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Effect of radiotherapy on angiogenesis of human pancreatic cancer transplanted tumor in nude mice*

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Abstract ***Objective:*** The aim of this study was to observe the effect of radiation on micro-vessel density (MVD) and vascular endothelial growth factor (VEGF) expression in an transplanted tumor of human pancreatic cancer in nude mice and to explore the role of radiotherapy on pancreatic cancer angiogenesis. ***Methods:*** After vaccinated pancreatic cancer PANC-1 cells, 20 nude mice were randomly divided into radiation group and control group. Radiation group received the radiation (dose of 20 Gy) and were killed 5 days later. The MVD and VEGF expression were determined by immunohistochemistry and then compared with the control group. ***Results:*** The number of MVD in the radiation group was significantly lower than that of the control group (8.30 ± 4.55 vs. 13.60 ± 4.28, $P < 0.01$). The optical density (OD) of VEGF in the radiation group was higher (2.11 ± 0.54 vs. 1.32 ± 0.61, $P < 0.05$) compared with the control group. ***Conclusion:*** The radiotherapy can reduce the number of vessels and increase the VEGF expression of human pancreatic cancer transplanted tumor in nude mice.

Key words pancreatic cancer; radiotherapy; angiogenesis; vascular endothelial growth factor (VEGF)

Radiation therapy is one of the treatments of advanced pancreatic cancer, but the efficacy is not satisfactory [1]. Previous studies concentrated on the killing capacity of radiation on cancer cells to improve the radiotherapy effect of pancreatic cancer. Recent studies find that the radiation on the tumor micro-vessel injury also play an important role in the inhibition of tumor growth and further study may provide a way to increase the effect of radiotherapy [2]. In this study, we observed the effect of radiation on micro-vessel density (MVD) and vascular endothelial growth factor (VEGF) expression in transplanted tumor of the human pancreatic cancer in nude mice by immunohistochemistry and explored the effect of radiotherapy on pancreatic cancer angiogenesis.

Materials and methods

Materials

Nude mice were purchased from the Animal Center of Xi'an Jiaotong University (Xi'an, China). The mice were 6–8 weeks old and 18–22 g weight, and the number of male and female was equal. All mice were raised in a sterile laminar flow chamber. Human pancreatic cancer cell lines PANC-1 cells were purchased from Shanghai Bohong Biotechnology Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) high glucose medium was purchased from Invitrogen Corp., (Carlsbad, CA, USA). VIII factor antibody, VEGF antibody and immunohistochemistry reagents were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). The linear accelerator was Varian 2300C/D linear accelerator (Varian, Stanford, CA, USA).

Tumor-bearing animal model

Cells in the period of logarithmic growing were collected and then injected into the subcutaneous of the right back of the nude mice at a density of 1×10^7 cells/mL and then the mice were returned to the sterile laminar flow chamber. The growth of the transplanted tumor was observed timing. Four weeks later, the nude mice were treated when the diameter of the tumor was about 1.5 cm.

Group and treatment

Mice were randomly divided into radiation group and control group (10 in each group). The radiation group was treated with 20 Gy X-ray irradiation at one time (6 MeV electronic line, the center of the radiation field was 4 cm × 4 cm and the irradiation source distance from the skin was 100 cm). Meanwhile, the control group did not re-

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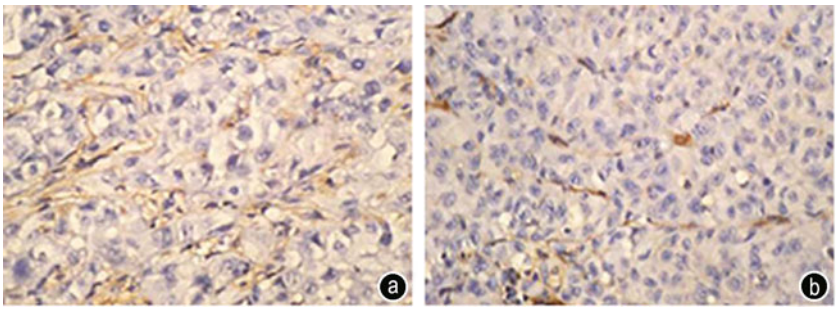

Fig. 1 Analysis of the micro-vessel density (MVD). The micro-vessels were stained brownish yellow and distributed in the surrounding of the pancreatic cancer cells. (a) Control group; (b) Radiation group. Slice thickness: 4 μm

ceive any radiation. Five days after radiation, the mice were killed and the tumor tissues were stripped completely and then fixed by 10% formaldehyde solution. Specimens were treated with conventional dehydration, fixed, embed in paraffin and serially sectioned (slice thickness 4 μm).

Immunohistochemical detection of blood vessels and VEGF

The sections were dewaxed, hydrated, and incubated in a 3% hydrogen peroxide solution to remove en-dogenous peroxide. And then tissue antigen retrieval was performed by heating the sections, which were then blocked with serum. The sections were incubated overnight at 4 ℃ in primary antibody and then incubated in secondary antibody and horseradish peroxidase-labeled streptavidin. The sections were visualized by incubating stable DAB and finally detected under an optical microscope. PBS was used instead of primary antibody as a negative control.

After treatment, tumor vascular endothelial cells could be labeled as brown. A tubular structure surrounded by positive staining of endothelial cells was counted as a positive blood vessel [2]. Then counted the number of MVD: firstly, selected the vision which was most densely stained in the vision field under a low magnification (× 100), and then counted the number of MVD under a high magnification (× 200). The value of MVD was presented as the mean of three fields.

The standard of positive VEGF expression was that an average of more than 20% of cells were stained obviously and positioned clearly, with the light yellow to brown particles appeared in the cytoplasm. The value of optical density (OD) was analyzed using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, USA) and presented as the mean of five horizons.

Statistical analysis

All data had been presented as the mean with standard deviation. To compare means between groups, Fisher's exact test and Student's *t*-test were used. *P* value of less than 0.05 was considered to be statistically significant. All statistical analyzes were carried out using the statistical analysis software SPSS 16 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1 The value of the MVD count and VEGF optical density

Group	Number	MVD	VEGF
Control	10	13.60 ± 4.28	1.32 ± 0.61
Radiation	10	8.30 ± 4.55**	2.11 ± 0.54*

Note: * $P < 0.05$ and ** $P < 0.01$ versus control group

Results

Decreased MVD in the radiation group by immunohistochemistry

The vessel stained spread the tissue sections of the transplanted tumor (Fig. 1). Fig. 1 also showed that the thickness and open degree of vessels was inconsistent.

The number of MVD in radiation group and control group was 8.30 ± 4.55 and 13.60 ± 4.28 respectively. The MVD in radiation group was significantly lower than that of the control group ($P < 0.01$, Table 1).

Increased VEGF expression in the radiation group by immunohistochemistry

VEGF was mainly found in the cytoplasm of pancreatic cancer and stromal cells. It was light to brownish yellow fine granular, which showed a focal or diffuse distribution. There was no obvious staining in the membrane and nucleus of the cells.

Compared with the control group, the OD value of VEGF of radiation group was higher (2.11 ± 0.54 vs. 1.32 ± 0.61, $P < 0.05$, Table 1).

Discussion

Tumor angiogenesis is a prerequisite for tumorigenesis, development and metastasis, which provide the necessary nutrition for the survival of tumor cells. Previous studies considered that tumor growth will not exceed 3 mm^3 without blood. Therefore, tumor angiogenesis blocking will make tumor "benign". Though hundreds of drug studies have involved in the suppression of tumor vessel recently, the effect is not satisfactory [3].

VEGF is a highly specific vascular endothelial cell mitogenic factor, which combines with its specific receptor (VEGFR), causing a series of signal transduction and releasing multiple of cytokines and growth factors. The cytokines and growth factors can promote endothelial cell proliferation, migration and angiogenesis and therefore plays an important role in tumor growth and metastasis [4].

The molecular mechanism of radiation-induced VEGF expression is a focus in tumor radiobiology research. In glioma, Knizetova *et al* [5] suggested that radiation could induce high expression of VEGF and caused the radiation resistance. Radiation-induced expression of VEGF has a protective effect on tumor blood vessels, and thus makes the tumor cells to escape radiation-mediated cytotoxicity, which promote cells more radio-resistant. On the other hand, the inhibition of VEGF expression can increase the radiosensitivity of tumor cells [6].

In our results, the VEGF expression of pancreatic cancer cells transplanted tumor increased after radiation, but the density of blood vessels declined. We considered that radiation can induce tumor cell expression of VEGF and promote formation of the blood vessel. But at the same time, it have a direct killing effect on cells, causing increased apoptosis of tumor vascular endothelial cells, reducing stimulation of VEGF target cells, at last resulting in lower density of blood vessels.

As a gene poison event, radiation therapy kills tumor cells and can lead to the activation of a variety of radiation effect genes at the same time. The activation of related gene can induce the secretion of various cytokines which can change the micro-environment of tumor cells. Studying the impact of radiation on the tumor cell proliferation and pro-angiogenic cytokine expression will further guide us to take more effective ways to increase the effect of radiotherapy in clinical practice.

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