ORIGINAL RESEARCH

Effect of Haishengsu on transplanted K562 and drug-resistant K562/ADM tumors: An experimental study

Ji-Zhu Liu¹

Ming Yang²

Da-Peng Li²

Bin Zhang¹

Shou-Guo Chen³

Chun-Bo Wang⁴

Guang-Yao Li¹

Le-Xin Wang⁵

¹ Liaocheng People's Hospital and Liaocheng Clinical School of Taishan Medical

University, Liaocheng Shandong, 252000, P. R. China

² State Key Laboratory of Experimental Hematology, Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin, P. R. China

³ Haisheng Oncology Hospital of Qingdao, Qingdao, P.R.China

⁴ Qingdao University Medical College, Qingdao, P. R. China

⁵ School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, Australia

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Abstract

Purpose: To evaluate the effect of Haishengsu (HSS) on transplanted K562 and drug-resistant K562/ADM tumors.

Methods: Mice were inoculated subcutaneously with K562 and K562/ADM cells, respectively. Tumour-bearing animals were divided into HSS, adriamycin, combination therapy (adriamycin plus HSS) and placebo groups. The anti-tumour effect was assessed by tumour growth curve and tumour inhibitory rate (IR).

Results: In animals inoculated with K562 cells, the inhibitory rates of high (1800mg/kg) and medium (900mg/kg) dose HSS groups were 100% and 96.4%, respectively, which was higher than that in the adriamycin (88.9%) or the combination therapy groups (85.8%, $P < 0.05$). The inhibitory rate in the low-dose HSS group (53.4%) was lower than in all other groups ($P < 0.01$). In mice inoculated with K562/ADM cells, the inhibitory rates in the high, medium and low dose HSS groups were 100%, 95.9%, and 44.1%, respectively. In the adriamycin group, the inhibitory rate

was 23.07%, which was lower than in the HSS group ($P < 0.01$). Pathological examination of tumour tissues from HSS-treated animals showed extensive necrosis and bleeding.

Conclusions: Haishengsu inhibits the growth of transplanted K562 tumours in mice. It is also effective in suppressing the growth of drug-resistant K562/ADM tumors in this animal model.

Tegillarca granosa L. (Chinese name “*xue han*”) is a species of seashell. It is widely distributed and used as a traditional Chinese medicine for cancer treatment in mainland China. Haishengsu (HSS) is a protein extract from *Tegillarca granosa* with a molecular weight of approximately 15 KDa. HSS has a potent suppressive effect on several types of tumor cells *in vivo* and

in vitro.¹⁻³ Recently, we found that HSS suppressed the growth of leukemia K562 cell by inhibiting the G0/G1 and S phases of the cell cycle.⁴ HSS also induced apoptosis in these leukemia cells by reducing the expression of apoptosis suppressor bcl-2, and increasing the expression of apoptosis promoting bax.⁴

The *in vivo* anticancer effects of HSS have been investigated in two small clinical trials.^{5,6} Daily intravenous administration of HSS for 4 weeks resulted in remission of non-small-cell lung cancer or renal cancer in approximately half of the patients.⁵ HSS was also found to enhance the efficacy of conventional chemotherapeutic regimens for non-small-cell lung cancer.⁶

K562 cell line is a blast crisis cell line of chronic myeloid leukemia.⁷ K562/ADM cells are drug resistant strains of K562 cells induced by adriamycin. The primary purpose of the present study was to investigate whether HSS could inhibit the growth of transplanted K562 and K562/ADM tumors in mice.

Materials and Methods

The study was approved by the institution review board of Liaocheng People's Hospital.

Sources of drugs, reagents and cells

HSS was purchased from Qingdao Haisheng Oncology Hospital (Shandong, China, batch number 990211); Adriamycin was from Pharmacia Cor. (New Jersey, USA). K562 and K562/ADM cell lines were provided by the Institute of Hematology, Chinese

Academy of Medical Sciences (Tianjin, China). Nude mice (female, 15-18g) were obtained from Beijing Vital River Laboratory Animal Co., Ltd (Beijing, China).

Cell culture

The drug-resistant leukemia cell line K562/ADM was derived from the parental K562 cells by continuous exposure to increasing concentrations of adriamycin (up to 20 µg/ml). K562 and K562/ADM cells were cultured in RPMI1640 culture medium containing 10% fetal calf serum (Gibco BRL, Gaithersburg, USA) at 37°C in an incubator (SANYO, Tokyo, Japan) of saturated humidity.

Establishment of mice model and drug administration

Twenty-four hours after receiving radiation treatment (4Gy), the nude mice were inoculated subcutaneously with 1×10^7 K562 (n=18) or 1×10^7 K562/ADM cells (n=18) in the buttocks. Five days after inoculation, the K562- or K562/ADM tumour-bearing mice were randomly divided into placebo, HSS, adriamycin and combination therapy (adriamycin + HSS) groups. Each group comprised 3 animals. HSS, adriamycin and placebo was intravenously administered via the tail vein of the K562 tumour- or K562/ADM tumour-bearing mice. The dosing regimens are listed in Table 1.

The antitumour effect of HSS was assessed by measuring the size of the tumour with a vernier caliper every three days. A tumour growth curve was drawn. Relative tumour volume (RTV) was calculated: tumour volume $V(\text{mm}^3) = 1/2 \times ab^2$ (a for

TABLE 1. Intravenous administration of drugs (3 animals in each group).

Groups	Drug	Dosages
Placebo	Normal saline	0.5ml, q3d×4d
Adriamycin	Adriamycin	2mg/kg, q3d×4d
Low HSS	HSS	450mg/kg, qd×10d
Medium HSS	HSS	900mg/kg, qd×10d
High HSS	HSS	1800mg/kg, qd×10d
Combination therapy group	Adriamycin+HSS	adriamycin 2mg/kg, q3d×4d; HSS 900mg/kg, qd×10d.

longer track, b for the short track); $RTV = V_t/V_0$ (V_0 was the tumour size measured for the first time, V_t was the tumour size at the end of therapy).

The inhibitory rate (IR) was calculated as following: $IR = (1 - T_{RTV}/C_{RTV}) \times 100\%$ (T_{RTV} : RTV of the treatment group, C_{RTV} : RTV of the control group).

Pathological examination of the tumour

On the 21st day of treatment, the mice were euthanized and the tumour tissues were dissected and fixed with 10% neutral formalin for pathological examination with light microscope.

Statistical analysis

Data are expressed as means $\pm$ SD. SAS8.2 software was used for data analysis. Numerical data were analyzed with one-way ANOVA. The tumour inhibitory rate between two groups (e.g. between adriamycin and medium or high dose HSS groups, or between low and medium or high dose HSS groups) was analysed with Chi-square test. Three group comparisons were performed with Tukey-Kramer multiple comparison tests. $P < 0.05$ was considered statistically significant.

Results

Effect of HSS on K562 tumors

All animals were alive at the end of the study. The inhibitory rate of medium and high dose HSS group was 96.4% and 100%, respectively (Table 2). The inhibitory rates in the adriamycin and the combination therapy groups were similar (88.9% vs 85.8%, $P > 0.05$); both were lower than in the medium and high dose HSS groups ($P < 0.05$). The inhibitory rate of low dose HSS group (53.4%) was lower than all other treatment groups ($P < 0.01$).

Tumour growth curves of the K562 tumor-bearing mice are shown in Fig 1. Medium and high HSS groups had the lowest growth curve. The curve of the low HSS group was below the placebo group but above all other treatment groups.

Effect of HSS on K562/ADM tumors

The inhibitory rates of the three HSS group (95.9% and 100% in medium and high HSS group) were higher than in the adriamycin group (23.1%, $P < 0.01$). The inhibitory rate in the low HSS group (44.1%) was also higher than in the adriamycin group ($P < 0.05$), but was less than in the other HSS groups

TABLE 2. Comparison of antitumour effects of HSS and adriamycin.

Groups		Tumour volume (mm ³)		RTV/CTV (%)	IR (%)
		Before therapy	After therapy		
Placebo	S	147.9 $\pm$ 16.8	4147.5 $\pm$ 3600.9	-	-
	R	124.5 $\pm$ 22.9	1667.1 $\pm$ 540.4	-	-
Adriamycin	S	157.8 $\pm$ 69.5	490.3 $\pm$ 443.3	11.0	88.9
	R	156.8 $\pm$ 86.5	1615.7 $\pm$ 576.5	76.9	23.1
Combination therapy	S	201.7 $\pm$ 36.7	807.3 $\pm$ 818.6	14.2	85.8
	R	173.2 $\pm$ 20.3	1093.9 $\pm$ 1126.6	47.1	52.9
Low HSS	S	148.4 $\pm$ 93.3	1948.9 $\pm$ 23.28.7	46.6	53.41
	R	173.94 $\pm$ 67.4	1301.9 $\pm$ 1030.8	55.9	44.13
Medium HSS	S	128.46 $\pm$ 37.3	129.9 $\pm$ 109.5	3.4	96.4*
	R	229.48 $\pm$ 119.5	126.2 $\pm$ 172.3	4.11	95.9**
High HSS	S	129.97 $\pm$ 56.7	1.3 $\pm$ 2.3	0	100.0*
	R	253.54 $\pm$ 88.9	1.3 $\pm$ 2.3	0	100.0**

S: group of mice inoculated with K562 cell, R: group of mice inoculated with K562/ADM cell; * $P < 0.05$, ** $P < 0.01$, compared with adriamycin and combination therapy group.

FIGURE 1. Growth curves of mice inoculated with K562 cells.

FIGURE 2. Growth curves of mice inoculated with K562/ADM cells.

FIGURE 3. Pathological examination of K562 tumour tissue from the placebo and the Haishengsu group.

($P < 0.01$).

The growth curves of these groups are shown in Fig 2. The curves from the three HSS groups and the combination group were below the placebo or adriamycin group.

Pathological examination of tumours

Examination of tumour tissue from the placebo group revealed a large number of tumour cells, with regular cell arrays but no bleeding or necrosis or inflammation (Fig 3). These tumour cells also had a larger proportion of nuclear/plasma ratio than the cells in the HSS group. In the tumour tissues from the K562-HSS group, there was extensive necrosis and bleeding. Inflammatory cells were readily seen (Fig 3). The appearances of the tumour tissues from the K562/ADM-HSS groups were similar to those in the K562-HSS group, showing a large number of necrotic tumour cells (Fig 4).

Discussion

The major findings of this study are: 1) HSS suppressed the growth of transplanted leukemia cell K562 in mice dose-dependently. At 900 and 1400mg/kg,

HSS exerted an inhibitory rate that was greater than adriamycin; 2) At a dose between 450 to 1400mg/kg, HSS suppressed the growth of adriamycin-resistant K562/ADM tumour; 3) The anti-tumour effects of HSS were associated with necrosis in the transplanted tumour tissues.

This is the first study to demonstrate the effectiveness of the seashell protein HSS in treating transplanted tumour *in vivo*. It is worth noting that the doses of HSS used in the present study were much higher than those used in the previous clinical trials on non-small cell lung cancer, or renal cancer, in which the doses were in the order of 2.4mg per day.^{5, 6} At this relatively low dose, HSS did not cause any clinically significant adverse effects in patients.^{5, 6} Although the high doses of HSS used in the mice were not associated with identifiable adverse effects, the safety profile and the effectiveness of these high doses HSS in treating human leukemia or solid tumors remain to be seen.

Resistance to chemotherapy is one of the major obstacles to the effective treatment of chronic myeloid leukemia. Patients with advanced myeloid leukemia have been less sensitive to therapy, and responses have been short lived.⁸ K562/ADM cells are drug re-

FIGURE 4. Pathological examination of adriamycin-resistant K562/ADM tumour tissue from the placebo and the Haishengsu groups.

sistant strains of K562 cells induced by adriamycin.⁹ In the present study, at the three doses tested, HSS was able to inhibit the growth of transplanted K562/ADM tumours in all animals. In contrast to adriamycin or placebo, HSS treatment was associated with extensive necrosis and cell damage in the tumour tissues. These results suggest that HSS is effective in treating drug resistant leukemia *in vivo*.

The therapeutic doses of intravenous adriamycin for tumour treatment in rats have been reported as being 1-6 mg/kg.^{10, 11} In our study, we used a moderate dose of 2 mg/kg for the treatment of the transplanted tumour. At this dose, there was suppression of adriamycin-sensitive K562 tumour, resulting in an inhibition rate of 88.9%. However, the therapeutic effects of the medium or high dose HSS appear to be superior to that of adriamycin, because with both adriamycin sensitive and resistant tumours, the inhibition rates in the two HSS groups were above 96.0% (Table 2). In the combination therapy (medium dose HSS and Adriamycin) group, the inhibition rate in the adriamycin resistant group (52.9%) was lower than in the medium dose HSS group (95.9%). The reason for the reduced therapeutic effect in the combined regimen is not clear. Whether it is due to the adverse drug

interaction between adriamycin and HSS requires further investigation.

The cellular or molecular mechanisms of HSS's anti-tumour actions observed in this study remain unclear. *In vitro* studies found that HSS causes apoptosis and suppress cell growth at the G0/G1 and S phases of the K562 cell cycle.⁴ Our recent study also demonstrated that with adriamycin-resistant K562/ADM cells, HSS also increased the rate of apoptosis.¹² In addition, HSS reduced the expressions of *p*-glycoprotein, a drug-resistance inducing protein in leukemia and other cells.¹² These actions may have contributed the anti-tumor effects as observed in the present study.

In conclusion, HSS exerts an anti-tumour effect on transplanted K562 tumours in mice. It is also effective in inhibiting the growth of adriamycin-resistant K562/ADM cancer in this animal model. These anti-tumour effects are accompanied by extensive tissue necrosis and bleeding in the tumour tissues. Clinical trials are required to ascertain whether high doses of HSS can be used safely and effectively to treat human cancer.

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References

1. Wang ZL, Chu X, Yang X, Chen SQ, Wang CB. Anti-tumor effects of extractive from *Arca Granosa Linnaeus*. *Shandong Med J* 2006;46:69-73.
2. Liu ZX, Zhan SM, Yao RY, Fang LH, Yang X, Wang CB. An experiment study on anti-tumor effect of *Haishengsu* injection. *Chin J Mar Drugs* 2004;23:35-7.
3. Yao RY, Chu X, Zhang YJ, Yang X, Liu XR, Wang CB. Anti-tumor effect of Haishengsu extracted from *Tegillarca granosa* in vitro and in vivo. *Chin J Mar Drugs* 2005;24:33-6.
4. Li GY, Liu JZ, Yu XM, et al. Effect of a seashell protein haishengsu on cell growth and expression of apoptosis genes in leukemia K562 Cells. *Clin Invest Med* 2008;31:E218-E221.
5. Zhang CX, Li GY, Han CS, et al. Effect of haishengsu preparation extracted from *Tegillarca granosa* in treatment of intermediate and late non-small-cell lung cancer (NSCLC) and late renal cancer. *Chin J Mar Drugs* 2005;24:55-8.
6. Li GY, Yu XM, Zhang HW, et al. Haishengsu as an adjunct therapy to conventional chemotherapy in patients with non-small cell lung cancer: a pilot randomized and placebo-controlled clinical trial. *Complement Ther Med* 2008;17:51-5.
7. Klein E, Ben-Bassat H, Neumann H, et al. Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *Int J Cancer* 1976;18:421-31.
8. Kantarjian HM, Talpaz M, Giles F, O'Brien S, Cortes J. New insights into the pathophysiology of chronic myeloid leukemia and imatinib resistance. *Ann Intern Med* 2006; 145:913-23.
9. Tsuruo T, Iida-Saito H, Kawabata H, Oh-hara T, Hamada H, Utakoji T. Characteristics of resistance to adriamycin in human myelogenous leukemia K562 resistant to adriamycin and in isolated clones. *Jpn J Cancer Res* 1986;77:682-92.
10. Friedlaender GE, Tross RB, Doganis AC, Kirkwood JM, Baron R. Effects of chemotherapeutic agents on bone. I. Short-term methotrexate and doxorubicin (adriamycin) treatment in a rat model. *J Bone Joint Surg Am.* 1984;66:602-7.
11. Pereverzeva E, Treschalin I, Bodyagin D, Maksimenko O, Kreuter J, Gelperina S. Intravenous tolerance of a nanoparticle-based formulation of doxorubicin in healthy rats. *Toxicol Lett* 2008;178:9-19.
12. Li GY, Li JZ, Zhang B, Wang LX, Wang CB, Chen SG. *Tegillarca granosa* extract Haishengsu inhibits the expression of P-glycoprotein and induces apoptosis in drug-resistant K562/ADM. *Pharm Biol* (in press)

Correspondence to:

Prof Lexin Wang,
School of Biomedical Sciences,
Charles Sturt University
Wagga Wagga, NSW 2678, Australia.
Phone: +61 2 69332905
Fax: +61 2 69 332587
E-mail: lwang@csu.edu.au
or
Li Guangyao,
Division of Hematology,
Liaocheng People's Hospital,
Liaocheng, Shandong Province, 252000, P. R. China.
Phone: +86-635-8276317
Fax: +86-635-8277306
E-mail: lcrmyy@163.com.