RESEARCH ARTICLE

Endostar Combined with Cisplatin Inhibits Tumor Growth and Lymphatic Metastasis of Lewis Lung Carcinoma Xenografts in Mice

Xiao-Peng Dong*, Tian-Hui Xiao, Hong Dong, Ning Jiang, Xiao-Gang Zhao

Abstract

Objective: To investigate the effects of endostar, a recombined humanized endostatin, plus cisplatin on the growth, lymphangiogenesis and lymphatic metastasis of the Lewis lung carcinoma (LLC) in mice. Methods: A tumor model were established in C57BL/6 mice by intravenious transplantation of LLC cells. Then the mice were randomized to receive administration with NS, endostar, cisplatin, or endostar plus cisplatin. After the mice were sacrificed, tumor multiplicity, tumor size and lymph node metastasis were assessed. Then the expression of vascular endothelial growth factor-c (VEGF-C) and podoplanin were determined by immunohistochemical staining. Results: Endostar plus cisplatin significantly suppressed tumor growth. lymphatic metastasis and prolonged survival time of the mice without obvious toxicity. The inhibition of lymphatic metastasis was associated with decreased microlymphatic vessel density (MLVD) and expression of VEGF-C. Conclusions: Endostar combined with cisplatin was more effective to suppress tumor growth and lymphatic metastasis than either agent alone. Thus this may provide a rational alternative for lung carcinoma treatment.

Keywords: Lung tumor - endostatin - cisplatin - lymphangiogenesis

Asian Pacific J Cancer Prev, **14 (5)**, 3079-3083

Introduction

Chemotherapy is the leading treatment for malignant tumor, and cisplatin has been administered frequently for lung cancer. In the past decade, some new cytotoxic drugs have come into application. Despite the progress, chemotherapy for malignant tumors is still insufficien, and lymphatic metastasis is a major prognostic factor in lung carcinoma.

In recent years, an approach combining chemotherapy with antiangiogenesis factors has been reported in treatment for established animal tumors (Huang et al., 2010). Endostatin is a internal fragment of the carboxyterminus of collagen XVIII, first produced by hemangioendothelioma (O'Reilly et al., 1997), capable of inhibiting endothelial cell proliferation and inducing endothelial cell apoptosis, which has been reported as one of the most potent endothelial cell inhibitors of angiogenesis and tumor growth (Boehm et al., 1997). Endostar is a recombinant humanized endostatin purified in an Escherichia coli system with an additional nine-amino acid sequence added to the N-terminal of the protein (Han et al., 2007), and this change cut down the preparation cost and improve the stability of protein without decreasing its antiangiogenic efficacy (Ling et al., 2007). Studies have showed that endostar can improve the response rate and progression-free survival when used in combination with chemotherapy regime in patients with advanced non-small-cell lung cancer (Sun et al., 2005; Rong et al., 2012).

In the present study, we used Lewis lung carcinoma cells and C57BL/6 mice to establish the lung carcinoma animal model, and investigated the effects of endostar plus cisplatin on the growth, lymphangiogenesis and lymphatic metastasis in mice implanted with Lewis lung carcinoma cells.

Materials and Methods

Cell culture, animals, and reagents

The Lewis lung carcinoma cell line was grown in RPMI-1640 (Gibco BRL, USA)supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. The cell line was incubated at 37°C in 5% CO_2.

48 female C57BL/6 mice weighing 20-24 g were purchased from the Division of Animals of Peking University, China. The mice were housed in the rooms of the Laboratory Animal Research Center of Shandong University with specified pathogen-free conditions. The room was maintained at 22±1 °C. All of the experiments were carried out according to guidelines approved by the Laboratory Animal Care Committee.

Endostar was provided by Simcere Pharmece Group

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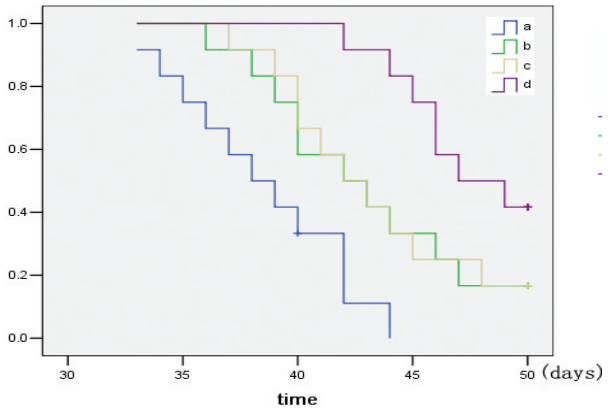

Figure 1. Survival Advantage in Lewis Lung Carcinoma Bearing Mice. Treatment with endostar or cisplatin resulted in prolonged survival time compared with the NS groups (P<0.05). And treatment with combination of cisplatin and endostar showed longer life span (P<0.05). a: NS; b: Cisplatin; c: Endostar; d: Endostar+cisplatin

(Yantai, Shandong Province, China).

Tumor model and therapeutic experiments

For in vivo implantation, LLC cells were washed in Hanks' balanced salt solution(HBSS) and injected intravenously at 1×10^6 cells in 0.2 ml HBSS in the caudal vein of C57BL/6 mice. On day 10 post cell injection, the mice were randomly assigned into four groups (n =12 animals/group): endostar group, caudal vein injection of 400 μg endostar; cisplatin group, caudal vein injection of 40 μg cisplatin; endostar plus cisplatin group, injection of 400μg endostar along with 40 μg cisplatin; NS group, injection of 0.2 ml of 0.9% sodium chloride. All the treatments were performed once daily and lasted for 10 days. The treated mice were closely monitored and sacrificed if any sign approaching death were shown. 50days after LLC cell injection, all mice were sacrificed. And a complete autopsy, including lymph node dissection, was performed. Tumor multiplicity (number of tumors >2 mm in longest diameter per mouse) and tumor size (sum of tumor diameters divided by tumor number) were recorded. All the animal experiments were approved by the Animal Care and Use Committee of the Shandong University.

Immunohistochemical staining and evaluation

Tumor tissue samples were fixed with 10% buffered formalin, and embedded in paraffin after routine dehydration. Consecutive 5-μm sections were cut from each block and were immunostained for VEGF-C, podoplanin. Antimouse VEGF-C, podoplanin (Santa Cruz Biotechnology, Inc)and S-P kits (DAKO Cytomation, Glostrup, Denmark) were used for immunohistochemical analysis. A biotinylated antirabbit antibody was used as a secondary antibody for detection. For negative controls, the primary antibodies were omitted.

Ten fields were selected for each section, and expression of VEGF-C in 1,000 tumor cells (100 cells/fields) was evaluated with high-power (x400) microscopy. The expression was evaluated semi-quantitatively as negative (<10% of tumor cells stained), positive (>10% of tumor cells stained) by two independent observers blinded to the mice's status.

Table 1. Effects of Endostar or/and Cisplatin on Tumor Growth, MLVD, Lymph Metastasis and VEGF-C

	NS	Endostar	Cisplatin	Endostar+ Cisplatin
Tumor Multiplicity	5.58±1.08	4.08±1.31	4.17±1.64	2.67±0.79
Tumor size(mm)	3.98±0.48	3.43±0.37	3.33±0.32	2.79±0.29
MLVD	8.67±1.72	6.25±1.55	8.33±1.56	6.08±1.51
Lymph Metastasis	5.58±0.99	4.17±1.27	4.25±0.97	2.67±1.07
VEGF-C				
Negative	2	9	3	10
Positive	10	3	9	2

Podoplanin positive vessels, found mainly in the marginal portion, had relatively large lumens. The number of podoplanin positive vessels was counted as follows: an area showing podoplanin positive vessels was examined first with low-power microscopy (x100), then with high power microscopy (x400). Five visual fields were selected to perform counting, average value was calculated as MLVD.

Toxicity Observation

Drug toxicity such as weight loss, ruffled fur, behavior change and feeding patterns were continuously observed. 10 days after treatment, blood samples were extracted from the tail vein. The white blood cell count, red blood cell count and platelet count were determined as measures of bone marrow toxicity, whereas AST plus ALT were recorded as measures of liver toxicity.

Statistical analysis

Statistical analysis was carried out with SPSS statistical software package. Differences in distribution were determined using the single factor analysis of variance or χ^2 test. Survival analysis was computed by the Kaplan-Meier method and compared by the log-rank test. Statistical significance was set up P<0.05.

Results

Effect of endostatin or/and cisplatin on tumor growth and life span

10 days after the Lewis lung cancer model was established, the C57BL/6 mice were randomized to receive administration with NS, endostar, cisplatin, or endostar plus cisplatin respectively. After 50 days, all the mice were sacrificed. No differences in tumor incidence were detected in four groups. Treatment with endostar or cisplatin as the single agent resulted in significant regression of tumor growth and prolonged survival time compared with the NS groups (P<0.05). Furthermore, the combination group showed more regression and longer life span than single agent (P<0.05) (Figure 1; Table 1).

Effect of endostatin or/and cisplatin on MLVD and lymph node metastasis

Lymphangiogenesis within tumor tissues was estimated in terms of MLVD on the section stained with anti-mouse podoplanin antibody. Podoplanin was mainly expressed in interstitial vascular endothelial cells around cancer nests, and positive reactions were occasionally seen

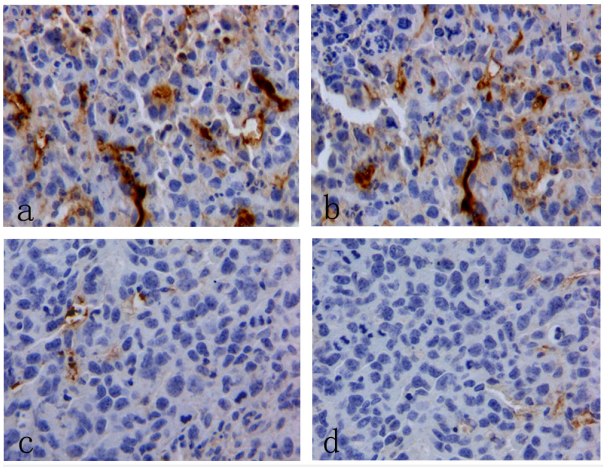

Figure 2. Podoplanin Positive Vessel in Lung Carcinoma (×400). Tumors of the group treated with NS or cisplatin, showed larger MLVD than the other two groups (P<0.05). However, There was no difference among the groups treated with endostar plus cisplatin or endostar alone. a: NS; b: Cisplatin; c: Endostar; d: Endostar+cisplatin

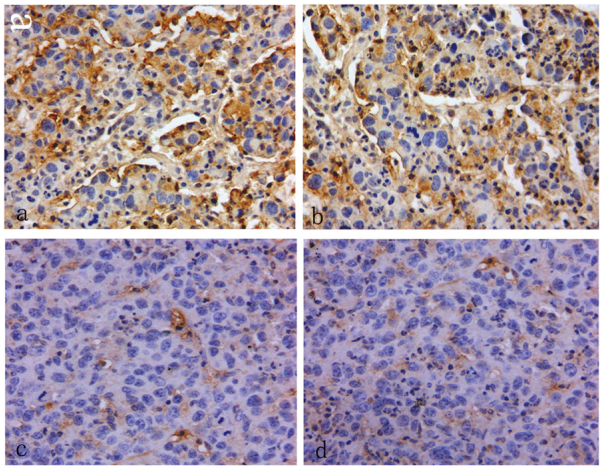

Figure 3. Expression of Vascular Endothelial Growth Factor-c in Lung Carcinoma (VEGF-C) (×400). Expression of VEGF-C in mice treated with NS or cisplatin was significantly higher than other groups (P< 0.05). No difference among the groups treated with endostar plus cisplatin or endostar alone was detected. a: NS; b: Cisplatin; c: Endostar; d: Endostar+cisplatin

Table 2. Toxicity of Endostar or/and Cisplatin on Mice

	NS	Endostar	Cisplatin	Endostar+ Cisplatin
White blood cell ($\times10^3$/mm^3)	7.87±0.73	7.33±0.79	7.61±0.87	7.30±0.70
Red blood cell ($\times10^4$/mm^3)	768.5±49.3	721.5±43.9	746.7±74.6	736.4±39.2
Platelet ($\times10^4$/mm^3)	36.2±5.8	30.6±5.1	34.4±5.6	31.7±4.7
AST (IU/I)	242.2±34.1	259.9±43.9	258.0±35.8	282.7±39.0
ALT (IU/I)	41.2±7.1	45.8±7.0	46.6±6.5	47.6±7.8

in tumor cells, with relatively weak staining. Tumors of the mice treated with NS or cisplatin, showed larger MLVD than the other two groups (P<0.05). However, There was no difference among the groups treated with endostar plus cisplatin or endostar alone (Figure 2; Table 1).

After all mice were sacrificed, a complete autopsy, including lymph node dissection, was performed. The mice treated with NS showed more lymph node metastases than the other three groups (P<0.05). In addition, the combination of endostar plus cisplatin showed stronger inhibition of lymphatic metastases than either agent alone (P<0.05) (Table 1).

Effect of endostatin or/and cisplatin on VEGF-C expression

VEGF-C positive substances appear as brownish-yellow fine particles mainly located in the cytoplasm of cancer cells. In addition, it was expressed in various degrees on cell membrane. The correlation with treatment factor was analyzed. Expression of VEGF-C in mice treated with NS or cisplatin was significantly higher than other groups (P< 0.05). However, no difference among the groups treated with endostar plus cisplatin or endostar alone was detected (Figure 3; Table1).

Toxicity of endostatin or/and cisplatin on mice

Compared with the control groups, no significant adverse consequences were observed such as weight loss, ruffled fur and behavior change. The white blood cell count, red blood cell count and platelet count as well as AST and ALT levels were all in the normal range,and none of the above parameters of the treatment groups showed significant difference (Table2).

Discussion

Cytotoxic agents are the first-line drugs used to kill tumor cells. However chemotherapy cannot prevent tumor growth and lymphatic metastasis completely. In addition, chemotherapy is often followed by serious side effects such as nausea, vomiting, neurotoxicity and myelosuppression (HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed?term=Cheng%20K%5BAuthor%5D&cauthor=true&cauthor_uid=23244110" Cheng et al., 2012). The present study demonstrated that recombinant endostatin plus cisplatin could evidently improve anti-tumor efficacy, including tumor growth suppression, survival time, and no serious adverse effects were found in the combination group. The possible mechanisms how the combined agents improved anti-tumor efficacy may be that cytotoxic treatment and antiangiogenesis therapy interact on each other. Endostatin can inhibits the proliferation and migration of endothelial cells, which leads to reduced tumor vascularization (Nyberg et al., 2005; Folkman et al., 2006). As a result, the increased vessel permeability leads to increased tumor exposure to cytotoxic drugs, and tumor cells are more vulnerable to the damage effects of chemotherapy. Meanwhile, cisplatin can influence the process of vascularization and to cause severe vasculotoxicity (Miller et al., 2001), which may strengthen the antiangiogenesis efficacy of endostatin. Therefore, combination of cytotoxic treatment and antiangiogenesis therapy was more effective to suppress tumor growth (Klement et al., 2002; HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed?term=Rong%20B%5BAuthor%5D&cauthor=true&cauthor_uid=22917490" Rong et al., 2012).

In the research, we investigated the effect of endostar plus cisplatin on the lymphangiogenesis and lymphatic metastasis of lung carcinoma. The data demonstrated that the MLVD and lymphatic metastasis significantly decreased in mice treated with combined agents compared to the controls. This suggests that the reduction in lymph node metastases may be due to the suppression of tumor lymphangiogenesis induced by endosar. Lymphangiogenesis plays important roles in the development, growth and metastasis of lung carcinoma, and correlates with lymph node metastases (Detmar et al., 2002). Endostatin can inhibits tumor-ralated angiogenesis, and study have showed that endostatin can also inhibits the proliferation and migration of lymphatic endothelial cells in vitro (Shao et al., 2005). Recent study demonstrated that endostar had an efficient anti-cancer effect in malignant pleural effusion of lung cancer through its suppressive effect on lymphangiogenesis (HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed?term=Ma%20X%5BAuthor%5D&cauthor=true&cauthor_uid=23285296" Ma et al., 2012).

In present study, we also found that the expression of VEGF-C in group treatment with endostatin was significantly lower than that in other groups. This suggests that endostar-mediated anti-lymphangiogenesis is due to the effect to inhibit the expression of VEGF-C partly. VEGF-C is a specific ligand of vascular endothelial growth factor receptor-3 (VEGFR-3) and VEGFR-2 (Yancopoulos et al., 2000). Recent study suggest that VEGF-C is associated with lymph node metastasis in malignant tumor (HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed?term=Wang%20Z%5BAuthor%5D&cauthor=true&cauthor_uid=22502683" Wang et al., 2012). Tumor cells could induce lymphangiogenesis and promote lymphatic metastasis of tumor cells by expressing VEGF-C (Makinen et al., 2001). In contrast, suppression of VEGFR-3 signaling inhibits lymph node metastasis (Shimizu et al., 2004), and inhibition of VEGF-C activity reduced the level of lymphangiogenesis and lymph node metastasis (Krishnan et al., 2003; HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed?term=Liu%20ZY%5BAuthor%5D&cauthor=true&cauthor_uid=22922710" Liu et al., 2012). Brideau found that endostatin can regulates the lymphangiogenesis by reducing the number of VEGF-C-producing inflammatory mast cells in the tumor tissue (Brideau et al., 2007). Therefore, these studies demonstrate that endostatin inhibits lymphangiogenesis and lymph expansion by regulating VEGF-C expression in tumor (Fukumoto et al., 2005; HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed?term=Wakisaka%20N%5BAuthor%5D&cauthor=true&cauthor_uid=22366442" Wakisaka et al., 2012).

In summary, the results of the study suggest that recombined humanized endostatin combined with cisplatin is more effective than either agent alone to suppress tumor growth and lymphatic metastasis, without obvious toxicity. The mechanism may in part concern the ability to inhibit the expression of VEGF-C. Endostar plus cisplatin is thus considered to be a effective therapeutic combination for lung carcinoma. Obviously, more clinical studies are requested.

Acknowledgements

We declare that there are no conflicts of interest.

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