Full Length Research Paper

Effects of combination gene therapy system pcDNA3.1(-)-shVEGF/yCDglyTK on proliferation of gastric cancer cells

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Accepted 14 December, 2010

The U6-shRNA expression cassette of pGenesil-1-hVEGF4-shRNA was subcloned into the skeleton vector, fusion suicide gene pCDNA3.1(-)-CV-yCDglyTK synthesized in preliminary studies to establish the combination gene therapy system pCDNA3.1(-)-shVEGF/yCDglyTK, so as to investigate the effects of the combination gene therapy system on proliferation and apoptosis of gastric cells. MTT assay showed that cell growth in the pcDNA3.1(-)-CV-yCDglyTK and pcDNA3.1(-)-shVEGF/yCDglyTK groups was significantly inhibited. Compared with cells transfected with pcDNA3.1(-)-CV-yCDglyTK, cells transfected with pcDNA3.1(-)-shVEGF/yCDglyTK were more sensitive to 5-fluorocytosine (5-FC) (P < 0.05). Flow cytometry indicated that the apoptotic rate of SGC7901 cells in the pcDNA3.1(-)-shVEGF/yCDglyTK group was as high as (67.9 ± 4.78)%, which was significantly higher than the other four groups (P < 0.05). The combination gene therapy system pcDNA3.1(-)-shVEGF/yCDglyTK can effectively suppress SGC7901 cell proliferation and induce apoptosis of these gastric cancer cells, the effects of which is higher than simple RNA interference (RNAi) or suicide gene therapy.

Key words: Suicide gene, RNA interference, vascular endothelial growth factor, gastric cancer, calcium phosphate nanoparticle.

INTRODUCTION

Gastric cancer is one of the most commonly seen malignant tumors in human beings, and is the fourth leading malignant tumor, with the second mortality among malignant tumors in the world (Parkin et al., 2005). Malignant tumors are a kind of multistage acquired genetic disease, the growth, invasion and apoptosis of which are related with genes. At least 60% of clinical trials have shown that malignant tumors are the best indications for gene therapy. Thus, gene therapy has become a research hotspot today for gastric cancer. In gene therapy strategies for gastric cancer, suicide gene therapy has considerable potential for clinical application.

RNA interference (RNAi) is also a new gene blocking technology developed in recent years on the basis of transcription, which has the characteristics of high efficiency, specificity and rapidity, as well as expands the targets for gene therapy. Vascular endothelial growth factor (VEGF) that is over-expressed in a variety of solid tumors can promote angiogenesis. Its expression level is closely related to tumor infiltration, microvessel density as well as tumor metastasis and recurrence. Research on angiogenesis and the related microcirculation of solid tumors and anti-tumor angiogenesis have become one of the hotspots in cancer research today. The technology of VEGF-targeted RNAi is an effective strategy for inhibiting tumor cell proliferation, migration, invasion and angiogenesis (Rosen, 2005). In this study, with calcium phosphate nanoparticles as vectors, the U6-shRNA expression cassette of pGenesil-1-hVEGF4-shRNA was subcloned into the skeleton vector, fusion suicide gene

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pCDNA3.1(-)-CV-yCDglyTK synthesized in preliminary studies to establish the combination gene therapy system pCDNA3.1(-)-shVEGF/yCDglyTK, so as to investigate the effects of the combination gene therapy system on proliferation and apoptosis of gastric cells.

MATERIALS AND METHODS

Materials

Cells and plasmids

Human gastric cancer cell line SGC7901 was preserved in the Medical Experiment Research Center of Xiangya Hospital Affiliated to Central South University and was preserved in the Medical Experiment Research Center of Xiangya Hospital Affiliated to Central South University and cultivated in RPMI-1640 cell culture medium containing 10% calf serum. The eukaryotic expression plasmids pCDNA3.1(-)-null, pGenesil-1-hVEGF4-shRNA, pCDNA3.1(-)-CV-yCDglyTK and pCDNA3.1(-)-shVEGF/yCDglyTK were all constructed in preliminary experiments(Liu et al., 2005, 2006).

Major reagents

Calcium phosphate nanoparticles were synthesized in preliminary experiments. Trizol was purchased from the Promega Corporation, RT-PCR Kit from TOYOBO, mouse anti-β-actin from Sigma, rabbit anti-yCDglyTK, mouse anti-VEGF and mouse secondary antibodies from Santa Cruz, and rabbit secondary antibodies from Amersham; 5-Fluorocytosine (5-FC) and dimethyl sulfoxide (DMSO) were purchased from the Sigma Corporation; modified RPMI-1640 and calf serum were both purchased from Hyclone.

Methods

Transfection

The experiment was carried out in five groups: (1) SGC7901 non-transfection group; (2)pCDNA3.1(-)-null group; (3) pGenesil-1-hVEGF4-shRNA group; (4) pCDNA3.1(-)-CV-yCDglyTK group; (5) pCDNA3.1(-)-shVEGF/yCDglyTK group. Transfection was conducted according to the method of calcium phosphate nanoparticle-based gene transfection (Boucher et al., 2006). Cells were seeded at a density of 2 x 105 cells/well onto a six-well culture plate, blended at the ratio of 1 : 15, 0.05 M CaCl2 and after 20 min blending, it was moved to well plate for transfection. At 6 h following transfection, complete culture medium containing 10% calf serum was employed instead of the previous culture medium. At 48 h following transfection, cells were harvested for reverse transcription polymerase chain reaction (RT-PCR) and at 72 h following transfection, cells were collected for Western blotting.

Detection of the relative survival rate using the methyl thiazolyl tetrazolium (MTT) assay

SGC7901 cells in the logarithmic growth phase were digested with trypsin and seeded at a density of 8 x 103 cells/well onto a 96-well culture plate containing four double wells. When the degree of cell fusion reached 70 to 80%, cells were transfected with the four kinds of plasmids, un-transfected SGC7901 cells as the control, and at 6 h following transfection, complete culture medium was used instead. At 24 h following transfection, complete culture medium containing 5-FC at a final concentration of 200 µg/ml was used instead. The MTT method was applied to do cell viability assays at day 1, 2, 3, 4, 5 and 6 after administration (20 µl MTT at a dose of 5 mg/ml was added to each well, after 4 h cultivation of cells, the supernatant was removed and then DMSO (200 µl/well) was added, so as to determine the light absorption value using the ELISA analyzer 10 min following oscillation away from light). Finally, the cell growth curve was plotted, time as abscissa and light absorption value as ordinate.

Analysis on SGC7901 cells in different transfection groups using flow cytometry

Cells were seeded at a density of 2 x 105 cells/well onto a 6-well plate and transfected for 6 h with the four kinds of plasmids when the degree of cell fusion reached 90%. Immediately after transfection, cells were incubated with 200 ug/ml 5-FC for 48 h, and then the supernatant and cells were collected, centrifuged, rinsed with PBS and fixed with 75% pre-cooled alcohol for over 24 h. The apoptotic rate of cells was detected using flow cytometry.

RT-PCR detection of the expression levels of CDglyTK mRNA and VEGF mRNA in SGC7901 cells of different transfection groups

The two-step method was adopted for RT-PCR. Primers used were as follows: yCDglyTK gene primers: p1 5'-GGGAGATTA GAGGAAATGTG-3'; p2 5'-ACGGCGTCGTCGTCGGCAATA-3', with the length of the PCR product being 707 bp. VEGF primers: p1 5'-TCTTTCAAGGCATCTCGTGTG-3'; p2 5'-ATCCGCTAATCTGCATGG T-3', with the product length of 112 bp. Internal control β-actin primers: p1 5'-AGCGAGCATCCCTAGGAAGT; p2 5'-GGGCAGAAGGCTCATCATT-3', with the product length of 285 bp. Reaction parameters: 95°C, 5 min; 98°C, 30 s; 55°C (including the VEGF gene annealing temperature of 53°C, 30 s; 72°C, 60 s; 32 cycles; 72°C, 7 min. After 1.5% agarose gel electrophoresis of PCR products, the image analysis software BandScan was applied to calculate the optical density ratio between the two gene bands and internal control band, representing the relative intensity of the content of corresponding genes.

Detection of the expression levels of yCDglyTK protein and VEGF protein in SGC7901 cells using Western blotting

Cell lysis buffer of SGC7901 cells was respectively collected 72 h after transfection, which was separated by 15% polyacrylamide gel electrophoresis, electrotransferred onto PVDF membranes, blocked at room temperature for 1 h as well as incubated at 4°C overnight using primary antibodies and at room temperature for 1 h using secondary antibodies for color development. The image analysis software BandScan was applied to analyze the results using β-actin as an internal control.

Statistical analysis

SPSS13.0 software was applied for statistical processing; specimens were compared using one-way ANOVA and the test for homogeneity of variance. When the variance was homogeneous, inter-group comparison was conducted using analysis of variance and the LSD-t test and when the variance was in homogeneous,
inter-group comparison was conducted using the Kruskal-Wallis H and Nemenyi tests. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

**MTT method**

The 5-FC displayed inhibitory effects on SGC7901 cells transfected with both pCDNA3.1(-)-CV-yCDglyTK and pCDNA3.1(-)-shVEGF/yCDglyTK, and the inhibitory effect was greater on SGC7901 cells transfected with the latter. The difference between the two groups was statistically significant ($P < 0.05$). There were no significant changes in the other three groups (Figure 1).

**SGC7901 cell apoptosis induced in different groups**

The apoptotic rate of group E, namely the pCDNA3.1(-)-shVEGF/yCDglyTK group, reached 72.4%, which was significantly higher than those of the other four groups. Differences were statistically significant ($P < 0.05$) (Table 1 and Figure 2).

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**Table 1. The ratio of SGC7901 cell apoptosis induced in different groups ($x \pm s$, n=3).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Rate of cell apoptosis (%)</th>
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<tbody>
<tr>
<td>(1) SGC7901 non-transfection group</td>
<td>3.67 ± 0.06</td>
</tr>
<tr>
<td>(2) pCDNA3.1(-)-null group</td>
<td>4.61 ± 0.05</td>
</tr>
<tr>
<td>(3) pGenesil-1-hVEGF4-shRNA group</td>
<td>21.5 ± 1.25※</td>
</tr>
<tr>
<td>(4) pCDNA3.1(-)-CV-yCDglyTK group</td>
<td>58.5 ± 2.24※</td>
</tr>
<tr>
<td>(5) pCDNA3.1(-)-shVEGF/yCDglyTK group</td>
<td>72.4 ± 3.72※</td>
</tr>
</tbody>
</table>

*vs (1) and (2), $P < 0.05$; ‘vs (1) and (2) and (3) and (4), $P < 0.05$.

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**Expression levels of yCDglyTK mRNA and VEGF mRNA in SGC7901 cells**

The yCDglyTK mRNA was expressed after transfection of pCDNA3.1(-)-CV-yCDglyTK and pCDNA3.1(-)-shVEGF/yCDglyTK into gastric cancer cells; no yCDglyTK-mRNA was expressed in the SGC7901 non-transfection group and the pCDNA3.1(-)-null group. Compared with SGC7901 non-transfection group and the pCDNA3.1(-)-null group, the pGenesil-1-hVEGF4-shRNA and pCDNA3.1(-)-shVEGF/yCDglyTK groups had lower expression levels of intracellular VEGF mRNA and the results were shown to be statistically different after analysis by the image analysis software BandScan ($P < 0.05$) (Table 2 and Figures 3A-B).

**Expression of yCDglyTK and VEGF proteins in SGC7901 cells by Western blotting**

The yCDglyTK protein was expressed in both the pCDNA3.1(-)-CV-yCDglyTK and pCDNA3.1(-)-shVEGF/yCDglyTK groups and no yCDglyTK protein was expressed in the SGC7901 non-transfection group and the pCDNA3.1(-)-null group. The expression levels of the
Figure 2. SGC7901 cell apoptosis induced in different groups. A. SGC7901 non-transfection group, B. pCDNA3.1(-)-null group, C. pGenesil-1-hVEGF4-shRNA group, D. pCDNA3.1(-)-CV-yCDglyTK group, E. pCDNA3.1(-)-shVEGF/yCDglyTK group.

Table 2. The expression levels of the VEGF protein.

<table>
<thead>
<tr>
<th>Groups</th>
<th>RT-PCR (VEGF/β-actin)</th>
<th>Western blotting (VEGF/β-actin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) SGC7901 non-transfection group</td>
<td>0.41 ± 0.02</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>(2) pCDNA3.1(-)-null</td>
<td>0.40 ± 0.02</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>(3) pGenesil-1-hVEGF4-shRNA</td>
<td>0.17 ± 0.01※</td>
<td>0.36 ± 0.01※</td>
</tr>
<tr>
<td>(4) pCDNA3.1(-)-CV-yCDglyTK</td>
<td>0.38 ± 0.02※</td>
<td>0.61 ± 0.02※</td>
</tr>
<tr>
<td>(5) pCDNA3.1(-)-shVEGF/yCDglyTK</td>
<td>0.15 ± 0.01▲</td>
<td>0.46 ± 0.02▲</td>
</tr>
</tbody>
</table>

※P < 0.05, vs (1) and (2); ▲P < 0.05, vs (1) or (2) or (4).
cells at the G0/G1 phase, decrease of cells at the S phase and cell cycle arrest at the G0/G1 phase. The expression level of VEGF mRNA in the VEGF-targeted RNAi group was greatly decreased and the corresponding level of VEGF protein was also significantly reduced. Some studies have shown that VEGF siRNA can effectively inhibit tumor cell proliferation, migration, invasion and angiogenesis (Ye et al., 2010).

In order to explore a more powerful gene therapy system of gastric cancer, our preliminary studies combining the fusion suicide gene and RNAi targeting VEGF, to construct the recombinant plasmid pCDNA3.1(-)VEGF-siRNA/yCDglyTK. Based on the pCDNA3.1(-)-CV-yCDglyTK skeleton, the U6-shRNA expression cassette of pGenesil-1-hVEGF4-shRNA was subcloned into the skeleton vector to construct the combination gene therapy system, so as to confirm the proliferation-inhibiting and apoptosis-inducing effects of this combination gene therapy system on gastric cancer cells. The expression levels of VEGF mRNA and protein were both significantly down-regulated in cells transfected with the combination plasmid pCDNA3.1(-)-shVEGF/yCDglyTK. The results of this experiment showed that 5-FC had an inhibitory effect on SGC7901 cells transfected with pCDNA3.1(-)-CV-yCDglyTK and pCDNA3.1(-)-shVEGF/yCDglyTK, and the inhibitory effect was more obvious on SGC7901 cells transfected with the latter, which were in line with what we had found in preliminary study (Wang et al., 2008). The rate of SGC7901 cell apoptosis within 48 h after transfection with pCDNA3.1(-)-shVEGF/yCDglyTK could reach 72.4% under the effect of 5-FC, which was significantly higher than those after simple transfection with the plasmid pGenesil-1-hVEGF4-shRNA or pCDNA3.1(-)-CV-yCDglyTK, indicating that the combination plasmid has a better apoptosis-inducing effect. It has been confirmed in this study that the plasmid pCDNA3.1(-)-CV-yCDglyTK and the combination expression plasmid pCDNA3.1(-)-shVEGF/yCDglyTK can successfully transfect gastric cancer cell line SGC7901 under the mediation of calcium phosphate nanoparticles, so as to induce its expression of the fusion suicide gene yCDglyTK. The above-mentioned experimental results further confirms the existence of synergy in such a combination gene therapy system which can obviously enhance the efficacies of inhibiting gastric cancer cell proliferation and inducing tumor cell apoptosis, which is in accord with the research results of other combination gene therapies in China (Liu et al., 2006).

The key to gene therapy lies in the vector. In this study, calcium phosphate nanoparticles are made use of due to their features of low toxicity, high transfection efficiency and target DNA protection etc (Boucher et al., 2006), so that they can mediate combination gene transfection and endow our combination gene therapy system with more superiorities. In order to enhance the targeting ability of gene therapy, the pCDNA3.1(-)-CV-yCDglyTK skeleton...
with the ability of target expression was employed during construction of the combination gene therapy system, which was driven by the CEA gene promoter to transform 5-FC to 5-FU only in CEA-positive cells. As for gastric cells with a high expression of CEA, such a killing effect has its specificity. Although, VEGF can exert certain impact on tumor cells through the VEGF/VEGFR-2 autocrine loop (Dias et al., 2000), it has been confirmed in our study that the survival curve of gastric cells transfected with pGenesil-1-hVEGF4-shRNA was different from that of the pCDNA3.1(-)-null group. The rate of SGC7901 cell apoptosis was 21.5% for the pGenesil-1-hVEGF4-shRNA group, as confirmed by flow cytometry. Nonetheless, the main effect of VEGF lies in the stimulation of tumor angiogenesis in tumor tissues and provides necessary blood supply for tumor tissue growth, so as to promote the progression of tumors. Since there was no blood vessel inhibition during the cell experiment, the effect of RNAi targeting VEGF still awaits further confirmation by animal experiments.

In conclusion, combination gene therapy mediated by calcium phosphate nanoparticles has better efficacy than simple gene therapy, in addition to its superiority in vector mediation of gene expression. Combination gene therapy can inhibit tumor cell proliferation and promote tumor cell apoptosis through its different mechanisms of action, providing new ideas for gene therapy of gastric cancer.

**ACKNOWLEDGEMENT**

This work was supported by grants from the National Natural Science Foundation of China (30800518) and The Ph.D. Programs Foundation Ministry of Education of China (200805331090).

**REFERENCES**


