Carcinoembryonic antigen-producing cell-specific oncolytic adenovirus, OV798, for colorectal cancer therapy

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Abstract
Human carcinoembryonic antigen (CEA) is overexpressed in most colorectal cancers and has been widely used as a clinical marker for the management of colon cancer patients. The transcriptional regulatory elements (TREs) of CEA include two enhancer elements and a promoter in the 5′-flanking region of the CEA gene. By using these elements in different combinations to control reporter gene expression and the replication of adenovirus variants in various tumor cells, we have identified an optimal CEA regulatory cassette that tightly controls gene expression and viral replication in CEA-producing colon cancer cells. One of these variants, OV798, in which this regulatory cassette controls E1A expression, was further characterized. OV798 preferentially replicates in and kills CEA-producing colorectal cancer cell lines such as LoVo and SW1463, but its replication is attenuated by 1000-fold in the CEA-negative cell lines Colo-320DM (colon cancer), PA-1 (ovarian cancer), G361 (melanoma), U118 MG (glioma), and HBL-100 (human breast epithelial cell). The antitumor activity of OV798 was further examined in BALB/c nu/nu mice carrying s.c. human colon tumor xenografts. A single intratumoral administration of OV798 resulted in growth inhibition of human LoVo colon cancer xenografts. Six weeks after treatment, relative tumor volume decreased to 90% of baseline for the OV798 treatment group, compared to an increase of 1200% of baseline at 4 weeks for the vehicle-treated group. In vitro and in vivo characterization indicate that OV798 could be used as a therapy for human colon cancer. (Mol Cancer Ther. 2003;2:1003–1009)

Introduction
Colorectal cancer claimed more than 56,000 lives in the United States in 2001, and is the second most common cause of cancer mortality (Cancer Facts and Figures 2001, American Cancer Society). Some of the recent research efforts for colorectal cancer therapy, especially for metastatic diseases, have focused on gene therapy approaches to express suicide genes carried by viral or nonviral vectors to cancer cells (1). The specific expression of these genes in tumor cells followed by produg treatment can restrict cytotoxicity of the converted drugs to tumor cells and provide a bystander killing effect. To achieve targeted gene expression, tumor-specific promoters or other regulatory elements are applied to drive suicide genes such as the Herpes Simplex Virus thymidine kinase (HSVtk) and bacterial or yeast cytosine deaminase (CD). One such commonly used tumor-specific promoter is from carci-noembryonic antigen (CEA) (2–5).

CEA is a cell surface glycoprotein normally expressed in fetal tissue and transcriptionally silent in adults. It is a member of the immunoglobulin supergene family found on chromosome 19. CEA functions as an intercellular adhesion molecule and plays an important role during development. However, CEA overexpression is associated with a variety of cancers of epithelial origin (6). Serum CEA elevation is observed in over 60% of patients with metastatic colorectal cancer (7), and, therefore, it has been widely used as a tumor marker for diagnosis and prognostic management. Expression of CEA in epithelial tumors occurs at the level of gene transcription, and the essential transcriptional regulatory elements (TREs) have been characterized and applied for targeted suicide gene expression (2, 3, 8). In the resulting gene therapy studies, adenoviruses and retroviruses were used as viral vectors to mediate restricted gene expression in the CEA-positive tumor cells via the transcriptional control of CEA TREs (8–12).

Recently, replication-competent adenoviruses have shown promising applications in cancer gene therapy (13, 14). Such vectors have specific gene mutations and/or are under tumor-specific transcriptional control that cause them to preferentially replicate in tumor cells. As a result of viral replication, the tumor cells will be destroyed and the viral oncolytic effect will be amplified through the generation and spread of progeny viruses. In this communication, we analyzed the CEA TREs and created an oncolytic adenovirus targeted to CEA-producing cancer cells by employing the CEA promoter and enhancer to control the expression of the adenoviral E1A gene. The resulting virus preferentially replicates in CEA-positive cell lines and is attenuated over 1000-fold in CEA-negative cell lines. In vivo characterization of this virus showed significant antitumor activity in CEA-positive xenografts.
liver, and breast epithelial cell HBL-100 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The human embryonic kidney cell line 293, which expresses adenovirus E1A and E1B genes, was purchased from Microbix Biosystems (Toronto, Canada). Human gastric cancer cell lines MKN45, MKN28, and MKN1 were provided by Dr. Omata (University of Tokyo, Japan). All cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS) and antibiotics.

**DNA Construction**

Human CEA promoter and enhancers were PCR amplified from human genomic DNA (Roche, Palo Alto, CA) by different primer combination (Table 1). Primers for promoter region were 76.13.1 and 76.13.2. Primers for the enhancer 1 region were 76.13.3 and 76.13.4, for the enhancer 2 were 76.16.1 and 76.22.2. All the cloning sites in the primers were to facilitate molecular cloning. CEA-Luciferase constructs were constructed from pGL2-Basic (Promega Inc., Madison, WI). Plasmid DNA was purified using Qiagen plasmid kits (Santa Clarita, CA).

A series of DNA constructs was generated using conventional molecular biology approaches (Fig. 1). All constructs except CP531 have the CEA promoters located from −299 to +68 bp of the transcription initiation site. CP531 has the short promoter repeat for driving luciferase gene expression (−90 to −40 bp of the transcription initiation site). This short CEA promoter was PCR amplified by primers 76.13.2 and 76.22.1 then followed by NruI and AvrII digestion. The NruI to AvrII fragment was repeatedly cloned to get CP531, which has four short CEA repeats before the luciferase gene.

**Transfection and Luciferase Assay**

DNA transfections were performed in six-well plates using Superfect (Qiagen). Equimolar amounts of CEA-Luciferase constructs were mixed with a CMV-β-gal construct at a ratio of 10:1 and used for transfection (15). The constructs CMV-Luc and pGL2-Basic were also mixed with CMV-β-gal and used as controls in the transfection of each cell type. Forty-eight hours posttransfection, cells were washed and lysed with lysis buffer to collect cell lysates for protein assay (Roche, Palo Alto, CA). Fifty microliters of lysates, normalized for total protein, from each transfection were used for luciferase and hGalactosidase assays. Luciferase assays were performed with an enhanced luciferase assay kit (PharMingen, San Diego, CA) on a luminometer (ML3000, Dynatech Laboratories, Chantilly, VA). β-Galactosidase luminescence assays were performed on a luminometer by Galacto-Light Plus (Tropix Inc., Bedford, MA). The β-galactosidase activity was used to normalize the transfection efficiency in all samples.

**Generation of Recombinant Adenoviruses**

All the recombinant adenoviruses were generated by cotransfection of low passage 293 cells with different DNA constructs and pBHGE3 as previously described (16). Potential recombinant viruses were picked and expanded by infecting 293 cells. To screen for expected recombinants, viral DNA was purified from crude plate lysates using the QIAamp Blood Kit as suggested by the manufacturer (Qiagen). PCR was performed using several primer pairs specific for the adenovirus E1 sequence that spanned the desired CEA inserts, and the PCR products were digested with restriction endonucleases to confirm the structure.

CP532 was derived by inserting the CEA promoter flanked by PinAI sites into CP321 (15) and used to produce OV799. The CEA promoter/enhancer 1 structure from CP520 was flanked by PinAI and XmaI sites and cloned into CP321. The subsequent construct, CP525, was used to generate OV798. For generating OV804, the multimerized, short CEA promoter from CP531 was cut out with PinAI/XmaI and cloned into CP321 to get CP540 (Fig. 1).

**Plateau Assay and Relative Plaque-Forming Index**

To characterize specific viral replication, cells were plated in six-well plates (BD Falcon, Franklin Lakes, NJ) at 5 x 10^5 cells per well, 24 h before infection. Viruses of interest were serially diluted and used to infect cells. Four hours

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**Table 1. Oligonucleotide sequence of primers used in the study**

<table>
<thead>
<tr>
<th>Oligonucleotide sequence of primers used in the study</th>
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<tbody>
<tr>
<td>76.13.1 5'-GATCTCGAGACCCGGGACCCTGCTGGTTTC 3'</td>
</tr>
<tr>
<td>76.13.2 5'-GATCCGCCGTCTTGGTTCAGGAACAGTTTGT 3'</td>
</tr>
<tr>
<td>76.13.3 5'-GATCACCGTTACCAATCTGAGACTTGGGAAGG 3'</td>
</tr>
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<td>76.16.1 5'-GATCACCGTTACCAATCTGAGACTTGGGAAGG 3'</td>
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<td>76.22.1 5'-GATCCGCCGTCTTGGTTCAGGAACAGTTTGT 3'</td>
</tr>
<tr>
<td>76.22.2 5'-GACCCGAGTGACTTGGATGATG 3'</td>
</tr>
<tr>
<td>35.88.1 5'-CCACACGTTTCCCTGTGAG 3'</td>
</tr>
<tr>
<td>35.88.2 5'-AACGGAAGTGACGAGTTTTGAG 3'</td>
</tr>
</tbody>
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**Figure 1.** Schematic structure of CEA transcriptional control elements and DNA constructs for reporter gene analysis. P, CEA promoter region from −299 to +68 bp of the transcription initiation site. p, short CEA promoter from −90 to −40 bp of the transcription initiation site. Enh1, CEA enhancer 1 from −3.9 to −6.1 kb. Enh2, CEA enhancer 2 from −10.7 to −13.6 kb. +, DNA fragment in original orientation. −, DNA fragment in reverse orientation. dl, enhancer 1 has about 500 bp deletion.
postinfection, the media were aspirated and each well was overlaid with 4 ml media containing 0.8% of low-melting temperature agarose. The plates were further incubated for 14 days for final score of plaque numbers and titers for each virus on each cell type.

For each virus, the titer on 293 cells was used to normalize the titers from other cells. The ratio between viral titer on specific cell and titer on 293 cell was used to calculate the relative plaque-forming index (PFI). The PFI of each virus was calculated as (viral titer on cell A/viral titer on 293 cell) and the relative PFI was expressed as a fraction of OV802 PFI, derived in an identical manner. OV802 is a recombinant wild-type adenovirus 5 (16). On permissive cells, viral titer will be close to the titer of OV802; therefore, the relative PFI is close to 1. However, on nonpermissive cells, the viral titer will be lower than OV802, resulting in PFI less than 1. The difference in such an index represents the ability of the virus to replicate in different cells. For an ideal replication-competent adenovirus, this index should be close to 1 in permissive cells and 0 in nonpermissive cells.

**Virus Yield Assay**

Six-well plates were seeded with 5 × 10^5 cells per well 24 h before infection. Cells were infected at a multiplicity of infection (MOI) of 2 PFU/cell for 4 h in serum-free media. After 4 h, the virus-containing media were removed, monolayers were washed with PBS, and 4 ml of complete media (RPMI 1640 with 10% FBS) were added to each well. Seventy-two hours postinfection, cells were scraped into the culture medium and lysed by three cycles of freeze-thaw. Two independent infections of each virus cell combination were titered in duplicate on 293 cells (15, 16).

**Detection of Viral DNA**

For further verification of viral replication, infected cells were processed for DNA extraction and slot hybridization. Briefly, infected cell lysate was mixed with lysis buffer from a Qiagen blood kit in the presence of proteinase K. The mixture was incubated at 55°C for 20 min and followed by adding RNase A and incubation at room temperature for 2 min. After mixing with ethanol, the suspension was carefully passed through the purification column and washed. DNA was recovered by water elution and applied to a membrane for slot blot hybridization to compare to an adenovirus 5 DNA standard. A 2.3-kb adenovirus 5 E4 region DNA fragment was PCR-amplified by primers 35.88.1 and 35.88.2 and used as probe.

**Animal Studies**

Six-to 8-week-old athymic nude mice were obtained from Simonson Laboratories (Gilroy, CA) and acclimated to laboratory conditions 1 week before tumor implantation. All animal studies were approved by an Animal Care and Use Committee. Xenografts were established by s.c. injection of 1 × 10^6 LoVo cells suspended in 100 μl of RPMI 1640 and 100 μl of Matrigel. Injections were made near the small of the back. When tumors reached between 200 and 300 mm^3, mice were randomized and dosed with 100 μl of test article (5 × 10^8 particles/mm^3) via intratumoral injection at days 1 and 3 after randomization. In each injection, the animal received approximately 2 × 10^11 viral particles; the control group received the same volume of PBS with 10% glycerol only. Tumors were measured in two dimensions by external caliper. Volume was estimated by the formula [length (mm) × width (mm)^2]/2. Animals were euthanized when their tumor volume reached about 2000 mm^3 in size in accordance with institute animal ethics standards. The difference in mean tumor volume between treatment groups was compared for statistical significance using the unpaired, two-tailed, t test.

**Results**

**Characterization of the CEA Promoter and Enhancers**

In the last decade, the CEA transcriptional control mechanism has been studied by several research groups and different CEA promoters/enhancers have been identified (3, 17, 18). To generate an optimal TRE consisting of the CEA regulatory elements for use in oncolytic adenoviruses, we produced a series of reporter constructs (Fig. 1). CP517 contains the CEA promoter region from −299 to +68 bp corresponding to the transcriptional initiation site, and this promoter structure is applied in most other constructs. CP520 and CP521 have the promoter and additional enhancer 1 from −3.9 to −6.1 kb in a different orientation. CP529 and CP530 contain the promoter and additional enhancer 2 from −10.7 to −13.6 kb in a different orientation. In CP524, a DNA fragment from −3.9 to −4.3 kb in the enhancer 1 region was deleted and the short enhancer 1 was linked to the promoter in reversed orientation. In CP531, a short CEA promoter, −40 to −90 bp, was repeated four times and used to control reporter gene expression. This group of DNA constructs was used to transfect CEA-positive and -negative cells for 2 days following which the cells were harvested for luciferase assays. In this study, pGL2-Basic was used as a negative control. CMV-Luc, with the CMV promoter driving the luciferase gene, was used as a positive control. To normalize the transfection efficiency of different cells, CMV-β-Gal was cotransfected with the CEA constructs and the resulting β-galactosidase activity was used to normalize the luciferase activity. The results shown in Fig. 2 indicated that all DNA constructs appeared to have significant luciferase activity that was about 1000 times greater than pGL2-Basic (data not shown). The reporter gene activity was consistently high in CEA-positive cells SW620, SW1463, and LoVo, but low in CEA-negative cells SW780, Chang liver, and HBL-100 cells. The difference in luciferase activity between permissive and nonpermissive cells ranged from 100- to 10,000-fold. The enhancer 2 and promoter combination constructs (CP529 and CP530) led to a low gene expression, whereas the enhancer 1 and promoter combination constructs gave higher activity and tissue selectivity (CP520, CP521, and CP524). Using promoter alone (CP517) or modified short promoter repeats (CP531) also led to a tissue-selective expression pattern. Among the CEA-positive cells tested in our study, LoVo appeared to have the highest CEA expression level (data not shown). In LoVo cells, CP520 with the promoter and
enhancer 1 (in reverse orientation) had the best expression. The level was close to CMV-Luc (data not shown). This result suggests that CP520 may represent the best CEA TRE structure for CEA regulatory expression. To further compare the tissue specificity of these combinations in adenovirus, CEA promoter alone, promoter/enhancer 1 (−) combination, and short promoter repeats were chosen for recombinant adenovirus construction.

Construction of CEA-TRE-Containing Adenovirus Variants

On the basis of the results from the in vitro reporter transfection experiment, we generated three recombinant adenovirus variants: OV799, OV798, and OV804 (Fig. 3). In OV799, the CEA promoter from −299 to +68 bp corresponding to the transcriptional initiation site was used to replace the endogenous E1A promoter. The E1A promoter in OV798 was replaced by the CEA enhancer 1 sequence from −6.1 to −3.9 kb in addition to the CEA promoter, whereas OV804 contained multiple copies of the short CEA promoter at the same site. After structure verification, these three viruses were compared for plating efficiency in different cell types. Fourteen days postinfection, the plaque number for each virus was determined, and viral titer was normalized against titer from 293 cells. The relative PFI was defined as ratio between the titer of the tested virus and the corresponding titer of OV802, a recombinant wild-type adenovirus 5 (16), tested on specific cell type. All three viruses selectively replicated on CEA-positive cells LoVo and SW1463 with a PFI near one, suggesting similar replication efficiency to OV802. In nonpermissive cells, their PFI dropped significantly due to attenuation. The overall difference of PFI between permissive and nonpermissive cells ranged from 10- to 1,000-fold. Additionally, between the two permissive cell lines LoVo and SW1463, all CEA-viruses had viral replication levels higher in LoVo due to higher level of CEA expression. From the comparison of their PFI in different cells, OV798 had the best selectivity profile (Fig. 4). In CEA-positive cells, OV798 replicated equally well as OV802 and had a relative PFI of approximately 1. However, in CEA-negative cells, OV798 had 100- to 1000-fold less replication than OV802. Therefore, this variant was chosen for further characterization.

Further in Vitro Characterization of OV798

The CEA-regulated replication-competent adenoviral variant OV798 displayed a clear selectivity profile in studies of relative PFI (Fig. 4). To further verify that the replication selectivity was due to CEA regulation and not the heterogeneity of different tumor types, three gastric cell lines with different CEA status were compared in a plaque assay (Fig. 5). Most gastric cancer cells express CEA. These cells were employed in the characterization of CEA-targeted gene expression (19, 20). In our study, two CEA-positive cell lines (MKN28, MKN45) and one CEA-negative (MKN1) were tested. All three CEA-positive cells, LoVo, MKN-28, and MKN-45, had OV798 PFI close to 1, whereas the PFI dropped to approximately 0.1 in the CEA-negative MKN-1 cell line (Fig. 5). This result further suggests that OV798 replication is CEA dependent.
In the virus yield assay, viral replication in various cell types was compared 72 h after infection (Fig. 6). In permissive cells, the testing virus should finish its productive life cycle and release large numbers of progeny virions. However, in nonpermissive cells, viral replication will be greatly restricted. Results shown in Fig. 6 indicated that two CEA-positive tumor cell lines LoVo and SW1463 support OV798 replication, whereas five other CEA-negative cells had over 1000-fold lower production. Because the initial adenoviral infection was set at an MOI of 2 in the virus yield assay, this result suggests that in Colo-320DM, PA-1, G361, U118 MG, and HBL-100 cells, OV798 has no replication activity. On the basis of the CEA characterization from these cells, both LoVo and SW1463 had high CEA production, whereas Colo-320DM had marginally detectable CEA (<1% compared to LoVo, data not shown), indicating that activation of the CEA promoter is primarily responsible for OV798 replication in these cells. This observation was further confirmed in the study when viral DNA was used as an indication of virus production in various cell lines. OV798 DNA in permissive and nonpermissive cell lines was determined by slot blot hybridization using adenovirus E4 DNA as a probe. A similar amount of viral DNA as with OV802 was detected in the OV798-infected 293 and CEA-producing LoVo cells, whereas the OV798 viral DNA was undetectable in the CEA-negative PA-1 and G361 cells (Fig. 7). This result was consistent with previous observations of plaquing efficiency and virus burst size, and suggests that OV798 only replicates in CEA-positive cells.

**Antitumor Efficacy Study of OV798 in Mouse Xenografts**

To determine the antitumor activity of OV798, the CEA-producing LoVo cell line was used to establish s.c. xenografts in athymic nude mice (Fig. 8). Tumor-bearing mice were grouped and treated on days 1 and 3 postrandomization via intratumoral injection of $2 \times 10^{11}$ viral particles of OV798 or an equivalent volume of saline. Tumors were measured weekly and closely monitored. In the control group, tumor grew aggressively. Animals were euthanized when their tumor burden became excessive after week 4 (average tumor size was over 2500 mm$^3$). In contrast, the OV798 treatment group showed clear growth inhibition. By week 6, the tumor volume in the OV798-treated group was about 90% of baseline, indicating clear tumor growth inhibition. Three out of seven animals had a reduction in tumor size of over 50%. Statistical analysis using the unpaired two-tailed t test indicated that the OV798 treatment had a significant antitumor activity ($P < 0.01$ compared to the control group). There was no sign of virus-related toxicity or weight loss associated with the treatment. All treated mice remained active and healthy during the study.

**Discussion**

CEA started to gain clinical significance in 1969 (21). In the last decade, many studies focused on using CEA transcriptional control to direct suicide gene expression in targeted...
In vivo efficacy of OV798. LoVo cell xenografts were raised in athymic nude mice. OV798 or vehicle control were administrated via i.t. injection at $5 \times 10^8$ viral particles/mm$^3$ on days 1 and 3 (indicated with arrows). Tumors were measured in two dimensions by external caliper and volume was estimated by the formula (length (mm) $\times$ width (mm)$^2$)/2. Animals were humanely killed when their tumor burden became excessive. The tumor volume was normalized to 100% at day of treatment. Error bars, $\pm$SE. The difference between treatment group and control group was compared using the unpaired, two-tailed, t test ($P < 0.01$ between control and OV798-treated group).

Figure 8. In vivo efficacy of OV798. LoVo cell xenografts were raised in athymic nude mice. OV798 or vehicle control were administrated via i.t. injection at $5 \times 10^8$ viral particles/mm$^3$ on days 1 and 3 (indicated with arrows). Tumors were measured in two dimensions by external caliper and volume was estimated by the formula (length (mm) $\times$ width (mm)$^2$)/2. Animals were humanely killed when their tumor burden became excessive. The tumor volume was normalized to 100% at day of treatment. Error bars, $\pm$SE. The difference between treatment group and control group was compared using the unpaired, two-tailed, t test ($P < 0.01$ between control and OV798-treated group).

CEA-positive cells for cancer gene therapy. For example, CEA promoter-controlled HSVtk or CD genes were used to kill CEA-positive gastric cancer and colorectal cancer cells (5, 10, 20). However, most of the studies applied replication-defective adenovirus or retrovirus vectors for the gene delivery. In these vectors, the CEA TREs were used to control the transgene expression. Recently, we achieved high tumor selectivity and significant antitumor efficacy in animals and in clinical setting when a tumor-specific TRE was used to control viral essential gene E1 in conditionally replication-competent adenoviruses such as CG7060 and CG7870 for prostate cancer, CG8900 for hepatocellular carcinoma, and CG8840 for bladder cancer (16, 22–24). Conditionally replicating adenoviruses for cancer therapy have advantages related to the amplification of antitumor effects through viral replication. By using CEA transcriptional control of adenoviral E1A expression, viral replication can be limited to CEA-positive tumor cells.

Studies of CEA transcriptional control have revealed different functional elements (3, 10, 17, 18). To assemble a well-regulated TRE, we compared combinations of a 0.36-kb promoter with different enhancers as well as a multimerization of a short promoter in reporter gene assays and in recombinant adenovirus studies. It was found that CEA promoter/enhancer 1 combination had a better tissue specificity profile in reporter gene assay. After further comparison of this strategy in recombinant adenovirus by controlling E1A expression, the viral construct OV798 showed the best specificity profile in a group of CEA-regulated adenoviral variants. Study of OV798 replication in different gastric cell lines confirmed that CEA expression controls viral replication specifically. This CEA-dependent replication profile was shown by not only the virus yield assay but also the DNA detection on a broader panel of cell lines.

It is known that transient gene expression by replication-defective adenovirus vectors restricts their in vivo applica-


Molecular Cancer Therapeutics

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