Adenovirus-mediated Prodrug Gene Therapy for Carcinoembryonic Antigen-producing Human Gastric Carcinoma Cells in Vitro

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ABSTRACT

We analyzed the ability of a recombinant replication-defective adenovirus vector with the carcinoembryonic antigen (CEA) promoter to transfer the thymidine kinase gene of herpes simplex virus (HSVtk) into gastric cancer cells to confer sensitivity to ganciclovir (GCV). CEA-producing gastric cancer cell lines (MKN28 and MKN45), a CEA-nonproducing gastric cancer cell line (MKN1), and a human uterine cervical cancer cell line (HeLa) were infected with a recombinant adenovirus carrying lacZ reporter gene coupled to the CEA promoter (AdCEAlacZ). The efficiency of AdCEAlacZ-mediated gene transfer was correlated with the amount of CEA produced by each cell line. Furthermore, the 50% growth inhibitory concentrations (IC50) of GCV were 21 and 5.8 μM for MKN28 and MKN45, respectively, when infected with a recombinant adenovirus carrying the HSVtk gene coupled to the CEA promoter (AdCEAtk). However, MKN1 and HeLa cells infected with AdCEAtk remained resistant to GCV (IC50 > 300 μM of GCV). In addition, a bystander killing effect was demonstrated against MKN45 cells when only 20% of AdCEAtk-infected cells were mixed with uninfected cells. These data indicate the potential for targeted gene therapy using the cell type-specific promoter of the CEA gene against gastric cancers that produce CEA.

INTRODUCTION

Gastric carcinoma is one of the most prevalent form of cancers in East Asia, including Korea and Japan, and is moderately prevalent in Europe (1, 2). Although extensive surgical operations have been combined with chemotherapy and immunotherapy, the mortality of gastric cancer is still high, due to carcinomatous peritonitis and liver metastasis at advanced stages (2, 3).

Various systems of somatic gene therapy for cancer have been developed, including drug sensitization, immunomodulation, genetic replacement, and antisense strategies (4, 5). One of the most promising methods is drug sensitization, which includes the transfer of suicide genes into tumor cells to render them sensitive to chemicals that are relatively nontoxic to normal tissues (6). HSVtk3 gene transfer, followed by the administration of GCV, is one drug sensitization system that has been studied extensively (6–8). GCV is an acyclic nucleoside that is normally metabolized at very low rates by mammalian cells. It is transformed by HSVtk into a monophosphate form that is subsequently converted by mammalian kinases to GCV triphosphate. The GCV triphosphate then competes with normal nucleotides for DNA replication in mammalian tumor cells, thus inhibiting cell growth and causing cell death (9). In addition, established tumors in experimental models completely regress, even when only a relatively small proportion of tumor cells is transferred with the HSVtk gene and subsequently exposed to GCV (10, 11). This phenomenon is called “the bystander effect.”

Retroviral vectors have the advantage of mediating stable gene transfer into replicating cells with low immunogenicity. However, this vector delivery system has limited in vivo use, because there are some difficulties in producing high titers of retrovirus and there is a risk of insertional mutagenesis (12). Alternatively, adenovirus vector systems that can produce high titers of viruses capable of efficient expression in target cells have delivered exogenous genes into a variety of cells and tissues (13).

The specific expression of transfected suicide genes in tumor cells may be induced using the promoter element of genes usually transcribed only by tumor cells. About 50% of gastric cancers are serologically positive for CEA and, most of the gastric cancer cells express CEA (3, 14). Schrwe et al. (15) cloned the genomic DNA of CEA and demonstrated that a 440-bp segment upstream from the transitional start exhibited an apparently higher promoter activity in CEA-producing cells than in nonproducing HeLa cells. Thus, tumor-specific gene expression can be achieved by coupling the CEA promoter to the desired gene.

Several studies describe retroviral vectors that contain transcriptional control elements to confine the expression of suicide genes only in tumor cells (16–18). However, there are few adenovirus vector-carrying suicide genes under the control of a tumor-specific promoter. In this study, we constructed a novel adenovirus vector that can efficiently transfer the HSVtk gene controlled by the CEA promoter and analyzed the specificity of this adenovirus vector system using CEA-producing and nonproducing gastric cancer cell lines.

MATERIALS AND METHODS

Cell Culture. The human gastric cancer cell lines, MKN1, MKN28, and MKN45, human uterine cervical cancer cell line HeLa, and human embryonal kidney cell line 293 were obtained from American Type Culture Collection. Human gastric cancer cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. HeLa and 293 cells were grown in DMEM containing 5% fetal bovine serum and penicillin/streptomycin.

CEA Production by the Cells. CEA produced by the cells was measured as described previously (19). In brief, human gastric cancer cells were seeded on 6-well plates (Corning, New York, NY) at a density of 5 × 10^4/well and cultured at 37°C in a 5% CO2 humidified atmosphere for 48 h. The medium was then replaced with new medium. After another 48 h incubation, the cells were counted, and the supernatant was collected. The CEA content in the supernatant was measured by RIA (RIA Beads kit; Special Reference Laboratory Co., Ltd., Tokyo, Japan). In this assay, the minimal detectable levels of CEA were 0.5 ng/ml. The results are represented as the means ± SD of quadruplicates from three independent experiments.

Preparation of Adenovirus Vectors. The recombinant replication defective adenoviruses, AdCEAtk (adenovirus vector with HSVtk driven by the CEA promoter). AdPGKtk (adenovirus vector with HSVtk driven by the PGK promoter), AdCEAlacZ (adenovirus vector with lacZ driven by the CEA promoter), were constructed by homologous recombination between an expression cosmid and the parental virus genome, as described (20, 21), and
detailed procedures will be published elsewhere.\(^4\) Briefly, the cis-acting sequences, which contain the putative CEA promoter region upstream of between −424 and −2 bp from the translational start, were amplified by PCR as described (16, 17). pCEAlacZ was constructed by subcloning the −3.1-kb nuclear localized lacZ gene [controlled under the CEA promoter (−440 bp)] between the XhoI and BamHI sites of pUHD15−I (22, 23). The pAdex1CEA lacZ cosmid was constructed by subcloning the CEAlacZ-poly(A) expression cassette into the ClaI site of the pAdex1cw cosmid, which contained a 31-kb adenovirus type 5 genome lacking E1A, E1B, and E3 genes (21).

The pCEAtk plasmid was formed by subcloning the −1140-bp HSVtk fragment under the control of the CEA promoter (−440 bp) between the EcoRI and BglII sites of pCAGGS (24). The pBluescriptKI(+)−CEAtk plasmid was constructed by subcloning the CEAtk−poly(A) expression cassette into the EcoRI and BglII sites of the pBluescriptKI(+) (Stratagen, La Jolla, CA). This CEAtk−poly(A) expression cassette was excised by ClaI digestion and subcloned into the ClaI sites of the pAdex1cw (pAxcw) cosmids, resulting in pAdex1CEAtk.

To construct pAdex1PGKtk, the PGK-HSVtk-poly(A) expression unit was cut from pGEMtk and subcloned into the ClaI site of the pAdex1cw (pAxcw) cosmids. To produce a recombinant adenovirus, the expression cosmids and adenovirus DNA terminal protein complex were cotransfected into the 293 cells by calcium phosphate precipitation. Recombinant adenovirus was isolated from a single plaque and expanded in the 293 cells; then the viral solution were stored at −80°C. The virus titer was determined by plaque assay on 293 cells as described (21).

In Vitro Transduction by AdCEA lacZ. During the exponential growth phase, gastric cancer and HeLa cells were plated in 6-well culture plates (Corning) at a density of 5 × 10⁴ cells/well before the AdCEA lacZ infection. Immediately before infection, the culture medium was aspirated, and suspensions of adenovirus at various moi (from 0 to 30 moi) were distributed over the monolayers. After a 24-h incubation, β-galactosidase expression was evaluated using X-gal as the substrate as described (21, 25). Blue precipitate in the cell nucleus indicates a β-D-galactosidase expression.

In Vitro GCV Sensitivity of the Cells Infected with AdCEAtk. The gastric cancer and HeLa cells were seeded and cultured in 96-well plates (Corning) at a density of 3 × 10⁵ cells in 100 μl of medium for 24 h. Immediately before infection, the culture medium was removed from the wells, and suspensions of AdPGKtk, AdCEAtk, or AdCEA lacZ at a moi of 30 were placed onto the cell monolayers. After an incubation for another 24 h, the medium containing virus was replaced with fresh medium containing various concentrations of GCV (0 to 400 μM). The cells were then cultured at 37°C and in a 5% CO₂ humidified atmosphere for another 6 days, and the medium containing GCV was changed every other day. Cell growth was then assessed by means of the MTT assay that measures viable cell dehydrogenase activity as described (26, 27). Cell proliferation was proportional to the absorbance at the test wavelength (570 nm), from which the reference wavelength (620 nm) was subtracted. The IC₅₀ of GCV was calculated using a curve-fitting parameter, and the results are represented as means ± SD from three independent experiments.

Analysis of in Vitro Bystander Effect. The bystander effect was determined by mixing the infected and uninfected cells at various ratios. Briefly, MKN45 cells were infected with AdCEAtk at a moi of 30. Twenty-four h later, the infected cells were mixed with various concentrations of uninfected cells. To examine the role of intimate cell–cell contact on the bystander effect, the mixed MKN45 cells were seeded into 96-well plates at a range of densities (5 × 10⁵, 1 × 10⁵, and 2 × 10⁵ cells/well). The cells were cultured at 37°C in a 5% CO₂ humidified atmosphere in the presence of 40 μM of GCV for 5 days. The number of viable cells was then measured by the MTT assay. The relative percentage of survival of the cells was calculated as follows:

\[
\% \text{ cell survival} = \frac{B}{A} \times 100
\]

where \(A\) was absorbance from uninfected cells incubated with medium alone, and \(B\) was absorbance from the cells infected with the adenovirus vector and incubated with medium containing varying concentrations of GCV. Data represent the means ± SD of three independent experiments.

RESULTS

CEA Secretion by Gastric Cancer Cell Lines. MKN45 cells secreted about 16-fold more CEA than the MKN28 cells (MKN45 cells, 34.7 ± 3.9 ng/10⁶ cells/day; MKN28 cells, 2.1 ± 0.1 ng/10⁶ cells/day). In contrast, CEA secretion was not detected in MKN1 nor in HeLa cell lines.

Adenovirus-mediated Gene Transfer Efficiency in Gastric Tumor Cell Lines. To test the adenovirus-mediated gene transfer efficiencies under the control of CEA promoter, three gastric cancer cell lines (MKN1, MKN28, and MKN45) and HeLa cells were infected with AdCEA lacZ at various moi. At an infection ratio of moi 30, the lacZ gene product was expressed in about 42 ± 4.9% of MKN45 cells and in 24 ± 6% of MKN28 cells when stained with X-gal but only in 2 ± 0.1% of the MKN1 cells. HeLa cells did not express the lacZ gene product (Fig. 1).

CEA-producing Gastric Cells Are Sensitized to GCV by AdCEA tk-mediated Gene Transfer. To analyze the specificity of the CEA promoter induced by HSVtk gene expression, MKN1, MKN28, MKN45, and HeLa cells were infected with AdCEAtk or AdCEA lacZ at a moi of 30 and exposed to various concentration of GCV for 6 days. The IC₅₀ was calculated using curve-fitting parameters.

Fig. 2 shows that among the four cell lines tested, MKN45 (highly CEA productive), was the most sensitive to GCV (IC₅₀, 5.8 μM). MKN28 cells (IC₅₀, 21 μM), which produce moderate amounts of CEA, were less sensitive to GCV than MKN45 cells. In contrast, the IC₅₀ of MKN1, in which CEA secretion was not detectable, was at most 320 μM of GCV and that of HeLa cells (a nongastric cancer cell line) was >400 μM.

On the other hand, the IC₅₀ of AdPGKtk-infected cancer cells was 3.8 μM in HeLa, 5.4 μM in MKN1, 11.2 μM in MKN28, and 3.7 μM in MKN45 (Fig. 2). These data indicated that AdPGKtk induced sensitivity to GCV in CEA-nonproducing cell lines (HeLa and MKN1) as well as in CEA-producing cell lines (MKN28 and MKN45).

The Bystander Effect of AdCEA tk-infected Gastric Cancer Cells in Vitro. To determine whether AdCEA tk-infected gastric cancer cells exert the bystander effect, the AdCEA tk-infected MKN45 cells were mixed with uninfected cells, then seeded at different cell densities, and cultured in medium containing GCV (40 μM) for 5 days. Fig. 3 shows the bystander effect in a cell density-dependent manner. At a density of 2 × 10⁴ cells/well, which allowed the most intimate cell contact, the cell survival was reduced to a level of 50 or 20% in the presence of GCV, when AdCEA tk-infected MKN45 cells accounted for 20 or 40% of the total cells, respectively. In contrast, at a sparse cell density of 5 × 10³ cells/well, the percentage of cell survival was slightly reduced to a level of 90 or 70% by GCV, when AdCEA tk-infected cells accounted for 20 or 40% of the total cells, respectively.

DISCUSSION

Several studies have demonstrated the efficacy of the HSVtk drug susceptibility system in vitro and in vivo models since the applications of this system to malignancy was described by Moolten et al. (6–8). This system has been mostly applied using recombinant retroviruses that only infect dividing cells (6–8, 10). Because direct intratumoral injection of infectious retrovirus supernatant does not have any effect on tumor growth in situ (28), retrovirus producer cells are required for their application in the tumor tissue (7, 10). In contrast, recombinant adenoviruses can deliver foreign genes to a variety of cells and tissues in vitro and in vivo with high efficiency (29, 30). The HSVtk gene has
gastric cancer cell lines, high levels of lacZ were expressed in CEA-producing cells (MKN45 and MKN28), but very little or none was found in CEA-nonproducing cells (MKN1 and HeLa). These results suggested that lacZ expression can be controlled by CEA promoter after adenovirus infection.

Fig. 1. Transduction efficiency of adenovirus in cancer cells in vitro. MKN45 (A), MKN28 (B), MKN1 (C), and HeLa (D) cells were plated on 6-well plates and infected with AdCEAlacZ at a moi of 30. Twenty-four h later, cells were fixed and stained with X-gal to demonstrate lacZ gene expression. ×100.

Fig. 2. The concentration of GCV yielding 50% inhibition of cell growth (IC50). Cells were seeded at a density of 3 × 10^3 cells in 96-multiwell plates infected with AdCEAlacZ (○), AdCEAtk (■), or AdPGKtk (●) at a moi of 30 and exposed to various concentrations (0 to 400 μM) of GCV for 6 days. The number of viable cells was measured by MTT assay, and the IC50 was calculated using curve-fitting parameters. Data are the means of three independent experiments; bars, SD.

Fig. 3. In vitro bystander effect in the AdCEAtk-infected MKN45 cell lines. Cells infected with AdCEAtk at a moi 30 were mixed with uninfected cells in the indicated proportions. Various densities of mixed cells were cultured for 5 days in 96-multiwell plates in the presence of 40 μM of GCV as indicated. The number of viable cells was measured by the MTT assay. The points on the ordinate correspond to the percentage of cells compared with uninfected cells in the absence of GCV as 100%. Values are represented as the means of three independent experiments; bars, SD.
In CEA-nonproducing cells, a small percentage of AdCEAlacZ-infected MKN1 cells were stained with X-gal, but HeLa cells were not stained. There are two explanations for this phenomenon. One is background expression of the lacZ gene by AdCEAlacZ in MKN1 cells. The other is that MKN1 may produce a small amount of CEA at an undetectable level. In fact, small amounts of protein and mRNA of CEA have been detected in normal gastrointestinal mucosa but not in HeLa cells (14, 15). Our results may be consistent with the latter notion.

After AdCEAtk infection, susceptibility to GCV was demonstrated only in CEA-producing cells. In parallel to the amount of CEA production, MKN28 cells exhibited a relatively higher IC50 of GCV than that of MKN45 cells. On the other hand, the IC50 of MKN1 and HeLa cells was extremely high. However AdPGKtk-infected MKN1 and HeLa cells were highly susceptible to GCV as well as AdPGKtk-infected MKN28 and MKN45 cells, indicating that all these cell lines are susceptible to the HSVtk-GCV system. Thus, our results suggested that expression of the HSVtk gene driven by the promoter sequence of the CEA gene leads to cell type-specific chemosensitivity to GCV in infected gastric cancer cells. Furthermore, the IC50 of GCV in the AdCEAtk-infected CEA-producing gastric cancer cells were within the range achievable by administering a clinical dose of GCV without toxic side effects (32), thus indicating that gene therapy using this vector might be beneficial in vivo.

It has been known that the adenovirus vector-mediated gene transfer system has several limitations for in vivo application, including transient expression of transferred gene and immunogenic response of the host against adenovirus (30, 33, 34). In fact, when inoculated gastric cancer cells in situ are currently under way.

REFERENCES


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