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ORIGINAL ARTICLE

Different combination schedules of gemcitabine with endostar affect antitumor efficacy

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Abstract
Purpose Antiangiogenic drugs inhibit tumor growth by decreasing blood supply and causing transient "normalization" of the tumor vasculature, thereby improving the delivery of systemic chemotherapy. A higher dose of antiangiogenic drugs may lead to a more marked decrease in intratumoral blood flow but may concomitantly cause a decrease in delivery of chemotherapeutic agents. The purpose of this study was to define an optimal schedule for the combination of gemcitabine with a recombinant endostatin, endostar.
Methods We evaluated the antitumor effects with different schedules of gemcitabine combined with or without endostar. The changes of vascular endothelial growth factor (VEGF) levels in tumor extracts and sera after gemcitabine treatment were examined. Endostar was also assessed for its abilities to inhibit the increase in VEGF levels. Apoptotic cells and microvessel density within tumor tissue were also examined.
Results Endostar administered simultaneously with or following gemcitabine improved the inhibition of tumor growth, compared with gemcitabine alone. VEGF levels decreased immediately after gemcitabine treatment, but increased in the following several days. Endostar administered simultaneously with or following gemcitabine could inhibit the increase in VEGF levels, thereby cause a decreased vessel density and an increased apoptosis in tumor tissue.
Conclusions Our finding suggested that endostar given simultaneously with or following gemcitabine might be optimal to enhance the antitumor effect.

Keywords Endostatin · Gemcitabine · VEGF · Antiangiogenesis · Combined therapy

Introduction

Tumors influence the surrounding host stroma by inducing angiogenesis to supply their nutrient needs and oxygen. As a new strategy for cancer therapy, antiangiogenic therapy attempts to inhibit new vessels from forming around a tumor and to break up the existing network of abnormal capillaries that feeds the cancerous mass [1–3]. With the role of angiogenesis in tumor growth and progression firmly established, antiangiogenic agents have received much widespread attention but strategies for their optimal use are still being developed [4]. As is well known, angiogenesis of tumor is tightly regulated by the balance between angiogenic and antiangiogenic factors [5, 6]. Biotherapy strategies leading to increased production of endogenous angiogenesis inhibitors would seem perfectly suited to support such an approach by tipping the balance toward a more antiangiogenic state [4, 7].

Xing-Chen Peng, Meng Qiu, and Meng Wei contributed equally to this work.

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Among the endogenous antiangiogenic molecules, endostatin, which is a 20-kDa C-terminal fragment derived from the C-terminal domain of collagen XVIII, is the most potent inhibitor of tumor angiogenesis. Although its mechanism of action is not yet completely defined, endostatin can induce apoptosis of endothelial cells [8], reduce endothelial cell motility by interfering with basic fibroblast growth factor-induced signal transduction [9], and block vascular endothelial growth factor (VEGF)-mediated signaling by directly interacting with the receptor VEGFR-2 [10]. In previous preclinical studies, endostatin has been proved to be effective in many animal models with no significant drug-related toxicity [11, 12]. Moreover, systemic endostatin therapy did not elicit drug-induced resistance after prolonged treatment in animals [13]. These promising results led to the rapid advance of this agent into the clinical arena [14]. Although promising, it is unlikely that antiangiogenic therapies are curative on their own because these agents, like endostatin, cannot kill tumor cells directly. Rather, their greatest potential may be realized when used in conjunction with conventional anticancer therapies, for example, chemotherapy [15, 16].

Antiangiogenic drugs (such as bevacizumab) may cause transient "normalization" of the tumor vasculature, improving the tumor perfusion and, consequently, the delivery of systemic chemotherapy. However, a higher dose of the antiangiogenic drugs may lead to a more marked decrease in intratumoral blood flow, but may concomitantly cause a decrease in delivery of chemotherapeutic agents [17–20], this might discount the combined effect. A rational combination schedule should be defined.

Endostar (marketed in China), a recombination endostatin protein, has a longer half-life (about 8 h) than endostatin and is at least twice as potent as endostatin in animal tumor models [21, 22]. In the present study, we attempted to define an optimal schedule for the combination of gemcitabine with a single high-dose endostar (300 μg once) in murine tumor model, also to investigate the possible mechanism responsible for the increased antitumor effects.

Materials and methods

Animal and tumor model

C57BL/6 mice, 6- to 8-week-old, were obtained from the experimental animal center of Sichuan University (Chengdu, Sichuan province, China) and were housed in our animal research facility. Mice were kept in groups of 3–5 per cage and fed with clean food and water. The animals were acclimatized for 1 week before use and maintained throughout at standard conditions: 24 ± 2°C temperature and 50 ± 10% relative humidity. Murine Lewis lung carcinoma cell line (LLC) was purchased from the American Type Culture Collection and was maintained in DMEM.

LLC cells (about 1×10^6) in 0.1 ml of PBS were injected subcutaneously into the right oxter of each mouse. Tumors growth were evaluated on alternate days by measurement of tumor diameters, and the volume of the tumor was determined using the formula: volume (mm^3) = length × width^2 × 0.52, as previously described [23]. All of the data are represented as means ± SE. The animal studies were approved by the Institutional Animal Care and Treatment Committee of Sichuan University.

Chemotherapy

Gemcitabine, as a single agent, has previously been reported to produce a delay in growth of murine LLC model. Gemcitabine was obtained from Eli Lilly (Indianapolis, Ind., USA). According to the previous studies [24], animals were injected intraperitoneally (i.p.) with gemcitabine at a dose of 30 mg/kg, diluted in 0.5 ml of sterile 0.9% normal saline (NS).

Recombinant endostatin protein

Endostar (Yantai Medgenn Co. Ltd. Shangdong province, China) was reconstituted in laminar air-flow hood under sterile conditions. The protein was stored at 4°C until used. The endostar was dissolved in 0.2 ml 0.9% NS and administered to each animal at a dose of 300 μg by tail vein injection.

Treatment groups

On day 18 after inoculation of LLC, when the diameter of tumors reached up to about 8–10 mm, animals were randomized into one of the following 5 groups ($n = 8$ in each group). Group Endo/Gem: endostar 300 μg was given via tail vein injection to each mouse on day 18 followed by i.p. injection of gemcitabine at a dose of 30 mg/kg on day 20. Group Endo + Gem: mice were treated with gemcitabine and endostar simultaneously using the methods described above on day 20. Group Gem/Endo: the i.p. injection of gemcitabine was given on day 20 followed by intravenous injection of endostar on day 22. Group Gem: the mice were only treated with gemcitabine on day 20. And Group NS: treated with NS control (Fig. 1a).

In order to assess the histologic changes, animals were killed on day 28 by cervical dislocation under halothane anesthesia. Tumors from killed mice were removed and fixed in the 4% formaldehyde solution for histologic analysis.

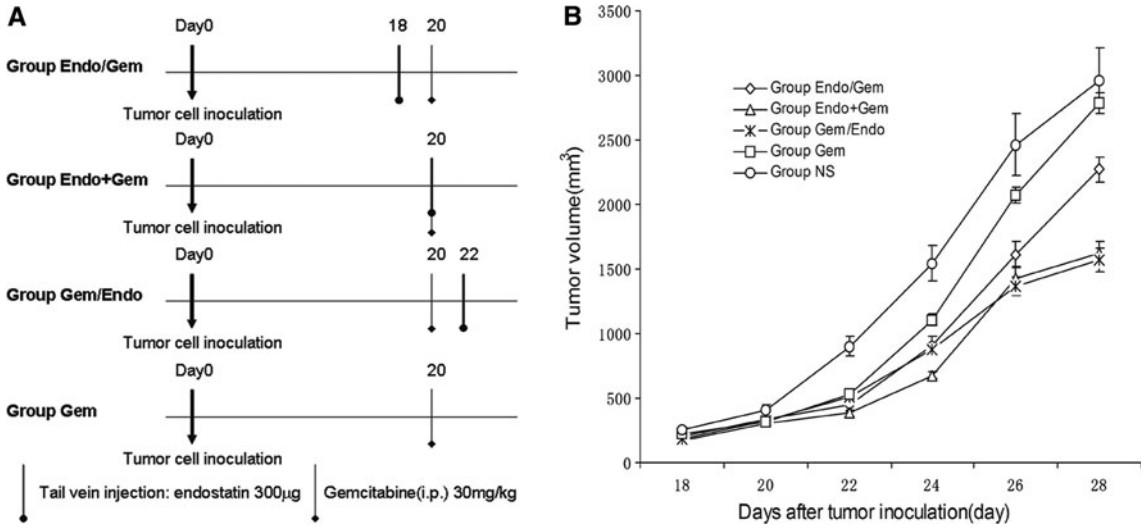

Fig. 1 Antitumor effect of endostar in combination with gemcitabine in LLC model. **a** Schematic diagram of different treatment groups. **b** The combination treatment, including Group Endo + Gem or Gem/Endo, enhanced tumor growth inhibition, compared with other groups. The results are expressed as means ± SE. The experiment was repeated three times with similar results

Measurement of VEGF levels in tumor extracts and serum

The tumors from killed mice were kept in liquid nitrogen immediately. Tumor extracts were prepared by homogenizing tumors in a buffer containing 150 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol (pH 7.5), 50 mM PMSF, 1 mg/ml leupeptin, and 2 mg/ml aprotinin. The homogenate was subjected to three freeze–thaw cycles in liquid nitrogen to lyse cells and then spun at 5,000 g at 4°C to pellet debris. VEGF levels were measured in tumor extract supernatants by ELISA (R&D Systems), according to the manufacturer's instructions. VEGF levels were normalized to total extract protein concentration as measured by Lowry assay and expressed as pg VEGF/mg total extract protein.

The blood samples were collected in microtainer plasma separating tubes (Becton–Dickinson, San Jose, CA) and centrifuged at 4°C, and plasma was frozen immediately and stored at −20°C until assayed.

Histologic analysis

Tumors were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 3–5 μm sections. Then, the sections were stained with hematoxylin and eosin (H&E). The presence of apoptotic cells within the tumor sections was evaluated by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) following the manufacturer's protocol. Percent apoptosis was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high-power fields/slide).

Immunohistochemistry analyses of microvessels expression were done with goat anti-mouse CD31 antibody (Santa Cruz Biotechnology) using the labeled streptavidin–biotin method. That is to say, sections were deparaffinized in xylol and rehydrated in graded alcohol series. Antigen retrieval was carried out by autoclaving sections in retrieval buffer (10 mM pH 6.0 EDTA citrate buffer) for 3 min in saturated steam after up-pressure gaining (126°C, 1.6 bar, 23 psi). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide at room temperature free of light for 20 min. Nonspecific binding of reagents was quenched by incubation of sections for 20 min in 5% normal rabbit serum. Sections were then incubated with goat anti-mouse CD31 (dilution 1/200) antibodies overnight at 4°C, followed by incubating with biotinylated rabbit anti-goat IgG and then streptavidin–biotin–horseradish peroxidase complex at 37°C for 1 h severally. Negative control was included with each run by substituting the primary antibody with non-immune rabbit serum. Cellular nuclei were counterstained with ameliorative Gill's hematoxylin.

According to the method of Weidner et al. [25], the quantification of microvessel density (MVD) was assessed. The sections were firstly screened at low magnifications (×40 and ×100) to identify the most vascular area of the tumor called hot spot. Within the hot spot area, the stained microvessel was counted in a single high-power (×400) field. MVD was expressed as the number of microvessel/field. Any CD31-stained endothelial cells or endothelial cell clusters that were clearly separated from adjacent

microvessel, tumor cells, or connective tissue elements were considered a single countable microvessel.

Statistical analysis

Statistical significance was determined using one-way ANOVA or Student's *t* test, as appropriate. A $P < 0.05$ was considered statistically significant.

Results

Effects of gemcitabine and endostar on LLC tumors

We investigated the combined effects of the single dose of endostar and gemcitabine on the growth of LLC in C57BL/6 mice and determined whether the inhibition of tumor growth depended on the sequence in which the drugs were administered. Mice bearing LLC tumors were divided into treatment groups as described above. When the diameter of tumors reached up to about 8–10 mm, the treatment was initiated. Consistent with previous observations [24], treatment with gemcitabine on day 20 showed significant inhibitory effects on tumor growth in Group Gem, compared with NS alone. Although gemcitabine inhibited tumor growth immediately after the treatment, the tumors resumed growth several days later. However, the combination therapy groups, including Group Endo + Gem and Gem/Endo, resulted in a pronounced inhibition of tumor development at the end of the observation period ($P < 0.05$ vs. Group Gem or NS). We unexpectedly discovered that there was no statistically significant difference between the Group Endo/Gem and Group Gem during all the period of treatment ($P > 0.05$). Compared with Group Endo/Gem, Group Endo + Gem and Gem/Endo had the most significant delay in tumor growth as determined by tumor volume by day 28 ($P < 0.05$). No statistically significant difference in tumor size was found between the Group Endo + Gem and Gem/Endo ($P > 0.05$). Thus, simultaneous therapy or sequential chemotherapy followed by endostar increase the effectiveness, compared with initial endostar followed by chemotherapy (Fig. 1b).

Effects of gemcitabine on VEGF levels in tumor extracts and serum

To explore the possible mechanism, we examined the production of VEGF after exposure to gemcitabine (30 mg/kg) in LLC tumor-bearing mice. When the diameter of tumors reached up to about 8–10 mm, the mice were then treated i.p. with a single dose of gemcitabine (the day of gemcitabine treatment was designated as day 0) and two of them were killed per day from day 1 to day 7. Mean VEGF levels in extracts from untreated tumors were 49.65 ± 4.59 pg/mg total protein. On day 1, the mean VEGF level in gemcitabine-treated tumors was obviously decreased (21.88 ± 3.11 pg/mg total protein). Interestingly, an increase in the VEGF level was observed from day 2, and it turned 2.1-fold higher than that in the untreated tumors (105.68 ± 13.71 pg/mg total protein) on day 3. The mean VEGF levels in extracts resumed its former levels on day 4 and remained unchanged for the following days (Fig. 2a). VEGF levels in the sera were also assessed by ELISA. Five mice were anesthetized with isofluorane and bled from the tail vein from day 1 to day 7 after the administration of intraperitoneal gemcitabine. We found that gemcitabine induced a similar significant decrease in plasma VEGF levels on day 1, with a concentration of 9.90 ± 4.62 pg/ml. VEGF levels were induced 1.54-fold 2 days after exposure to chemotherapy and remained elevated for the following days (Fig. 2b). Intraperitoneal injection of NS was used as a control at the same time. VEGF levels in both tumor extracts and sera remained unchanged after injection of NS (data not shown).

Down-regulation of VEGF expression by endostar

Having established the change of VEGF levels in response to gemcitabine, we next sought to investigate the possible mechanism of the combined effects observed in vivo and assess the effects of endostar on VEGF levels mediated by gemcitabine. Twenty LLC-bearing mice were randomized into 4 groups ($n = 5$ in each group), Groups Endo/Gem, Endo + Gem, Gem/Endo or Gem were treated, respectively, as described above. Serum and tumor tissue were prepared and stored as described above in order to study the changes of VEGF levels on day 23. In extracts from LLC tumors, an obvious inhibition of VEGF expression in Groups Endo + Gem and Gem/Endo was observed on day 3 after injection of gemcitabine. No significant modulation of VEGF expression was observed in Group Endo/Gem (Fig. 3a). The ability of endostar to inhibit VEGF expression after gemcitabine treatment was also observed in sera. The average value of VEGF levels was far lower in the Groups Endo + Gem and Gem/Endo compared with Groups Endo/Gem and Gem (Fig. 3b).

Inhibition of tumor angiogenesis and increase in tumor cell apoptosis

LLC tumors from Groups Endo/Gem, Endo + Gem, Gem/Endo, Gem and NS were harvested on day 28 and were prepared as described above. Angiogenesis within tumor tissue was determined by immunohistochemical staining for CD31 and estimation of angiogenesis via microvessel counts. Treatment with Endo + Gem and Gem/Endo

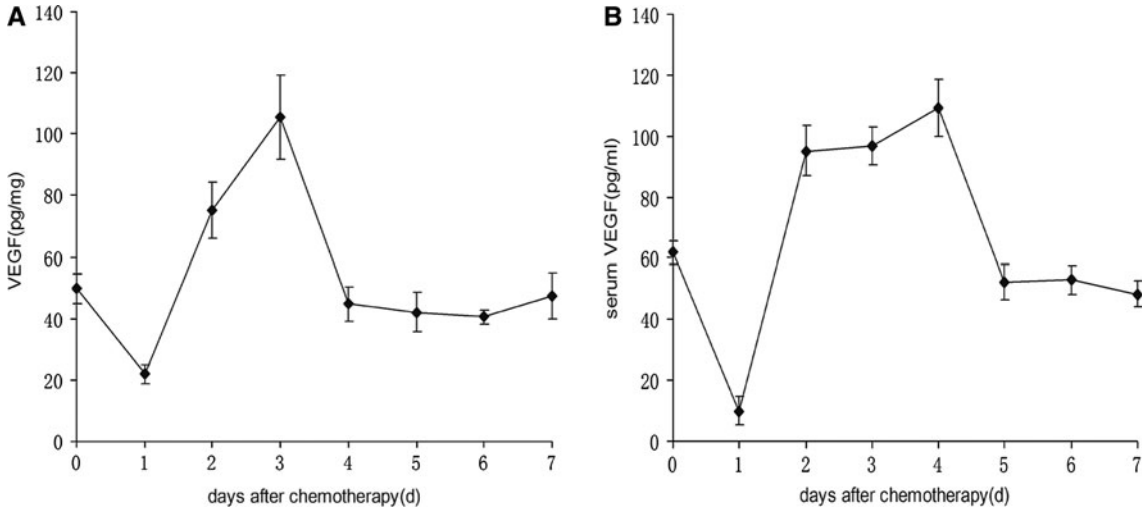

Fig. 2 VEGF levels in extracts or serum after single exposure to gemcitabine in LLC-bearing mice. **a** The changes of VEGF levels in extracts. LLC tumors were exposed to a single dose of intraperitoneal gemcitabine at a dose of 30 mg/kg. Total protein was isolated from the tumors as described above, and VEGF levels were measured in tumor extract supernatants by ELISA. The results are expressed as means ± SE. **b** the changes of VEGF levels in serum after a single injection of gemcitabine and the results are expressed as means ± SE

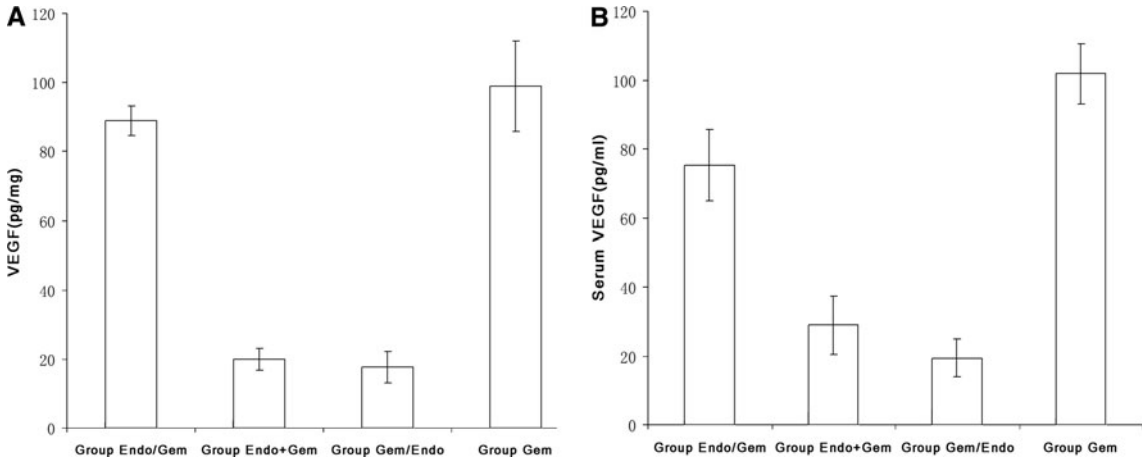

Fig. 3 Effects of endostar on reproduction of VEGF by a single dose of gemcitabine. The VEGF levels in serum and extracts were detected by ELISA in Group Endo/Gem, Endo + Gem, Gem/Endo and GEM on day 23, respectively. **a** A comparative study on VEGF levels in extracts. Group Endo + Gem and Gem/Endo contributed to a significant inhibition of chemotherapy-induced VEGF up-regulation while Group Endo/Gem did not show any obvious differences from Group GEM. **b** a comparative study on VEGF levels in serum. Compared with the controls, a significant reduction could be found in Group Endo + Gem and Gem/Endo, which was in line with the findings in extracts

resulted in an obvious inhibition of the angiogenesis in tumors compared with the other three groups. The average number of vessels per high-power field was also lower for the Group Gem/Endo or Group Endo + Gem than for controls: 5.95 ± 1.41 (Gem/Endo), 8.51 ± 1.09 (Endo + Gem) versus 43.04 ± 5.72 (Endo/Gem), 41.64 ± 4.63 (Gem), and 53.7 ± 6.31 (NS) (Fig. 4).

To further investigate the mechanism of the combined effect of gemcitabine and endostar on LLC tumors, a TUNEL assay was performed to study tumor cell apoptosis. As shown in Fig. 5, the TUNEL assay revealed a markedly higher increase in apoptosis within tumor tissues from the Groups Gem/Endo or Endo + Gem, when compared with the other Groups: 61.53 ± 8.72 (Gem/Endo), 56.94 ± 4.64 (Endo + Gem) versus 29.13 ± 4.61 (Endo/Gem), 32.52 ± 6.42 (Gem), and 5.31 ± 1.69 (NS).

Discussion

The understanding that the growth of tumors depends on the acquisition of a blood supply has led to the development of new therapies for cancer based on inhibition of neovascularization [26]. Antiangiogenic agents have had striking

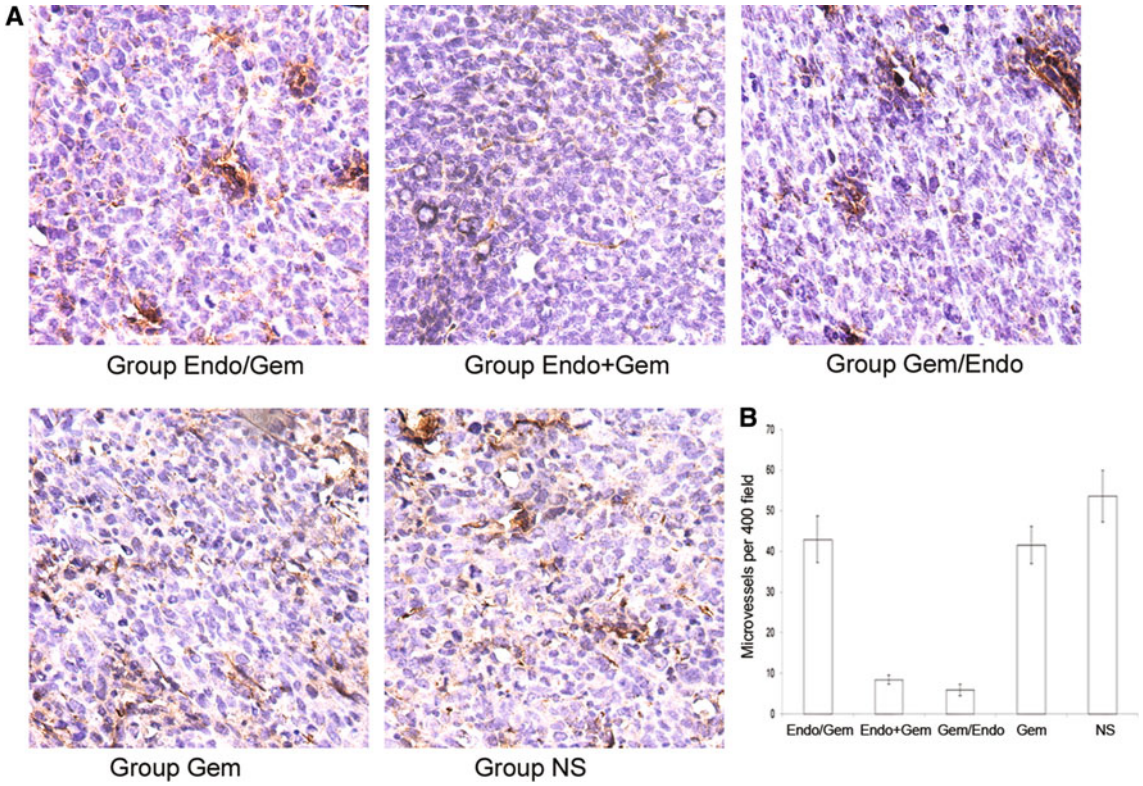

Fig. 4 Inhibition of angiogenesis within tumors was estimated by immunohistochemical analysis. **a** microvessels were stained with an antibody reactive to CD31. The sections of LLC tumor tissues were obtained from mice in Group Endo/Gem, Endo + Gem, Gem/Endo, Gem and NS. **b** Vessel density was determined by counting the number of the microvessels per high-power field (hpf) in the section, as described in "Materials and methods"

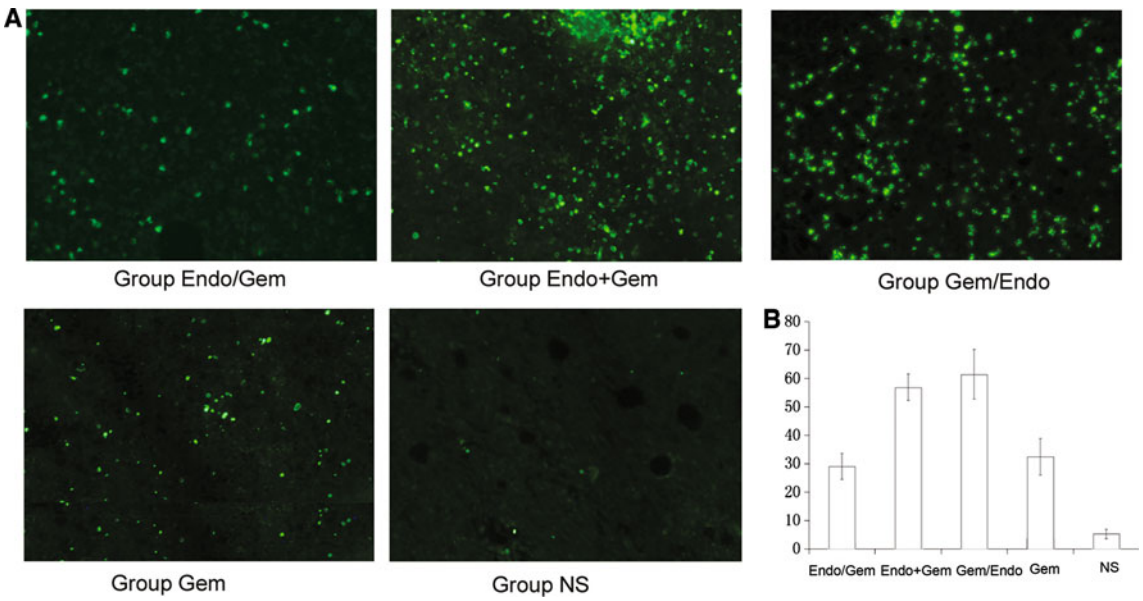

Fig. 5 Induction of tumor cell apoptosis was estimated by TUNEL staining. **a** The sections of LLC tumor tissues were obtained from mice at day 28 after tumor cell injection. Apoptotic tumor cells were elevated within tumor tissues obtained from Group Gem/Endo or Group Endo + Gem mice, compared with Group Endo/Gem, Gem and NS mice. **b** The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each field

success in preclinical models, and new agents are rapidly entering into clinical trials [27]. Current data suggest cytotoxic chemotherapy, and angiogenesis inhibitors used in combination may produce complementary therapeutic benefits in the treatment of cancer [15, 16]. However, this raised questions regarding the optimal scheduling of chemotherapy in combination with antiangiogenic therapies.

Several observations have been made in this study concerning the drug-administration schedules to investigate better efficacy of endostar used in combination with gemcitabine in the murine LLC model. Interestingly, the concomitant treatment of tumor-bearing mice with endostar and gemcitabine, including Group Endo + Gem and Gem/Endo, resulted in enhanced inhibition of tumor growth while Group Endo/Gem did not show statistically significant difference, compared with the group treated with gemcitabine alone. Our study suggested that the antitumor activity of the combination in LLC tumor model was schedule dependent.

Recent studies showed an increased release of cytokines by the tumor cells due to anti-cancer drugs [28]. Based on the reasonable assumption that after chemotherapy, surviving and repopulating tumor cells would require angiogenesis for optimal tumor cell survival conditions, we investigated the hypothesis that chemotherapeutic drugs may affect angiogenic factor secretion, such as VEGF, one of the most important angiogenic factors. We discovered that after a single injection of gemcitabine (30 mg/kg), the VEGF levels declined sharply in extracts of LLC tumors on day 1. A significant induction of VEGF production could be found in gemcitabine-treated tumors on day 2, but the level of VEGF increased in the following several days. Despite some mechanistic insights into endostatin actions, a putative link between the antiangiogenic protein and the regulation of VEGF had not been clearly established. We determined whether the changes of VEGF levels caused by chemotherapeutic agents in LLC can be blocked by endostar, which was responsible for the finding that the efficacy of antitumor activity in LLC tumor models was schedule dependent. We found that endostar treatment could offset chemotherapy-induced VEGF up-regulation.

The phenomenon observed in this study was assumed as the consequence of the initial damage and following surviving and repopulating of tumor cells which require angiogenesis for survival conditions. At this time, the administration of endostar might interfere the tumor angiogenesis. Our finding that endostatin administered simultaneously with or following gemcitabine could abolish the increase in VEGF supported the hypotheses. On the other hand, the Endo/Gem schedule did not significantly improve the antitumor effect, compared with gemcitabine alone, which hinted that the "normalization" of the tumor vasculature might not be the main mechanism for the improved antitumor effect in this dose of endostar.

Besides gemcitabine, other cytotoxic modalities were also found to induce the expression of VEGF in diverse tumor types. For example, radiation was demonstrated to induce VEGF expression by melanoma cells, rendering the tumor-associated endothelial cells more resistant to radiation's effects. In these studies, endothelial cell survival and proliferation after irradiation was enhanced by supplementation of VEGF, whereas anti-VEGF antibody enhanced the cytotoxic effects of irradiation [29, 30]. Previous data also showed that radioimmunotherapy induced VEGF expression in colon cancer-bearing nude mice [31]. Although some studies have reported inhibition of VEGF expression by traditional chemotherapeutic drugs, such as paclitaxel [32] and docetaxel [33], suggesting that these drugs are antiangiogenic in vivo, more evidence indicates induction rather than suppression of VEGF after chemotherapy, which leads to a possible escape mechanism from chemotherapy. Chemodrugs, including cisplatin [34], 5-fluorouracil [35], dacarbazine [36], and anthracyclines [37], have been reported to induce VEGF expression.

Nonetheless, our observation was a preliminary exploratory study and many questions are still waiting for answers. Further studies are on-going to investigate the distribution of chemotherapeutic agents, as well as the comparison of conventional dose and higher dose of endostatin in combination with chemotherapy.

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Conflict of interest None.

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