Dendritic cell-tumor cell fusion vaccine prevents growth of subcutaneous transplanted esophageal carcinomas

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Abstract Background and Objective: Our previous studies have shown that dendritic cell (DC)-tumor cell fusion vaccine can induce specific antitumor response against esophageal carcinoma cells. This study was to investigate the inhibitory effect of intratumor injection of the antigen-specific cytotoxic T lymphocytes (CTLs) induced by DC-tumor cell fusion vaccine against subcutaneously transplanted esophageal carcinoma cells in nude mice, and to analyze the influence of DC/tumor cell fusion vaccine on proliferation and apoptosis of esophageal carcinoma cells. Methods: Fusion cell vaccine of mature DCs with EC109 cells were generated by the polyethylene glycol (PEG) protocol and the antigen-specific CTLs were induced. The models of transplanted human esophageal carcinoma in nude mouse were established using EC109 cell line. Thirty-three nude mice with subcutaneous tumors were randomly divided into three groups. Subcutaneous tumors of group A (n=11), group B (n=11) and group C (n=11) were intratumorally injected with the CTLs induced by DC/tumor fusion vaccine, T lymphocytes and RPMI-1640 medium respectively once a week. After four weeks of intratumor injection, the nude mice were killed and the nodules were anatomized. The mean volume and weight of tumors of each group were measured, and the tumor inhibitory rates of the Group A and the Group B were calculated and compared. The expression of proliferating cell nuclear antigen (PCNA) was detected by immunohistochemistry (S-P method). The mean PCNA-label index (LI) of three groups was compared. The cell cycle and cell apoptosis of the xenograft tumor cells were analyzed by flow cytometry. The mean S-phase fraction (SPF) and the mean rate of cell apoptosis of three groups was compared respectively. Results: Both the mean volume and the mean weight of xenograft tumors in group A ([(881.45± 31.14 ) mm$^3$ and (0.88± 0.04)g] were significantly smaller than those of group B [ (1493.37± 51.67) mm$^3$ and (1.38± 0.07)g] and group C [(2065.77± 87.55) mm$^3$ and (2.04 ± 0.11)g]. The tumor inhibitory rates of Group A was significantly higher than that of group B (56.86% vs. 32.35%, F=1218.08, P=0.001). The mean PCNA-LI of xenograft tumors was less in the group A (26.83± 0.95%) than in the group B (51.82± 1.51%) and group C (68.93± 2.40%) (F=1528.39, P=0.000). The mean SPF of xenograft tumors was less in the group A (12.46± 0.36%) than in the group B (29.39± 0.96%) and the group C (42.25± 1.43%) (P=0.05). The mean apoptotic rate of xenograft tumors was less in the group A (38.03± 1.21%) than in the group B (17.75± 0.56%) and the group C (6.59± 0.22%) (P<0.05). Conclusion: The model of subcutaneous xenograft tumors in nude mice using human esophageal carcinoma cell line EC-109 has been successfully established. CTLs induced by DC/tumor fusion vaccine has specific antitumor immunity efficacy in vivo. CTLs can inhibit the
proliferation of tumor cells and induce apoptosis of tumor cells in local tumors.

**Key words:** esophageal neoplasm, dendritic cells, fusion vaccine, transplanted tumor, cytotoxic T lymphocytes

Currently, as the efficacy of the comprehensive treatment for esophageal carcinoma is inadequate, other effective treatments for esophageal carcinoma are thus required. Immunobiological cancer therapy is a novel treatment for cancer, following surgery, radiotherapy and chemotherapy for cancer.\(^1\) and the regimen of dendritic cell (DC)/tumor cell fusion vaccine, especially, is a hot spot in research. The previous study found that DC/tumor cell fusion vaccine effectively induced specific anti–tumor immune response against esophageal carcinoma cells in vitro.\(^2\) In order to verify the specific killing effect of DC/tumor cell fusion vaccine for esophageal carcinoma in vivo, the research investigated the inhibitory effect of intratumor injection of antigen–specific cytotoxic T lymphocytes (CTLs) induced by DC/tumor cell fusion vaccine against subcutaneously transplanted esophageal carcinoma cells in nude mice, and to analyzed the influence of DC/tumor cell fusion vaccine on proliferation and apoptosis of esophageal carcinoma cells.

**Materials and Methods**

**Materials.** Cell lines and experimental animals. The human esophageal carcinoma cell line, EC109 was preserved by the State Key Laboratory of Oncology in South China, Sun Yat–sen University (Guangzhou, Guangdong, China). Forty male BALB/c nude mice in two batches were provided by the Center of Experimental Animals, Sun Yat–sen University (Guangzhou, Guangdong, China). Each batch included 20 mice ranging in age from 4 to 5 weeks and in weight from 18 to 20 g. Under the specific pathogen free (SPF) conditions, the mice were raised in the Center of Experimental Animals. The qualification number for the mice is SCXK (Yue) 2004–0011.

Main reagents. The FITC–labeled mouse anti–human CD80, CD83, CD86, MHC–Class I, MHC–Class II and IgG monoclonal antibodies, and PE–labeled mouse anti–human MUC1 monoclonal antibody were purchased from BD Sciences (Franklin Lakes, New Jersey, USA). IL–2, IL–4, recombinant human granulocyte colony–stimulating factor (rhGM–CSF), fetal bovine serum (FCS) were obtained from Hangzhou Sijiqing Biological Engineering Materials, Co., Ltd (Hangzhou, Zhejiang, China). RPMI–1640 was purchased from Invitrogen (San Diego, California, USA). Polyethylene glycol (PEG) and lactate dehydrogenase (LDH) were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). The proliferating cell nuclear antigen (PCNA) and the immunohistochemistry (IHC) kit were purchased from Maxim. Bio (Fuzhou, Fujian, China). The cell cycle analysis kit was purchased from BD Sciences (Franklin Lakes, New Jersey, USA).

**Methods.** Preparation of DC/EC109 fusion cell vaccine and proliferation of T lymphocytes. EC109 was cultured, DCs and T lymphocytes were isolated and cultured, DC/EC109 fusion cell vaccine were prepared, and T lymphocytes were proliferated in reference to the previous study.\(^2\)

Establishment of nude mice with subcutaneously transplanted esophageal carcinoma. EC 109 cells were cultured routinely and those in the logarithmic phase of growth were collected into 50 ml centrifuge tubes. According to the literature,\(^3\) \(1 \times 10^7/mL\) esophageal carcinoma cell suspension was prepared with the collected EC 109 cells plus normal saline. Under sterile conditions, the carcinoma cell suspension was injected subcutaneously into the right axillary region of the nude mice using the 1 mL syringe, each with 0.2 mL (containing approximately \(2 \times 10^6\) cells).

Experiment grouping and treatment. Two batches of mice were included in the research, each with 20 mice. The model of subcutaneously transplanted human esophageal carcinoma was successfully established in 33 nude mice. They were randomly divided into three groups with 11 in each. In Group A, the mice were treated with activated T lymphocytes (CTLs) as the effector cells; in Group B, the mice were treated with T lymphocytes as the effector cells; in Group C (the blank control group), the mice were treated with serum–free RPMI1640 medium. According to literature,\(^4\) the T lymphocytes (activated or non–activated) were adjusted to \(5 \times 10^6/mL\) as the effector cells, prepared with the serum–free RPMI–1640 medium, and injected into multiple sites of the implanted esophageal carcinoma with 1 mL (equivalent to \(5 \times 10^6/mL\) T lymphocytes) for each mouse. The mice in the blank control were injected with the identical volume of serum–free RPMI–1640 medium. Both the serum–free RPMI1640 medium and the RPMI1640 cell culture medium were added to maintain the optimal environment for tumor cell growth.
medium. Starting from two weeks after implantation of esophageal carcinoma, the mice were injected with T lymphocytes or serum –free RPMI –1640 medium once a week for four weeks consecutively. The mice that died during treatment were excluded.

The volume and the weight of transplanted esophageal carcinoma and the tumor inhibitory rate. 

The mice were killed by cervical spine dislocation after four weeks. Following the removal of in –situ esophageal carcinoma, the blood and fat were cleared. The volume and the weight of transplanted esophageal carcinoma were measured. The tumor inhibitory rate was calculated according to the following formula: the tumor inhibitory rate = (1 – the mean weight in the treatment group/ the mean weight in the control group) ×100%.

Detection of PCNA in esophageal carcinoma. 

Expression of PCNA in three groups of nude mice was detected using the IHC assay (the S–P method) as per the manufacturers instructions. The primary antibody was mouse anti-human PCNA monoclonal antibody (with a concentration of 1:100). The negative control was regarded as replacement of the primary antibody by PBS, while the positive control was the positive section provided in the IHC kit. The sections were stained using the IHC S–P method, colored with DAB, re-stained with hematoxylin, dehydrated with ethanol, cleared, and blocked with neutral balsam. Ten high–power (HPF, 400x) fields were randomly selected with 100 cancer cells in each to visualize brown or buffy granules as positive PCNA under the microscope. The PCNA Label Index (PCNA –LI) was considered as the percentage of PCNA–positive cells: PCNA–LI=PCNA positive cells/1000×100%.

Analysis of cell cycle and detection of cell apoptosis. Fresh esophageal carcinoma tissues, after being rinsed in PBS, were minced into pieces of less than 0.5mm, digested with 0.25% pancreatin at 37°C for 10 min and filtered through a 300–mesh nylon net followed by centrifugation. The tissues were made into monolayer suspension using normal saline. The monolayer suspension was rinsed in PBS, fixed with 70% cooled ethanol, and stored at 4°C. The suspension was penetrated with Triton X –100 and RNA enzyme was added to remove RNA. 200 μl out of 1 mL 0.5% pre–cooled propidium iodide (PI) of was added for staining for 30 min in dark, followed by centrifugation and cleansing in PBS. The apoptotic rate of cells and cell cycle were detected using flow cytometry. The cell proliferation activity was expressed as the S–phase fraction (SPF): SPF= S/(G1/G0+S+G1/M)×100%. The changes in cell cycle and the cell apoptotic rate were analyzed using multicycle software.

Statistical analysis. All analyses were performed using the SPSS version 10. The volume and the weight of transplanted esophageal carcinoma were expressed as mean ± SD. The between–group mean values were compared using univariate analysis. A statistically significant difference was considered if a P value was < 0.05.

Results

DC –EC109 cell fusion vaccine and T lymphocytes. The findings of the phenotype of DCs, the esophageal carcinoma cells, the DC –EC 109 fusion cells, and the results of T lymphocytes were consistent with the previous outcomes.

Growth of implanted esophageal carcinoma in nude mice and survival post after the treatment. Successful establishment of the esophageal carcinoma model was considered if the diameter of the subcutaneous nodule was over 5mm. In the first batch of 20 nude mice with subcutaneously implanted esophageal carcinoma, the esophageal carcinoma was successfully established in 15 nude mice and one mouse died in Group A during the treatment. In the second batch of 20 nude mice, 18 survived and none died during the treatment. In the two batches, a total of 33 mice bore successfully established esophageal carcinoma. The period of esophageal carcinoma establishment was consistent in the 33 mice and the overall success rate of carcinoma establishment was 82.5%.

Comparison of the mean volume and the mean weight of transplanted esophageal carcinoma, and the tumor inhibitory rate. The macroscopic views of esophageal carcinoma in each group are specified in Figure 1. The mean volume and the mean weight are detailed in Table 1. Statistical analysis showed that the mean volume and the mean weight of the esophageal carcinoma in Group A were significantly lower than those in Groups B and C. Statistically significant differences were noted (Table 1). The tumor inhibitory rates were 56.86% and 32.35%, respectively, in Group A and Group B, implying that the activated CTLs induced by DC/EC109 fusion vaccine have better tumor inhibitory effects than the
Table 1  The mean volume and the mean weight of xenograft tumors in groups A, B and C

<table>
<thead>
<tr>
<th></th>
<th>Group A (n=10)</th>
<th>Group B (n=11)</th>
<th>Group C (n=11)</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean volume (mm³)</td>
<td>881.45±31.14</td>
<td>1493.37±51.67</td>
<td>2065.77±87.55</td>
<td>950.67</td>
<td>0.000</td>
</tr>
<tr>
<td>Mean weight (g)</td>
<td>0.88±0.04</td>
<td>1.38±0.07</td>
<td>2.04±0.11</td>
<td>1335.90</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*One nude mouse died during the treatment.

![Figure 1 Macroscopic view of subcutaneously transplanted tumors in nude mice for the first time (A) and the second time (B)](image)

Results of PCNA in transplanted esophageal carcinoma. The IHC staining results indicated that PCNA was expressed in all three groups highlighted as brown or buffy granules. The mean PCNA-LI in Group A, Group B and Group C were (26.83±0.95)%, (51.82±1.51)% and (68.93±2.40)%, respectively. Statistical analysis showed that PCNA-LI in Group A was statistically significantly lower than that in Group B and Group C (F=1528.39, P=0.000).

Results of cell cycle and cell apoptotic rate. The proportions of cells in the G0/G1 phase, the S phase and the G2/M phase were apparently different in the A, B and C groups (Fig. 2). The SPF was lower in Group A than in Group B and Group C, with statistically significant differences. The mean apoptotic rates in Group A, Group B and Group C were (38.03±1.21)%, (17.75±0.56)% and (6.59±0.22)% respectively. The mean apoptotic rate in Group A was higher than that in Group B and Group C, with statistically significant differences (Table 2).

Table 2  Comparison of the mean SPF and the mean rate of cell apoptosis among groups A, B and C

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38.03±1.21</td>
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<tr>
<td></td>
<td>76.67±2.26</td>
<td>12.46±0.36</td>
<td>11.32±0.41</td>
<td>17.75±0.56</td>
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<tr>
<td></td>
<td>61.25±2.13</td>
<td>29.39±0.96</td>
<td>9.15±0.23</td>
<td>6.59±0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51.23±1.86</td>
<td>42.25±1.43</td>
<td>7.26±0.34</td>
<td>4.56±0.51</td>
<td></td>
</tr>
<tr>
<td>F value</td>
<td>–</td>
<td>–</td>
<td></td>
<td>–</td>
<td>0.001</td>
</tr>
<tr>
<td>P value</td>
<td>–</td>
<td>–</td>
<td></td>
<td>–</td>
<td>0.001</td>
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</tbody>
</table>

SPF: S-phase fraction.

![Figure 2 Cell cycle distribution of subcutaneously transplanted tumor cells of groups A, B and C](image)

The mean S-phase fraction of cell cycles of xenograft tumors in group A is (12.46±0.36)% (A), in group B is (29.39±0.96)% (B), and in group C is (42.25±1.43)% (C).
Discussion

Growth inhibitory effects of intra–tumor injection of CTLs on transplanted esophageal carcinoma cells. The previous study demonstrated that CTLs induced by DC/EC109 cell fusion vaccine significantly kill human esophageal cancer EC109 cells in vitro. The current research, using intra–tumor injection of CTLs induced by DC/EC109 cell fusion vaccine in the esophageal carcinoma established in the T lymphocytes–immunodeficient mice, found that the mean volume and the mean weight of the transplanted esophageal carcinoma, as well as the tumor inhibitory rate in the group treated with activated T lymphocytes as the effector cells were significantly lower than those in the group treated with non–activated T lymphocytes and in the control group. The findings further demonstrated that the antigen–specificity of CTLs induced by DC/EC109 cell fusion vaccine stayed stable in vitro and the antigen–specific CTLs significantly suppressed tumor growth, implying that the CTLs have consistently specific killing effects on esophageal carcinoma both in vitro and in vivo, in line with the findings of other studies. Sabzevari et al. established human melanoma subcutaneously in immunodeficient nude mice and inject it using activated CTLs and interleukin –2. They found that CTLs significantly suppressed growth and metastasis of transplanted tumors. Compared with this research, though in vitro amplification inactivation of CTLs were different in the current research, the CTLs with different resultant characteristics in two studies both had strong anti–tumor activity.

Influence of antigen–specific CTLs on tumor cell proliferation. Uncontrolled cell proliferation and apoptosis are major causes of development and progression of tumor. PCNA is a coenzyme of DNA polymerase that participates in synthesis of nuclear DNA and is closely correlated with cell proliferation. As PCNA reflects cell proliferation, it is normally used as a cell proliferation marker to evaluate malignancy and proliferation potential of the tumor. Chemotherapy or biotherapy directly kills tumor cells in the mitotic phase, and further has an impact on expression of PCNA. Consequently, PCNA in tumor cells serves as an index representing tumor proliferation and evaluating efficacy of cancer treatment. In the current research, the mean PCNA–LI was significantly lower in the treatment groups than that in the control group, implying that intra–tumor injection of CTLs induced by DC/EC109 cell fusion vaccine assists to suppress tumor proliferation and enables the tumor in a growth–inhibitory condition. The findings in the current research demonstrated at the cellular and molecular level that CTLs induced by DC/EC109 cell fusion vaccine have significant inhibitory effects on EC109 subcutaneously implanted in nude mice.

Influence of antigen–specific CTLs on tumor cell cycle and cell apoptosis. In the cell cycle, S phase reflects tumor cell proliferation and the level of malignancy. The chemotherapy or biotherapy directly influences proportions of all phases in the tumor cell cycle. Consequently, detection of SPF in tumor cells helps reflect the cell proliferation capacity and proliferation rate, and evaluate efficacy of cancer therapy. The current research found that the G2/M phase was prolonged and the SPF was decreased significantly in the treatment group of activated CTLs, with the mean apoptotic rate significantly higher than that in the control group, implying that following intra–tumor injection of CTLs induced by DC/EC109 cell fusion vaccine, the tumor growth was suppressed, the activity of tumor proliferation was decreased, the cells in the mitotic phase was decreased and the apoptotic cells were increased. All these indicate that CTLs induced by DC/EC109 cell fusion vaccine suppress growth of tumor cells through inhibiting cell cycle and inducing tumor cell apoptosis.

In conclusion, the current research successfully established the nude mouse model subcutaneously implanted with human esophageal carcinoma EC–109 cell line and demonstrated that subcutaneous injection of the antigen–specific CTLs induced by DC/EC–109 cell fusion vaccine suppressed growth of transplanted esophageal carcinoma. The study indicates at the cell biological level that the antigen–specific CTLs induced by DC/EC109 cell fusion vaccine inhibit tumor cell proliferation and induce tumor apoptosis for anti–tumor effects, which solidifies the experimental basis for application of fusion vaccine in clinical practice.
References


