Synergistic anti-tumor effect of recombinant human endostatin adenovirus combined with gemcitabine

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Endostatin is an important endogenous inhibitor of neovascularization, which has been widely used in anti-angiogenesis therapy for cancer. To fully explore the potential of endostatin, we evaluated the anti-tumor efficacy of the combination of recombinant human endostatin adenovirus and low-dose gemcitabine in nude mice. We injected recombinant human endostatin adenovirus intratumorally plus a low dose of gemcitabine i.p. routinely. The combination treatment produced no side-effects, and resulted in marked suppression in tumor formation and growth of established human lung carcinoma xenografts in nude mice, with decreased microvessel density and increased apoptosis percentage. Our data support the idea of synergistic anti-tumor properties of endostatin plus low-dose chemotherapy against human lung cancer *in vivo*. *Anti-Cancer Drugs* 16:551–557 © 2005 Lippincott Williams & Wilkins.

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Introduction

Generation of new blood vessels plays a crucial role in a large variety of biological and pathological processes. It is well established that the growth and progression of most solid cancers are angiogenesis dependent. Targeting tumor vasculature has been a new therapeutic strategy [1,2]. New blood vessel development is a complex process, and quite a few growth factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs) and basic fibroblast growth factor (bFGF) participate in the formation of new vessels. Tumor angiogenesis is highly orchestrated by a balance between positive and negative regulators. One of the potent anti-angiogenesis agents, endostatin, primarily purified from the conditioned media of EOMA (murine hemangioendothelioma) cells, is the proteolytically cleaved fragment containing the C-terminal 184 amino acids derived from the non-collagenous (NC1) domain of the basement membrane collagen XVIII, which inhibits endothelial cell migration and proliferation [3]. Anti-angiogenesis therapy for cancer is aimed at producing a 'dormant' state in which tumor cell proliferation and tumor expansion is stalled by inhibiting tumor-related angiogenesis, thus depriving tumors of essential nutrients and oxygen [4].

Lung cancer is a major cause of cancer-related death worldwide [5]. Several new cytotoxic drugs have been introduced to be active against non-small cell lung cancer (NSCLC). Gemcitabine is a deoxycytidine analog applied clinically against several solid tumors, such as ovarian cancer, NSCLC and bladder cancer [6].

As a biologic therapy, anti-angiogenesis agents offer potential for the treatment of lung cancer; however, emerging data suggest that anti-angiogenesis alone may be limited in advanced tumors. In recent years there has been an increasing interest in attempting to combine radiation or chemotherapy with angiogenesis inhibitors for tumor suppression—the combination may be more appropriate to produce improved efficacy and reduced toxicity by transcending each limitation. Endostatin and thalidomide can both cause direct inhibition of endothelial cells. Treatments combining thalidomide with carboplatin, paclitaxel, etc., have been applied in phase II or III studies for advanced NSCLC [7]. At the same time, preliminary results of a phase I study of recombinant endostatin for solid tumors have been published. However, the effect on the combination of endostatin with cytotoxic drugs remains to be evaluated.

Widely utilized to inhibit tumor neovascularization, gene therapy, especially with adenovirus vectors, has shown success in surmounting the shortcomings of recombinant protein injection [8,9]. Since endostatin and gemcitabine

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are both effectively used for NSCLC separately, in the present study we evaluated the efficacy of the combination of local recombinant endostatin adenovirus and low-dose gemcitabine for the treatment of established human lung carcinoma in animal models. Our findings showed that low-dose cytotoxic drug combined with an anti-angiogenic agent can be successfully used against human lung cancer without obvious side-effects and perhaps other solid tumors as well.

Materials and methods

Recombinant adenoviral vector construction

To clone human endostatin, total RNA was extracted from human liver tissue (TRIzol; Invitrogen, Carlsbad, CA). The full-length human endostatin cDNA (about 570 bp) was amplified by RT-PCR using the primer (5′-CGGGATCCACAGCCACCGCGACTTCCAGCC-3′ and 5′-GCGGATCCTACT TGGAGGCAGTCATGAAGC-3′) with a *Bam*HI restriction site in both primers (Titan One Tube RT-PCR kit; Roche, Mannheim, Germany). After sequence confirmation, the cDNA was cloned into cloning vector PUC18 and then into a shuttle vector for the rescue of the recombinant adenovirus (AdEasy system) as described before [10]. The viral particles were amplified in 293 cells, purified by CsCl gradient ultracentrifugation and measured by absorption (A_{260}). The virus titer was quantified using a standard $TCID_{50}$ assay.

Cell culture

The human lung adenocarcinoma cell line A549, the murine hemangioendothelioma cell line EOMA and the human embryonic kidney cell line (HEK293) were obtained from ATCC (Manassas, VA), and cultured in DMEM supplemented with 10% fetal bovine serum plus ampicillin and streptomycin routinely. Culture were split 1:4 every 4 days.

Western blotting analysis

A549 cells were transduced at a multiplicity of infection (m.o.i.) of 100 (10^8 p.f.u./10^6 cells in 1.0 ml of complete media) with Ad-hEndo and control adenovirus (Ad-null) or no transduction. Cells were conditioned at 37°C for 48 h, supernatant was harvested and concentrated by ultrafiltration (Centricon YM-3; Millipore, Bedford, MA), and analyzed by Western blotting. EOMA is a positive control [3]. Samples (10 µl) were separated on a 12% SDS–PAGE gel and transblotted onto a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked by TTBS (including 0.1% Tween 20 in TBS) with 5% non-fat milk and later it was probed with rabbit polyclonal anti-endostatin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:400 at 4°C overnight. Blots were then incubated with 1:5000 horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Sigma, St Louis, MO). Protein bands were visualized by the DAB detection kit (Sigma).

Animal studies

Female (6–8 weeks old) athymic nude mice (purchased from the Laboratory Animal Center of Sichuan University) were acclimatized for 1 week. Mice were housed under pathogen-free conditions, and fed with animal chow and water *ad libitum*. Animals were anesthetized using sodium phenobarbitone prior to all procedures and observed until fully recovered.

In vivo activity of endostatin

Human lung adenocarcinomas were established by injecting nude mice s.c. with 1.5×10^7 A549 cells in 100 µl PBS in the right flank. Seven days later, when the tumors were palpable, mice were randomly divided into five groups ($n = 5$ animals/group): Ad-hEndo (ADE), intratumoral injection of 1×10^9 p.f.u. recombinant adenovirus twice a week; gemcitabine (GEM), twice i.p. treatment of 125 mg/kg every injection in a week; Ad-hEndo plus gemcitabine (E + G), Ad-hEndo delivery on Monday and Tuesday, along with viral injection, gemcitabine administration on Wednesday and Saturday; Ad-null (ADC), intratumoral injection of 1×10^9 p.f.u. non-recombinant virus twice a week; and NS, equal volume of normal saline on the same schedule as above. All of the treatments lasted for 2 weeks [11]. Tumor diameters were callipered every 4 days. The tumor volume = $0.52 \times L \times W^2$, where W is the width of the tumor and L is the length of the tumor. Treated mice were closely monitored and killed if any signs of death were seen. Mice in all groups were sacrificed 60 days after tumor establishment. We looked for possible side-effects during the treatment.

Detection of microvessel density and apoptosis ball

Frozen tissues were sectioned (5 µm) and fixed in acetone at 4°C. For detection of CD31 (platelet/endothelial cell adhesion molecule-1), sections were probed with a monoclonal rat anti-mouse CD31 antibody (1:400; Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with biotinylated polyclonal rabbit anti-rat antibody (1:200; Vector, Burlingame, CA). Positive reaction was visualized using 3,3-diaminobenzidine as chromagen (DAB substrate kit; Vector). Sections were counterstained with hematoxylin and mounted with glass coverslips. Then sections were visualized in an Olympus microscope at × 10 magnification. Apoptosis cells were identified by the fluorescent *in situ* terminal deoxynucleotidyltransferase-mediated nick end-labeling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche) following the manufacturer's guide. Images were captured by fluorescence microscope at × 20 magnification (Olympus). The blood vessels or the apoptosis cells were counted from three areas in each section in a blinded manner.

Synergistic index calculation

Mean values of tumor volume (MVD) cell apoptosis were used for calculation of corresponding synergistic indexes

using the methods described previously [12]. Briefly, the synergistic index of tumor volume (FTV, fractional tumor volume), MVD (compared to the untreated control groups, the resultant value was decreased) was obtained by dividing the expected relative ratio by the observed relative ratio, whereas the synergistic index of cell apoptosis (compared to the untreated control group, the resultant value was increased) was obtained by dividing the observed relative ratio by the expected relative ratio. An index greater than 1 indicates a synergistic effect; an index less than 1 indicates a less than additive effect.

Statistics

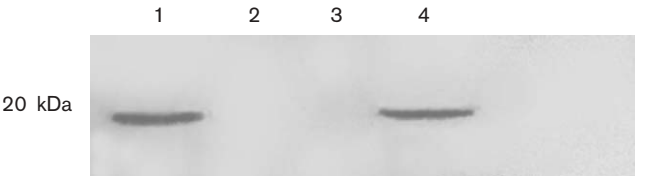
The results of the statistical analyses are presented as means ± SD. For comparison of individual time points, differences between the groups were tested by performing ANOVA and an unpaired Student's *t*-test. All p values were two-sided and statistical significance was defined as $p < 0.05$.

Results

Recombinant human endostatin expression *in vitro*

A human lung cancer cell line A549 was transduced with 100 m.o.i. of Ad-hEndo or Ad-null, respectively. After 48 h, conditioned supernatant was concentrated and mixed with 2 times sample buffer. Samples were separated on a 12% SDS–PAGE gel and transferred onto PVDF membranes. The membrane blots were probed with primary antibody and followed by second antibody. A distinct band of about 20 kDa, corresponding to the size of endostatin, was visualized in the Ad-hEndo-treated cells and EOMA, but not in Ad-null-transduced and non-transduced cells (Fig. 1).

Fig. 1



Expression of endostatin protein determined by Western blot. Recombinant human endostatin was expressed as a single band of approximately 20 kDa in Ad-hEndo transfected A549 cells (lane1), while no band was detected in empty virus (Ad-null) (lane2) transfected or untreated (lane4) tumor cells. EOMA was the positive control (lane3).

Combination treatment significantly reduces tumor growth *in vivo*

Seven days after the human lung cancer model was established, nude mice were randomized to receive administration of gemcitabine, Ad-hEndo, gemcitabine plus Ad-hEndo, Ad-null or normal saline separately (Fig. 2A). All mice were monitored every 4 days for a change in tumor growth. At day 60, all the animals were sacrificed. Tumors from Ad-null or normal saline-treated mice did not differ in tumor size. Treatment with gemcitabine or Ad-hEndo as a single agent resulted in tumor growth regression of 34 and 43%, respectively, compared with NS groups. The combination group showed enhanced efficacy in tumor volume suppression by approximately 73% (Fig. 2B).

Toxicity assays

To measure systemic toxicity of the treatments, body weights of mice were monitored twice a week using a digital balance (Setra EL-2000S). No signs of cumulative adverse results were observed in gross measures such as weight loss, ruffling of fur, change in behavior and feeding. In addition, pathologic inspection was assessed by hematoxylin & eosin staining of sections. No organ hemorrhaging in kidney (Fig. 3A), liver (Fig. 3B) or lung (Fig. 3C) was found by microscopic examination. As most adenoviruses will infect liver tissue through i.v. injection, we delivered viruses at 1×10^9 or 2×10^9 p.f.u./mouse i.v., and evaluated the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels 2 days after injection. The results of the serum liver enzyme assays of the animals treated with 1×10^9 p.f.u./mouse Ad-hEndo or Ad-null were all in the normal ranges. However, at a dose of 2×10^9 p.f.u./mouse, we detected high serum levels in both groups (AST > 400 IU/l and ALT > 200 IU/l), suggesting acute liver damage after high-dose adenovirus infection.

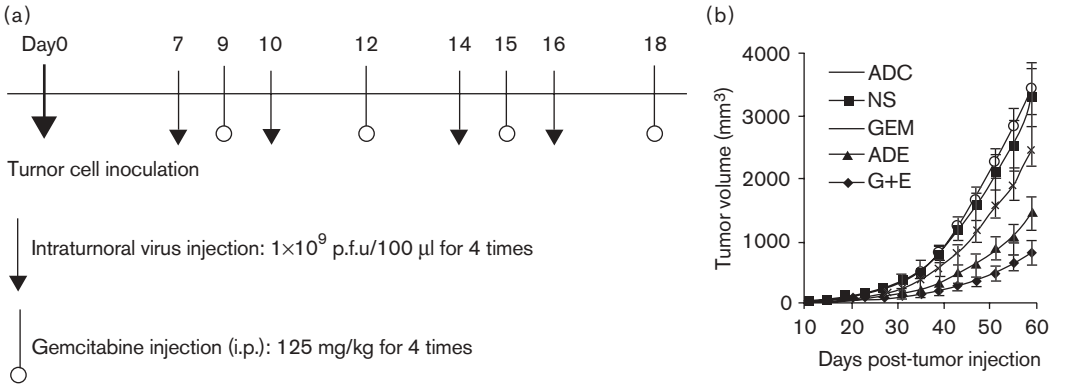
Inhibition of tumor-induced angiogenesis (CD31) and increase of apoptosis (TUNEL)

Tumor sections from each group were stained with anti-CD31 antibody (Fig. 4A) and TUNEL reagent (Fig. 5A) to determine microvessel density (MVD) and apoptosis rate. Tumors of the control groups, including Ad-null and saline, showed high microvessel density and those of the gemcitabine group had intermediate values. Those submitted to Ad-hEndo alone or combination treatment showed decreased microvessels, especially in the combination group (Fig. 4B). There was no difference in apoptosis count between controls, but more apoptotic cells were seen in those from the chemotherapy or anti-angiogenic treatment group alone. Furthermore, tumors from animals that received Ad-hEndo plus gemcitabine showed the highest apoptotic indices (Fig. 5B).

Endostatin and gemcitabine act synergistically in tumor suppression

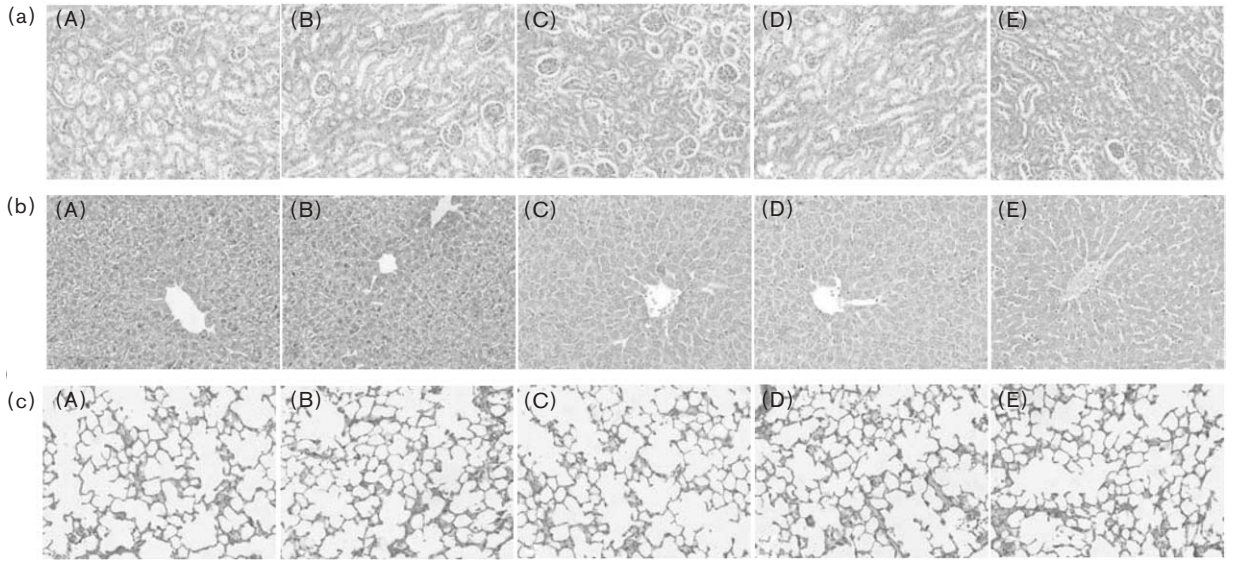
Treatment was not associated with any side-effects. We also found an interaction between anti-angiogenesis and cytotoxic drug. Combination therapy of endostatin with low-dose gemcitabine resulted in more significant tumor growth inhibition than either endostatin or gemcitabine administration separately. The FTV of the combination group showed a synergistic effect about 35 days after

Fig. 2



In vivo combination therapy with Ad-hEndo and gemcitabine. (a) The schedule of combination therapy for tumor-bearing mice. (b) Significantly decreased tumor volume in nude mice after combination therapy. Animals were treated with ADC, NS, GEM, ADE or E + G (as indicated). Plotted is the mean measurement of the diameters. Bars = SE. $p < 0.05$ as determined by unpaired Student's *t*-test between groups.

Fig. 3



H & E staining of kidney (a), liver (b) and lung (c) in recipient mice. No organic hemorrhage apperaed in the combination group and no differences were seen between groups. (A) NS. (B) ADC. (C) GEM. (D) ADE. (E) E + G.

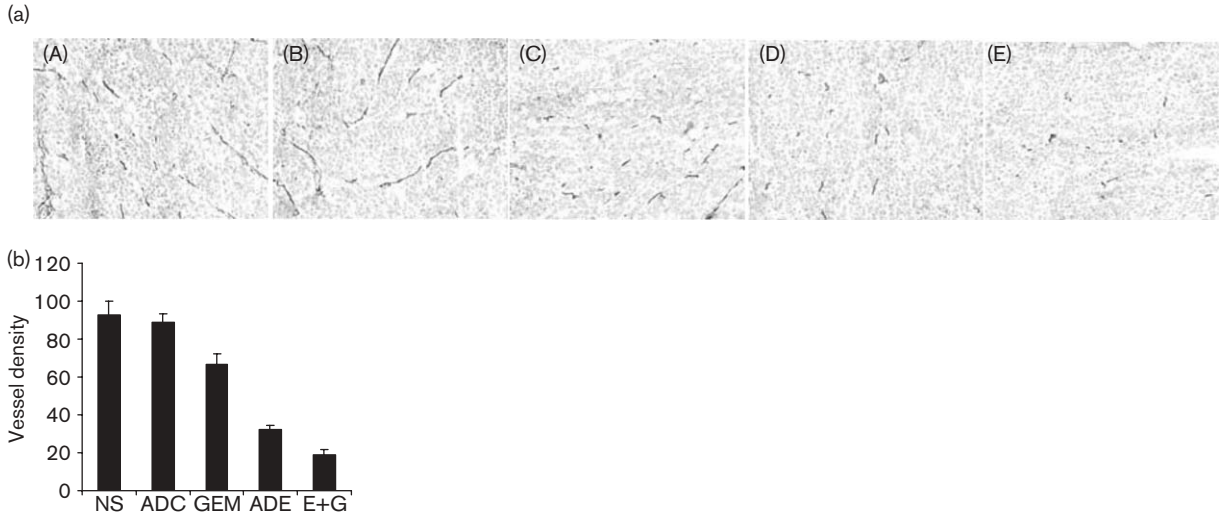
tumor cell transplantation (Table 1). Angiogenesis and apoptosis within tumor tissue were estimated in tumor sections. The combination treatment revealed a synergistic relationship in tumor MVD (synergistic index = 1.27) (Table 1) and apoptosis indices (synergistic index = 1.23) (Table 1).

Discussion

In lung cancer, the extent of angiogenesis seems important for prognostic stage and survival [13]. Endostatin is a natural cleavage polypeptide derived from its parent protein, collagen type XVIII, and studies have shown that the 20-kDa protein is a potent anti-angiogenesis agent [14]. The treatment of neovascularization inhibition has gained particular attention to suppress tumor growth due to the fact that tumor-associated endothelial cells proliferate chronically during tumor development.

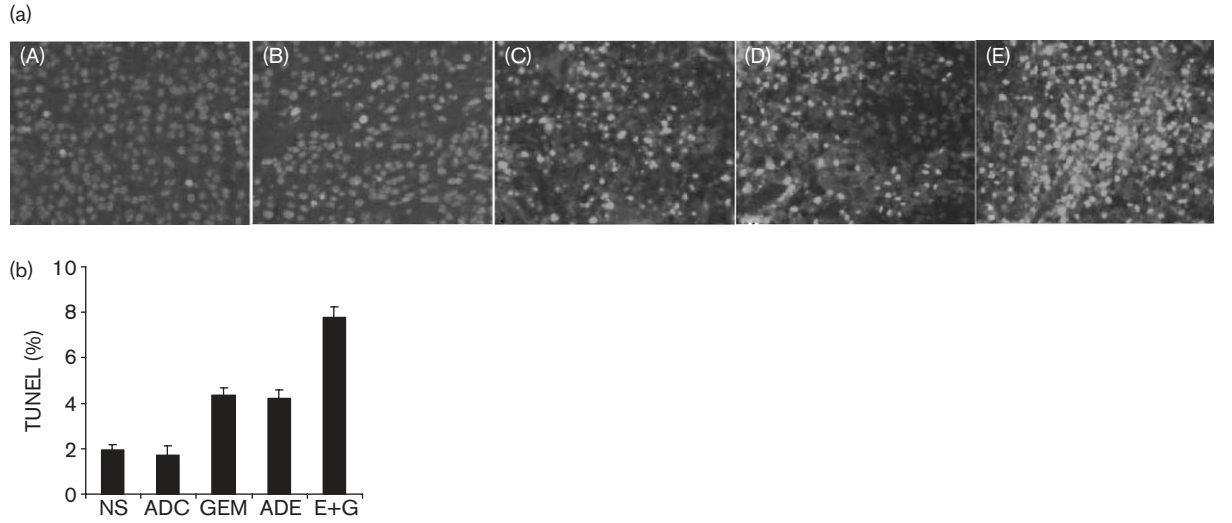
Chemotherapy schemes for the treatment of NSCLC mostly produce a limited improvement, associated with considerable toxicities and acquired drug resistance.

Fig. 4



Inhibition of tumor vascularization by combination administration. Frozen tissue sections were fixed and treated with rat anti-CD31 antibody. The stained sections were visualized under $\times 100$ magnification (a). (b) Significantly decreased microvessels in the combination group (E) and Ad-hEndo (D)-treated alone group compared with the NS (A), ADC (B) and GEM (C) groups.

Fig. 5



Combination treatment results in increased apoptosis of human lung cancer xenograft. Histological sections of tumors were stained using the TUNEL kit and detected by immunofluorescence microscopy ($\times 200$) (a). (b) The data showed no significant differences between the control, saline (A) and Ad-null (B) groups, while an increased apoptosis rate was seen in the gemicitabine (C)- or Ad-hEndo (D)-treated groups, especially in the combination group (E).

Gemcitabine (dFdC) is commonly used in NSCLC and other cancers [11]. dFdC is phosphorylated to its triphosphate (dFdCTP) after being taken up by cells, which is effective in stopping DNA polymerization. dFdC is also capable of inhibiting ribonucleotide reductase (RR), an enzyme with a key role in DNA repair procedures [6].

Studies combining low-dose chemotherapy with anti-angiogenesis therapy for solid tumors have been reported in recent years and can suppress tumor growth more effectively than conventional chemotherapy or anti-angiogenic biotherapy alone [9,12,15]. Our studies show that the association between recombinant human endostatin adenovirus and gemcitabine inhibits the growth

Table 1 Combination therapy of endostatin with gemcitabine fraction relative[a] to NS control group

Day[b]	Endostatin	Gemcitabine	Combination treatment		
			Expected[c]	Observed	Ratio[d]
Tumor volume index					
35	0.45	0.68	0.31	0.29	1.06
39	0.43	0.72	0.31	0.27	1.13
43	0.40	0.66	0.26	0.23	1.15
47	0.40	0.74	0.29	0.23	1.29
51	0.42	0.75	0.31	0.23	1.37
55	0.41	0.75	0.31	0.26	1.19
59	0.44	0.77	0.34	0.25	1.36
Microvessel density index	0.35	0.72	0.26	0.20	1.23
Apoptosis cells index	0.46	0.41	0.19	0.24	1.27

[a]Relative fraction, in the analysis of tumor volume, FTV = mean tumor volume experimental/mean tumor volume control; in MVD, mean microvessel count experimental/mean microvessel count control; in apoptosis index, mean apoptosis index experimental/mean apoptosis index control.
[b]Day after tumor cell transplantation.
[c]Mean fraction of endostatin × mean fraction of chemotherapy group.
[d]Obtained by dividing the expected fraction by the observed fraction (or dividing the observed fraction by the expected fraction, in apoptosis analysis). A ratio of greater than 1 indicates a synergistic effect; a ratio of less than 1 indicates a less than additive effect

and metastasis of human lung cancer established in athymic nude mice, and yields decreased microvessel counts and an increased apoptosis index to an enhanced extent. Intratumoral injection of recombinant adenovirus and i.p. treatment of low-dose cytotoxic agents can be applied safely without serious systematic reactions.

Conventional chemotherapy for NSCLC usually causes side-effects such as nausea, hair loss, neurotoxicity and myelosuppression [16], and leads to the development of acquired resistance to the cytotoxic drug of some heterogeneous survival cells [17]. Conventional dosing chemotherapy calls for episodic application of a cytotoxic drug near the maximum tolerated dose and interrupted by a period of rest to let normal cells recover. With the low rate of replication and cell division (the proliferation index of endothelial cells in tumor vessels is usually less than 3% [18]), tumor-associated endothelial cells are only weakly disrupted by standard chemotherapy. In combination studies, however, cytotoxic drugs were administered routinely at low dosage without a long interval, which can target endothelial cells in the tumor, and block their repair and recovery during the rest periods. In the context of tumor angiogenesis, angiogenesis inhibitors do not act directly on cancer cells, suggesting that anti-angiogenesis treatment alone is not sufficient for complete eradication of the tumor. Regular administration of chemotherapy and anti-angiogenesis in combination produced regression of tumors, minor side-effects and prolonged non-recurrence periods [12,15]. The interaction between anti-angiogenesis and chemotherapy may lie in several facts. First, some chemotherapeutic agents have anti-angiogenic properties. Some traditional cytotoxics have been shown to have inhibitory effects on endothelial cell proliferation, migration and tubule formation [19,20]. Moreover, if endothelial cells are inhibited from migrating or proliferating, they would lack certain adhesive contact with the matrix and themselves. Non-adhesive endothelial cells are more susceptible to a cytotoxic agent and this results in a higher apoptosis rate [21]. Third, the initial disruption of angiogenesis could hamper and delay the repair of inflicted dividing cells, and the increased vessel permeability may lead to increased tumor exposure to cytotoxic drugs. Thus, tumor cells are more vulnerable to the damaging effects of chemotherapy, especially when the cytotoxic drug is given at a low dose [22]. Therefore, a coordinated approach targeting multiple tumor-associated cell properties seems to be a promising strategy for marked inhibition of tumor growth [23,24].

In summary, the present study suggests that the combination of recombinant human endostatin adenovirus with gemcitabine produces an apparent decrease in tumor volume and microvessel density, and an increase of apoptosis without obvious undesired toxicity in the treatment of experimental human lung cancer. Selecting the optimal anti-angiogenic and chemotherapeutic therapy doses and application schedule may prove difficult [17,25], and further studies should be carried out to elucidate the molecular mechanism of anti-tumor effects on this combination therapy.

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