{-1}), imported *L*-asparaginase (8000 IU·kg^{-1}·d^{-1}), and cyclophosphamide (30 mg·kg^{-1}·d^{-1}) had significant inhibitory effect on the growth of inoculated S_{180} in mice (Tab 4).

DISCUSSION

It was demonstrated that treatments of plasma cells (from either human or mice) with recombinant *L*-asparaginase had profound effect on the morphology of the cells. Apoptosis was observed with cells treated with recombinant *L*-asparaginase. Quantitative DNA analysis with flow-cytometer indicated that reduction of DNA synthesis upon recombinant *L*-asparaginase

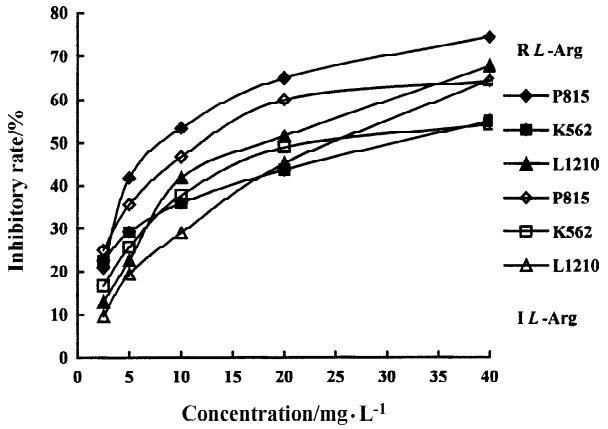

Fig 2. Inhibitory effect of recombinant (closed: R *L*-Arg) and imported (open: I *L*-Arg) *L*-asparaginase on the growth of tumor cells.

Tab 2. Effect of ip administration of *L*-asparaginase on life span of mice transplanted with L1210 or P388. *n*=10 DBA/2 mice. Mean±SD. ᶜ*P*<0.01 *vs* control.

Group	Dose/ IU·kg^{-1}·d^{-1}	L1210 Survival days	L1210 Pro-longed /%	P388 Survival days	P388 Pro-longed /%
Control	–	9.0±1.2		9.2±1.0	–
Recombinant *L*-asparaginase					
	5400	21±6ᶜ	136	21±5ᶜ	130
	2700	16±4ᶜ	73	16±4ᶜ	72
	1350	12±3ᶜ	34	12±3ᶜ	26
Imported *L*-asparaginase					
	5400	21±6ᶜ	132	22±6ᶜ	134
	2700	16±4ᶜ	76	16±5ᶜ	73
Doxorubicin	2 mg·kg^{-1}·d^{-1}	31±7ᶜ	246	32±8ᶜ	246

treatments was a result of interception at the G_0/G_1 stages. Meanwhile, apoptosis was considered responsible for the appearance of the sub-peak at the G_1 stage before the G_0/G_1 stages. Results reported here suggested that the accelerated tumor cell death was accomplished through cell apoptosis from treatment with *L*-asparaginases.

Tab 3. Inhibitory effect of ip administration of recombinant *L*-asparaginase on the growth of Heps in mice. *n*=10 Kunming mice. Mean±SD. ᶜ*P*<0.01 *vs* control.

Group	Dose/IU·kg^{-1}·d^{-1}	Weight of body/g Pre-dose	Weight of body/g After dose	Weight of tumor/g	Inhibitory/%
Control		19.9±1.2	26.2±1.6	1.71±0.24	
Recombinant *L*-asparaginase	5400	19.8±1.3	24.5±1.0	1.0±0.3ᶜ	39
	2700	20.2±1.2	25.0±1.2	1.2±0.4ᶜ	33
	1350	19.9±1.2	26.6±1.3	1.54±0.20	10
Imported *L*-asparaginase	5400	20.1±1.2	24.4±1.1ᶜ	1.02±0.27ᶜ	40
Cyclophosphamide	20 mg·kg^{-1}·d^{-1}	19.8±1.3	22.5±1.4ᶜ	0.47±0.13	73

Tab 4. The inhibitory effort of iv administration of recombinant *L*-asparaginase on the growth of Heps or S_{180} transplanted into mice. *n*=10 Kunming mice. Mean±SD. ᶜ*P*<0.01 *vs* control.

Group	Dose/IU·kg^{-1}·d^{-1}	Heps Weight of tumor/g	Heps Inhibitory rate/%	S_{180} Weight of tumor/g	S_{180} Inhibitory rate/%
Control		1.64±0.24	–	1.75±0.22	
Recombinant *L*-asparaginase	8000	0.92±0.15ᶜ	43.9	0.96±0.19ᶜ	45.5
	4000	1.11±0.18ᶜ	32.3	1.17±0.27	33.5
	2000	1.45±0.23	11.6	1.56±0.24	11.0
Imported *L*-asparaginase	8000	0.92±0.19ᶜ	43.9	0.93±0.19ᶜ	47.0
Cyclophosphamide	30 mg·kg^{-1}·d^{-1}	0.42±0.10ᶜ	74.4	0.45±0.13ᶜ	74.2

Results from *in vivo* studies indicated that recombinant *L*-asparaginase displayed significant inhibitory effects on the growth of several tumor cells. Our study showed that ip administration of recombinant *L*-asparaginase dramatically increased the life span of mice with L1210 lymphocyte leukemia and mice with P388 lymphocyte leukocyte. Both ip and iv administration of recombinant *L*-asparaginase significantly inhibited the growth of Heps transplanted into mice. Meanwhile, iv administration of *L*-asparaginase alone could dramatically inhibit the growth of S_{180} transplanted into mice. The inhibitory efficiency to all tumor cells was directly proportional to the dosage applied.

This study provides direct proof that recombinant *L*-asparaginase is a potent drug for clinical treatment of leukemia.

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