Tissue Specific Cytotoxicity of Colon Cancer Cells Mediated by Nanoparticle-delivered Suicide Gene In vitro and In vivo

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Abstract Purpose: This study aimed to develop an efficient and safe strategy to introduce suicide genes into colon cancer cells. Experimental Design: In this study, we fused an enhanced carcinoembryonic antigen promoter (CEA) to a suicide gene, cytosine deaminase (CD). This construct was delivered into colon cancer cells using calcium phosphate nanoparticles (CPNP). The cells were then treated with the prodrug 5-FC. The therapeutic effect was evaluated in vitro and in vivo. Results: Our study showed that the CEA promoter–driven, CPNP-delivered suicide gene was only expressed in CEA-positive colon cancer cells, and resulted in significant cytotoxicity after administration of the prodrug 5-FC in vitro. Moreover, our in vivo study showed that CPNP-mediated CEA-CD delivery, together with 5-FC treatment, resulted in significant tumor growth delay in xenograft human colon carcinoma. Conclusions: Our study indicates that the combination of CPNP and CEA-CD gene expression represents a novel approach for CEA-positive tumor gene therapy.

Colon cancer is one of the most frequently diagnosed malignancies in the world (1). The yearly incidence of colon cancer is estimated to be 1.0 million, and approximately 500,000 people die as a result of colon cancer worldwide (2, 3). Despite recent advances in chemotherapy, radiotherapy, and surgery, the survival rate of advanced colon cancer is still low. Therefore, better treatment options need to be explored in order to treat advanced colon cancer. Among various approaches, suicide gene therapy seems to be a very promising method. Suicide gene therapy is based on the intracellular delivery of a gene coding for an enzyme that transforms a prodrug into a cytotoxic product (4, 5). The most commonly used suicide genes are the herpes simplex virus type 1 thymidine kinase (TK) and Escherichia coli cytosine deaminase (CD). TK phosphorylates nucleotide analogues such as ganciclovir to monophosphate metabolites, which are further phosphorylated by endogenous kinase to form cytotoxic ganciclovir triphosphate (6). CD deaminates 5-fluorocytosine (5-FC) into the cytotoxic drug 5-fluorouracil (5-FU; ref. 7). Tumors can be eradicated even where only 10% of the neoplastic cells are transduced with the suicide gene, because of the bystander effect. For example, when the prodrug 5-FC is administrated systemically, CD expressed in localized areas of tumor can produce very high local concentrations of 5-FU, thus killing the neighboring tumor cells (8–10). Present suicide gene therapy, however, has had only limited success because it is prone to cause side effects due to lack of tumor tissue–specific expression (11). To reduce the side effects and achieve targeted gene therapy, tumor tissue–specific promoters have been applied to drive the expression of suicide genes. One commonly used tumor tissue–specific promoter is the carcinoembryonic antigen (CEA), which is overexpressed in most colon cancers (12). Therefore, targeted gene therapy of colon cancer can be achieved by coupling the CEA promoter with the suicide genes.

For the cancer gene therapy to become a practical clinical reality, it is necessary to develop delivery vectors that can transport therapeutic genes to the target site either locally or systemically (13). Viral vectors are commonly used in the delivery of therapeutic genes. However, viral vector transfection can induce severe immunotoxity as well as inadvertent gene expression changes after random integration into the host genome (14, 15). The safety issues of viral vectors have generated a surge in the development of nonviral gene delivery vectors over the last few years (16, 17). Among them, nanoparticulate systems are expected to have a major impact leading to the development of new types of therapeutic tools because of their versatility, ease of preparation, encapsulation of various sizes of plasmids, and protection of the encapsulated plasmid DNA (18, 19). One focus in nanobiotechnology is the development and use of nanoparticles for safe and efficient...
gene delivery in vivo (20, 21). Recent work by our group and others has established the feasibility of using the virus-like-sized calcium phosphate nanoparticles (CPNP) as a novel nonviral vector for in vivo gene transfection. The CPNP-DNA complex can enter the cells efficiently, therefore resulting in enhanced gene transfer (22, 23). In addition, the CPNP-DNA complex overcomes many of the limitations that often occurred with cationic lipid and polymer-based nanoparticles, including high toxicity on repeated use and potent inflammatory activity in vivo (24–26). However, the use of CPNP for delivery of therapeutic genes in vivo for colon cancer therapy has not yet been established. In the present study, we combined CPNP with tumor-specific expression of CD to evaluate the therapeutic efficacy in CEA-expressing human colon carcinoma cells, as well as in the xenograft human colon carcinoma (Fig. 1).

Materials and Methods

Cell lines and cell culture. We used the following cells in our studies: Lovo, a human colon cancer cell line (CEA positive), and HeLa, a human adenocarcinoma cell line (CEA negative). All of the cell lines were obtained from the Cancer Research Institute, Central South University. Cells were cultured in RPMI 1640 medium (Invitrogen Inc.) with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin at 37°C, 5% CO2.

Construction of tumor tissue–specific CD expression cassette. Human CEA promoter was amplified by PCR from human genomic DNA using forward primer 5'-CGACCCTAAATGCAGCTTGACGGTTCGTTGTG-3' and reverse primer with Xho I site (italic): 5'-AATCAGGCCGTTGTTACTGTTGTTTTGTCGTTGAC-3'. Cytomegalovirus (CMV) enhancer was amplified from pEGFP-N1 vector (Clontech, Takara Bio, Inc.) using the forward primer containing Xba I site (italic): 5'-AATCTGAACCATGCTGCGGTCCGTTGACT-3' and reverse primer: 5'-GCTAGAGCAACCCTACCCCGGTTCGTTGACT-3'. These two fragments were then fused by overlap PCR using the forward primer of CMV enhancer and reverse primer of CEA to produce a chimeric CMV-enhanced CEA promoter. To produce the CEA-CD cassette, CEA promoter and yeast CD were cloned into pcDNA3.1 (-) vector.

Construction of CPNP. The protocol of constructing CPNP has been previously reported (22). In brief, 50 mL of aqueous solution of calcium chloride, 1% SDS, and 1% F68 were dissolved by continuous stirring (300 rpm) for 24 h to form microemulsion A. Fifty milliliters of aqueous solution of disodium hydrogen phosphate, 50 mL of aqueous solution of sodium citrate, 1% SDS, and 1% F68 were dissolved by continuous stirring (300 rpm) for 24 h to form microemulsion B. Microemulsion B was slowly added to microemulsion A at the rate of 10 mL/h with continuous stirring at 35°C. The pelletted nanoparticles were washed with 75% ethanol and sonicated for 1 h. The dispersed nanoparticles were frozen at -70°C for 2 h, and then moved to the freeze drier.

Transfection in vitro. DNA was mixed with CPNP to generate the CPNP-DNA complex at the combination ratio of 2 µg DNA to 20 µg CPNP. Cells were seeded in a 6-well plate at a density of 2 × 105 cells per well. When cells reached 70% to 80% confluence, CPNP/CEA-CD or CPNP/CMV-CD was added into each well. To establish stable expression of CD in Lovo and HeLa cells, G418 (600 µg/mL) was applied after 48 h. Fifteen days later, a single colony was transferred to 12-well plates and screened for CD expression by reverse transcription-PCR (RT-PCR). CD-expressing Lovo and HeLa colonies were maintained under the selective pressure of G418 (200 µg/mL). For the cytotoxicity assay, 48 h after transfection, cells were treated with 5-FC for 5 d, and MTT assay was carried out to analyze cell viability.

RT-PCR assay. RT-PCR assays were done as described previously (27, 28). Total RNA was extracted using TRIzol reagent (Invitrogen). RT-PCR of CD was done using the AMV reverse transcription Kit (Promega Corporation) and primers: 5'-GGGAGTATAGGGAAAGTGT-3' and 5'-ACGGCTTCGTCGTAAGTAA-3'. Human β-actin gene was amplified as an internal control using the primers of 5'-ACCCCGCGAGCTCACC-3' and 5'-GGGGTGTTGAAGGTCTCAAA-3'.

Immunofluorescence assay. Cells were fixed in 2% formaldehyde-PBS buffer (pH 7.4) for 15 min at 20°C. After washing with PBS 3 × 10 min, cells were treated with PBS (pH 7.3) containing 0.2% Triton X-100 and 1% normal goat serum wash for 3 min, and then the cells were incubated with rabbit anti-CD antibody (1: 50 dilution; Promega) for 1 h at room temperature following the subsequent incubation of goat antirabbit IgG-FITC antibody (1: 50 dilution; Promega) for 1 h at 37°C in a humidified chamber. After washing with PBS for 3 × 10 min, a cover slip was mounted with a drop of mounting medium and sealed with clear nail polish.

MTT assay. To determine the cytotoxicity of prodrug 5-FC, Lovo cells stably expressing CEA-CD or untransfected Lovo cells were seeded in 96-well plates at 3,000 cells per well and incubated at 37°C for 24 h. The cells were then treated with 5-FC at different concentrations (0, 20, 50, 100, 200, 400, 800, and 1,200 µg/mL) for 5 d. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay was carried
out as described previously (28). In brief, 20 μL of MIT solution (20 mg/mL) were added to each well and incubated for 4 h. Then the reaction was stopped by DMSO (Promega) and the optical density was determined at wavelength 490 nm on a multwell plate reader (Bio-Rad model 3550, Bio-Rad).

Flow cytometry. Lovo cells stably expressing CEA-CD or CMV-CD and untransfected Lovo cells were seeded in 10-cm dishes. 5-FC was added when the cells reached 90% to 95% confluence. Cells were then pelleted, washed, and resuspended in staining buffer (HEPES-buffered PBS supplemented with 2.5 mmol/L CaCl2) prior to addition of FITC-labeled annexin V (15 min at 4°C). After that, flow cytometry was done on eosinophils gate on the basis of their forward and side light scatter with the exclusion of all cell debris. The flow cytometric analyzer was also used as a measurement of eosinophil apoptosis through the differences in the degree of red autofluorescence in the two populations.

Xenograft studies. Balb/c nude mice between 4 and 6 weeks of age were purchased from Shanghai Experimental Animal Center, China. The mice were fed and watered ad libitum and maintained according to established guidelines. For in vivo analysis of tumor growth, 1 × 107 untransfected HeLa or Lovo cells (24 mice), HeLa or Lovo cells stably expressing CEA-CD (6 mice) were injected s.c. into the flanks of Balb/C nude mice. When the tumor grew to the volume of 200 mm3, the mice inoculated with untransfected cells were randomized into four groups. According to our previously established protocol, the nanoparticles complex was formulated with a ratio of 10 μg of nanoparticles and 1 μg of DNA (22). Nanoparticle complexes (CPNP-vector, CPNP/CEA-CD or CPNP/CMV-CD) were injected into the tumor mass in three groups. After 3 d, 15 consecutive daily i.p. injections of 5-FC (500 mg/kg) were given to all of the five groups (6 mice per group). The growth curves were plotted as the mean of tumor volume ± SE (first day of 5-FC injection), and the formula \( V = \frac{1}{2} W^2 L \) (W, the shortest dimension; L, the longest dimension) was used to calculate tumor volume.

Statistical analysis. Statistical analysis was done by one-way ANOVA and the interactive term was evaluated by Bonferroni corrected pair-wise comparisons. \( P < 0.05 \) was considered to be statistically significant.

Immunohistochemical assays. Immunohistochemical staining was done using the streptavidin-peroxidase method. Serial 4-μm-thick sections were collected from the maximum cross-section of the colon xenografts. Sections were blocked with 10% normal goat serum in PBS supplemented with 2.5 mmol/L CaCl2) prior to addition of FITC-labeled annexin V (15 min at 4°C). After that, flow cytometry was done on eosinophils gate on the basis of their forward and side light scatter with the exclusion of all cell debris. The flow cytometric analyzer was also used as a measurement of eosinophil apoptosis through the differences in the degree of red autofluorescence in the two populations.

Results

CPNP effectively delivers DNA into Lovo cells in vitro. To check the transfection efficiency using CPNP, Lovo cells were transfected with CPNP/green fluorescent protein complex. Green fluorescent protein expressing Lovo cells were detected using flow cytometry 48 hours after transfection. As shown in Fig. 2, the transfection efficiency using CPNP is 53.38%. These data indicate that CPNP is an efficient strategy to deliver DNA into colon cancer cells.

CEA-CD is specifically expressed in CEA-positive Lovo cells. The expression of the fusion gene CD was analyzed by RT-PCR. CD was amplified from Lovo cells stably expressing CEA-CD and CMV-CD, and from HeLa cells stably expressing CMV-CD, but not from HeLa cells transfected with CPNP/CEA-CD (Fig. 3A). Furthermore, immunofluorescence experiments also showed a similar pattern (Fig. 3B). These data indicate that CEA-CD is expressed in a tissue-specific manner; that is, it is expressed only in CEA-positive cells.

CEA-CD/5-FC system results in cytotoxicity in Lovo cells. To test cytotoxicity induced by the CEA-CD/5-FC system, the unmodified Lovo cells and CEA-CD stably transfected Lovo cells were treated with various concentrations of 5-FC. Cytotoxicity was induced in a dose-dependent manner, and CEA-CD greatly sensitized the cytotoxicity (Fig. 4). For example, in the presence of 100 μg/mL 5-FC, the cell viability of unmodified Lovo cells was 74.12%, whereas the viability of CEA-CD stably transfected Lovo cells was 23.62%; in the presence of 200 μg/mL 5-FC, the cell viability of unmodified and CEA-CD stably transfected Lovo cells was 70.35% and 11.02%, respectively.

CEA-CD/5-FC system induces cell apoptosis. To determine if the CEA-CD/5-FC–induced cytotoxicity is associated with cell apoptosis, CEA-CD, CMV-CD stably transfected Lovo cells, and unmodified Lovo cells were treated with 5-FC (200 μg/mL) for 24 hours, and then analyzed by flow cytometry. The analysis found 38.68% and 29.25% apoptotic cells in CEA-CD and CMV-CD stably transfected Lovo cells, respectively. In contrast, only 0.42% apoptotic cells appeared in the unmodified Lovo cells (Fig. 5). These results suggest that 5-FC treatment could induce cell apoptosis in CD stably transfected Lovo cells.

CPNP/CEA-CD /5-FC induces cytotoxicity in vitro and in vivo. To evaluate the ability of CPNP/CEA-CD/5-FC to induce cytotoxicity in vitro, cell viability was analyzed in CPNP/CEA-CD, CPNP/CMV-CD transiently transfected Lovo cells, CEA-CD stably transfected Lovo cells (as positive control), and untransfected Lovo cells (as negative control) in response to
the treatment of 200 μg/mL 5-FC. As shown in Fig. 6A, in HeLa cells, the transfection of CEA-CD did not sensitise the cytotoxicity caused by 5-FC, whereas the transfection of CMV-CD did; in Lovo cells, the mean cell survival is 10.6% in CEA-CD-positive Lovo cells, 27.3% in the cells transiently transfected with CPNP/CEA-CD, 32.2% in cells transiently transfected with CPNP/CMV-CD, and 98% in untransfected Lovo cells. These results indicate that CPNP/CEA-CD/5-FC efficiently induced cytotoxicity in CEA-positive cells in vitro.

We further tested the effect of the CPNP/CEA-CD/5-FC system on the growth of xenograft colon carcinoma in vivo. With the treatment of 5-FC, the injection of CPNP/CMV-CD inhibited HeLa xenograft tumor growth, whereas the injection of CPNP/CEA-CD had little effect on HeLa xenograft tumor growth (Fig. 6B, upper panel). In contrast, both the CPNP/CMV-CD injection and CPNP/CEA-CD injection inhibited Lovo xenograft tumor growth, and the CPNP/CEA-CD injection seemed to be relatively more effective (Fig. 6B, lower panel). The average tumor volume of the CPNP/CEA-CD-injected group on the 36th day after tumor implantation was <47% of the tumor volume in the PBS-injected control group (P < 0.05). These data suggest that the CPNP/CEA-CD/5-FC system specifically inhibits CEA-positive tumor growth in vivo.

To study the expression and distribution of CD in the xenograft tumors, we did immunohistochemical assays on the xenograft tumor sections using anti-CD antibody. As shown in Fig. 7, there was no CD expression in the PBS-injected or CPNP/vector-injected xenograft tumor. By contrast, CD expression was detected in xenograft tumor injected with CPNP/CMV-CD or CPNP/CEA-CD.

**Discussion**

Strategies using tumor-specific promoters have been attempted in adenovirus-based gene therapy of colon and prostate cancers (29, 30). In the present study, the development of tumor-specific expression of suicide gene and the subsequent delivery with nonviral CPNP has been shown to be effective in inhibiting tumor growth and decreasing side effects. This conclusion is based on our findings that the complex of CPNP/CEA-CD was transfected into Lovo cells effectively and the fused suicide gene was successfully expressed at both mRNA and protein levels. The expression of CEA-CD triggers the subsequent cell death in CEA-positive Lovo cells but not in CEA-negative HeLa cells. CPNP efficiently delivers the CEA-driven CD gene into tumor cells in vitro and in colorectal cancer xenograft in vivo. The chimeric enhanced CEA promoter (CV) conducted a tissue-specific expression of the fused suicide genes CD, which was as effective as expression from the CMV promoter. Therefore, targeted gene therapy of colon cancer could be achieved by coupling the CEA promoter with the fused suicide gene. The combination of CPNP with the CEA-CD vector could represent a novel approach for colon cancer gene therapy.

Increasing tumor cell sensitivity to cytotoxic agents by transducing a gene coding a drug-activating enzyme is a promising strategy for cancer gene therapy. Currently, two suicide genes are being considered for human colon cancer gene therapy: the herpes-simplex-virus thymidine kinase (HSV-tk) and E. coli CD genes. Two fusion suicide genes, CD/UPRT (uracil phosphoribosyl transferase) and E. coli CD, were thought to be the most effective in converting nontoxic prodrugs into cytotoxic metabolites (29, 31, 32). After systemic administration of the produg, the cells that express the suicide gene can convert the produg into a toxic metabolite and cause death not only in the cells expressing the gene, but also in cells in close proximity, a phenomenon called the bystander effect (33, 34). However, present cancer therapy using suicide genes is limited due to side effects owing to lack of tumor tissue.

![Fig. 3: CEA-CD is specifically expressed in CEA-positive Lovo cells. A, RT-PCR detection of CD expression in Lovo and HeLa cells. Lane 1, DNA ladder; lane 2, untransfected Lovo cells; lanes 3 to 4, Lovo cells transfected with CPNP/CEA-CD or CPNP/CMV-CD; lane 5, untransfected HeLa cells; lanes 6 to 7, HeLa cells transfected with CPNP/CEA-CD or CPNP/CMV-CD. The β-actin serves as an internal control. B, immunofluorescence assay of CD expression. a, representative immunofluorescence stain of CEA-CD-transfected Lovo cells; b, representative IF of CMV-CD-transfected Lovo cells; c, representative IF of unmodified Lovo cells; d, representative IF of CPNP/CEA-CD-transfected HeLa cells; e, representative IF of CMV-CD-transfected HeLa cells; f, representative IF of unmodified HeLa cells.](image)

![Fig. 4: CEA-CD/5-FC system results in cytotoxicity in Lovo cells. Unmodified Lovo cells and CEA-CD stably transfected Lovo cells were treated with different concentration of 5-FC. After 5 d of incubation, the cell samples were subjected to MTT assay.](image)
specificity. Although tumor tissue–specific, promoter-driven suicide genes have been shown to be effective in adenovirus-based gene therapy of colon and prostate cancers (29, 30), the disadvantages of viral vectors, such as immunotoxicity, risk of recombination, and unwanted mutagenesis, limit its application in the clinic (35–37). The development of a safe and efficient nonviral gene delivery vector is a major challenge that needs to be overcome in order for gene therapy to become a clinical reality (19). Recently, the nanoparticulates system has become an attractive therapeutic tool for future tumor therapy. In this study, we first formulated the CPNP and suicide gene CD, which is driven by tissue specific CEA promoter, for local delivery into CEA-positive colon cancer xenograft.

Nanoparticles with a positive surface charge are taken preferentially in the tumor and retained for longer duration as compared with negatively charged or neutral particles (38). CPNP are virus-like sized particles with 23.5 to 34.5 nm diameters and have positive surface charges. Thus CPNP can transfer DNA into tumor cells with high transfection efficiency (22). Over the past couple of years, many toxicology reports have shown that exposure to nanotechnology derived particles poses serious risks to biological systems. This issue becomes even more serious for i.v. injected nanoparticles (25, 39). Our previous study has shown that CPNP have minimal cytotoxicity (22). In addition, a recent study by our group showed that CPNP efficiently delivered vascular endothelial growth factor-C small interfering RNA, and effectively inhibited lymphangiogenesis and growth of gastric cancer in vivo (40).

Although the present study was established in the xenograft tumor model by intratumoral injection of CPNP/CEA-CD complex, the properties of CPNP, such as positive surface charges, protection of the encapsulated plasmid DNA, and low toxicity, imply that systemic administration will also be efficient. We are evaluating the efficacy of the systemic administration of CPNP/CEA-CD complex in our laboratory.

By considering that nanoparticles are preferentially taken up by the liver (19, 41), and colorectal cancer usually results in liver metastases, nanoparticle-delivered CEA-CD could be more efficient in treating colon cancer with liver metastases, which usually does not adequately respond to conventional therapy. The specificity of this system would also make it suitable for...
preventive therapy, as the suicide genes would only be expressed in CEA-positive cells.

Our CPNP/CEA-CD /5-FC system may surmount four key issues for cancer gene therapy: efficiency, specificity, and safety. First, the use of the fusion suicide gene CD has been shown to be highly efficient in killing cancer cells. Second, we showed that CPNP could efficiently deliver the CEA-CD gene in vitro and in vivo. Third, CEA promoter–driven CD is only expressed in CEA-positive cells, such as many colon cancer cells, but not in CEA-negative cells, such as normal cells. Fourth, CPNP overcome the host innate immune responses compared with the viral vector, and have minimal cytotoxicity compared with the other nanoparticulates systems. Thus, our strategy of using CPNP and the CEA promoter–mediated CD gene expression represents a novel approach for future CEA-positive tumor gene therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


Clinical Cancer Research

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