RESEARCH ARTICLE

Experimental Study of Endostar Injection Concomitant with Cryoablation on Lung Adenocarcinoma A549 Xenografts

Chun-Hua Ma[&], Rong Jiang[&*], Jin-Duo Li, Bin Wang, Li-Wei Sun, Yuan Lv

Abstract

Objective: To explore the inhibiting effect and mechanism of Endostar injection concomitant with cryoablation on lung adenocarcinoma A549 xenografts in nude mice. Materials and Methods: A total of 24 nude mice with subcutaneous xenografts of the A549 cell line were established and divided into 4 groups when the maximal diameters of tumors became 1 cm: control group, Endostar group, cryoablation group and combination group (Endostar concomitant with cryoablation). The nude mice were sacrificed after 21-days treatment, tumour tissues were removed to measure their volume, in situ test of TdT-mediated dUTP nick end labeling (TUNEL) was adopted to determine the cellular apoptosis around freezing injury zones, and immunohistochemical SP test was applied for the detection of micro-vessel density (MVD) and vascular endothelial growth factor (VEGF) expression levels. Results: At 21-days after treatment, the growth velocities of control group, Endostar group, cryoablation group and combination group were 236.7±51.2%, 220.0±30.6%, 159.5±29.3% and 103.3±25.5% (P<0.01), while cellular apoptosis rates of tumors were 21.7±2.34%, (22.17±1.47)%, 38.3±1.37% and 49.2±1.72%, (P<0.01), respectively, according to the immunohistochemical test. MVD and VEGF expression levels in the combination group were both lower than in other groups (P<0.01), also being positively related (r=0.925, P<0.01). Conclusions: Endostar can significantly improve the inhibitory effects of cryoablation on xenografts of lung adenocarcinoma A549, and the mechanism is probably associated with its function as an inhibitor of tumour neo-angiogenesis through down-regulating VEGF expression.

Keywords: Endostar - cryoablation - lung adenocarcinoma A549 xenografts - VEGF - micro-vessel density

Asian Pac J Cancer Prev, **14 (11)**, 6697-6701

Introduction

Recombinant Human Endostatin Injection, also named as Endostar Injection, is a new targeted drug developed independently by Chinese researchers, which can strongly inhibit tumor angiogenesis (Folkman, 2006) and has certain effect on middle and advanced non-small cell lung cancer (NSCLC) when concomitant with chemotherapies (Wang et al., 2005; Fei et al., 2013; Yan et al., 2013; Liu et al., 2013; Lu et al., 2013; Yin et al., 2013). Thus, it has been included in Chinese Version of Clinical Practice Guideline of Non-small Cell Lung Cancer of National Comprehensive Cancer Network (NCCN). As one of the minimally invasive therapies for tumor, argon-helium cryoablation has many advantages, such as safe, effective, minimally invasive and slight in adverse reactions, etc., which has been applied to the treatment of multiple solid tumors like prostate cancer, lung cancer and liver cancer, etc., with gradually conformed efficacy in clinic (Gary et al., 2008; Hu et al., 2007; Guo et al., 2010). However, there were few reports about the experimental studies of Endostar concomitant with cryoablation on middle and advanced NSCLC. Therefore, this study, through establishing the nude mice model of xenograft of human lung adeno-carcinoma A549, observed the anti-tumor effect of Endostar concomitant with cryoablation, so as to further investigate its mechanism, providing references for clinical treatment of middle and advanced NSCLC.

Materials and Methods

Experimental materials

Lung adeno-carcinoma A549 clones were purchased from Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences, 24 BALB/c nu female nude mice aged 5~6 weeks were brought from Scientific Department of Experimental Animals of Medical School of Beijing University, with license number being SCXK (Jing) 2006-0008, and all mice were raised in Animal Research Center of Tianjin Key Laboratory of Cerebral Vessels and Neural Degeneration (SPF grade). Endostar Injection was obtained from Yantai Maidejin Biotechnology Ltd. Co. Immunohistochemical kits were brought from Abcam Company, anti-human

*Department of Intervention, Tianjin Huanhu Hospital, Tianjin Cerebral Vascular and Neural Degenerative Disease Key Laboratory, Tianjin, China [&]Equal contributors *For correspondence: jiangrong1989@sina.com*

VEGF monoclonal antibody of mice were taken from Beijing Zhongshan Jinqiao Company, primary imminohistochemical kits of TUNEL were obtained from American Roche Company, while argon-helium freezing system and 1.7 mm argon-helium knife were purchased from American Endocare Company.

Experimental methods

Cells culture: Lung adeno-carcinoma A549 cells were incubated into McCoy'5A culture medium containing 10% fetal bovine serum, and cultured in absolute humidity with 5% CO_2 at 37°C. The cells were grew with adherence, digested and generated by 0.125% trypsin, and the medium solution was changed every 48 h~72 h.

Establishment and experimental methods of nude mice model of xenograft of lung adeno-carcinoma A549: (1) Appropriate amount of PBS solution was added to A549 cells in logarithmic growth to make 2×10^7/mL cell suspension. (2) Implantation of A549 cells: 0.2 mL cell suspension was subcutaneously injected to the posterior armpit on the right side of each nude mouse that was 6 weeks old and 20 g in weight, and the tumor would developed in 7 d. (3) Animal division and therapeutic methods: nude mice were divided into 4 groups when the diameter of subcutaneous lump was 1 cm, 6 mice for each. In control and Endostar groups, the tumors were punctured without cryoablation, and 0.9% equivoluminal normal saline (NS) and 20 mg·kg-1·d-1 Endostar were injected into abdominal cavity 2 h after puncture for totally 14 d, respectively. Whereas in cryoablation and combination (Endostar concomitant with cryoablation) groups, after tumor cryoablation, 0.9% equivoluminal NS and 20 mg·kg-1·d-1 Endostar were injected into abdominal cavity 2 h after cryoablation for totally 14 d, respectively. 21 d after treatment, the nude mice were sacrificed by chloral hydrate to remove tumor tissues which were then fixed by 4% para-formaldehyde solution. (5) Cryoablation therapy: Nude mice were anesthetized by abdominal injection of 3.5 mg·kg^{-1} 10% chloral hydrate, fixed and sterilized on skin. Then the frozen points of cryocare knives were inserted in the center of tumors. Cryoablation of double circulation was adopted to reduce the temperature to -120°C for 10 s, which was restored to 0°C and repeated.

Observation of tumor growth on growth: During treatment, the longest diameter (L) and the widest transverse diameter (W) were measured by vernier caliper every 2 d. Tumor volume was calculated by V (mm^3)=$LW^2/2$, while the growth velocity was expressed by f =V/V_0, in which V and V_0 represented the tumor volumes measured at different time points and those before treatment, respectively.

In situ detection of cellular apoptosis around freezing injury area by TUNEL method: Paraffin sections of tumor tissues were made and added with 25~50 uL horseradish peroxidase antibodies, which were counterstained comparatively with hematoxylin after DAB coloration, and then dehydrated, made transparent and fixed tightly. The positive expression was marked by brown nucleus, which was manifested by the ratio of cells with positive expression in each high-power field in the total amount of cancer cells after 5 different fields were chosen under 400-fold microscope in each section, and its mean value was selected as the terminal value for statistical analysis.

MVD count in tumor tissue tested by immunohistochemical SP test: Paraffin sections of tumor tissues were made, with primary and secondary antibodies being anti-mouse CD34 monoclonal antibody in rats and anti-rate IgG of biotinylation goat, respectively, which were counterstained comparatively with hematoxylin after DAB coloration, and then dehydrated, made transparent and fixed tightly. The positive expression was marked by black and streak staining of cellular matrix. Tumor tissues in each section was put under low-fold microscope (×40) to select high-density area of vessels and then transferred to high-fold microscope (×200) to precisely count the amount of micro-vessels, and its mean value of micro-vessels in 5 different fields was considered as the terminal value for statistical analysis.

VEGF expression level in tumor tissue tested by immunohistochemical SP test: Paraffin sections of tumor tissues were made, with primary and secondary antibodies being anti-mouse CD34 monoclonal antibody in rats and anti-rate IgG of biotinylation goat, respectively, which were counterstained comparatively with hematoxylin after DAB coloration, and then dehydrated, made transparent and fixed tightly. VEGF expressed in cytoplasm under optical microscope (×200), which showed clear black or brown granules or masses, which was manifested by the ratio of cells with positive expression in each high-power field in the total amount of cancer cells after 5 different fields were chosen under 400-fold microscope in each section, and its mean value of micro-vessels in 5 different fields was considered as the terminal value for statistical analysis.

Statistical data analysis

SPSS 17.0 statistical software was applied to deal with all data in each group. Measurement data was expressed by ($\overline{\chi}\pm s$), comparisons between groups were analyzed by ANOVA variance analysis, while LSD and Spearman correlation coefficient were adopted to compare data between two groups and observe the relevance between indexes, respectively. $P<0.05$ was regarded as significant.

Results

Observation of anti-tumor effect of Cryoablation on xenograft of lung adeno-carcinoma A549

Tumor cells in the center of freezing area were necrotized and disintegrated after cryoablation, with split nucleus being in coagulative necrosis. Damaged area was obviously observed around necrotic zone, in which partial tumor cells shrank with nuclear and chromatin condensations being characterized by the properties of apoptotic cells. And peripheral area of damaged area was observed with tumor cells in normal shape. Tumor growth velocities of control group, Endostar group, cryoablation group and combination group were (236.68±51.23)%, (220.02±30.61)%, (159.46±29.33)% and (103.34±25.50)% respectively, and there were statistically significant differences among groups ($P<0.01$), as shown in Table 1.

Table 1. Comparison of Tumor Volume, Growth Velocity and Cellular Apoptosis Rate in Each Group ($\bar{\chi}$±s)

Groups	Tumor volume (cm^3)		Tumor growth velocity (%)	Tumor cellular apoptosis rate (%)
	Before treatment	21-day after treatment		
Control group	0.65±0.12	1.48±0.14	236.68±51.23	21.67±2.34
Endostar group	0.65±0.09	1.40±0.12	220.02±77.41	22.17±1.47
Cryoablation group	0.70±0.14	0.99±0.06	159.46±29.33	38.33±1.37
Combination group	0.67±0.13	0.63±0.05	103.34±25.50	49.17±1.72
F value	0.761	93.340	17.528	343.636
P value	>0.05	<0.01	<0.01	<0.01

Table 2. Comparison of MVD and VEGF Expression Levels in Each Tumor Tissue ($\bar{\chi}$±s)

Groups	MVD	VEGF (%)
Control group	21.10±0.86	36.17±1.72
Endostar group	20.43±0.79	35.17±1.17
Cryoablation group	29.17±0.96	50.83±2.14
Combination group	12.40±1.40	26.33±1.37
F value	264.051	229.691
P value	<0.01	<0.01

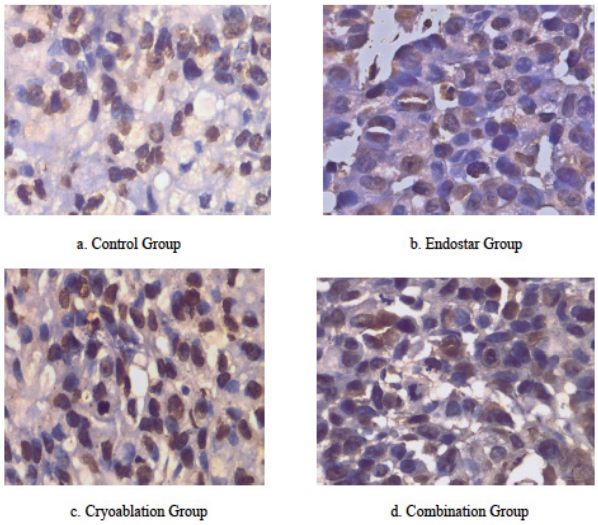

Figure 1. Immunohistochemical Staining of Tumor Cellular Apoptosis (×400)

Cellular apoptosis in freezing area tested by in situ test of TUNEL

After cryoablation, TUNEL staining results of tumor tissues in freezing area showed that apoptotic cells were distributed mainly around necrotic zone, with condensed nucleus brown in color under optical microscope, and chromatin near to nuclear membrane was condensed as broad bean- or new-moon-like shape. In control and Endostar groups, small amount of apoptotic cells were distributed in tumor cells (Figures 1a-1d), and the apoptotic rates of tumor cells in each group were (21.67±2.34)%, (22.17±1.47)%, (38.33±1.37)% and (49.17±1.72)% respectively, and there were significant differences among groups (P<0.01), as shown in Table 1. Expression levels of MVD and VEGF detected by immunohistochemical test (Table 2)

Amounts of VEGF-positive cells in dispersive and spotted distribution were found in tumor cells around freezing area after cryoablation, and MVD also increased apparently. Optical microscope showed that cellular matrix was black in color and streak in shape in micro-

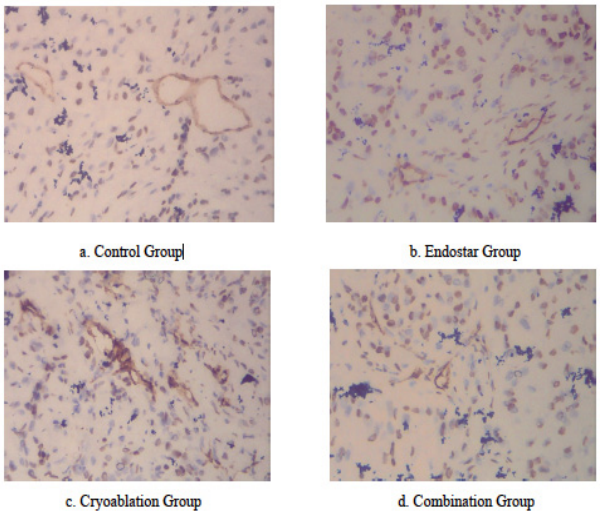

Figure 2. MVD Immunohistochemical Staining (×200)

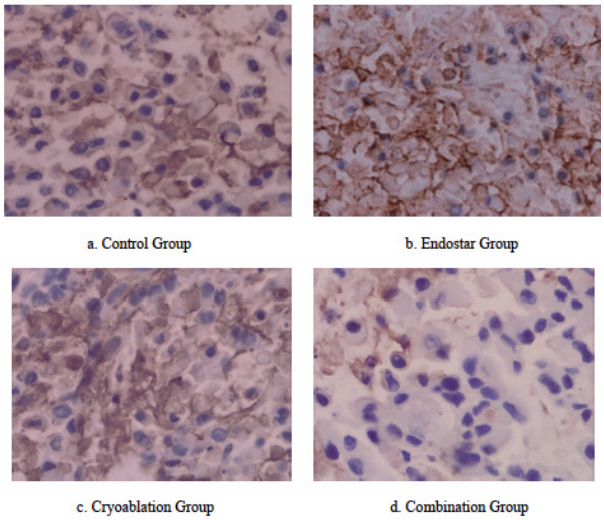

Figure 3. VEGF Immunohistochemical Staining (×400)

vessels of tumors (Figures 2a-2d), while VEGF-positive cytoplasm was black or brown in color and granular or lumpy in shape (Figures 3a-3d). MVD and VEGF expression level in tumor tissues in combination group were evidently lower than in other groups (P<0.01), and VEGF expression severity was in positive relation with MVD (r=0.906, P<0.01), according to Spearman correlation analysis.

Discussion

Endostatin is an endogenous angiogenesis inhibitor extracted and purified from supernatant of cultured

endothelioma in mice by O'Reilly et al in 1997, which can inhibit the development and metastasis of tumor via suppressing the tumor angiogenesis by restraining the growth of vascular endothelial cells specifically. Endostar is a new human endostatin independently created and developed by Chinese scholars through modifying the amino acid sequence of endostatin, which has favorable anti-tumor angiogenesis, with its objective effective rate being 3%~5% in single use, and has synergistic and favorable clinical effects when concomitant with chemotherapy in treating advanced NSCLC (Wang et al., 2005). Cryoablation is a minimally invasive therapy developed in recent years, which can lead to coagulative necrosis of tumor cells through forming ice crystals in tumor cells by quick freezing so as to make convulsion of capillary and small vessels in local tumor tissues, damage tumor vessels, block local blood circulation, form thrombus, and thus result in ischemia and anoxia of tumor tissues. Moreover, cryoablation can bring about specific antigens after necrosis of tumor cells and stimulate body to produce specific antibodies, which can wipe out the residual tumor cells by immunological function of antibody to tumor cells. Guo Z and Xing WG, et al., reported that cryoablation could reduce tumor burden quickly, control tumor progression effectively and improve the quality of life to patients (Hu et al., 2007; Xing et al., 2008). However, its long-term efficacy needs further observations.

Endostar has lower controlling effect on tumor in single use, but has certain influence when combined with chemotherapy in treating advanced NSCLC. Thus, it was still dubious whether there was synergistic effect between Endostar and cryoablation. Based on this thought, this study, through comparing control group, Endostar group, cryoablation group and combination group, hoped to improve the short-term effect by increasing the local controlling rate of tumor, which showed that after cryoablation, most tumor cells were observed with coagulative necrosis with apoptotic tumor cells distributing in freezing area, that the tumor growth velocity was lower in combination group than in other groups ($P<0.01$), suggesting that Endostar concomitant with cryoablation could evidently promote tumor controlling rate with synergistic effect.

The recurrence and metastasis of residual tumor after cryoablation are two important factors influencing the long-term efficacy. Kimura et al., found that after cryoablation of mice model with RM-9 prostate carcinoma, MVD increased markedly in residual tumor tissues (Kimura et al., 2010). Ueda et al.() believed that MVD could be considered as a reliable index for the metastasis and prognosis of tumors (Ueda et al., 1999). And this study indicated that MVD increased apparently in tumor tissues in damaged area after cryoablation, and VEGF expression also increased in area where there were more MVD, which were in positive association with each other, demonstrating that the increase of MVD was in correlation with the up-regulation of VEGF expression level. VEGF is the proverbial contributing factors for tumor micro-angiogenesis at present, a common marker for measuring the severity of tumor angiogenesis. Endostar can prevent the combination of VEGF with endothelial cells in order to block VEGF action directly; reduce tumor angiogenesis via inhibiting the activation, proliferation and metastasis of vascular endothelium; improve the transient "normalization" of tumor cells to keep them in dormant state, so as to induce the apoptosis and promote the sensitivity of tumor cells on physical and chemical damages (Mauceri et al., 1998; Lee et al., 2000). This study revealed that MVD and VEGF expression in residual tumor cells decreased significantly in combination group, predicating that Endostar could inhibit tumor angiogenesis through down-regulating VEGF expression level and suppress the activation, proliferation and metastasis of tumor vascular endothelium, so as to inhibit the recurrence of residual tumor tissues. Cellular apoptosis rate of tumor cells in combination group was evidently lower than in other groups, which was predicated to be associated with the synergistic effect of Endostar in improving the transient "normalization" of tumor cells to keep them in dormant state when combined with cryoablation, so as to improve the tumor cell apoptosis.

To sum up, the experimental results of nude mice with subcutaneous xenograft of lung adeno-carcinoma A549 demonstrated that Endostar concomitant with cryoablation have synergistic effect, whose mechanism is in association with the down-regulation of VEGF expression level in residual tumor tissues so as to further inhibit tumor angiogenesis and induce cellular apoptosis, providing experimental basis for clinical therapies with certain clinical guidance.

References

Fei ZH, Yao CY, Yang XL, et al (2013). Serum BMP-2 Up-regulation as an Indicator of Poor Survival in Advanced Non-small Cell Lung Cancer Patients. *Asian Pac J Cancer Prev*, **14**, 5293-9.

Folkman J (2006). Antiangiogenesis in cancer therapy-endostatin and its mechamisms of action. *Exp Cell Res*, **312**, 594-607.

Gary Onik (2008). Rationale for a "Male Lumpectomy" a Prostate Cancer targeted approach using cryoablation: results in 21 patients with at Least 2 years of follow-up. *Cardiovasc Intervent Radiol*, **31**, 98-106.

Guo Z, Wang HT, Xing WG, et al (2010). A preliminary clinical study of targeted cryoablation of prostate in the treatment of T3N0M0 prostate cancer. *National Med J Chin*, **90**, 2815-9.

Hu KW, Li QW, Zuo MH, et al (2007). Clinical observation on the combined treatment of 57 cases of non-small cell lung cancer using argon-helium cryosurgery and Chinese herbal medicine. *Chin J Integr Med*, **13**, 224-7.

Kimura M, Rabbani Z, Mouraviev V, et al (2010). Morphology of hypoxia following cryoablation in a prostate cancer murine model: its relationship to necrosis, apoptosis and, microvessel density. *Cryobiology*, **61**, 148-54.

Lee CG, Heijn M, di Tomaso E, et al (2000). Anti-vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. *Cancer Res*, **60**, 5565-70.

Liu YC, Zhou SB, Gao F, et al (2013).Chemotherapy and late course three dimensional conformal radiotherapy for treatment of patients with stage III non- small cell lung cancer. *Asian Pac J Cancer Prev*, **14**, 2663-5.

Lu YY, Huang XE, Xu L, et al (2013). Potential predictors of sensitivity to pemetrexed as first-line chemotherapy for

patients with advanced non-squamous NSCLCs. *Asian Pac J Cancer Prev*, **14**, 2005-8.

Mauceri HJ, Hanna NN, Beckett MA, et al (1998). Combined effects of angiostatin and ionizing radiation in antitumourtherapy. *Nature*, **394**, 287- 91.

Ueda M,Terai Y,Kurnagai K, et a1 (1999).Correlation between tumor angiogenesis and expression of thymidine phosphorylase, and patient outcome in uterine cervical carcinoma. *Hum Pathol*, **30**, 1389-94.

Wang JW, Sun Y, Liu YY, et al (2005). A random, double-blind, control, multi-center phase III clinical study of Endostatin concomitant with NP protocol on advanced NSCLC. *Chin J Lung Cancer*, **48**, 283-90.

Xing WG, Guo Z, Wang HT, et al (2008). Percutaneous cryoablation of prostate cancer guided by rectal ultrasound: a retrospective analysis of 42 cases. *Chin J Radio*, **42**, 807-11.

Yan HA, Shen K, Huang XE (2013). Clinical study on mannan peptide combined with TP regimen in treating patients with non-small cell lung cancer. *Asian Pac J Cancer Prev*, **14**, 4801-4.

Yin HT, Tian QZ, Guan L (2013). In vitro and in vivo evaluation of the antitumor efficiency of resveratrol against lung cancer. *Asian Pac J Cancer Prev*, **14**, 1703-6.