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Animal study of rh−endostatin combined with ginsenoside Rg3 on inhibiting growth of breast cancer xenografts

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ABSTRACT

Objective: To study the inhibiting effect of rh-endostatin combined with ginsenoside Rg3 on growth of breast cancer xenografts. **Methods:** C57BL/6 nude mice were selected as research subjects, animal models with breast cancer xenografts were established through subcutaneous injection of breast cancer cells in the neck and randomly divided into A-D groups, and normal saline, rh-endostatin, ginsenoside Rg3 and rh-endostatin combined with ginsenoside Rg3 were injected respectively. Tumor growth, serum angiogenesis factor contents, percentages of cell cycles and expression levels of cell cycle-related molecules in tumor tissue of four groups were compared. **Results:** 7 d, 14 d and 21 d after treatment, tumor volume as well as serum VEGF and bFGF contents of B group, C group and D group were significantly lower than those of A group, and tumor volume as well as serum VEGF and bFGF contents of D group were significantly lower than those of B group and C group; 21 d after treatment, tumor weight, percentages of S phase and G_2/M phase as well as mRNA contents and protein contents of cyclin E and CDC25A in tumor tissue of B group, C group and D group were lower than those of A group, and G_0/G_1 phase percentages were higher than that of A group; tumor weight, percentages of S phase and G_2/M phase as well as mRNA contents and protein contents of cyclin E and CDC25A in tumor tissue of D group were significantly lower than those of B group and C group, and G_0/G_1 phase percentage was lower than those of B group and C group. **Conclusion:** rh-endostatin combined with ginsenoside Rg3 can more effectively inhibit growth of breast cancer xenografts, make cell cycle arrest and down-regulate expression of cell cycle-related molecules.

1. Introduction

Breast cancer is one of the primary malignant tumors that threaten female health and safety, the incidence ranks first in female malignant tumors in our country, and surgical resection combined with postoperative chemotherapy, endocrine therapy, targeted therapy and so on is the most common clinical treatment option[1]. rh-endostatin is a targeted therapy drug developed by Chinese scientists, it has inhibiting effect on tumor angiogenesis and it has shown positive value in treatment of malignant tumors[2]. Ginsenoside Rg3 is the extract of Chinese herbal medicine ginseng root, and its antitumor effect has received increasing attention and concern[3]. Although the molecular mechanisms of anti-tumor effect of ginsenoside Rg3 are still to be elucidated, the effect of the drug on inhibiting malignant tumor cell proliferation and migration at level in vitro has been confirmed by many studies. Therefore it was speculated that ginsenoside Rg3 had inhibiting effect on breast cancer growth, and its combination with rh-endostatin could more effectively inhibit breast cancer growth. In the following research, the inhibiting effect of rh-endostatin combined with ginsenoside Rg3 on growth of breast cancer xenografts was analyzed.

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2. Materials and methods

2.1. Experimental materials

C57BL/6 nude mice were purchased from Shanghai Slac Laboratory Animal Company, RNA detection-related kits were purchased from Takara Company, Western-blotting-related reagents and antibodies were purchased from Sigma Company and flow cytometry kits were purchased from Roche Company.

2.2. Experimental methods

2.2.1. Cell culture and amplification methods

Cell lines were taken out of liquid nitrogen container, then incubated and shaken in water bath at 37 ℃, melted, then added to 1650 media containing 10% serum and cultured, culture conditions were 37 ℃ and 5% CO_2, and after cells grew and overspreaded, 0.125% trypsinase was used for digestion and passage, continuing to inoculate, culture and amplify.

2.2.2. xenograft model establishment and medication methods

Sub-cultured breast cancer cells were taken, the density was adjusted to 1×10^7 /mL, 0.2 mL of cell suspension was subcutaneously inoculated in nape of nude mice, tumor growth was continuously observed, after tumor volume grew to 200-300 mm^3, mice were used for subsequent grouping and medicating, they were divided into A-D groups, A group received intraperitoneal injection of normal saline, B group received intraperitoneal injection of 10 mg/kg rh-endostatin, C group received intraperitoneal injection of 5 mg/kg ginsenoside Rg3 and D group received intraperitoneal injection of 10 mg/kg rh-endostatin and 5mg/kg ginsenoside Rg3. They received medication once every other day.

2.2.3. Tumor growth measurement methods

After model establishment, before treatment as well as 7 d, 14 d and 21 d after treatment, vernier caliper was used to measure the major diameters and minor diameters of tumors of two groups, and tumor volume equaled 0.5 × major diameter × minor diameter. On the 21 d, animals were killed, and xenografts were anatomized and weighed.

2.2.4. Serum collection and index detection methods

After model establishment, before treatment as well as 7 d, 14 d and 21 d after treatment, blood specimens were collected from orbit and centrifuged to get serum, and ELISA kits were used to detect vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) contents.

2.2.5. Tumor tissue specimen collection and index detection methods

21 d after treatment, nude mice were killed via cervical dislocation, tumor tissue was anatomized and equally divided into three that were added to Trizol lysate, protein lysate and PBS respectively, and homogenized specimens were used for detection of RNA, protein and cell cycle respectively. RNA detection method was PCR, protein detection method was Western-blotting and cell cycle detection method was flow cytometry.

2.3. Statistical process methods

SPSS 21.0 software was used to input and statistically process data, comparison among groups was by variance analysis, and differences were considered to be statistically significant at a level of $P<0.05$.

3. Results

3.1. Tumor growth

After model establishment and before treatment, tumor volume of four groups had no differences; 7 d, 14 d and 21 d after treatment, tumor volume of A group continuously increased, and tumor volume of B group, C group and D group continuously decreased; tumor volume and tumor weight of B group, C group and D group were significantly lower than those of A group, and tumor volume and tumor weight of D group were significantly lower than those of B group and C group.

Table 1.
Comparison of tumor growth of two groups.

Group	*n*	Tumor volume (mm^3)				Tumor weight (mg)
		Before treatment	7 d after treatment	14 d after treatment	21 d after treatment	
A	8	257.5±25.3	324.7±35.5	371.5±41.3	423.5±56.1	1 024.2±115.6
B	8	255.4±27.8	224.7±24.2[a]	210.4±20.7[a]	182.7±18.7[a]	894.5±95.6[a]
C	8	257.6±26.5	230.1±26.5[a]	216.7±22.5[a]	187.7±20.3[a]	854.7±94.5[a]
D	8	256.2±25.6	208.5±22.5[abc]	193.4±20.3[abc]	170.5±19.4[abc]	657.8±71.4[abc]
F		0.184	5.977	8.684	13.844	8.912
P		>0.05	<0.05	<0.05	<0.05	<0.05

Note: (1) A group: normal saline treatment group; B group: rh-endostatin treatment group; C group: ginsenoside Rg3 treatment group; D group: rh-endostatin combined with ginsenoside Rg3 treatment group; (2) a: compared with A group, there were differences; b: compared with B group, there were differences; c: compared with C group, there were differences;

3.2. Dynamic changes of serum angiogenesis molecule contents

After model establishment and before treatment, serum VEGF and bFGF contents of four groups had no differences; 7 d, 14 d and 21 d after treatment, serum VEGF and bFGF contents of A group continuously increased, and serum VEGF and bFGF contents of B group, C group and D group continuously decreased; serum VEGF and bFGF contents of B group, C group and D group were significantly lower than those of A group, and serum VEGF and bFGF contents of D group were significantly lower than those of B group and C group.

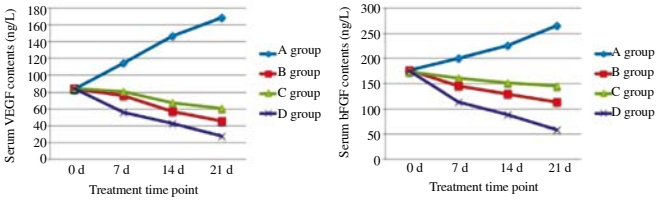

Figure 1. Dynamic changes of serum angiogenesis molecule contents of four groups; note: A group: normal saline treatment group; B group: rh-endostatin treatment group; C group: ginsenoside Rg3 treatment group; D group: rh-endostatin combined with ginsenoside Rg3 treatment group.

3.3. Cell cycle percentages

Cell cycle percentages in tumor tissue of four groups had differences. Results of pair wise comparison were as follows: G_0/G_1 phase percentages in tumor tissue of B group, C group and D group were higher than those of A group, and percentages of S phase and G_2/M phase were lower than those of A group; G_0/G_1 phase percentage in tumor tissue of D group was higher than that of B group and C group, and percentages of S phase and G_2/M phase were lower than those of B group and C group.

Table 2.
Comparison of cell cycle percentages in tumor tissue of four groups.

Group	*n*	G_0/G_1 phase	S phase	G_2/M phase
A	8	54.85±6.58	24.23±2.56	20.92±2.26
B	8	62.35±7.65[a]	18.69±1.86[a]	18.96±1.94[a]
C	8	63.57±6.97[a]	19.41±2.05[a]	17.02±1.89[a]
D	8	78.12±7.89[abc]	13.86±1.62[abc]	8.02±0.93[abc]
F		5.971	7.977	13.822
P		<0.05	<0.05	<0.05

Note: a: compared with A group, there were differences; b: compared with B group, there were differences; c: compared with C group, there were differences;

3.4. Cell cycle-related molecule expression levels

mRNA contents and protein contents of cyclin E and CDC25A in tumor tissue of four groups had differences. Results of pair wise comparison were as follows: mRNA contents and protein contents of cyclin E and CDC25A in tumor tissue of B group, C group and D group were lower than those of A group; mRNA contents and protein contents of cyclin E and CDC25A in tumor tissue of D group were significantly lower than those of B group and C group.

Table 3.
Comparison of cell cycle-related molecule expression levels in tumor tissue of four groups

Group	*n*	mRNA content		Protein content	
		Cyclin E	CDC25A	Cyclin E	CDC25A
A	8	100.00±12.58	100.00±14.12	100.00±13.95	100.00±13.28
B	8	76.78±8.19[a]	71.32±7.85[a]	74.96±7.97[a]	80.23±9.04[a]
C	8	73.54±7.65[a]	75.23±8.12[a]	69.79±7.14[a]	75.67±8.32[a]
D	8	45.68±5.68[abc]	42.45±5.91[abc]	39.69±4.12[abc]	40.49±4.68[abc]
F		9.392	11.346	14.722	12.376
P		<0.05	<0.05	<0.05	<0.05

Note: (1) A group: normal saline treatment group; B group: rh-endostatin treatment group; C group: ginsenoside Rg3 treatment group; D group: rh-endostatin combined with ginsenoside Rg3 treatment group; (2) a: compared with A group, there were differences; b: compared with B group, there were differences; c: compared with C group, there were differences;

4. Discussion

Recombinant human endostatin (rh-endostatin), also known as endostar, is the improved product of endostatin by Chinese scientists, which adds 9 amino acid residue sequences in endostatin N-terminal[4]. Production of endostar uses *Escherichia coli* as protein expression system, protein replication rate is higher, physicochemical properties are more stable, half-life in serum is longer and functioning duration is longer[5]. Structural improvement of rh-endostatin enhances the inhibiting effect of endostatin on vascular endothelial cell migration, and it can reduce the number of new blood vessels in tumor tissue and block nutrition supply needed for tumor cell metabolism[6]. Endostar has been used for the treatment of lung cancer, breast cancer, gastric cancer and many other advanced malignant tumors and has displayed positive effect[7-9]. Nonetheless, the illness of some patients continues to develop after they receive rh-endostatin treatment, and this indicates that there is still room for enhancing and improving efficacy of rh-endostatin in treatment of malignant tumors.

Tumor growth process involves a variety of links and targets, and the treatment should also be combined with various drugs against different targets. Ginsenoside Rg3 is an ingredient with antitumor activity extracted from extracting solution of Chinese herbal medicine ginseng root, and it is a kind of tetracyclic triterpenoids saponin[10]. Studies in vitro have confirmed that ginsenoside Rg3 has inhibiting effect on malignant tumor cell proliferation, invasion and other processes. Therefore it was speculated that ginsenoside Rg3 and rh-endostatin could inhibit tumor growth from three links: cell proliferation, invasion and angiogenesis[11,12]. In order to verify the speculation, animal models with breast cancer xenografts were established and treated through intraperitoneal injection of ginsenoside Rg3 and rh-endostatin, and then tumor volume and tumor weight were measured to reflect tumor growth. Analysis of results showed that tumor volume and tumor weight of B group, C group and D group were significantly lower than those of A group, and tumor volume and tumor weight of D group were significantly

lower than those of B group and C group, which indicated that both ginsenoside Rg3 and rh-endostatin could inhibit breast cancer growth, and combined use of the two drugs could enhance the inhibiting effect on tumor growth.

Local generation of large amounts of new blood vessels is the most basic link of breast cancer development, and new blood vessels can on the one hand provide energy and nutrition for tumor cell proliferation, division, differentiation and other biological processes, and on the other hand provide paths for distant metastasis of tumor cells after invasion. Currently known cytokines closely related to angiogenesis include VEGF and bFGF, and tumor cells themselves can synthesize and secrete VEGF and bFGF and increase the number of new blood vessels through local direct effect[13,14]. Analysis of dynamic changes of VEGF and bFGF contents in serum of four groups showed that 7 d, 14 d and 21 d after treatment, serum VEGF and bFGF contents of A group continuously increased, and serum VEGF and bFGF contents of B group, C group and D group continuously decreased, which indicated that both ginsenoside Rg3 and rh-endostatin could inhibit the generation of VEGF and bFGF. Further analysis of differences among B group, C group and D group showed that serum VEGF and bFGF contents of D group were significantly lower than those of B group and C group, which indicated that combined use of ginsenoside Rg3 and rh-endostatin had stronger inhibiting effect on the generation of VEGF and bFGF than that of single drug alone.

Studies about the antitumor effect of ginsenoside Rg3 in recent years believe that the functioning molecular pathway of the drug is blocking the development of cell cycle. Development of cell cycle depends on a variety of cell cycle-related proteins, and cyclin E that is expressed in G_1-S phase can be combined with cyclin dependent kinase 2 (CDK2) to form complex and promote the development of cell cycle[15]; CDC25A is a dual-specific phosphatase expressed in middle and late stage of G_1, and it can catalyze dephosphorylation of CDK2 molecule and prompt cell transition from G_1 phase to S phase[16]. After ginsenoside Rg3 and rh-endostatin treatment, percentages of different cell cycles and contents of related molecules in tumor tissue were detected, and results showed that G_0/G_1 phase percentages in tumor tissue of B group, C group and D group were higher than that of A group, and percentages of S phase and G_2/M phase as well as mRNA contents and protein contents of cyclin E and CDC25A were lower than those of A group; above changes in D group were more significant than those in B group and C group. It indicated that combined use of ginsenoside Rg3 and rh-endostatin could more effectively inhibit cell cycle and make cell cycle arrest in G_0/G_1 phase.

Based on above discussion, it can be concluded that rh-endostatin combined with ginsenoside Rg3 can more effectively inhibit growth of breast cancer xenografts, make cell cycle arrest and down-regulate expression of cell cycle-related molecules.

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