Application of the Cre Recombinase/loxP System Further Enhances Antitumor Effects in Cell Type-specific Gene Therapy against Carcinoembryonic Antigen-producing Cancer

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ABSTRACT

A considerable number of studies of cancer have shown that the cell type-specific promoter is an effective tool for selective expression of foreign genes in tumor cells. However, few reports have demonstrated significant in vivo antitumor effects using this strategy thus far, possibly because the low activity of such a promoter results in insufficient expression of genes in cancer cells as well as in insignificant antitumor effects, even when the cells are infected by highly efficient gene transfer methods.

To overcome this problem, we used the Cre/loxP system for the cell type-specific gene therapy against carcinoembryonic antigen (CEA)-producing cancer. We constructed a pair of recombinant Ads. One expresses the Cre recombinase (Cre) gene under the control of the CEA promoter (Ad.CEA-Cre). The other contains the herpes simplex virus thymidine kinase (HSV-TK) gene separated from the strong CAG promoter by insertion of the neomycin resistance (neo) gene (Ad.Iox-TK). The HSV-TK gene of the latter Ad is designed to be activated through excisional deletion of the neo gene by Cre enzyme released from the former one only when CEA-producing cells are infected simultaneously with these Ads.

Coinfection by these Ads rendered a human CEA-producing cancer cell line 8.4-fold more sensitive to ganciclovir (GCV) compared with infection by Ad.CEA-TK alone, the HSV-TK gene of which is directly regulated by the CEA promoter. On the other hand, coinfection with these Ads did not significantly change the GCV sensitivity of non-CEA-producing cells. Intratumoral injection of Ad.CEA-Cre combined with Ad.Iox-TK followed by GCV treatment almost completely eradicated CEA-producing tumors established in the subcutis of athymic mice, whereas intratumoral injection of Ad.CEA-TK with GCV administration at most retarded the growth of inoculated tumors. These results suggest distinct advantages of the Cre/loxP system applied in the conventional cell type-specific gene therapy against cancer.

INTRODUCTION

CEA is an oncofetal protein that is often detected at high levels in the serum of cancer patients, including those with cancers of the lung, colon, stomach, and pancreas (1–4). Many cases of these cancers are found to be already advanced at initial diagnosis, and are refractory to conventional chemoradiotherapy, and show poor prognosis (3, 5, 6).

Recently, a strategy called virus-directed enzyme/prodrug therapy has been used as an alternative treatment for localized brain tumors in clinical trials and has demonstrated promising results (7, 8). In applying virus-directed enzyme/prodrug therapy to refractory CEA-producing cancer, disseminated cancer cells must be controlled without affecting normal cells. Cell type-specific promoters have been shown to be effective tools for targeting tumor cells (10–17). They induce tumor cell-specific death by selective expression of the CD or the HSV-TK genes in tumor cells. However, few reports have demonstrated sufficient antitumor effects, even when tumor cells were introduced with specific promoter-suicide chimera genes in vivo by an Ad, the most efficient vector for introducing foreign genes into target cells (13, 16). It is conceivable that the activity of cell type-specific promoters, including the CEA promoter, is too low to induce sufficient drug sensitivity in vivo. Richards et al. (18) reported that multimerization of a part of the CEA promoter alone or ligated with its specific enhancer sequences resulted in copy number-related increases in promoter activity. We also analyzed by the CAT assay whether the activity of the CEA promoter was augmented by ligation of the enhancer sequence which extends from −6.1 to −4.0 kb upstream regions from the transcriptional start of the CEA gene or by tandem ligation of four copies of the 420-bp fragment of the CEA promoter sequence. These fragments, however, did not contribute to a significant increase in the CAT activity in CEA-producing LoVo cells. Cre recombinase (Cre) derived from bacteriophage P1 is a 38,000 protein and mediates the site-specific excisional deletion of a DNA sequence that is flanked by a pair of loxP sites and is composed of 34 nucleotides (19). In α-fetoprotein-producing hepatocellular carcinoma cells, the Cre/loxP system contributed to an ~100-fold increase in the magnitude of the LacZ gene expression compared with that obtained by infection with an Ad expressing the LacZ gene directly driven by the α-fetoprotein promoter (20). To obtain enhanced expression of the HSV-TK gene exclusively in tumor cells and subsequent significant in vivo antitumor effects, we applied the Cre/loxP system to HSV-TK/GCV therapy for CEA-producing cancer. We constructed a Cre-producing Ad driven by the CEA promoter (Ad.CEA-Cre) and another Ad designed for inducible expression of the HSV-TK gene by Cre (Ad.Iox-TK). Coinfection with these Ads induced selective GCV sensitivity at a higher level in vitro and augmented in vivo antitumor effects in human CEA-producing tumors, as compared with those obtained by infection with Ad.CEA-TK alone, an Ad that expresses the HSV-TK gene under the control of the CEA promoter.

Here, we demonstrate that the Cre/loxP system significantly enhances antitumor effects of the cell type-specific gene therapy against CEA-producing cancer.
MATERIALS AND METHODS

Cell Lines. Two human adenocarcinoma cell lines of the lung, VMRC-LCD and A549, and a human colon cancer cell line, LoVo, were all obtained from Japan Cancer Research Resources (Tokyo, Japan). A human embryonal kidney cell line, 293, was purchased from American Type Culture Collection (Manassas, VA). Three cancer cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. 293 cells were grown in DMEM containing 10% FBS and penicillin/streptomycin.

CEA Production by Tumor Cells. Cells grown in culture medium were washed, suspended in PBS, and homogenized with a sonicator at 50 W for 2 min. The CEA content in the cell lysate was measured by solid-phase RIA. The lower limit of detection of CEA was 0.5 ng/ml. The total protein concentration was assayed by Lowry’s method (Special Reference Laboratory, Tokyo, Japan). Each value represents the former concentration divided by the latter one (ng/mg protein).

CAT Assay. The CAT assay was carried out as described previously (11). In brief, 5 μg of a plasmid with the SV40 promoter-CAT gene (pSV2CAT) or a plasmid with the CEA promoter-CAT gene (pCEACAT) was cotransfected with 10 μg of pSV-β-galactosidase, a plasmid with the LacZ gene driven by the SV40 promoter as an internal control for transfection, into each cell line. 14C-labeled chloramphenicol and its acetyl form were quantitated in duplicate by liquid scintillation counting in Aquasol (DuPont-NEN, Boston, MA). The ratio of the count for the acetyl form to total count was calculated as the CAT activity. The CAT activity of pCEACAT was expressed as a percentage of that of pSV2CAT, a positive control plasmid.

Construction of Ads. The recombinant replication-defective Ads were constructed by the cosmid-adenoviral DNA terminal protein complex method (21). The CEATK fragment prepared as the BglII-HindIII fragment from pCEATK (11) was ligated into the Sall site of pAXcawt and designated pAXcEAxT. pAXcAosTK and pAXcCALNLoxTK were constructed by subcloning the TK fragment from the BglII-PvuII site of ptk4 (11) into the Sall site of pAXcAw and pAXcALNw (20), respectively. The BglII-HindIII CEA promoter fragment from pCEACAT (11) was also inserted into the Sall site of pAXcAw and designated pAXcCEATK. pAXcAosTK and pAXcCALNLoxTK were constructed as described previously (11). The recombinant Ads were constructed by the cosmid-adenoviral DNA terminal protein complex method (21). The CEATK fragment prepared as the BglII-I site of pCEATK (11) was ligated into the Sall site of pAXcAw and designated pAXcCEATK. The recloning of the CEATK fragment from pCEACAT into pSVII-CAT (11) was also inserted into the Sall site of pAXcAw and designated pAXcCEATK. The recloning of the CEATK fragment from pCEACAT into pSVII-CAT (11) was also inserted into the Sall site of pAXcAw and designated pAXcCEATK.

RESULTS

CEA Production and CEA-specific Promoter Activity in Human Cancer Cell Lines. The amount of CEA protein in cell homogenate was measured (Table 1). LoVo and VMRC-LCD cells produced significant amounts of CEA protein (32.2 ± 12.7 and 204.1 ± 43.5 ng/mg protein, respectively), whereas secretion from A549 cells was below the detectable level. The activity of the CEA promoter was also analyzed using CAT assay (Table 1). The CAT activities of pCEACAT in LoVo and VMRC-LCD were 8.2 ± 0.7 and 61.7 ± 3.4% of that of pSV2CAT, respectively, whereas it was very low in A549 cells (4.2 ± 0.4%). A549 produced a significant amount of CEA in our previous experiments (11), but it became a non-CEA-producing cell line during a long-term cultivation. These results suggest that the CEA promoter shows selective activity in CEA-producing cancer cells, although the activity is not so strong.

Mechanisms Activating the HSV-TK Gene Specifically in CEA-producing Cancer Cells by the Cre/IoxP System. Fig. 1B illustrates the mechanisms activating the HSV-TK gene specifically in CEA-producing cancer cells. When CEA-producing cells are infected simultaneously with Ad.CEA-Cre and Ad.Iox-TK, the former Ad produces Cre recombinase under the control of the CEA promoter. Then Cre recombinase catalyzes the excision of the HSV-TK gene in CEA-negative cells. This leads to an increase in the HSV-TK gene expression and the HSV-TK promoter activity in CEA-negative cells. The HSV-TK promoter activity in CEA-negative cells is significantly higher than that in CEA-expressing cells. This is the mechanism by which Cre recombinase can activate the HSV-TK gene specifically in CEA-negative cells.

Table 1 CEA production and CEA-specific promoter activity in human cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CEA production (ng/mg protein)</th>
<th>CEA promoter activity (% of SV40 promoter)</th>
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<tr>
<td>LoVo</td>
<td>77.2 ± 12.7</td>
<td>98.2 ± 0.7</td>
</tr>
<tr>
<td>VMRC-LCD</td>
<td>204.1 ± 43.5</td>
<td>61.7 ± 3.4</td>
</tr>
<tr>
<td>A549</td>
<td>Not detectable</td>
<td>4.2 ± 0.4</td>
</tr>
</tbody>
</table>

a Level of CEA production was calculated as the concentration of CEA protein divided by that of the total protein in cell lysates. Results represent the means ± SD of three independent isolated measurements.

b The CAT activity of pCEACAT was expressed as a percentage of that of pSV2CAT. Results represent the means ± SD of three independent experiments. Each experiment was performed in duplicate.

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the produced Cre excises the neomycin resistance gene between a pair of loxP sites out of the latter Ad and activates the HSV-TK gene driven by the strong CAG promoter. Therefore, administered GCV is phosphorylated and induces cell death exclusively in CEA-producing cells.

Determination of the Ideal Ratio of the Paired Ads to Achieve Maximum Expression of the LacZ Gene Using the Cre/loxP System. To maximize the expression of the LacZ gene using the Cre/loxP system in vitro, we analyzed the ideal ratio of Ad.CEA-Cre to Ad.lox-LacZ. Cre released from Ad.CEA-Cre activates the LacZ gene driven by the CAG promoter. Human adenocarcinoma cell lines, LoVo and A549, were infected simultaneously with these coupled Ads at various ratios at a total moi of 30. The LacZ gene expression was quantified by β-galactosidase assay (Fig. 2). In CEA-producing LoVo cells, the strongest β-galactosidase activity (7.1 ± 0.3 μunits/cell) was obtained with Ads at a ratio of 1:1. No nonspecific β-galactosidase activity was detected on infection with Ad.lox-LacZ alone. Moreover, in non-CEA-producing A549 cells, the β-galactosidase activity remained at very low levels, regardless of the ratios of the paired Ads. The β-galactosidase activity at each ratio increased in proportion to the total moi and reached the maximum level at a total moi of 30 (data not shown). Significant cell toxicity caused by infection with Ads was observed at a moi higher than 30 (data not shown). These results show that the Cre/loxP system allows specific expression of the LacZ gene in CEA-producing cells and confers the highest level of expression when these coupled Ads are used at a ratio of 1:1.

**In Vitro Enhanced GCV Sensitivity of CEA-producing Cancer Cells by Coinfection with Ad.CEA-Cre and Ad.lox-TK.** To yield maximum expression of the HSV-TK gene in CEA-producing cancer cells using the Cre/loxP system, we coinfected LoVo, VMRC-LCD, and A549 cells with Ad.CEA-Cre and Ad.lox-TK at various ratios. There was no significant difference in transduction efficiency of Ads among these three cell lines, as determined by infection with Ad.CAG-LacZ (data not shown). GCV sensitivity was evaluated using MTT assay (Fig. 3). The lowest IC50 was obtained in both LoVo and VMRC-LCD cells when coinfection with the paired Ads was performed at a ratio of 1:1. These results were compatible with those obtained by infection with Ad.CEA-Cre and Ad.lox-LacZ (Fig. 2). The IC50 of each cell line (0.17 ± 0.04 and 1.40 ± 0.50 μM, respectively) were 8.4- and 3.3-fold lower than that obtained by infection with Ad.CEA-TK alone (1.43 ± 0.21 and 4.63 ± 0.71 μM, respectively; P < 0.01) and 2488- and 316-fold lower than the values for nontransduced cell lines (423.4 ± 40.4 and 443.3 ± 55.1 μM, respectively). The IC50 of these CEA-producing cells conferred by the Cre/loxP system were comparable with those obtained by infection with Ad.CAG-TK, which rendered all of these cell lines highly sensitive to GCV, regardless of CEA production. In LoVo and VMRC-LCD cells, the lowest IC50 were only 2.1- and 1.5-fold higher than the value for those infected with Ad.CAG-TK (0.17 ± 0.04 and 0.08 ± 0.02 μM, respectively). Infection with Ad.lox-TK alone did not significantly change GCV sensitivity in CEA-producing cells. Moreover, coinfection with Ad.CEA-Cre and Ad.lox-TK did not induce significant change in sensitivity of non-CEA-producing A549 cells. These results show that coinfection with Ad.CEA-Cre and Ad.lox-TK enables Cre-mediated transgene expression in CEA-producing cells and confers high GCV sensitivity to these cells.

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Fig. 1. A. structure of recombinant Ads. Ad.CAG-TK expresses the HSV-TK gene driven by the CAG promoter. Ad.CEA-TK and Ad.CEA-Cre express the HSV-TK gene and the Cre gene, respectively, under the control of the CEA promoter. Ad.lox-TK is designed to express the HSV-TK gene under the CAG promoter only after Cre recombination excises the neomycin resistance (neo) gene. ▲, the adenoviral genome; ○, adenoviral DNA terminal protein complexes; △, deletions of adenoviral early regions. CAG, CAG promoter; CEA, CEA promoter; NCα, NLS-tagged Cre gene; SpA, SV40 early poly(A) signal; GpA, rabbit β-globin poly(A) signal; *, loxP site. B, the Cre/loxP system mediates high level expression of the HSV-TK gene specifically in CEA-producing cancer cells. Cre recombinase expressed by Ad.CEA-Cre excises the neo gene with SpA at the loxP sites out of Ad.lox-TK and activates the HSV-TK gene driven by the strong CAG promoter.

Fig. 2. Coinfection with Ad.CEA-Cre and Ad.lox-LacZ at a ratio of 1:1 maximizes selective expression of the LacZ gene in CEA-producing cell lines. One hundred thousand LoVo or A549 cells were coinfected with these Ads at various ratios at a total moi of 30. Three days after infection, cell lysates were prepared and subjected to β-galactosidase assay. Columns, mean β-galactosidase activity; bars, SD. Numbers below columns indicate the moi of Ads used for infection. The same experiment was repeated twice more with similar results.

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Ad.CEA-Cre and Ad.lox-TK remarkably augments selective GCV sensitivity in CEA-producing cancer cells compared with infection by Ad.CEA-TK alone and that the magnitude of enhancement in GCV sensitivity correlates with the activity of the CEA promoter rather than the amount of CEA protein produced (Table 1).

Immediate Rejection of Established CEA-producing Tumors by Coinfection with Ad.CEA-Cre and Ad.lox-TK Followed by GCV Treatment. To evaluate in vivo antitumor effects on CEA-producing tumors by coinfection with Ad.CEA-Cre and Ad.lox-TK, we injected tumors of LoVo cells established in the subcutis of athymic mice i.t. with Ads, followed by GCV administration when the average tumor area reached 20 mm² (Fig. 4). When a low dose of Ads (2×10⁸ pfu per injection) was injected (Fig. 4A), significant regression of tumor growth was observed only in the mice treated with Ad.CEA-Cre and Ad.lox-TK followed by GCV (group I; group I versus groups III-VIII, P < 0.005). In three of five mice (60%) in this group, tumors were completely eradicated, and two other mice.
showed transient regression of tumor during GCV treatment. Nevertheless, the other treatment including Ad.CEA-TK/GCV (group III) did not have any significant effects on tumor growth (group III versus group VII, P > 0.05). To further augment antitumor effects, we administered an increased dose of Ads to the mice (5 × 10⁶ pfu per injection; Fig. 4B). All mice treated with Ad.CEA-Cre and Ad.Iox-TK followed by GCV injection (group I) showed immediate regression of inoculated tumors. Six of seven mice (86%) became free of palpable tumor by the end of the treatment schedule, and the tumor in the rest of the mice in this group remained a small nodule. On the other hand, Ad.CEA-TK/GCV (group III) as well as Ad.CEA-Cre and Ad.Iox-TK/saline treatment (group II) slightly retarded the growth of tumors but did not eradicate them (group III versus group IV, P < 0.05, from day 24 to day 44, and group II versus group VI, P < 0.01, from day 24 to day 44, respectively). Thus, antitumor effects observed in the Ad.CEA-Cre and Ad.Iox-TK/GCV treatment group (group I) were more remarkable compared with those of the Ad.CEA-TK/GCV treatment group (group III; group I versus group III, P < 0.0001). We did not observe any apparent side effects including body weight loss or death in these treatment cycles. These results suggest that the Cre/loxP system remarkably enhances antitumor effects on CEA-producing tumors in this cancer gene therapy model.

**DISCUSSION**

This is the first report demonstrating that application of the Cre/loxP system remarkably enhances selective expression of the HSV-TK gene in CEA-producing tumor cells and subsequent in vivo antitumor effects.

The activity of the CEA promoter in CEA-producing LoVo cells was comparable with that of the SV40 promoter determined by CAT assay (Table 1). Ad.CEA-TK rendered LoVo cells 296-fold more sensitive to GCV in vitro in comparison with uninfected cells (Fig. 3). However, repeated i.t. injection of Ad.CEA-TK followed by administration of GCV at most retarded the growth of the inoculated tumors (Fig. 4). On the other hand, i.t. injection of Ad.CAG-TK, which has the strong CAG promoter, with GCV treatment completely eradicated s.c. LoVo tumors in all animals. It is conceivable that expression of the HSV-TK gene at a higher level is necessary for complete rejection of tumors. To augment selective expression of the HSV-TK gene in CEA-producing cells, we applied the Cre/loxP system. Coinfection with Ad.CEA-Cre and Ad.Iox-TK significantly enhanced the sensitivity of CEA-producing LoVo and VMRC-LCD cells to GCV. The IC₅₀ of these cells were only 2.1- and 1.5-times as high as those of Ad.CAG-TK-transfected LoVo and VMRC-LCD cells, respectively. Moreover, infection with Ad.Iox-TK alone did not induce nonspecific GCV sensitivity in CEA-producing cells, nor did coinfection with these Ads significantly change GCV sensitivity in Non-CEA-producing cells (Fig. 3). GCV treatment following i.t. injection of Ad.CEA-Cre combined with Ad.Iox-TK induced immediate regression of established CEA-producing LoVo tumors and resulted in complete tumor rejection in six of seven animals (Fig. 4B). This treatment significantly suppressed the growth of the other CEA-producing VMRC-LCD tumors as well in all mice but did not show any antitumor effects on non-CEA-producing A549 tumors (data not shown). The following reasons might account for the enhanced antitumor effects. (a) A limited amount of induced Cre recombinase effectively processes a large number of molecules on the target Ad genome (23, 26). (b) The activity of the CAG promoter is far stronger than that of the CEA promoter. In fact, Ad.CAG-TK made LoVo cells 18-fold more sensitive to GCV compared with Ad.CEA-TK (Fig. 3). (c) Cre is efficiently activated inside the nucleus by NLS with an effective initiator codon of Ad.CEA-Cre (23).

Marginal retardation of tumor growth was observed in mice injected i.t. with a higher dose of Ad.CEA-Cre combined with Ad.Iox-TK then administered saline (Fig. 4B). When LoVo cells were coinfected with Ad.CEA-Cre and Ad.Iox-TK or infected with Ad.CAG-TK alone in vitro at a total moi of 30, the viability of the infected cells decreased to 70 or 50% of that of uninfected cells, respectively (data not shown). Other reports also showed that i.t. injection of Ads containing the CAG promoter significantly retarded the growth of s.c. tumors (16). We speculate that injection by a higher dose of Ads with the CAG promoter interferes with the transcription of genes necessary for cell survival and induces apoptosis (12, 16).

Death related to severe hepatic toxicity is reported when animals receive administration of Ad expressing the HSV-TK gene driven by the cytomegalovirus promoter followed by GCV treatment (27, 28). In another report, reverse transcriptase-PCR analysis detected expression of transduced genes predominantly in the liver, the kidneys, and the lung, and histological examination revealed zonal necrosis in the periporal area of the liver when an Ad expressing the CD gene under the control of the CAG promoter was i.p. administered then 5-fluorocytosine given (29). We did not observe any side effects including elevation of transaminase or creatinine in the serum of animals treated with i.p. injection of Ad.CEA-Cre and Ad.Iox-TK followed by GCV administration (data not shown). Thus, application of the Cre/loxP system is expected to not only enhance antitumor effects at a reduced dosage of Ads but also avoid damage to normal tissues. Another advantage in application of this system is that it might be no longer needed to specify the enhancer or silencer sequences in the genome for each cell type-specific promoter. Our ultimate goal is to control metastasized tumor cells without affecting normal cells. We are now planning to evaluate the efficacy and side effects of this system in the peritonitis carcinomatosa model in mice, mimicking pleuritis for lung cancer and peritonitis for gastrointestinal cancers in future clinical applications to human beings.

Recently, a combination of HSV-TK/GCV gene therapy with cytokines, including interleukin 2 (30, 31), interleukin 4 (32), and granulocyte-macrophage colony-stimulating factor (31), has been shown to enhance antitumor effects. Modified Ad.Iox-TK in which the neomycin resistance gene is replaced by these cytokine genes would enhance antitumor effects. When these Ads are used simultaneously with Ad.CEA-Cre, not only is the HSV-TK gene expressed specifically in CEA-producing tumor cells, but the cytokines released nonspecifically from surrounding normal cells also induce antitumor immunity.

In conclusion, the Cre/loxP system augments possible clinical application of gene therapy specific for CEA-producing cancer.

**ACKNOWLEDGMENTS**

We are grateful to Dr. Izumia Saito for providing materials used for viral constructs and for helpful technical support. We also thank Dr. Jun-ichi Miyazaki for the CAG promoter and Yoko Habe for technical and secretarial assistance.

**REFERENCES**

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