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Endostatin gene therapy enhances the efficacy of paclitaxel to suppress breast cancers and metastases in mice

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Abstract

Chemotherapy combined with antiangiogenic therapy is more effective than chemotherapy alone. The aim of this study was to investigate whether endostatin, a potent anti-angiogenic agent, could enhance the efficacy of paclitaxel to combat breast cancer. An expression plasmid encoding mouse endostatin (End-pcDNA3.1) was constructed, which produced intense expression of endostatin and inhibited angiogenesis in the chorioallantoic membrane assay. 4T1 breast tumors were established in BALB/c mice by subcutaneous injection of 1×10^5 4T1 cells. The End-pcDNA3.1 plasmid diluted in the transfection reagent FuGENE^{TM} was injected into the tumors (around 100 mm^2), and paclitaxel was injected i.p. into the mice. Endostatin gene therapy synergized with paclitaxel in suppressing the growth of 4T1 tumors and their metastasis to the lung and liver. Both endostatin and paclitaxel inhibited tumor angiogenesis and induced cell apoptosis. Despite the finding that endostatin was superior to paclitaxel at inhibiting tumor angiogenesis, paclitaxel was nevertheless more effective at inducing tumor apoptosis. The combination of paclitaxel and endostatin was more effective in suppressing tumor growth, metastases, angiogenesis, and inducing apoptosis than the respective monotherapies. The combinational therapy with endostatin and paclitaxel warrants future investigation as a therapeutic strategy to combat breast cancer.

Introduction

Breast cancer is the second leading cause of cancer-related deaths among women throughout the world [1]. Despite extensive exploration for novel therapies, the success of current treatments is still unsatisfied, and the side effects due to some forms of treatment can be particularly harsh. Surgery remains the mainstay of treatment for primary breast cancer, but is ineffective once the cancer has spread to distant sites. Surgery may paradoxically enhance tumor cell dissemination into the bloodstream resulting in the seeding of tumor cells in distant organs [2, 3]. Chemotherapy and endocrine therapy comprise the main adjuvant treatments to prevent or delay metastases following local management [4, 5]. Paclitaxel has been show to be a potent drug to treat metastatic breast cancer, ovarian cancer and other forms of cancer [6]. Paclitaxel prevents division of cancer cells by stabilizing the mitotic spindle and inhibiting daughter cell separation, thereby causing toxicity and cell death [7]. However, several toxic side-effects and clinically significant implications, including severe anaphylactic hypersensitivity

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reactions and peripheral neuropathy, have been reported in paclitaxel treated patients [8]. Therefore, potential therapeutic strategies to improve the efficacy of paclitaxel are needed.

Anti-angiogenic therapy targeting the tumor blood supply has become a promising approach to combat cancer, given that solid tumors including breast cancer must establish an adequate vascular network in order to acquire the nutrition necessary for growth and metastasis [9]. Endostatin, an M_r 20,000 proteolytic fragment of the C-terminus of collagen XVIII, is one of the most potent angiogenesis inhibitors [10]. Its antiangiogenic activity involves binding to integrin $\alpha5\beta1$ [11] or E-selectin [12], inhibiting metalloproteinases [13], or influencing a set of growth-associated gene networks in endothelial cells [14, 15]. Endostatin either suppresses or induces the regression of a wide variety of established tumors [16], including breast cancers and metastases in animal models [17–20]. Furthermore, endostatin stabilized tumor growth after chemotherapy in a NOD/SCID mouse model of human high-grade non-Hodgkin lymphoma [21]. It also helped low dose carboplatin to prevent the metastasis of human testicular germ cell tumor xenografts [22], enhanced the antitumor efficacy of paclitaxel to prevent liver metastasis in a model of colorectal cancer model [23], and synergized with adriamycin to suppress tumor progression in an orthotopic murine mammary carcinoma model [24]. Endostatin gene therapy prevented the formation of tumor blood vessels, decreased tumor growth, and synergized with chemotherapy to suppress ovarian and lung cancers [25, 26]. Here we tested whether endostatin gene therapy would enhance the efficacy of paclitaxel to combat highly metastatic 4T1 breast cancers [27].

Materials and methods

Mice, cell lines and reagents

Female 6–8 week-old BALB/c mice were obtained from the Key Laboratory of General Surgery in Shandong Province, China. Throughout the study period mice were kept in an air-conditioned room with controlled humidity, temperature, and 12 h light: dark cycle. The 4T1 breast cancer cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained as monolayer cultures at 37°C in RPMI 1640 medium (Gibco BRL Inc., Grand Island, NY, USA), supplemented with 10% fetal calf serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM pyruvate. Tumor cell suspensions of greater than 90% viability were prepared from subconfluent cultures with 0.25% Trypsin-0.02% EDTA. The anti-endostatin (PA1-601) and anti-CD31 (MEC13.3) antibodies were purchased from Affinity BioReagents Inc. (Golden, CO, USA) and Pharmingen (CA, USA), respectively. Paclitaxel injectable solution was purchased from Bristol-Myers Squibb Company, Shanghai, China

Endostatin expression vectors

Complementary DNA encoding mouse endostatin was released from an endostatin plasmid [28], and subcloned into pcDNA3.1 to construct the endostatin expression vector End-pcDNA3.1.A secretion signal from the mouse immunoglobulin κ chain was fused to the N-terminus of endostatin, and endostatin expression was driven by a cytomegalovirus immediate-early promoter. DNA sequence analysis confirmed that no mutation had been incorporated.

Endostatin expression in COS-1 cells

The methodology has been described previously [29]. Briefly, COS-1 cells were grown to 60–70% confluence, and transfected with 4 μg of End-pcDNA3.1 plasmid using lipofectamine PLUS (Life Technologies, China). Empty pcDNA3.1 vector served as a control. The COS-1 cells and supernatants were collected 48 h following transfection, and the concentrated conditioned media and cell lysates were subjected to Western blot analysis as below.

Chorioallantoic membrane (CAM) assay

The chicken embryo CAM assay is widely used to measure the activity of anti-angiogenic agents [30]. Briefly, eggs were incubated for 7 days at 37°C and 60% humidity. A square window was opened in the shell and the membrane of the gas chamber was carefully removed to expose the chorioallantoic membrane. Twelve eggs were randomly

divided into two groups, and were doped with 50 μL conditioned media from COS-1 cell that had been transfected with either End-pcDNA3.1 or pcDNA3.1. The windows were sealed and the eggs further incubated for 72 h. The CAMs were photographed and the number of vessel branch-points formed by the blood vessels was counted.

Animal model and treatments

All surgical procedures and care administered to the animals were in accordance with institutional guidelines. Tumors were established by subcutaneous injection of 1×10^5 4T1 cells into the mice. Tumor volumes were estimated according to the formula: $\pi/6 \times a^2 \times b$, where a is the short axis, and b the long axis. When tumors reached around 100 mm^3 in about 15 days, the mice were randomly assigned to 4 treatment groups: pcDNA3.1, End-pcDNA3.1, paclitaxel, and End-pcDNA3.1 + paclitaxel. To standardize the experiments, mice in each group received both intratumoral and i.p. injections. In the pcDNA3.1 and End-pcDNA3.1 groups, mice received i.p. injection of 200 μL of PBS, and intratumoral injection of 200 μg of either pcDNA3.1 or End-pcDNA3.1 diluted in 100 μL of FuGENE^{TM} 6 transfection reagent (Roche, Shanghai, China), respectively. In the paclitaxel group, mice received i.p. injection of 200 μL of paclitaxel solution at the dose of 15 mg/kg weight and intratumoral injection of 200 μg of pcDNA3.1 diluted in 100 μL of FuGENE^{TM} 6. In the End-pcDNA3.1 + paclitaxel group, mice received i.p. injection of 200 μL of paclitaxel solution at the dose of 15 mg/kg and intratumoral injection of 200 μg of End-pcDNA3.1 diluted in 100 μl of FuGENE^{TM} 6. All experiments included 15 mice per treatment group. FuGENE^{TM} 6 was shown to be an efficient *in vivo* transfection reagent in our previous study [31].

Measuring metastases

The mice were euthanized at the end of experiments, and the lungs and livers were removed. The numbers of metastases on the lung surface were counted with a magnifying lens. The livers were fixed with 4% paraformaldehyde and transverse 5 μm sections made at 5 different levels to cover the entire liver. The sections were stained with hematoxylin and eosin. Metastatic nodules containing more than 6 cancer cells were counted.

Immunohistochemistry

Tumor cryosections (6 μm) were fixed with acetone, rinsed with PBS, blocked with 3% BSA for 2 h, and incubated overnight with primary antibodies. They were subsequently incubated for 30 min with appropriate secondary antibodies using the Ultra Sensitive TMS-P kit (Zhongshan Co., Beijing, China), and immunoreactivity developed with Sigma FAST DAB (3,3′-diaminobenzidine tetrahydrochloride) and $CoCl_2$ enhancer tablets (Sigma-Aldrich, Shanghai, China). Sections were counterstained with hematoxylin, mounted, and examined by microscopy.

Assessment of tumor vascularity

The methodology to determine tumor vascularity has been described previously [29]. Briefly, 6 μm tumor sections were immunostained with an anti-CD31 Ab, as described above. Stained vessels were counted in ten blindly chosen random fields at $400 \times$ magnification, and the mean microvessel density was recorded.

In situ detection of apoptotic cells

The methodology has been described previously [28]. Briefly, serial sections of 6 μm thickness were prepared from tumors 3 weeks following treatment, stained with the TUNEL agent (Boehringer Mannheim, Germany), and examined by fluorescence microscopy. Adjacent sections were counterstained with hematoxylin and eosin. The total number of apoptotic cells in 10 randomly selected fields was counted. The apoptosis index was calculated as the percentage of positive staining cells according to the formula: number of apoptotic cells $\times$ 100/total number of nucleated cells.

Western blot analysis

The tumor tissues and cultured cells were homogenized in protein lysate buffer. Debris was removed by centrifugation at $10{,}000 \times g$ for 10 min at 4°C. The lysates were resolved on 12% polyacrylamide SDS gels, and electrophoretically transferred to polyvinylidene difluoride (PVDF)

membranes. The membranes were blocked with 3% BSA overnight, incubated with primary antibodies, and subsequently with alkaline phosphatase-conjugated secondary antibody. They were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (Tiangen Biotech Co. Ltd., Beijing, China). Blots were stained with an anti-tubulin antibody to confirm that each lane contained similar amounts of tumor homogenate.

Statistical analysis

Kruskal–Wallis tests were performed to test the significance of the treatment effect in relation to numbers of metastases. For other data, results were expressed as mean values ± standard deviation (SD), and a Student's t-test was used for evaluating statistical significance. A value of less than 0.05 ($p < 0.05$) was used for statistical significance.

Results

Expression of recombinant endostatin in COS-1 cells

Western blot analysis using an anti-endostatin antibody demonstrated that recombinant endostatin was present in the cell lysate and conditioned medium of COS-1 cells transfected with End-pcDNA3.1. The anti-endostatin Ab detected an endostatin protein band of 20 kDa in both the cell lysate and conditioned medium of End-pcDNA3.1 transfectants, whereas no such band was present in the cell lysate or conditioned medium of COS-1 cells transfected with the empty vector pcDNA3.1 (Figure 1 A).

Recombinant endostatin inhibits angiogenesis

The conditioned media of COS-1 cells transfected with the End-pcDNA3.1 plasmid, which contained recombinant endostatin as shown above, significantly inhibited angiogenesis in the CAM, compared with treatment with empty vector (Figure 1 B vs C). The number of vessel branch-points was significantly reduced in CAMs treated with recombinant endostatin, compared with control ($p < 0.05$) (Figure 1 D).

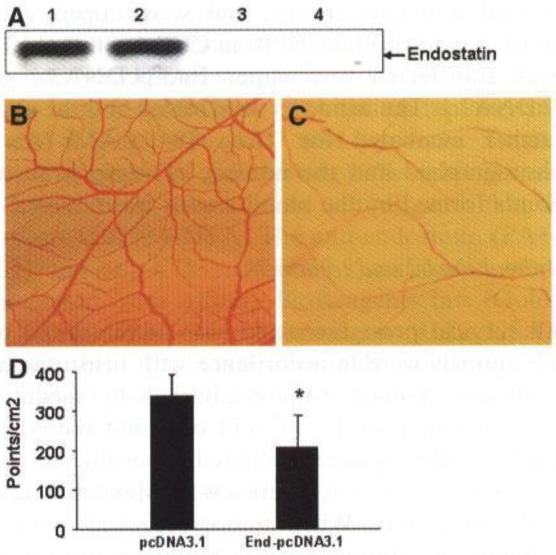

Figure 1. Recombinant endostatin protein inhibits angiogenesis in the chorioallantoic membrane of chicken eggs. (A) Western blot analysis of endostatin expression. Cell lysates (lanes 1 and 3) and concentrated conditioned media (lanes 2 and 4) of COS-1 cells transfected with End-pcDNA3.1 (lanes 1 and 2) and empty pcDNA3.1 plasmid (lanes 3 and 4) were Western blotted with anti-endostatin Ab. (B, C) Illustrated are representative photographs of chorioallantoic membranes treated with conditioned media from cultures of COS-1 cells transfected with pcDNA3.1 (B) and End-pcDNA3.1 plasmids (C). (D) The numbers of branch-points of the blood vessels were counted. A significant difference between the End-pcDNA3.1 and pcDNA3.1 groups is denoted by "*".

Endostatin gene transfer results in intense in situ transgene expression

4T1 tumors injected with the End-pcDNA3.1 plasmid were sectioned 4 days following gene transfer. Representative photographs revealed intense expression of endostatin throughout tumors treated with the End-pcDNA3.1 plasmid, whereas control sections from pcDNA3.1-treated tumors were only slightly stained by the anti-endostatin Ab due to weak expression of endogenous endostatin (Figure 2 A vs B). An endostatin protein of 20 kDa was detected by Western blot analysis of homogenates of transfected tumors 4 days after gene injection, but was absent from homogenates of control tumors (Figure 2 C).

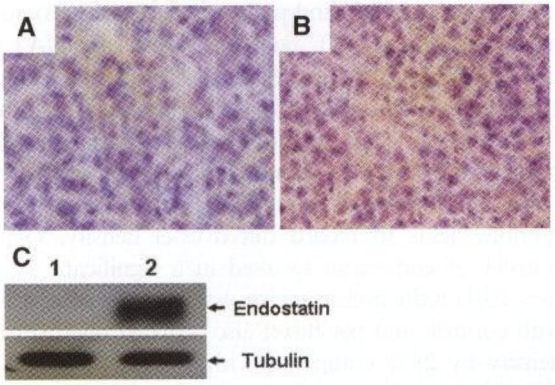

Figure 2. Intense expression of endostatin *in situ* after intratumoral gene transfection. Illustrated are representative 4T1 tumor sections prepared 4 days following intratumoral gene transfer of pcDNA3.1 (A) and End-pcDNA3.1 (B) plasmids. The sections were immunostained with an anti-endostatin antibody. (C) Homogenates of tumors 4 days after intratumoral injection of pcDNA3.1 (lane 1) and End-pcDNA3.1 (lane 2) plasmids were Western blotted with anti-endostatin (upper panel) and tubulin (lower panel) antibodies.

Endostatin synergizes with paclitaxel to suppress subcutaneous tumors

Tumors were established by subcutaneous injection of 4T1 tumor cells into the flanks of mice. Fifteen days later, when the tumors reached around 100 mm^3, the mice were randomly assigned to 4 treatment groups: pcDNA3.1, End-pcDNA3.1, paclitaxel and End-pcDNA3.1 + paclitaxel. As shown in Figure 3A, the tumors treated with empty vector pcDNA3.1 grew rapidly, reaching 1798 ± 279 mm^3 in volume 35 days after implantation, a growth rate which was not significantly different from that of untreated tumors (1902 ± 330 mm^3, $p > 0.05$). In contrast, in the paclitaxel group, the tumors had reached only 862 ± 125 mm^3 in volume 35 days after implantation, being significantly smaller than the control tumors ($p < 0.01$). Endostatin gene therapy also resulted in a significant reduction in tumor volumes (1245 ± 265 mm^3), compared with control tumors ($p < 0.05$). A combination of End-pcDNA3.1 and paclitaxel further suppressed tumor growth such that tumors reached only 374 ± 135 mm^3 in size, which was highly significantly smaller than the control tumors ($p < 0.001$), and significantly smaller than the tumors treated with endostatin or paclitaxel monotherapies (both $p < 0.05$) (Figure 3A).

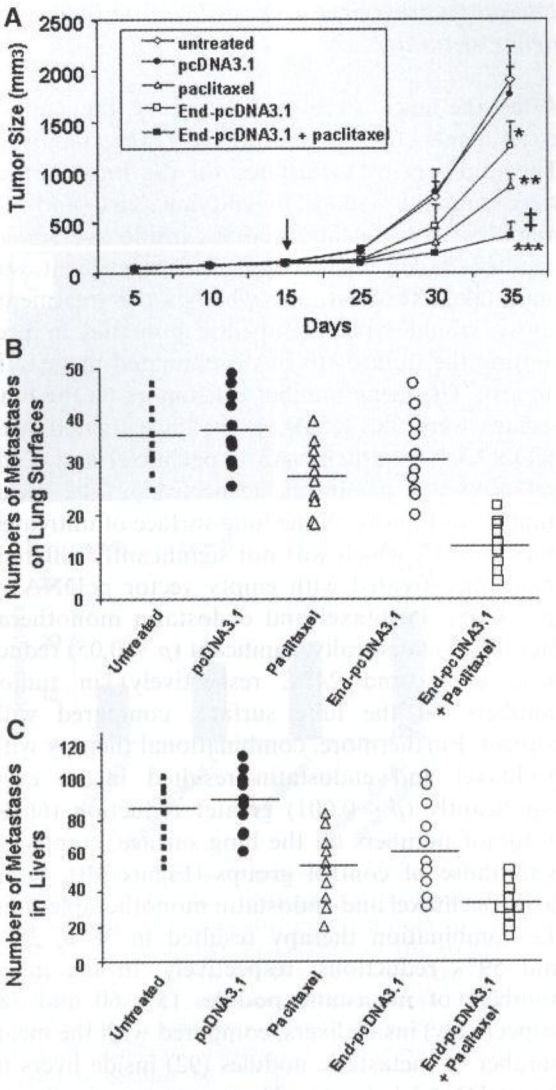

Figure 3. Endostatin gene therapy synergizes with paclitaxel to suppress breast tumor growth and metastases. (A) 4T1 breast tumors were established in mice by subcutaneous injection of 4T1 cells. When the tumors reached around 100 mm^3 (indicated by a vertical arrow), they received pcDNA3.1, paclitaxel, End-pcDNA3.1, or End-pcDNA3.1 + paclitaxel treatments. Untreated tumors served as controls. The sizes (mm^3) of tumors were monitored and recorded. A significant difference in tumor volumes from control is denoted by "*" ($p < 0.05$), "**" ($p < 0.01$), or "***" ($p < 0.001$). "‡" Indicates significant difference from End-pcDNA3.1 or paclitaxel treatments. (B) The mice were euthanized 35 days after 4T1 tumor implantation, and the numbers of tumors on the surface of lungs were counted. (C) The mice were hepatectomized, and livers were sectioned and stained with hematoxylin/eosin. The numbers of metastatic nodules inside livers were counted. Each point represents a single animal, and the mean number of metastases is indicated by a transverse line.

Endostatin synergizes with paclitaxel to suppress tumor metastases

After the mice were euthanized at the end of experiments, the lungs and livers were removed. The numbers of metastases on the lung surface were counted with a magnifying lens, and the numbers of metastatic nodules inside livers were counted under microscopy. The experiment was undertaken to determine whether the treatments above would have therapeutic potential in preventing the outgrowth of disseminated metastatic tumors. The mean numbers of tumors on the lung surface were 38, 26, 29, 13 in mice treated with pcDNA3.1, End-pcDNA3.1, paclitaxel and End-pcDNA3.1 + paclitaxel, respectively. The mean number of tumors on the lung surface of untreated mice was 37, which was not significantly different from mice treated with empty vector pcDNA3.1 ($p > 0.05$). Paclitaxel and endostatin monotherapies led to statistically significant ($p < 0.05$) reductions (32% and 24%, respectively) in tumor numbers on the lung surface, compared with control. Furthermore, combinational therapy with paclitaxel and endostatin resulted in an even significantly ($p < 0.001$) greater reduction (66%) in tumor numbers on the lung surface, compared with those of control groups (Figure 3B). Similarly, paclitaxel and endostatin monotherapies and the combination therapy resulted in 38%, 35% and 59% reductions, respectively, in the mean numbers of metastatic nodules (57, 60 and 38, respectively) inside livers, compared with the mean number of metastatic nodules (92) inside livers in the pcDNA3.1 group. There was no significant difference in the mean number of metastatic nodules inside livers between pcDNA3.1-treated and untreated mice (Figure 3C).

Endostatin synergizes with paclitaxel to inhibit tumor angiogenesis

Five mice were sacrificed two weeks after treatment from each of the four groups of mice (pcDNA3.1, End-pcDNA3.1, paclitaxel and End-pcDNA3.1 + paclitaxel). As there was no significant difference in tumor growth and metastases between pcDNA3.1-treated and untreated tumors, the untreated mice were excluded from the experiments below. The tumors were sectioned and stained with an anti-CD31 Ab to visualize microvessels. There were fewer microvessels in tumors treated with End-pcDNA3.1 (Figure 4B) and paclitaxel (Figure 4C), compared with pcDNA3.1-treated tumors (Figure 4A). Furthermore, there were even fewer microvessels in tumors treated by the combinational therapy (Figure 4D), compared with tumors treated by the monotherapies. Tumor microvessels in sections were counted in blindly chosen random fields to record microvessel density. Gene transfer of endostatin resulted in a significant 39% ($p < 0.01$) reduction in microvessel density compared with control, and paclitaxel also reduced microvessel density by 28% compared with control ($p < 0.05$). Furthermore, the combinational therapy with End-pcDNA3.1 and paclitaxel highly significantly ($p < 0.001$) reduced microvessel density by 51%, compared with mock treatment. The microvessel density of tumors in the combination treatment group was also significantly less than that of the End-pcDNA3.1 or paclitaxel treatment groups (both $p < 0.05$), demonstrating synergism between endostatin and paclitaxel in inhibiting tumor angiogenesis (Figure 4 E).

Endostatin synergizes with paclitaxel to induce cell apoptosis

We next examined whether the tumor cells underwent programmed death as measured by *in situ* labeling of fragmented DNA using the TUNEL method. A small number of apoptotic cells were detected in tumors injected with empty plasmid (Figure 5A), whereas tumor apoptosis was increased following intratumoral injection of End-pcDNA3.1 (Figure 5 B), and paclitaxel treatment (Figure 5 C). Despite the finding that End-pcDNA3.1 was superior at inhibiting tumor angiogenesis, paclitaxel was more effective at inducing tumor apoptosis, but once again the combination of End-pcDNA3.1 and paclitaxel was the most effective (Figure 5D). Thus, the apoptosis index for tumors treated with either End-pcDNA3.1 ($p < 0.05$) or paclitaxel ($p < 0.01$) was significantly higher than that of tumors treated with the empty vector pCDNA3.1. Furthermore, the apoptosis index for tumors treated with a combination of End-pcDNA3.1 and paclitaxel was highly significantly ($p < 0.001$) different from that of the control tumors (Figure 5 E), and significantly ($p < 0.05$) different from that of endostatin- or paclitaxel-treated tumors.

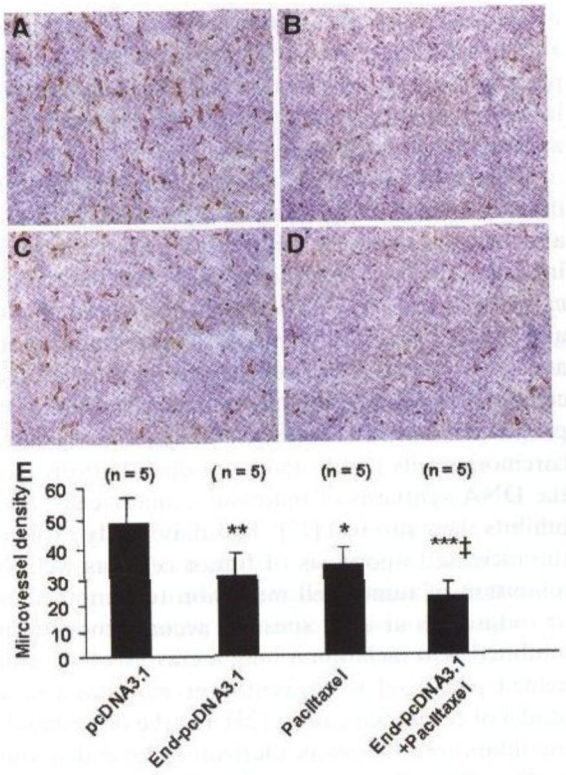

Figure 4. Endostatin gene transfer synergizes with paclitaxel to inhibit tumor angiogenesis. Illustrated are representative tumor sections prepared 2 weeks after treatment from mice receiving pcDNA3.1 (control) (A), End-pcDNA3.1 (B), paclitaxel (C), or paclitaxel + End-pcDNA3.1 treatments (D). Sections were stained with the anti-CD31 Ab. (E) Tumor microvessels in sections were counted in blindly chosen random fields to record microvessel density. n, number of tumors assessed. A significant difference in microvessel density from control is denoted by "*" ($p < 0.05$), "**" ($p < 0.01$), or "***" ($p < 0.001$). "‡" Indicates significant difference from End-pcDNA3.1 or paclitaxel treatments.

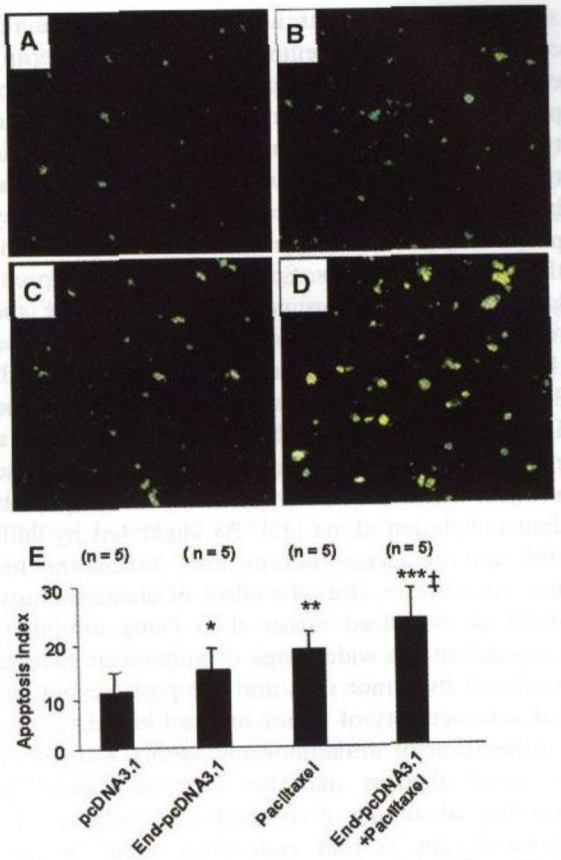

Figure 5. Endostatin gene transfer synergizes with paclitaxel to induce cell apoptosis. Illustrated are representative tumor sections prepared 2 weeks after treatment from mice receiving pcDNA3.1 (control) (A), End-pcDNA3.1 (B), paclitaxel (C), or paclitaxel + End-pcDNA3.1 treatments (D). They were stained by TUNEL analysis to detect apoptotic cells. (E) TUNEL-positive cells were counted to record the apoptosis index. A significant difference in microvessel density from control is denoted by "*" ($p < 0.05$), "**" ($p < 0.01$), or "***" ($p < 0.001$). "‡" Indicates significant difference from End-pcDNA3.1 or paclitaxel treatments. n, numbers of tumors assessed.

Discussion

The present study has demonstrated that concomitant treatment of mice bearing subcutaneous 4T1 breast cancer with endostatin gene therapy and paclitaxel results in increased inhibition of growth of both primary and metastatic tumors. Both endostatin and paclitaxel exert their anti-tumor effects by inhibiting tumor angiogenesis and inducing apoptosis. The combinational therapy with endostatin and paclitaxel showed a stronger effect than monotherapies. It is debatable whether the interaction between endostatin and chemotherapeutic drugs is synergistic or additive. Velde et al. [23] reported that combined treatment with recombinant endostatin protein and doxorubicin resulted in additive anti-tumor effects in a liver metastasis model, whereas Plum et al. [24] reported that this combination had a synergistic effect in an orthotopically implanted mammary tumor model. The enhanced therapeutic efficacy of chemotherapy by endostatin has advantages over other chemotherapeutic synergies. Combining paclitaxel with other cytotoxics can lead to nonoverlapping or overlapping toxicities, which hinder clinical implementation. In contrast, antiangiogenic therapy is relatively less toxic compared with cytotoxic drugs [21–24],

and holds promise as a means to improve the clinical utility of chemotherapy. Chemotherapy can mobilize endothelial precursor cells, and hence play an important role in neovascularization and tumor growth [32]. Hence, early antiangiogenic therapy in combination with chemotherapy could be beneficial for the success of cancer therapy by preventing neovascularization by mobilized endothelial precursor cells. Some authors have speculated that antiangiogenic therapy targeting the tumor vasculature could hamper blood-borne therapeutic agents from reaching tumor cells [33, 34]. However, a combination of the antiangiogenic agent, tumor necrosis factor (TNF) α, and a cytotoxic agent, melphalan, was shown to generate a higher intratumoral concentration of melphalan than melphalan alone [35]. As suggested by Jain [36], antiangiogenic therapy may 'normalize' tumor vasculature, thus the effect of chemotherapy might be enhanced rather than being inhibited. Considering the wide range of angiogenic factors produced by tumor cells and the possible biological heterogeneity of tumor induced blood vessels, combination of antiangiogenic agents with other forms of therapy may be more effective [37]. Another advantage of combining endostatin with chemotherapy is that endostatin could be employed to reduce the dose of chemotherapeutic agents to spare the patient the side-effects of cytotoxic drugs, without impairing antitumor efficacy.

Despite many successful experiments with antiangiogenic therapies to treat tumors in animal models, several disappointing results have reported [38, 39], and potential tumor resistance mechanisms to antiangiogenic therapy have been revealed, indicating that single-agent therapeutic strategies are not desirable [37]. The obvious example is in a rat tumor model of Yoshida sarcoma, where intravenous administration of TNP-470, a classical angiogenesis inhibitor, suppressed the growth of primary tumors, but increased the growth of metastatic foci in distant lymph nodes [40]. The lesson from this study is that angiogenesis inhibitors need to be administered at relatively high levels over a long-term period. In this regard, gene therapy appears to be more powerful than other forms of anti-angiogenic therapy, as gene therapy has the potential to produce the therapeutic agents at high concentration in a local area for a sustained period, and avoid the problems encountered with long-term administration of recombinant proteins. In the present study gene expression vectors were injected intratumorally, resulting in intense expression of antiangiogenic protein *in situ*.

It has recently been reported that endostatin directly inhibits migration and invasion of head and neck squamous cell carcinoma cells [41]; inhibits *in vitro* growth of C51 murine colon cancer cells and HT29 human colon cancer cells, and induces their apoptosis through accumulation at the G1 phase [42]; and directly modulates the cellular function of lung cancer cells, inhibits proliferation, and induces apoptosis of Lewis lung carcinoma cells [43]. Endostatin directly decreases the DNA synthesis of mammary cancer cells, and inhibits their survival [17]. This may partly explain the increased apoptosis of tumor cells, as well as inhibition of tumor cell migration to remote sites or outgrowth at such sites. In accord, endostatin inhibited B16 melanoma lung metastasis [44], and helped paclitaxel to prevent liver metastasis in a model of colorectal cancer [23]. On the other hand, the inhibition of neovascularization by endostatin may restrict the supply of tumor cell survival factors provided either by endothelial cells or by the circulation. The mechanism by which endostatin mediates tumor cell apoptosis may be due, in part, to the loss of an adequate vasculature, which would deprive the tumor of oxygen and nutrients. Several studies indicate that angiogenesis inhibitors can induce tumor cell apoptosis by decreasing the levels of endothelial-cell-derived paracrine factors that promote cell survival. At least 20 such proteins have been reported to be produced by endothelial cells, including PDGF, IL-6 and heparin-binding epithelial growth factor (HB-EGF) [45]. Production of paracrine factors is decreased, in part, because angiogenesis inhibitors can inhibit endothelial-cell proliferation [46]. In addition, endostatin inhibits the production of vascular endothelial growth factor (VEGF) [15, 47], which has been shown to act as a survival factor for 4T1 cells [48]. Combining the data together, the antitumor and anti-metastatic activity of endostatin relies on its anti-angiogenesis and pro-apoptosis properties.

A number of chemotherapeutic drugs have been shown to inhibit the tumor angiogenesis of several different types of cancers when administered at low doses in a metronomic approach to

therapy [49]. In the present study we demonstrated that paclitaxel inhibited the angiogenesis of 4T1 breast tumors. Metronomic therapy using cytotoxic drugs is not currently accepted in clinical practice, as continuous administration at low doses might induce acquired drug resistance. In the present study, we used a clinically relevant dose of paclitaxel to target both the tumor cells and the tumor endothelium. Combination of metronomic chemotherapy and antiangiogenic therapy enhances antitumor activity by targeting multiple facets of endothelial cells [50]. The enhanced antitumor activity displayed by the combination of endostatin and paclitaxel may be exerted by targeting both the tumor-associated endothelium and the tumor cells themselves, as evidenced by inhibition of tumor angiogenesis and promotion of cell death, resulting in inhibition of tumor growth and metastases. Several mechanisms may be operating to cause increased tumor cell apoptosis in response to combination therapy, which cannot be explained simply by the decrease in microvessel density. The action of both agents has been shown, at least in part, to involve their stimulation of an anti-tumor immune response, hence the reason for conducting our experiments in immunocompetent wild-type Balb/c mice. Endostatin protein, when injected subcutaneously, was found to be more effective against highly immunogenic 3LL-C75 tumors, than against weakly immunogenic 3LL tumors. Its antitumor activity against 3LL-C75 tumors was impaired in immunodeficient mice, suggesting that the activity of endostatin is due, in part, to stimulation of an anti-tumor immune response [51]. CpG sequences present in the DNA of plasmids used for gene therapy are themselves immunostimulatory, in that they induce pro-inflammatory cytokines including IL-12, TNF-α, IFN-γ and IFN-α [52]. IFN-α activates NK cells and assists in eliminating metastatic foci, and IL-12 and the combination of TNF-α and IFN-γ exert anti-angiogenic activity [53, 54]. Thus, cyclophosphamide combined with plasmid DNA resulted in a reduction in the size and number of malignant melanoma metastases in mouse lungs, whereas both agents alone were not effective [52]. In the present study, control vector alone had no affect on tumor growth, indicating that the anti-tumor effects of the endostatin vector End-pcDNA3.1 were primarily due to the expression of endostatin, and not due to CpG. We cannot, however, discount the possibility that CpG-induced expression of proinflammatory cytokines by the End-pcDNA3.1 plasmid contributes partly to the synergy with paclitaxel. In an additional mechanism, endostatin may indirectly stimulate an anti-tumor immune response by causing apoptosis of tumor cells by depriving them of nutrition and oxygen, thereby leading to the release of tumor antigens, and cross-presentation by infiltrating professional antigen presenting cells to induce specific anti-tumor immunity [29, 55, 56]. In addition to its anti-neoplastic toxicity, paclitaxel has been shown to exert immune-activating properties by activating NK cells [57], T cells [58], and macrophages [59], and by enhancing the production of the cytotoxic mediators, such as TNF-α and IL-12 [59], or by acting as a second signal for the activation of the tumoricidal activity mediated by IFNγ-activated macrophages [60, 61].

In summary, the combination of paclitaxel and endostatin is more effective than either agent alone in suppressing breast cancer growth, and metastasis. The combinational therapy with endostatin and paclitaxel warrants future investigation as a therapeutic strategy to combat breast cancer.

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