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Inhibitory effect of endostatin combined with paclitaxel-cisplatin on breast cancer in xenograft-bearing mice

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Abstract. In the present study, we aimed to investigate the tumor-inhibiting effects of recombinant human endostatin (rhES) combined with paclitaxel-cisplatin (TP regimen) on human breast cancer in xenograft-bearing nude mice. A total of 24 mice bearing human breast cancer xenografts were administered both rhES and TP, TP alone, rhES alone or saline. The tumor growth inhibition was observed. Serum vascular endothelial growth factor (VEGF) levels and microvessel density (MVD) were determined by ELISA and immunohistochemistry, respectively. Cell apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining. Survival time was observed in another 24 nude mice with the same treatment. MVD expression in the group administered rhES and TP was lower than that in the other groups ($P<0.05$); serum VEGF levels in the combined drug group were lower compared to the other groups; the apoptotic index increased in the combined drug group. We conclude that the effect of the TP regimen combined with rhES on breast cancer is better than that of the TP regimen alone.

Introduction

Breast cancer is a common cancer among women, and its morbidity is increasing over time. The resection rate is unlikely to be markedly increased by improving surgical techniques. Chemotherapy, radiotherapy and endocrine treatment have certain limitations and side-effects. The overall cure rate of breast cancer has not improved significantly over the past 10 years. Treatment failure is mainly due to tumor recurrence and metastasis. Today, bio-targeted therapy has become a new hotspot in breast cancer research.

In 1971, Folkman reported the theory of angiopoiesis: If there is no blood vessel, the diameter of the tumor will not exceed 2-3 mm (1). A large amount of research on anti-angiogenesis has been carried out. Dozens of anti-angiogenic drugs have been studied in order to find a drug which can have an effect on the angiogenesis pathway and vascular endothelial cells. However, the majority of these drug studies have had to be halted due to their serious side-effects and poor efficacy. It is well known that endostatin is one of the potent endogenous anti-angiogenic factors, which is composed of 184 amino acids and has a molecular weight of 22 kD (2).

It is not clear how endostatin functions *in vivo*. Kim *et al* (3) reported that endostatin blocked the expression of endothelial cell-specific E-selectin, regulated vascular endothelial cell adhesion and cytoskeleton formation, and directly interfered with tyrosine phosphorylation of the vascular endothelial growth factor (VEGF) receptor. The results revealed that endostatin blocked the VEGF-mediated signaling pathway, inhibited the proliferation of endothelial cells, and promoted the apoptosis of endothelial cells. Hanai *et al* (4) found that endostatin decreased the activity of β-catenin, inhibited cyclin D1 expression and induced G1 arrest of endothelial cells. MacDonald *et al* (5) also reported that endostatin could remodel the blood vessels. The possible reason for this was that endostatin binds to tropomyosin, which regulates the actin cytoskeleton so as to contract endothelial cells, thereby remodeling blood vessels. This mechanism also explains the decrease in diameter of the tumor blood vessels. Another study showed that the endostatin gene polymorphism can affect endostatin activity. The endostatin 4349A allele was associated with invasive breast cancer (6).

Endostar (rh-endostatin, YH-16) is a new recombinant human endostatin (rhES) developed by Medgenn Bioengineering Co. Ltd., Yantai, Shandong, China, in 2000. It was the first large-scale success of the refolding of endostatin in the world. Endostar creatively adds 9 amino acids to the N-terminal of endostatin peptides. This change solves the problems of high cost, low purity and poor stability in traditional methods of endostatin production. Endostar was admitted to a Phase I clinical study as a first-class anti-tumor drug in July 2001, and was produced in September 2005. A total of 493 histologically or cytologically confirmed stage IIIB and IV non-small cell

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lung cancer (NSCLC) patients were enrolled in a randomized, double-blind, placebo-controlled and multicenter trial. The combination of endostar with the vinorelbine and cisplatin (NP) regimen significantly increased the response rate, median time to tumor progression and clinical benefit rate, compared with NP alone in advanced NSCLC patients. Endostar combined with chemotherapy showed a synergistic activity and a favorable toxicity profile in advanced cancer patients (7,8).

Numerous studies in different laboratories have also obtained exciting results over the years. Endostatin has also been reported in breast cancer. The research findings are as follows: i) Plasma endostatin levels increased in patients who had the primary breast tumor removed (9), ii) a new angiogenic balance is formed in patients following surgery and such a resultant balance may be beneficial for the prognosis of breast cancer (10), iii) endostatin expression in MDA-MB-435 breast cancer cells effectively suppressed breast tumor growth by inhibiting angiogenesis and increasing apoptosis (11), and iv) another study showed that intravenous injection of liposomes complexed to plasma encoding endostatin reduced tumor growth in the nude mice by almost 40% (12).

From these assays, we hypothesized that elevated levels of plasma endostatin could block angiogenesis in breast cancer and improve the efficacy of chemotherapy. The combination of endostar, the first endostatin anti-cancer drug in the world, with chemotherapy for breast cancer has not yet been reported. In the present study, the MCF-7 cell line and nude mice with breast cancer were treated with rhES to observe the inhibition of proliferation and the induction of apoptosis. The results could provide experimental evidence for the clinical use of endostar combined with chemotherapeutic drugs in patients with breast cancer.

Materials and methods

Animal models of breast cancer. The human breast cancer cell line MCF-7 (ATCC, Manassas, VA, USA) was cultured in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA, USA). The tumor cells in log phase were digested with 0.25% trypsin (Gibco, Grand Island, New York, USA) and suspended in 0.2 ml of Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS). Subsequently, $1x10^7$ tumor cells (0.3 ml) were injected into the mammary fat pad at the right armpit. The 52 female BALB/c-nu/nu mice (Experimental Animal Center of Beijing Medical University, No. SCXK-2007001) were all 4-5 weeks old and weighed 18-25 g. The 52 nude mice had borne lesions 10 days later. After measuring the long axis (a) and short axis (b) of the tumor, tumor volume was calculated using $V=0.5 \times ab^2$. By excluding too large or too small tumors, a total of 48 tumor-bearing mice were used in the experiment. All animal procedures were carried out with the approval of the Animal Ethics Committee of the Third Military Medical University.

Animal experiments. A total of 48 tumor-bearing mice were randomly divided into Group A and B by means of a random number table. Groups A and B were then randomly divided into 4 subgroups (n=6 each). The mean tumor volume in each subgroup showed no significant difference ($P>0.05$). Drugs in the experiment included taxol (T; ShanXi PuDe Pharmaceutical Co., Ltd; No. 20070101), cisplatin (P; ShanDong Qilu Pharmaceutical Co., Ltd; No. 703014CF) and endostar (rhES; JiangSu XianSheng Pharmaceutical Co., Ltd). The 4 subgroups were as follows: i) Combined drugs group (TP + rhES); taxol 20 mg/kg, administered intraperitoneally (i.p.) on days 1, 7 and 14, cisplatin 5 mg/kg, administered i.p. on days 1 and 7, endostar 10 mg/kg, administered intravenously (i.v.) from day 1 and continued for 14 days. ii) Chemotherapy group (TP alone); paclitaxel 20 mg/kg, administered i.p. on days 1, 7 and 14, cisplatin 5 mg/kg, administered i.p. on days 1 and 7. iii) Endostar group (rhES alone); endostar 10 mg/kg, administered i.v. for 14 days. iv) Saline group (control); the same amount of sterile saline, administered i.v. for 14 days. Group A was detected by several indicators, and Group B was observed for the survival period.

Tumor volume and the tumor growth curve. Tumor volumes of BALB/c mice were measured by vernier caliper every 3 days for 21 days in total. The average tumor volume of each group was calculated, and tumor growth curves were drawn accordingly. The rates of anti-tumor activity were then calculated as usual.

Serum VEGF by ELISA. Serum VEGF levels were determined by ELISA in Group A on day 21. Blood was obtained by removing the eyeballs and the serum was collected by centrifugation for 8 min at 5,000 rpm. Serum VEGF levels were measured by the double antibody sandwich ELISA method (VEGF ELISA test kit; Invitrogen, Carlsbad, California, USA). The specimens were diluted at 1:100. The detailed steps were carried out according to the manufacturer's instructions.

Microvessel density (MVD). MVD in tumors was assessed by immunohistochemistry as suggested by Weidner (13). Nude mice in Group A were sacrificed by cervical dislocation after the eyeballs were removed. Immunohistochemical stainings were performed on tissues fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections (5 μm) were dewaxed and rehydrated. For light microscopy, peroxidase was quenched with methanol and 3% H_2O_2 for 15 min. Antigen retrieval was performed in 0.1 mol/l citrate buffer (pH 6.0) in an 800 W microwave for 15 min. Following washing in PBS, the rabbit anti-mouse CD34 polyclonal antibody (No. BAO532; Wuhan Boster Co., Ltd., Wuhan City, China) was used as the primary antibody. Diaminobenzidine (DAB) showing color steps were followed according to the manufacturer's instructions. The immunostained sections were first scanned at a low magnification (x40), and the areas with the greatest number of microvessels ('hotspots') were selected for further evaluation. The microvessel count was then determined by counting all immunostained vessels in five separate hotspots at a high magnification (x200). The average MVD in the 5 selected vessel hotspots was then calculated.

Apoptotic index (AI). AI was detected in paraffin-embedded sections of Group A by TdT-mediated dUTP nick end-labeling (TUNEL staining kit; Roche Co., Ltd., Germany). Enzyme solution (containing TdT) and labeling solution (containing biotin-dUTP) were added to the TUNEL reaction solution at a dilution of 1:9. Experimental procedures were carried out

according to the manufacturer's instructions. The nuclei of normal cells were blue. By contrast, the nuclei of apoptotic cells were yellow and brown. The proportion of positive cells among all cancer cells in 5 random cancer areas was calculated at a high magnification (x400).

Survival period of tumor-bearing mice. BALB/c mice in Group B were normally fed following a 14-day consecutive treatment until natural death. Survival period was recorded from the beginning of innoculation to the time of death, and survival curves were drawn accordingly.

Statistical analysis. All the data including tumor volumes, VEGF, MVD and AI were expressed as the means ± SD. The relationship between tumor volumes and VEGF, MVD and AI indicators was analyzed by one-way ANOVA. Overall survival analyses were performed using the Kaplan-Meier method. Differences were considered to be statistically significant when $P<0.05$. All statistical analyses were performed using the statistical package SPSS 13.0.

Results

Tumor growth and inhibition rate. Tumors occurred on the 10th day following implantation in all 52 BALB/c mice. A total of 48 tumor-bearing mice were used in the following experiment. Among them, 24 mice from Group A were alive until being sacrificed to detect a number of indicators. Tumor volumes of BALB/c mice were measured by vernier caliper every 3 days for 21 days in total. The average tumor volumes of each group were calculated, and tumor growth curves were drawn accordingly. Tumor inhibition rates were then calculated as usual.

In each subgroup, animals were lively, and daily observation revealed no abnormality in eating or any marked weight loss. During the first 3 days after the treatment, the tumors grew at a moderate and steady rate, showing no significant difference in volume ($P>0.05$) among the different groups. Three days later, the different groups began to exhibit a difference in tumor growth. The drug combination group and chemotherapy group showed a flat but extending growth curve. By contrast, the tumor grew at a rapid rate in the saline group, which resulted in a steep curve. Tumor volumes were measured in each group (mm^3) at the end of the experiment. Tumor volumes were 343.6057±214.55573 in the drug combination group, 809.2204±196.46286 in the chemotherapy group, 1085.8786±157.46491 in the endostar group and 1227.9323±350.45055 in the saline group, with statistically significant differences ($P<0.05$). Tumors grew more slowly in the drug combination group than in the other groups.

Similarly, tumor weights were measured in each subgroup (g) at the end of the experiment. Tumor weights were 0.5233±0.04759 in the drug combination group, 0.8633±0.11448 in the chemotherapy group, 1.055±0.17986 in the endostatin group and 1.3633±0.3793 in the saline group, with statistically significant differences ($P<0.05$). Tumors grew more slowly in the drug combination group than in the other groups.

Tumor inhibition rates were 61.62% in the drug combination group, 36.765% in the chemotherapy group and 22.61% in the endostatin group. A comparison of the tumor volumes in a number of subgroups and growth curves is shown in Fig. 1A and B.

Detection of serum VEGF. Serum VEGF levels ($pg \cdot ml^{-1}$) were 33.50±2.17 in the drug combination group, 45.67±3.56 in the chemotherapy group, 41.33±4.93 in the endostatin group and 73.67±3.50 in the saline group. Statistical analysis showed that the serum VEGF levels in the drug combination group were significantly lower than those in the other groups ($P<0.05$).

MVD counts with different treatments. CD34-positive cells were stained brown, and located in the cell membrane and (or) cytoplasm of the microvascular endothelial cells with clear boundaries. In each group, CD34-positive cells with different levels of staining were observed. MVD counts were 22.17±5.98 in the drug combination group, 37.33±6.80 in the chemotherapy group, 32.00±3.35 in the endostatin group and 48.00±3.41 in the saline group. MVD counts were significantly reduced in the drug combination group compared with those in the other 3 groups ($P<0.05$; Fig. 2). There was no significant difference in MVD counts between the chemotherapy group and the endostatin group ($P>0.05$).

AI. With a light microscope, TUNEL staining showed a brown positive signal, which was located in the nucleus and concentrated in the nucleoplasm close to the nuclear membrane. In each group, TUNEL-positive cells with different staining levels could be observed. The AI (%) was 60.00±5.33 in the drug combination group, 38.00±7.21 in the chemotherapy group, 33.83±7.21 in the endostatin group and 11.50±3.78 in the saline group. The AI was significantly increased in the drug combination group compared with that in the other 3 groups ($P<0.05$; Fig. 3).

Survival of tumor-bearing nude mice and growth curves. A total of 24 mice from Group B of the BALB/c mice were normally fed until natural death occurred. The survival of experimental animals and growth curves are shown in Fig. 4. Average survival time (days) was 79.50±3.15 in the drug combination group, 73.17±1.28 in the chemotherapy group, 69.33±2.22 in the endostatin group and 50.50±3.04 in the saline group. Statistical analysis showed that there was no significant difference in survival between the drug combination group and the chemotherapy or endostatin group ($P>0.05$). However, the average survival time was significantly prolonged in the drug combination group compared with that in the saline group ($P<0.05$).

Discussion

Breast cancer development is a complex multi-step process in which angiogenesis (or neovascularization) plays a very essential role. It was found that tumor MVD was closely related to relapse-free survival (RFS) and overall survival (OS) of breast cancer patients. Uzzan *et al* (14) found that high MVD predicted poor survival [RR, 1.99 for RFS (95% CI, 1.33-2.98) and RR, 1.54 for OS (95% CI, 1.01-2.33)], and MVD may be a better prognostic factor when assessed by CD31 or CD34. Byrnes *et al* (15) drew the similar conclusion that high VEGF levels and MVD always led to poor clinical prognosis.

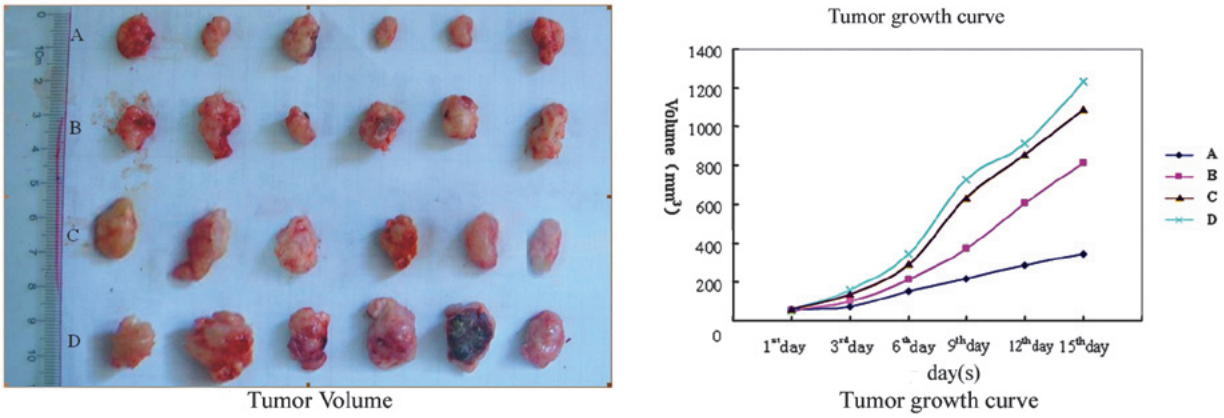

Figure 1. Tumor growth and inhibition rate. The left panel shows tumor sizes in each group. Samples in A, B, C and D were taken from the drug combination group (TP + endostar), the chemotherapy group (TP alone), the endostar group (endostar alone) and the saline group (control), respectively. Tumor volume in the saline group was significantly ($P<0.05$) larger than that in the other experimental groups. The right panel shows the tumor growth curve in each group. It could be observed that tumors in the drug combination group grew more slowly than those in the other groups.

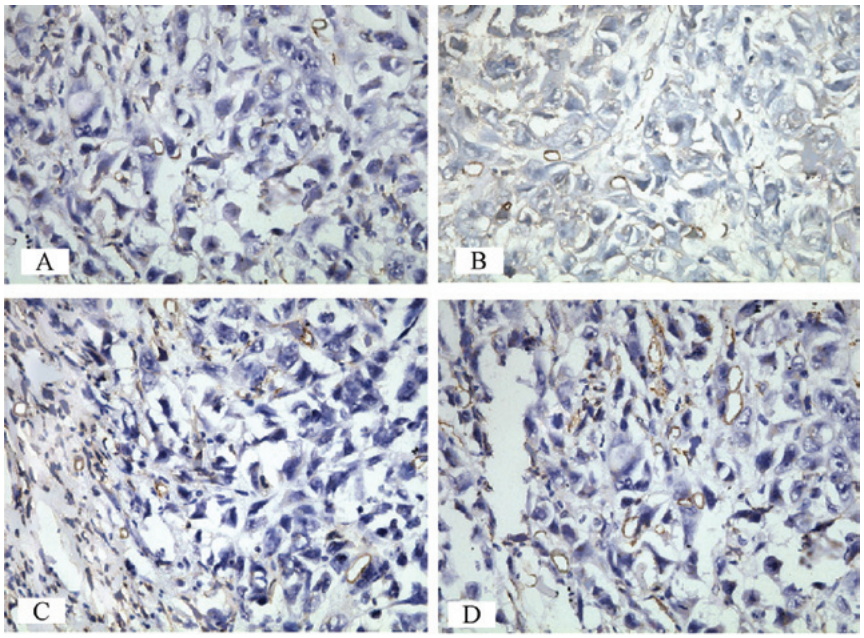

Figure 2. CD34 staining and microvessel density (MVD) count (SP, x400). CD34-positive cells were stained brown, located in microvascular endothelial cells, tumor cell membrane and (or) cytoplasm, with clear boundaries to other organizations. In each group, there were different levels of CD34-positive stained cells. Samples in A, B, C and D were taken from the drug combination group (TP + endostar), the chemotherapy group (TP alone), the endostar group (endostar alone) and the saline group (control), respectively. There were significantly reduced MVD counts in the combination drug group compared with the other 3 groups ($P<0.05$). There was no significant difference between the chemotherapy group and the endostatin group ($P>0.05$).

Bachelot *et al* (16) reported that patients with local breast cancer had higher serum levels of endostatin and longer median survival than those with metastasis, which suggests that endostatin may inhibit breast cancer invasion and metastasis, thereby improving the prognosis. We could treat breast cancer by blocking angiogenesis during its various stages.

Pro-angiogenic and anti-angiogenic proteins, such as VEGF and endostatin, contribute to the local angiogenic balance. Certain studies have shown that endostatin inhibits tumor growth and angiogenesis. Jia *et al* (17) found that rhES combined with chemotherapy reduced tumor angiogenesis and increased the apoptosis of B16F10 melanoma cells. Endostatin combined with radiotherapy enhanced the anti-tumor effect in the Lewis lung cancer model according to Luo *et al* (18).

The human breast cancer cell line, MCF-7, chosen in this study showed a wild-type genotype p53, was ER- and PR-positive, and sensitive to chemotherapy. Paclitaxel, with its excellent anti-tumor efficacy, has been recognized as first-line chemotherapy for breast cancer. As an agent in cell microtubules, paclitaxel is capable of arresting cells in the G2 phase and M phase and inducing apoptosis of tumor cells (19).

In this study, we found that rhES combined with TP chemotherapy slowed tumor growth significantly in nude mice bearing breast cancer xenografts, compared with TP chemotherapy, endostar alone or saline alone. By the end of the experiment, the volume and weight of the tumors in each group were significantly different ($P<0.05$). Combination therapy is superior to single chemotherapy. Zhao *et al* (20)

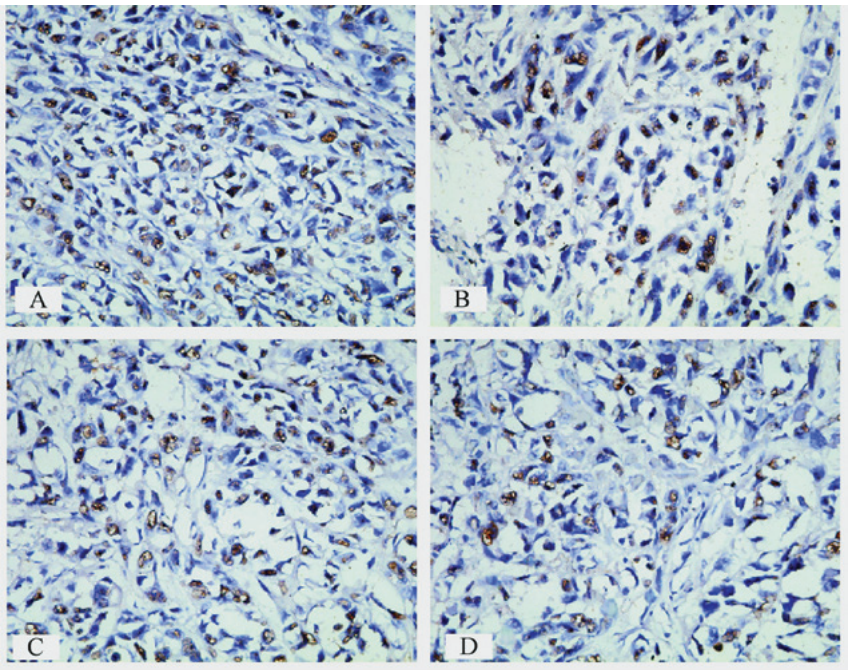

Figure 3. Apoptotic index of cancer cells (TUNEL, x400). TUNEL-stained cells showed a brown positive signal located in the nucleus and concentrated in the nucleoplasm close to the nuclear membrane. In each group, TUNEL-positive cells could be observed in different levels. Samples in A, B, C and D were taken from the drug combination group (TP + endostar), the chemotherapy group (TP alone), the endostar group (endostar alone) and the saline group (control), respectively. There was a significantly increased apoptotic index in the combination drug group compared with the other 3 groups (P<0.05).

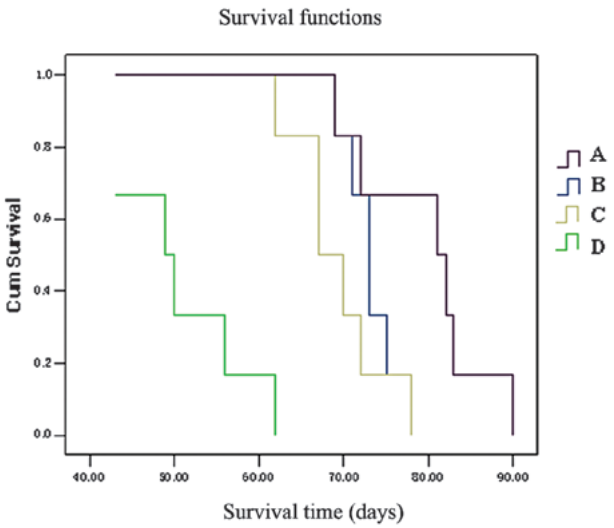

Figure 4. Survival of nude mice and growth curves. Survival of 24 experimental animals and growth curves are shown. Average survival time (days) in each group differed. Samples in A, B, C and D were taken from the drug combination group (TP + endostar), the chemotherapy group (TP alone), the endostar group (endostar alone) and the saline group (control), respectively. There was no significant difference between the combination drug group and the chemotherapy or endostatin group (P>0.05), but the average survival time was significantly prolonged in the combination drug group compared with the saline group (P<0.05).

observed the same result showing that endostar combined with recombinant p53 adenovirus inhibited human breast cancer xenografts better than treatment with a single agent.

From the perspective of evidence-based medicine, the endpoint of therapeutic efficacy in cancer patients should be extended to prolonged survival time or improved quality of life. The present experiment was carried out in early tumors and we observed inhibition of tumor growth. Compared with chemotherapy, the long-term effect of combination therapy was not satisfactory, and it failed to prolong the survival time (P>0.05). The reasons were analyzed as follows: First, anti-cancer drugs follow the law of 'first-order kinetics' that a certain amount of anti-cancer drugs killed a certain percentage, rather than a fixed number, of tumor cells. That is the reason to recommend the use of sequential chemotherapy in conservative cancer treatment. Second, Compertzian law states that the tumor cells grow quickly to the original size after a non-complete elimination. The present study only lasted for one cycle of treatment without total inhibition of residual tumor cells, which affected the long-term effect. Thus, we propose that, as for the combination therapy, drug intervention should last at least for 2 cycles to maintain the effect of the treatment and to prolong survival time in tumor-bearing mice.

Numerous growth factors with the ability to promote tumor angiogenesis have been found. Among them, VEGF is the most essential angiogenic factor, which can increase vascular permeability, and promote endothelial cell migration, proliferation and tumor angiogenesis. Thus, it is capable of promoting tumor growth and metastasis. In this experiment, we observed that serum VEGF levels in the endostar group were significantly lower than those in the saline group (P<0.05) and that serum VEGF levels in the drug combination group were significantly lower than those in the other groups (P<0.05).

We also observed in the tumor biopsy that endostatin reduced the tumor MVD in vascular endothelial cells stained by CD34, and combination therapy significantly inhibited angiogenesis compared to endostar alone (P<0.05). Therefore, endostatin directly affected angiogenesis, and indirectly led to VEGF reduction. Thus, it eventually destroyed the

'nutrition pipeline' of the tumor and inhibited the tumor growth. The results showed that serum VEGF levels may serve as laboratory indicators to judge the clinical validity of endostar.

Endostar (a new rhES) is a new anti-angiogenesis drug developed by Chinese scientists. Further studies are required in order to fully evaluate its efficacy in various malignancies, treatment timing and treatment programs. The present research for the clinical use of endostatin combined with chemotherapy in breast carcinoma provides an experimental basis and expands its use to clinical indications.

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