RESEARCH COMMUNICATION

Adenovirus-mediated Double Suicide Gene Selectively Kills Gastric Cancer Cells

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Abstract

   The aim of this study was to evaluate the effect of the adenovirus-mediated double suicide gene (CD/TK) for selective killing of gastric cancer cells. Gastric cancer cells SCG7901 and normal gastric epithelial cell lines were infected by adenoviruses Ad-survivin/GFP and Ad-survivin/CD/TK. GFP expression and CD-TK were detected by fluorescence microscopy and reverse transcriptase polymerase chain reaction (RT-PCR), respectively. After treatment of the infected cells with the pro-drugs ganciclovir (GCV) and/or 5-FC, the cell growth status was evaluated by methyl thiazolyl tetrazolium assay. Cell cycle changes were detected using flow cytometry. In nude mice bearing human gastric cancer, the recombinant adenovirus vector was injected directly into the tumor followed by an intraperitoneal injection of GCV and/or 5-FC. The subsequent tumor growth was then observed. The GFP gene driven by survivin could be expressed within the gastric cancer line SCG7901, but not in normal gastric epithelial cells. RT-PCR demonstrated the presence of the CD/TK gene product in the infected SCG7901 cells, but not in the infected normal gastric epithelial cells. The infected gastric cancer SCG7901, but not the gastric cells, was highly sensitive to the pro-drugs. The CD/TK fusion gene system showed significantly greater efficiency than either of the single suicide genes in killing the target cells (P<0.01). Treatment of the infected cells with the pro-drugs resulted in increased cell percentage in G0-Gl phase and decreased percentage in S phase. In nude mice bearing SCG7901 cells, treatment with the double suicide gene system significantly inhibited tumor growth, showing much stronger effects than either of the single suicide genes (P<0.01). The adenovirus-mediated CD/TK double suicide gene driven by survivin promoter combined with GCV an 5-FC treatment could be an effective therapy against experimental gastric cancer with much greater efficacy than the single suicide gene CD/TK combined with GCV or 5-FC.

Keywords: Suicide gene therapy - adenovirus - survivin promoter - gastric cancer

Introduction

   Gastric cancer is the most common malignancy of the digestive tract. More than 100 million new cancer patients arise each year worldwide, and some areas in China have ranked first in the number of deaths due to gastric cancer (Thompson et al., 1993; Patino, 1994; Topuz et al., 2002; Dicken et al., 2005; Shang and Pena, 2005). In recent years, with the advancement of cancer research, the pathogenesis, clinical diagnosis, and treatment of gastric cancer have progressed greatly. However, the diagnosis and treatment of gastric cancer are still beset with many difficulties and the overall treatment effect is not satisfactory (Kuramoto et al., 2009; Yamamoto et al., 2009; Li et al., 2010).

   Cancer gene therapy typically refers to exogenous genes used as gene products transfected into the patient to kill tumor cells. Cancer gene therapy, specifically suicide gene therapy, has become a new anti-cancer treatment after traditional surgery, radiation therapy, chemotherapy, and other treatments (Girald et al., 2011; Qiu et al., 2011). The suicide gene, also known as the pro-drug-sensitive gene, is a new gene developed for gene therapy. Generally, the suicide gene refers to a pro-drug convert gene or the cytotoxic gene receptor gene from prokaryotic organisms (viruses and bacteria) (Goya et al., 2004; Sharma et al., 2009). Gastric cancer suicide gene therapy refers to the suicide gene transfected into gastric cancer cells to produce the specific enzyme that converts the nontoxic pro-drug (or cytotoxic factor) to toxic products (or cytotoxic factor) to kill tumor cells. There are a variety of suicide genes, including herpes simplex virus-thymidine kinase (HSV-TK) and Escherichia coli cytosine deaminase gene (E. coli-cytosine deaminase, CD) The HSV-TK gene encodes thymidine kinase which can convert nucleoside analogs into diphosphate compounds, further metabolizing to toxic triphosphate, thereby inhibiting DNA polymerase from exerting its anti-tumor effect. The CD gene encodes cytosine deaminase, which can be metabolized into uracil, and 5-Fc, which can be metabolized into the

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cytotoxic 5-Fu. Therefore, the syntheses of RNA and DNA are inhibited, and in this sense, the CD gene plays a cytotoxic role. In breast cancer, glioma, colon cancer, and other tumors, treatments have conferred significant effects (Zwacka and Dunlop, 1998; Moriuchi et al., 2002; Brade et al., 2003). Due to the shortcomings of single suicide gene therapy, gene combination therapy has been used, but lack of high activity specific promoter limits its role in targeting tumors (Boulaiz et al., 2005; Eisold et al., 2006). Studies have shown that the survivin gene is highly expressed in gastric cancer (Luo et al., 2011), not in normal gastric tissues. Hence, this gene can be used as a cancer-specific promoter in cancer gene therapy.

Materials and Methods

Cell and animals

SGC7901 human gastric cancer cell lines, normal gastric epithelial cell line GES-1, and adenovirus Ad (GFP+) was obtained from our laboratory. The cells were cultured in RPMI1640 medium (GIBCO) supplemented with 10% newborn calf serum at 37 °C in a 5% CO_2-humidified incubator. SPF BALB/C female nude mice 4 to 6 weeks old, weighing 18 g to 20 g, were cultured in a clean environment and kept in good health.

Adenovirus packaging, amplification, purification, and titer determination

The procedure was performed according to the methods described in a previous report (Ma et al., 2006).

Expression of GFP driven by survivin promoter

Cells at the logarithmic growth phase were collected, and 4×10^5 cells were seeded in a 6-well plate until the cells reached approximately 90% confluence. Different infections (multiplicity of infection, MOI) of the recombinant adenovirus Ad-survivin-GFP and a little medium were added, after which the mixture was shook slowly. About 2 h to 3 h later, the medium was supplied and culturing was continued at 37 °C in a 5% CO_2 incubator. After 72 h, the green fluorescent protein (GFP)-positive cell percentage was counted under a fluorescence microscope.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted with Trizol reagent according to the protocol described by the supplier (TakaRa, Dalian, China). cDNA of CD/TK was obtained from the total RNA by RT-PCR according to standard protocols (Mosmann, 1983). The PCR primers were designed as follows: CD/TK (496 bp) upstream: 5'-CTGGAAGTGAAGCAGGAAGTCGCGCC-3' and downstream: 5'-GCATCTCTTTAACGCGCGCGTGATG-3'. The targeted DNA amplified specifically was confirmed by electrophoresis and sequencing. PCR products were analyzed using the GelWorks software after scanning the ethidium bromide-stained 1.5% agarose gel.

Cell viability

Cell viability was examined using a colorimetric assay based on the methyl thiazolyl tetrazolium (MTT) labeling reagent (Jiang et al., 2010). Human gastric cancer cell lines SGC-7901 were seeded in 96-well plates (6×10^3 cells/well) 24 h prior to serum starvation, followed by serum starvation for 24 h and incubation in serum-free medium. The cells were then incubated with different groups of pro-drugs (gancyclovir, GCV; 5-FC; GCV+5-FC; and control) for 48 h. After incubation at 37 °C for 3 h, the cells were lysed in 50% dimethylformamide and 20% SDS at 37°C. The optical densities (ODs) at 550 and 670 nm were measured using a plate reader, and the differential ODs between 550 and 670 nm (OD 550 nm to 670 nm) were determined. Experiments were repeated three times.

Flow cytometry analysis

Human gastric cancer cell lines SGC-7901 transfected with or without double suicide gene at a density of 1×10^6 were incubated in serum-free medium and in pro-drug GCV+5-FC for 48 h. The cells were collected and treated with RNase. The DNA was stained with propidium iodide. The cell cycle phase distribution was analyzed with CXP Cytometer 1.0 (BeckmanCoulter Cytomics 500).

Tumor xenograft model

Thirty BALB/c female mice aged 4 to 6 weeks were selected and respectively implanted with 2×10^6 SCG7901 cells. When the tumor grew to 0.5 cm in diameter, they were randomly divided into six groups (n=5). Group A was the negative control, group B was injected only with adenovirus, group C was injected with the pro-drugs GCV and 5-FC, group D was injected with adenovirus and pro-drugs GCV and 5-FC, group E was injected with adenovirus and pro-drug GCV, and group F was injected with adenovirus and 5-FC. The mice were observed daily. The time of tumor emergence in each group was recorded to compare the different tumor-forming times after transfection among the six groups. Five weeks later, the tumors were taken out and weighed to compare the different weights and volumes of tumors after transfection among the six groups. The formula used is as follows: inhibition rate = (average tumor weight in control group-average tumor weight treated group) / average tumor weight control group × 100%.

Statistical analysis

SPSS 13.0 software was used for statistical analysis and t test was used in the comparison between two groups. One-way ANOVA was used for multiple comparisons. P <0.05 was considered significantly different.

Results

Adenovirus packaging, amplification, purification, and titer determination

After the recombinant plasmid was transfected with 293 cells with Lipofectamine reagent for 24 h, the expression of GFP fluorescence was visible and reached a peak at 3 d to 5 d. The cells were collected at 7 d to 10 d, amplified repeatedly, and then centrifuged by CsCL density gradient to produce the adenovirus which has a 2×10^{11} pfu/ml titer.
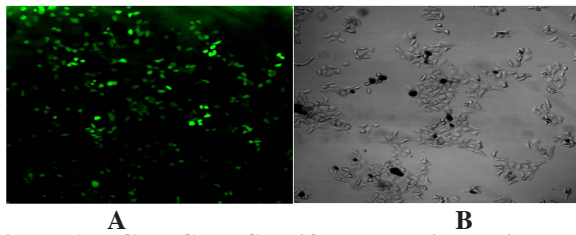
Figure 1. EGFP Gene Specific Expression Driven by Surivivin Promoter in the Gastric Cancer SCG7901 Cells. A: Gastric cancer SCG7901 cells; B: Gastric mucosal epithelial GES-1 cell
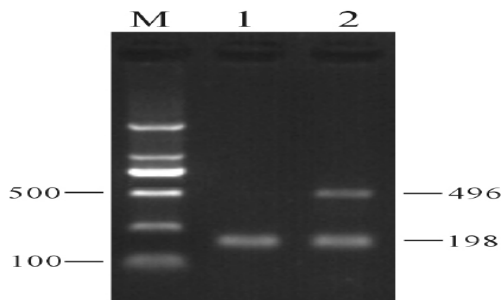
Figure 2. RT-PCR analysis of CD / TK Expression Detected in Gastric Cancer Cells Infected by Ad-survivin-CD/TK Fusion Gene

GFP expression

SCG7901 cells and normal gastric epithelial cell line GES-1 were infected by Ad-survivin-GFP. About 72 h later, fluorescence expression was detected in the SCG7901 cells, but not in the control GES-1 cells, indicating that survivin only expressed in gastric cancer cells, not in the normal gastric epithelial cells (Figure 1).

CD/TK expression

RT-PCR analysis revealed that CD / TK expression was only detected in gastric cancer cells infected by Ad-survivin-CD/TK fusion gene, whereas gene expression was undetectable in the control GES-1 cells (Figure 2).

Cell viability

Gastric epithelial cells infected by Ad-survivin-CD/TK were not sensitive to the pro-drugs. At concentrations of 100 mg/L GCV and 320 mg/L 5-FC, cell survival was 89.6%. SCG7901 cell survival was only 7.5% under the same concentration, which showed statistically significant difference (P <0.01). Cell cytotoxicity was dose-dependent using pro-drug GCV +5- FC on SCG7901. Cell survival decreased with increased drug concentration, whereas the growth of gastric epithelial cells was not significantly inhibited. Using only the pro-drug GCV (10 mg/L), the SCG7901 cell survival rate was 62%. Using 5-FC (160 mg/L), the survival rate was 65% and the combined survival rate was 15.6%, whereas the normal gastric epithelial cell survival was not significantly affected in the three groups (Figure 3).

Cell cycle analysis

After the SCG7901 cells were treated by the pro-drugs GCV (1 mg/L) and 5-FC (80 mg/L) for 48 h, the cell cycle was measured by flow cytometry. The results showed that the ratio of the G0–G1 phase in the negative
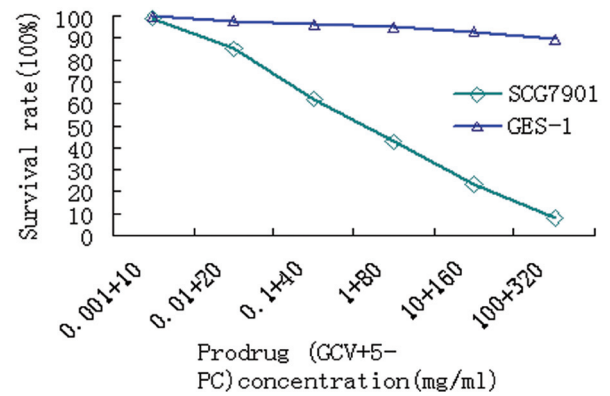
Figure 3. Cell Viability of Gastric Epithelial Cells and SCG7901 Infected by Ad-survivin-CD/TK at Different Concentrations of GCV and 5-FC

control group was 52.3% and that of the S phase was 24.6%. The G0-G1 phase ratio of the treatment group was 826% and that of the S phase was 16.5%. The percentage of cells blocked in the G0-G1 phase in the treatment group increased significantly, whereas the S phase cells decreased significantly. The difference was statistically significant (P <0.05).

Tumor-forming ability

The weights and inhibition rates of the tumor specimens in all groups were as follows: A, (585. 14 ± 19.75) mg; B, (576.43 ± l6.38) mg, inhibition rate of 1.48%; C, (581.1l ± l3.42) mg, inhibition rate of 0.69%; D, (102.04 ± 6.38) mg, inhibition rate of 82.56%; E, (225.13 ± 25.02) mg, inhibition rate of 61.52%; and F, (217.00 ± 16.53) mg, inhibition rate of 62.90%. The differences in the tumor weight between the groups were statistically significant (P<0.01). The difference among the D, E, F, and control (A) groups was statistically significant (P <0.01), suggesting the growth inhibition of gastric cancer in the three groups. Comparing B and C groups with the control group (A), the differences in tumor weight between the groups were not statistically significant (P>0.05), suggesting that adenovirus vector or pro-drug alone had no inhibitory effect on the tumor. The tumor volume in group D was the smallest, significantly smaller than those in groups E and F (P<0.01), suggesting that the inhibitory effect in the combination therapy was more significant than that in the single suicide gene.

Discussion

The application of a single enzyme/pro-drug system barely achieves the purpose of cancer cure, and multiple suicide gene combinations are some of the ways to improve the effect of such systems (Rittner et al., 2007). Therefore, the current study used the pAdEasy system to construct a CD/TK double suicide fusion recombinant adenovirus. The exact mechanism of the treatment system is that the thymidine kinase generated by the TK gene encoding could phosphorylate the nucleoside pro-drug (GCV) to transform into GCV triphosphate, which inhibits DNA polymerase functions or penetrates competitively into cells to terminate DNA synthesis, leading to cell death. In cancer gene therapy, target genes placed under

tumor-specific gene regulatory sequences can selectively express in host cells without affecting other cells (Bilsland et al., 2007). The survivin promoter-mediated gene could only express in the vast majority of gastric cancer cells, but not in normal gastric epithelial cells. Thus, survivin can be used as an ideal target for tumor biological therapy.

In the current study, SCG7901 and normal gastric epithelial cell GES-1 were infected by the suicide CD/ TK gene system. We found that pro-drug GCV+5-FC could induce SCG7901 non-necrotic cell injury in vitro when the transgenic cells were transfected with different concentrations of the pro-drug GCV and/or 5-FC. MTT showed that SCG7901 cell growth inhibition was dose-dependent on the pro-drug within a certain dose range. The CD/TK fusion gene in gastric cancer was more effective than any single suicide gene, but the gastric epithelium was not sensitive to the pro-drugs, suggesting that the suicide gene system regulated by the survivin gene was cell-specific. The flow cytometry results showed that the transgenic cells were blocked at the G0-G1 phase, suggesting that DNA synthesis was inhibited. Therefore, the double suicide gene system can specifically inhibit SCG7901 cell proliferation, which is related to cell cycle arrest. The animal experiments showed that the GCV 5-FC, GCV, and 5-FC group suicide gene system can effectively inhibit the growth of gastric cancer xenografts in nude mice, but the combination therapy performed significantly better than the single suicide gene, which further confirmed the effectiveness of double suicide gene system. The double suicide gene adenoviruses constructed in the current study can efficiently infect and kill the target cells. The application of the CD/TK gene prevented single suicide gene deficiencies. The survivin promoter optimized the efficacy of the system, which selectively affected the tumor cells. The survivin promoter had a non-toxic effect on normal gastric epithelial cells. The experimental system provided a foundation for further studies and clinical applications of such systems.

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