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Abstract

**Purpose:** The aim of the present study was to investigate the anti-tumor effect of a pEgr-1-endostatin-TNF-α recombinant plasmid induced by ionizing radiation. **Method:** Three hundred and twenty mice bearing Lewis lung carcinomas were divided into four experimental groups: blank control, irradiation treatment, plasmid treatment and plasmid combined irradiation treatment. Twenty-four hours after the recombinant plasmid was injected locally into the tumors of the mice, they were irradiated with 10 Gy γ-rays. The concentration of TNF-α and endostatin in the serum of mice was measured by ELISA and tumor growth in each group was compared. The tumor microvessel density was examined by H&E staining and immunohistochemistry analysis of CD31 positive cells. **Results:** Radiation could induce the expression of pEgr-1-endostatin-TNFα. The levels of endostatin and TNF-α could express steadily for about 4 weeks, with concentrations of 52.6±4.19 and 12.0±0.87 ng/ml respectively, in the second week in combined therapy group and maintained at relative higher level in the fourth week than other groups (F=29.7, P<0.05). Compared with the control group, the tumor micro vessel density was significantly depressed (P<0.05) and tumor growth was significantly inhibited (5907.2±78.6 mm³ vs. 763.5±12.3 mm³, P<0.05). **Conclusions:** The expression of pEgr-1-endostatin-TNF-α could be induced in mice in vivo and exhibited more significant anti-tumor and anti-angiogenesis effects than irradiation alone.

Keywords: Endostatin - TNF-α - gene-radiotherapy - irradiation

Materials and Methods

**Materials**

Reconstructed pEgr-1-endostatin-TNF-α plasmid and Lewis lung carcinoma cell line were kindly provided from Department of Radiation Biology, Medical Center in Memorial of Norman Bethune, China. E. coli strain JM109 was provided from the Central Laboratory of The Affiliated Hospital of Medical College of Qingdao University. Lipo2000 liposome was purchased from Gibco BRL, Life. Technologies Inc., Grand Island, NY, USA. Rabbit anti-mouse TNF-α antibody and endostatin ELISA kit were purchased from Jingmei BioTech Co.Ltd. Female Kunming strain mouse (n=320, 4-6 weeks, 18±2 g) were purchased from Laboratory Animal Department, National Institute for the Control of Pharmaceutical and Biological Products..

**Establishment of animal model**

Lewis lung cancer cells (2.0×10⁶/ml) were subcutaneously injected into the right lower limb. When the diameter of tumor reaches 0.5 cm, the mice bearing Lewis lung carcinoma were divided into four
groups randomly. There were control group without any treatment, irradiation treatment group which will be exposed to the γ-ray locally, the plasmid treatment group and the liposome combined irradiation treatment. Each group contained 80 animals.

**The intratumoral injection of plasmid**

The liposome and the reconstructed plasmid were mixed with 1:1 to a volume of 150 μl in which the plasmid was 80 μg. The injection of plasmid was performed on three different sites averagely.

**Irradiation treatment and sample collection**

Single exposure to a 60Coγ source at a dose rate of 35 cGy/min at 80 cm distance. The dosage for each mouse was 10 Gy. After infection of plasmid for 24 h, the mice were also locally exposed to 60Coγ under the same condition and the dosage for each mouse was 10 Gy. After irradiation for 24 h, the orbital blood of ten mice was taken at a total volume of 1x2 ml. Then the orbital blood was also taken every three days. The serum was separated by centrifugation at 1200 r/min for 5 min. The concentration of TNF-α and endostatin in the serum at different time points was examined by ELISA assay.

**Measurement of tumor volume**

The maximal diameter of tumor (d) was determined by vernier caliper on the day of irradiation and every three days after irradiation. The volume of tumor (V, mm3) was calculated using the following formula: \( V = \frac{1}{6} \pi d^3 - 100 \) (Herbst et al., 2002). The tumor growth time (GT) was defined as the time when the tumor reaches the four-fold larger than the prime volume on the day of irradiation. The absolute growth delay (AGD) was defined as the subtraction of GT between treatment group and control group.

**Measurement of tumor micro vessel density (MVD)**

After 30 days of irradiation, the mice were sacrificed and the tumor tissues were subjected to Hematoxylin and Eosin (H&E) staining. The growth of tumor vessel of observed under the microscope at the magnification of 10 ×10. The average count of cells of the ten fields was calculated. In addition, the CD31 in the tumor tissues were detected by immunohistochemistry using anti CD31 antibodies. The MVD was determined.

**Statistical analysis**

The tumor volum, the concentration of molecular and the density of tumor vessel were expressed as means ± SD. Analysis of variance was performed using SPSS 10.0. The compare between two groups was performed using student’s t test with significance determined as P < 0.05.

**Results**

**Expression of endostatin in serum**

There are not significant changes in the concentration of endostatin in control group, irradiation group and plasmid group in four weeks. However, the concentration of endostatin in Irradiation+Plasmid group was significantly increased after 24 h of irradiation treatment. In the second week, the concentration reaches the peak value (52.64±4.19 ng/ml). Moreover, the difference was still significant in the fourth week (F=19.023, P<0.05, Figure 1).

**Expression of TNF-α in serum**

After irradiation, different concentration of TNF-α in mice of four groups was observed. Compared to the control group, the concentration of TNF-α in plasmid group was not changed significantly (F=1.073, P>0.05). However, in the irradiation treatment group, the concentration of serum was obviously decreased after two weeks of irradiation. And in the fourth week, the serum concentration in irradiation treatment group was recovered, which may be due to the effect of irradiation on the immunity function of mice. Maybe some immunity factors including TNF-α were downregulated.

**The growth of tumor**

The initial tumor volume was not significant changed during the four groups after plasmid injection or irradiation treatment. After 5 days of irradiation, the growth of tumor was significantly inhibited. The tumor growth was significantly slower in irradiation or combination treatment group than in control group (F=112.893, P<0.05). The tumor volume in the irradiation and plasmid combined group was not significantly different from the irradiation single treatment group during the first 12 days (F=0.344, P>0.05). However, during the following days, significant difference was observed between the
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Table 1. The Micro Vessel Density Evidenced by H&E Staining and CD31 Positive Cells Evidenced by Immunohistochemistry in Different Treatment Groups (cells/field, means ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>H&amp;E staining cells</th>
<th>CD31 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.2±1.5</td>
<td>15.1±1.3</td>
</tr>
<tr>
<td>Irradiation</td>
<td>9.5±0.6</td>
<td>12.8±1.2</td>
</tr>
<tr>
<td>Plasmid</td>
<td>10.4±0.5</td>
<td>14.8±1.0</td>
</tr>
<tr>
<td>Irradiation+Plasmid</td>
<td>4.3±0.3a</td>
<td>6.9±1.5a</td>
</tr>
</tbody>
</table>

a, t=14.063 and 10.465, P<0.05, in comparison to control

Discussion

Gene-radiotherapy, a combination of gene therapy and radiotherapy, is a new paradigm for cancer treatment (Senzer et al., 2004; Wang et al., 2004; Wu and Li, 2004; Skovset et al., 2005). Gene-radiotherapy may represent a useful addition to neoadjuvant management of cancer, considering that it can improve the therapeutic effectiveness of radiotherapy and help solve those problems involved in gene therapy, such as low efficiency of gene transduction and deficiency of an available regulation method to control the target gene expression in vivo. In 1992, Weichselbaum, a tumor radiotherapist from U.S., proposed a combinative application of gene therapy and radiotherapy for the tumor therapy that is the presumption of gene-radiotherapy (Herbst et al., 2002). In his study, an adenovirus vector for Ad.EGR-TNF was constructed. The expression of TNF-α, a broad-spectrum anti-tumor factor, was triggered by radiotherapy and showed remarkable anti-neoplastic activity in animal models (Ponnazhagan et al., 2004). Moreover, the dosage of TNF-α diminished via modulated gene transduction, which resulted in decreased toxicity by systemic administration. In addition, several studies demonstrated that reconstructed adenovirus expressing endostatin (a direct angiogenesis inhibitor) effectively inhibited the angiogenesis of tumor tissue and prevented the growth and metastasis of malignancy. However, there is a dosage- and time- dependent effect of adenovirus expressing endostatin, and the inhibition of tumor is not satisfactory (Li et al., 2006). On the other hand, Maucer et al reported that endostatin and irradiation showed combined cytotoxicity and the combination of endostatin and irradiation was more potent in inhibiting tumor growth than either single treatment (Mauceri et al., 2009). Endostatin induced cell apoptosis by inhibiting angiogenesis, afterward blocked the malignant proliferation of cancer. Further studies showed that the in vivo expression of angiogenesis inhibitor via reconstructed...
vector caused efficacious anti-tumor effect during a long period. Currently, the domestic and foreign researches just focused on single-gene-therapy for cancer. However, there is less reported on the combination of gene-therapy and radiation mediated by the radiosensitive promoter. Because multi-factors and a variety of genes are involved in the development of malignancy, the combination treatment for tumor therapy should bring more efficacious anti-tumor effect. Therefore, we designed two different target genes in our experiment, which can produce direct and indirect anti-neoplastic effects for certain different modalities.

On the basis of radiation-induced features of Egr-1, we construct an irradiation induced endostatin and TNF-α dual gene expression regulation system (PEgr-1-endostatin-TNF-α) in use of the cis-acting element of the promoter of Egr-1 gene. In this system, TNF-α possesses anti-tumor activity and endostatin possesses anti-neoplastic activity. After transduction both genes in vivo, irradiation was performed locally to kill the tumor. At the same time, the expression of anti-tumor gene was induced, which resulted in killing effect of double suicide genes. Thus, we can relieve the unnecessary injury to the normal tissue due to the decrease dose of irradiation. Moreover, the specific expression of tumor-killing gene can be regulated by gamma-ray local irradiation. There are many advantages for gene-irradiation therapy. The expression of both genes can be effectively regulated by irradiation. Compared to systemic administration of gene therapy, the toxicity and adverse reaction can be significantly minimized.

The results of the present study demonstrated that tumor growth in the control group was faster and the tumor volume displayed exponential trends. During the 30-day observation period, their volume enlarged by nearly 30 times. The single radiotherapy with a large dosage may effectively inhibit the growth of tumor. Such inhibition, however, would remain about 2 weeks at most. After the treatment, the tumor growth velocity would recover. The growth of tumor in the simply plasmid-injected group showed no significant difference in comparison to control. That is because in the absent of irradiation, anti-neoplastic genes could not express. As we associated radiotherapy with the injection of plasmid PEgr-1-endostatin-TNF-α, which can be induced by the irradiation and then expressed in mice, the expression of endostatin and TNF-α was evidently up-regulated. Statistics significance was observed when compared to the other groups (F=19.023, P<0.05). The expression of both genes reached peak concentration during the second or third week after therapy, and remains at high level for a long time. The radiotherapy associated with plasmid injection group displayed better curative effectiveness than radiotherapy or plasmid injection alone (F=128.042, P<0.05). In the former group, the tumor growth was prominently delayed. After H&E staining, the neoangiogenesis in the tumor tissue was observed. We can found that micro vascular density and the number of CD31 marked endothelial cells was significantly inhibited (t=14.063, 16.465, P<0.05). It suggested that the expression of endostatin inhibited the angiogenesis, and thus after radiotherapy strongly inhibits tumor growth. Compared to the gene therapy of single endostatin gene, the radiotherapy associated with PEgr-1-endostatin-TNF-α plasmid injection showed significantly inhibition effect to tumor growth.

Along with the rapid development of gene engineering technology, the gene-therapy for malignant tumor has opened up a new field of cancer treatment (Herbst et al, 2002; Ponnazhagan et al, 2004). Target genes can be transferred into tumor cells or tumor vascular endothelial cells by gene transduction system and their expression can be induced effectively. By autocrine and paracrine pathways, the tumor cell growth or the angiogenesis was inhibited. By using thus strategy, the therapeutic cost was decreased, and the inconvenience brought about by drugs injection was avoided. Furthermore, it also improved the therapeutic efficacy due to the formation of the high drug dense in local tumor tissue. Nowadays, animal experiments in vivo have been carried out. The clinical evaluation, however, calls for more deep research about the gene transduction system with better efficiency, specificity and safety (Tjin et al, 2006). The comprehensive therapy involving multi-genes transduction can inhibit the tumor in different stages, which may result in more conspicuous curative effect (Liu et al, 2009). Associating the traditional oncotherapy, such as chemotherapy, radiotherapy and surgery as well as immune therapy and so on, with the gene therapy might have a promising future in therapy for malignancy.

References


Skovseth DK, VeugerMJ, Sorensen DR, et al (2005). Endostatin dramatically inhibits endothelial cell migration, vascular...


