RESEARCH ARTICLE

Experimental Study on Residual Tumor Angiogenesis after Cryoablation

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Abstract

Objective: To explore the mechanism and significance of tumor angiogenesis by observing changes of microvessel density (MVD) and expression of vascular endothelial growth factor (VEGF) in residual tumor tissues after cryoablation. Materials and Methods: A total of 18 nude mice xenograft models with transplanted lung adenocarcinoma cell line A549 were established and randomly divided into 3 groups when the maximum diameter of tumor reached 1 cm: control, cisplatin (DDP) and cryoablation. The nude mice were sacrificed after 21-d cryoablation to obtain the tumor tissues. Then immunohistochemistry was applied to determine MVD and the expression of VEGF in tumor tissues. Results: The tumor volumes of control group, DDP group and cryoablation group were 1.48±0.14 cm^3, 1.03±0.12 cm^3 and 0.99±0.06 cm^3 respectively and the differences were significant (P<0.01), whereas MVD values were 21.1±0.86, 24.7±0.72 and 29.2±0.96 (P<0.01) and the positive expression rates of VEGF were 36.2±1.72%, 39.0±1.79% and 50.8±2.14% (P<0.01), respectively, showing that MVD was proportional to the positive expression of VEGF (r=0.928, P<0.01). Conclusions: Cryoablation can effectively inhibit tumor growth, but tumor angiogenesis significantly increases in residual tumors, with high expression of VEGF playing an important role in the residual tumor angiogenesis.

Keywords: Cryoablation - transplanted tumor - A549 lung adenocarcinoma cells - VEGF - microvessel density

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Introduction

As a new minimally invasive technique for tumors, cryoablation is safe, effective and minimally invasive with slight adverse responses, which can form ice crystals in tumor cells by quick freezing, develop spasms of capillaries and small vessels in local tumor tissues, damage tumor vessels and blockage local blood circulation to promote the ischemia and anoxia so as to improve the necrosis and apoptosis of tumor cells (Guo, et al., 2005). It is difficult to cryoablate malignant tumors due to their huge volume and infiltrative growth, so the recurrence and metastasis of residual tumor becomes a critical influencing factor for the long-term efficacy of cryoablation. Studies showed that local tumor tissues could promote a series of high expressions of angiogenesis factors and induce tumor angiogenesis under conditions of ischemia, anoxia and low PH value, etc., which could result in the recurrence and metastasis of tumors. Therefore, the author predicated that cyroablation-induced tumor angiogenesis might be an important cause for the tumor recurrence. In this study, nude mice models with transplanted human lung adenocarcinoma cell line A549 were established and performed with cryoablation to explore the mechanism and significance of tumor angiogenesis by observing the changes of microvessel density (MVD) and expression of vascular endothelial growth factor (VEGF) in residual tumor angiogenesis tissues after cryoablation.

Materials and Methods

Experimental materials

Lung adenocarcinoma cell line A549 were purchased from Institutes of Basic Medical Sciences of Chinese Academy of Medical Sciences. A total of 18 5~6-week female BALB/c nude mice were obtained from Department of Laboratory Animal Science of Pecking University Health Science Center, with license number being SCXK (Jing) 2006-0008, which were supported in Animal Experimental Center of Cerebrovascular and Neurodegenerative Key Laboratory (SPF degree) in Tianjin. Endostar was donated by Yantai Medgenn Biological Engineering Co., Ltd, while immunehistochemical kits of rat anti-mice CD34 monoclonal antibody and mouse anti-human VEGF monoclonal antibody were taken from Abcam company and Beijing Zhongshan Jinqiao company respectively. Cryocare refrigeration system and 1.7 mm frozen cutting head were bought from American Endocare Company.

Experimental methods

Cell culture: Lung adenocarcinoma A549 cells were inoculated in McCoy'5A culture medium containing 10%

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Table 1. Comparisons of Tumor Volume and Growth Rate Before and after Treatment in Each Group ($\bar{\chi}$±s)

Groups	Tumor volume (cm^3)		Tumor growth
	Before treatment	21 d after treatment	rate (%)
Control group	0.65±0.16	1.48±0.14	236.68±51.23
DDP group	0.66±0.09	1.03±0.12	160.12±35.58
Cryoablation group	0.64±0.13	0.99±0.06	159.46±29.33
F value	0.026	35.804	7.466
P value	0.925	<0.001	<0.001

fetal calf and were cultured in 5% CO_2 incubator at 37°C with absolute humidity. The adherent cells were digested by 0.125% trypsin and sub-cultured, whose culture solution was changed once every 48~72 h.

Establishment and experimental methods of transplanted lung adenocarcinoma A549 nude mice models: (1) cell line A549 in exponential phase were added with appropriate amount of PBS solution to prepare 2×10^7/mL cell suspension; (2) A549 cell implantation: 0.2 mL cell suspension was injected subcutaneously to the dextral posterior armpit of each mouse (age: 6 weeks; weight: 20 g), and the tumors would develop in 7 d; (3) Animal grouping and administrative methods: All mice were randomly divided into 3 groups when the subcutaneous tumor was about 1 cm in diameter, 6 cases for each. Control group was given tumor puncture without cryoablation, DDP group was intraperitoneally injected with 5 $mg\cdot kg^{-1}$ DDP after tumor puncture without cryoablation while cryoablation group was performed with cryoablation. According to pre-experimental results, nude mice were sacrificed by chloral hydrate after 21-d treatment. Then, tumor tissues were collected and fixed by 4% paraformaldehyde solution; (4) Cryoablation: Nude mice were anaesthetized by 10% chloral hydrate, fixed by 3.5 $mg\cdot kg^{-1}$ paraformaldehyde solution, sterilized on skin, and inserted with a 1.7 mm frozen cutting head of cryocare knife. Dual-cycle cyroablation was applied, in which the temperature was reduced to -120°C for 10 s and then recovered to 0°C. Then, the above cryoablation was repeated once again.

Observation of tumor growth: Tumor volume and growth rate were expressed by V and f respectively. $V=LW^2/2$ and $f=V/V_0$, in which L and W expressed the longest diameter of tumor and the widest transverse diameter in vertical direction measured every 2 d by vernier caliper, while V and V_0 expressed the tumor volumes measured at different times and before treatment, respectively.

MVD counts in tumor tissues detected by immunohistochemical SP test: Paraffin sections of tumor tissues were prepared. The primary antibody was mice anti-human VEGF monoclonal antibody and the secondary one was biotinylation goat anti-mice IgG. After DAB developing, the sections were contrastively re-stained by hematoxylin, dehydrated, made transparent and tightly fixed. The positive expression was manifested as brown and streak staining of cell matrix. The vascular high-density area of each tumor tissue section was selected under low-fold scope (×40) and transformed to high-fold microscope (×200) to calculate the amounts of micro-vessels accurately. The mean values of micro-vessel amounts in 5 different fields were considered as the terminal value of each case for the statistical analysis.

Table 2. Comparisons of MVD and VEGF Expressions in TumorTissues of Each Group ($\bar{\chi}$±s)

Groups	MVD	VEGF (%)
Control group	21.10±0.86	36.17±1.72
DDP group	24.70±0.72	39.00±1.79
Cryoablation group	29.17±0.96	50.83±2.14
F value	134.181	101.506
P value	<0.001	<0.001

VEGF expression in tumor tissues detected by immunohistochemical SP method: Paraffin sections of tumor tissues were prepared. The primary antibody was mice anti-human VEGF monoclonal antibody and the secondary one was biotinylation goat anti-mice IgG. After DAB developing, the sections were contrastively re-stained by hematoxylin, dehydrated, made transparent and fixed. Under optical microscope (×200), VEGF expression was located in cytoplasm, marked by clear brown orange or brown granules or clumps. Five different fields of each section were selected under high-fold microscope (×400) to calculate the ratio of cells with positive expression in all cancer cells, and the mean value was obtained to conduct the statistical analysis.

Statistical data analysis

SPSS 17.0 software was adopted for all data analysis. Measurement data was expressed by mean ± standard deviation ($\bar{\chi}$±s) and analyzed by ANOVA while LSD was used for the pair-wise comparisons. The relevance between two indexes were observed by Spearman correlation coefficients, and $P<0.05$ was regarded to be statistically significant.

Results

General data and tumor growth of animals

During the formation of tumors, there were no significant differences in food intake, defecation, activities and other general conditions of nude mice. However, after cryoablation, compared with nude mice in control and crypablation groups, those in DDP group had decreased food intake and activities, as well as dull and unconcerned responses to outside stimuli, most of which had emaciation. 21 d after cryoablation, the tumor volumes of control group, DDP group and cryoablation group were (1.48±0.14) cm^3, (1.03±0.12) cm^3 and (0.99±0.06) cm^3 respectively and the differences were significant ($P<0.01$), but the differences between cryoablation and DDP groups were not significant ($P=0.546$). The tumor growth rates of control group, DDP group and cryoablation group were (236.68±51.23)%, (160.12±35.58)% and (159.46±29.33)% respectively and the differences were significant ($P<0.01$), but the differences between cryoablation and DDP groups had no statistical significance ($P=0.978$) (Table 1).

Immunohistochemical staining of MVD and VEGF expressions in each group (Table 2)

After cryoablation, VEGF positive cells in tumor

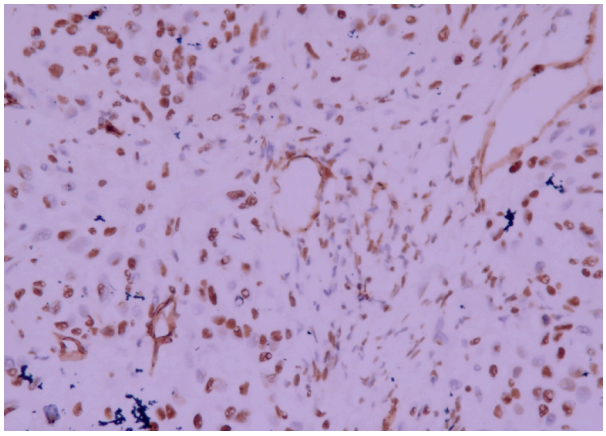

Figure 1. Immunohistochemical Staining of MVD Expression in Cryoablation Group (×200)

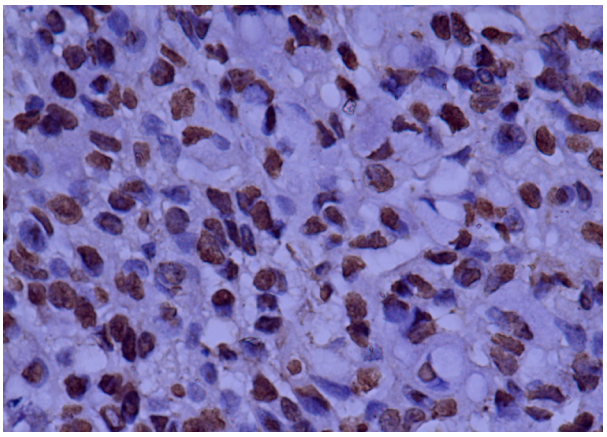

Figure 2. Immunohistochemical Staining of VEGF Expression in Cryoablation Group (×400)

tissues around freezing damage area were large in amount and dispersive and focal in distribution, with evidently increased MVD distribution. Under optical microscope, the cellular matrixes of tumor micro-vessels were brown in color and streak in shape (Figure 1), while those in VEGF positive cells were in clear brown orange or brown color and granular or clumpy shape (Figure 2). MVD were (21.10±0.86), (24.70±0.72) and (29.17±0.96) (P<0.01) while the positive expression rates of VEGF were (36.17±1.72)%, (39.00±1.79)% and (50.83±2.14)% (P<0.01) in control, DDP and cryoablation groups , respectively, and MVD expression was proportional to the positive expression of VEGF (r=0.928, P<0.01) by Spearman correlation test.

Discussion

In cryoablation, a minimally invasive technique, ultra-low temperature produced by cryogenic materials acts on tumor cells to produce ice crystals in and out of cells and cellular dehydration, leading to cellular necrosis of tumor tissues. Additionally, cryoablation also serves as a "frozen embolism" in that the freezing and temperature-increasing processes in short term can give rise to the formation of ice crystals in and out of tumor cells to induce cellular burst while ultra-low temperature may cause cellular dehydration and shrinkage, bringing about the increase of cellular electrolyte toxicity and the sharply decrease of intra-cellular PH value, the blood flow stagnation in cryoablation and its surrounding areas and the formation of micro-vessels (Gage et al., 1998). The superposition of above effects can damage tumor cells to the maximal extent, however, the tumor tissues cannot be completely cleared because of multiple influencing factors, such as the huge volume and infiltrative growth of tumor body, the "hot pools" of tumor vessels and the limited freezing areas, etc. (Cao et al., 2013; Liang et al., 2013). After cryoablation, the residual tumors become the origins of tumor recurrence and metastasis, and the important factors influencing the short-term efficacy of cryoablation. The recurrence and metastasis processes of residual tumors are complicated, including increased tumor proliferation activity, expressions of anti-apoptotic genes and angiogenesis factors, formation of angiogenesis as well as abnormal expressions of adhesion factors, multi-drug resistance genes and telomerase, etc., in which angiogenesis factor expression and angiogenesis formation exert considerable functions (Li et al., 2002; Diao et al., 2014; Erdogan et al., 2014; Madjd et al., 2014; Tang et al., 2014; Wong et al., 2014). The severity of tumor angiogenesis indirectly reflects the ability of tumor infiltration, recurrence and metastasis. Tumor MVD is a common index used to measure the formation severity of tumor angiogenesis and evaluate the tumor recurrence and prognosis (Ueda et al, 1999).

The results of this study indicated that cryoablation could effectively inhibit tumor growth with definite efficacy and had slight influence on the daily lives of nude mice. MVD in tumor tissues of cryoablation group was evidently higher than in other 2 groups, and the differences were both significant, demonstrating that tumor angiogenesis increased obviously in residual tumor tissues after cyroablation, which was predicated to be closely connected with the slight necrosis severity of tumor tissues around cryoablation injury area. The residual tumor cells could induce tumor angiogenesis after cryoablation under the stimulation of ischemia, anoxia and low PH value, which could protect the tumor cells from being damaged by cryoablation and increase their survival chances, providing basis for the tumor recurrence and metastasis. In addition, this study showed similar results with Kimura M's research, in which MVD also increased markedly in residual tumor tissues in mice with RM-9 prostate cancer cells after cryoablation (Kimura et al., 2010).

As to the regulation of tumor angiogenesis, Hanahan et al proposed the "Switch Balance Hypothesis of Angiogenesis", who believed that angiogenesis was regulated by switch (Hanahan et al, 1996). Promoting and inhibiting factors are in dynamic equilibrium in the angiogenesis of normal tissues, which lost the equilibrium physically in tumor tissues. With the enlargement of tumor body, ischemia and anoxia could be observed in internal cells of tumors as well as necrosis of partial tumor cells. The residual tumor cells could up-regulate promoting angiogenesis factors and (or) down-regulate inhibiting angiogenesis factors under conditions of ischemia, anoxia and low PH value, causing the imbalance of positive and

negative angiogenesis factors and the increased tumor angiogenesis. In promoting factors of tumor angiogenesis, VEGF is the most strong and specific growth factor consisted of tumor cells, vascular endothelial cells and macrophages, which could act on the receptors of vascular endothelial cells through autocrine and paracrine, and is effective in improving the growth, proliferation, metastasis of endothelial cells, extracellular matrix degeneration and the formation of vascular tube structure, etc. (Folkman et al., 1990; Das et al., 2012), suggesting that VEGF and its receptors exert significant functions in the development, progression and prognosis of tumors. Therefore, VEGF is considered to be an important detection indicator in the promoting factors of tumor angiogenesis (Li et al., 2013). This research showed that VEGF level was remarkably higher in cryoablation group than in other 2 groups after cryoablation, and the dependency detection demonstrated that VEGF expression level was in positive relation with MVD. Moreover, the positive cells of VEGF and angiogenesis were distributed around tumor tissues and necrosis areas in this study, indicating that VEGF was of great significance in promoting residual tumor angiogenesis.

In one word, cryoablation can effectively inhibit tumor growth. However, the high expression of VEGF plays an important role in residual tumor angiogenesis, giving rise to significantly increased tumor angiogenesis after cryoablation, which means that the blockage and down-regulation of VEGF expression may reduce residual tumor angiogenesis and promote the long-term efficacy of cryoablation, providing partial theoretical basis for the combined protocols in clinic.

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