In Vivo Gene Therapy for α-Fetoprotein-producing Hepatocellular Carcinoma by Adenovirus-mediated Transfer of Cytosine Deaminase Gene

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ABSTRACT

The α-fetoprotein (AFP) gene is normally expressed in fetal liver and is transcriptionally silent in adult liver but overexpressed in human hepatocellular carcinoma (HCC). Here, we demonstrate that replication defective recombinant adenoviral vectors, containing the human AFP promoter/enhancer, can be used to express the Escherichia coli cytosine deaminase (CD) gene (AdAFP/CD) and the β-galactosidase gene (AdAF-PlacZ) in AFP-producing HCC cell lines. Expression of the CD gene by adenovirus from the AFP promoter/enhancer (AdAFP/CD) induced cells sensitive to 5-fluorocytosine (5FC) in the AFP-producing cells but not in the AFP-nonproducing cells. Transduction by an adenoviral vector harboring an ubiquitous strong promoter and CD gene showed enzymatic activity and 5FC killing in all cell lines. When AdAFPlacZ was injected into the s.c. established hepatoma in vivo, expression of the β-galactosidase gene was confined to AFP-producing HCC xenografts. Moreover, HCC xenografts regressed by transduction with AdAFP/CD and subsequently with 5FC treatment in vivo. These findings suggest that utilization of the AFP promoter/enhancer in an adenoviral vector can confer selective expression of a heterologous suicide gene in hepatocellular carcinoma cells in vitro and in vivo.

INTRODUCTION

HCC is one of the most common malignancies with poor prognosis in eastern Asia, especially China, Japan, Taiwan, Singapore, Korea, and sub-Saharan Africa. The annual number of deaths worldwide is evaluated at 1,250,000. Although multiple treatment modalities have been applied to HCC, it remains one of the most difficult tumors to treat when multiple foci of tumor or distant metastases are observed (1–4). HCC is highly amenable to the development of gene therapy (5–7). Since the majority of patients are not candidates for curative surgery, chemotherapy is often the only option; however, the high rate of serious events of drug prevents an effective chemotherapeutic treatment. 5FU is often used for the treatment of HCC in combination with another chemotherapeutic agent. It causes cell death by inhibition of both RNA and DNA, inhibiting both RNA and DNA syntheses; however, its effectiveness is limited by systemic toxicity associated with high doses (8, 9). Therefore, it may be desirable and feasible to produce a high local concentration of 5FU at the tumor site by targeting the drug in a tumor cell-specific manner to allow targeted killing of cancer cells (10). CD is a nonmammalian enzyme found in bacteria and fungi but absent in eukaryotes, which catalyzes the hydrolytic deamination of cytosine and 5FC to uracil and 5FU, respectively (11–13). Since 5FC is an antifungal drug that is relatively nontoxic in humans (14), it seemed possible to induce higher concentrations of 5FU at tumor sites in the presence of 5FC by means of transduction through a vector encoded with the CD gene.

Adenovirus vectors have been used to efficiently deliver exogenous genes into a variety of cells and tissues (15–18). Recently, adenovirus vectors containing transcriptional control elements which express the suicide gene preferentially to tumor cells in vitro and in vivo have been reported (19–24).

AFP is a tumor-associated marker for HCC which can be observed at elevated serum levels in advanced stage patients (25). Several cis- and trans-acting elements which regulate the human AFP gene have been identified. Previous studies have located the hepatocyte-specific enhancers in a far upstream region (~4.0 kb and ~3.3 kb) of the AFP gene and have shown the existence of the hepatocyte-specific silencer between the enhancer and hepatocyte-specific promoter region (26, 27).

In this study, we constructed replication-defective adenoviral vectors containing the CD gene or lacZ gene under control of the AFP promoter/enhancer, evaluated the expression of the reporter gene in AFP-producing cells in vitro and in vivo, and assessed the ability of AdAFP/CD to confer the sensitivity to 5FC in athymic nude mouse HCC models in vivo. Our data demonstrate that infection of AFP-producing HCC cells with an adenovirus vector containing the CD gene with 5FC treatment subsequently effectively suppresses the growth of these cells in vitro and in vivo.

MATERIALS AND METHODS

Cell Cultures. Human HCC cell lines, HuH-7 and HLF, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Human HCC cell line PLC/PRF/5 was obtained from the RIKEN Cell Bank (Tsukuba, Japan). Human embryonal kidney cell line 293 cells were purchased from American Type Culture Collection (Rockville, MD). HuH-7 cells were cultured in RPMI 1640 containing 0.5% FBS and 0.2% lactalbumin hydrosylate, and PLC/PRF/5 cells were cultured in DMEM with 10% FBS. HLF cells were grown in MEM with 20% FBS, and 293 cells were maintained in DMEM with 5% FBS. AFP secretion of cells was measured using the Serodia AFP mono kit (Fujirebio, Tokyo, Japan) as described previously (23).

Recombinant Adenovirus Preparation. The recombinant adenovirus vector AdAFP/CD (adenovirus vector containing the CD gene driven by AFP enhancer/promoter) was prepared in this study according to the method described previously (22–24). The pBluescript II SK-AF(AB) (23) contains the Apal-BgIII fragment including the AFP enhancer domains A and B (~4.0 to ~3.3 kb) and the BgIII-HindIII fragment covering the 0.17-kb AFP promoter. The ~1.4-kb Escherichia coli CD gene fragment and ~0.5- kb SV40 early mRNA polyadenylation signal sequence [poly(A)] were blunt ended and subcloned into the EcoRV site of the pBluescript II SK-AF(AB), resulting in pBluescript II SK-AF(AB)CD. The pBluescript II SK-AF(AB)CD was digested with XbaI, the ends were made blunt, and the AF(AB)CD-poly(A) expression cassette was subcloned into the Cleal site of the pAdex1 cosmid, resulting in pAdex1AFFP/CD. The pAdex1cosmid is a 42-kb cosmid containing a 31-kb adenovirus type 5 genome lacking EIA, EIB, and E3 genes as described previously (23).
described previously (28, 29). The expression cosmid cassette and adenovirus DNA-terminal protein complex were cotransfected into 293 cells by calcium phosphate precipitation. Incorporation of the expression cassette into the isolated recombinant virus was confirmed by digestion with appropriate restriction enzymes. The recombinant adenoviruses AdAFPPlac2 (lacZ gene driven by the AFP enhancer/promoter), AdCAlacZ (lacZ gene driven by the CAG promoter (30)), and AdCACD (CD gene driven by the CAG promoter) have been prepared previously (22, 23). The recombinant viruses were propagated with 293 cells and the viral solution was stored at −80°C. The titers of viral stocks were determined by plaque assay on 293 cells as described (27, 28). None of the stocks of virus used in the experiments contained detectable replication-competent viruses as evaluated using the PCR assay, which uses two pairs of primers in the same reaction to detect adenoviral E1A DNA with coamplification of E2B DNA as an internal control (31).

Adenovirus-mediated LacZ Expression in Vitro. The hepatoma cell lines were plated at a density of 5 × 10^4 cells/well in 6-well culture plates (Iwaki Glass, Tokyo, Japan) 24 h before AdAPFlacZ or AdCAlacZ infection. Immediately before infection, culture medium was aspirated and varying amounts of adenovirus were distributed over the cell monolayer. The ratio of the number of adenovirus per cell is expressed as moi. After 24 h of culture, the cells were stained with X-gal (Wako Pure Chemical Industries, Ltd.) to demonstrate the transduced lacZ gene product, and the number of β-galactosidase-positive cells was counted as described previously (23).

CD Enzymatic Activity. Enzymatic activity of CD was measured according to the method of Richards et al. (32) with minor modifications. Briefly, hepatoma cell lines were infected with either AdAFPPlacZ, AdAPFlacZ, AdCACD, or AdCAlacZ at a moi of 3 pfu/cell. After 24 h, the cells were washed three times with cold PBS and then resuspended in 1 ml of 100 mM Tris (pH 7.8) and 1 mM EDTA. Soluble cell extracts were prepared by sonication of the cells followed by centrifugation to remove cellular debris. Reaction solution (15 µl) containing 10 µg of extract in 3 mM cytosine (0.14 mCi/mmol [6-3H]cytosine; Moravek Biochemicals) was incubated at 37°C for 60 min and then terminated by the addition of 345 µl of 1 M acetic acid. The reaction product [3H]uracil was separated from [3H]cytosine using SCX Bond Elute (Varian) that had been previously rinsed with 1 ml of 1 M acetic acid. The radioactivity of AdAFPPlacZ- or AdCAlacZ-infected parental cell lysates from the radioactivity of AdAFPPlacZ- or AdCAlacZ-infected cell lysates was measured.

In Vitro SFC or SFU Sensitivity. Cells were plated at a density of 5 × 10^4 cells/well in 96-well culture plates (Iwaki Glass) and were infected with either AdAFPPlacZ, AdAPFlacZ, AdCACD, or AdCAlacZ at a moi of 3 pfu/cell. After incubation for a subsequent 24 h, the medium was aspirated and replaced with new medium containing various concentrations of 5FC. The cells were then cultured at 37°C for 6 days, and the number of viable cells was measured using the MTT assay as described previously (23). Inhibition of cell growth in the presence of various concentrations of SFC or SFU was analyzed as described previously (33). The IC_{50} of SFC or SFU was calculated using a curve-fitting parameter based on the Marquardt method (34).

In Vivo Tumor Treatment. To evaluate the ability of AdAFPPlacZ or AdCAlacZ to deliver transgenes in the tumors in situ, established tumors (100 mm³) were transduced with adenovirally directed gene transfer under control of the AFP promoter/enhancer, and AdCACD-infected human hepatoma cells were transduced with lacZ gene product, and the number of β-galactosidase-positive cells was counted as described previously (23).

RESULTS

Adenovirally Directed LacZ Gene Transfer in HCC Cell Lines in Vitro. Three human hepatoma cell lines, HuH-7, PLC/PRF/5, and HLF, were used as target cells for recombinant adenovirus infection. As shown in Table 1, HuH-7 and PLC/PRF/5 cells secrete AFP; however, HLF cells do not. To analyze the specificity of adenovirally directed gene transfer under control of the AFP promoter/enhancer, three HCC cell lines were infected with AdAFPPlacZ at different mois. At a moi of 10, expression of the lacZ gene product was demonstrated in almost all HuH-7 cells when stained with X-gal, but not in HLF cells. The lacZ gene product was expressed in two AFP-producing cell lines, HuH-7 and PLC/PRF/5, and the lacZ expression in these cells increased in parallel with the increase in moi. In contrast, lacZ gene expression was not detected in AFP-nonproducing HLF cells, even at a high moi. When these cells were infected with AdCAlacZ (lacz gene is driven by an ubiquitously strong CAG promoter), the lacZ gene was expressed in all three HCC cell lines. There was no statistical difference in gene transfer efficacy among these three cell lines, indicating that human HCC cells are susceptible to adenovirus infection.

CD Enzymatic Activity of AdAFPPlacZ- and AdCAlacZ-infected Hepatoma Cell Lines. To evaluate whether a functional CD enzyme was produced in cells infected with CD expressing adenovirus, cell lysates prepared from either AdAFPPlacZ-, AdAPFlacZ-, AdCACD-, or AdCAlacZ-infected and parental HCC cell lines were incubated with [6-3H]cytosine. Conversion from cytosine to uracil was determined by the relative amount of [6-3H]cytosine to [6-3H]uracil using an ion exchange column. The CD enzymatic activity of AdAFPPlacZ-infected cells was significantly higher in the cell lysates of HuH-7 and PLC/PRF/5 cell lines (163.7 pmol/min/mg protein and 125.9 pmol/min/mg protein, respectively) in contrast to that of the cell lysate of the HLF cell line (19.0 pmol/min/mg protein). Higher CD activity was found in all three cell lines when infected with AdCACD, and there was no statistical difference in CD enzyme activity among AdCAlacZ-infected cells (HuH-7, 50.755 pmol/min/mg protein; PLC/PRF/5, 53.128 pmol/min/mg protein; HLF, 53.879 pmol/min/mg protein). Furthermore, none of the AdAPFlacZ- or AdCAlacZ-infected cells produced [6-3H]uracil as compared to the counts of parental cells (data not shown). These results indicate the functional CD enzyme production of CD-transduced cells and the marked enzyme activity increase of AFP-producing cell lines when infected with AdAFPPlacZ.

Tumor Cells Expressing the CD Gene Are Sensitive to SFC in Vitro. To test whether adenovirus-mediated CD gene expression in the cells confer sensitivity to SFC, HCC cells were infected with either

<table>
<thead>
<tr>
<th>Table 1</th>
<th>AFP secretion and CD enzymatic activities of parental, AdAFPPlacZ-infected, or AdCAlacZ-infected human hepatoma cell lines</th>
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<tbody>
<tr>
<td>Cell lines</td>
<td>AFP secretion (ng/24 h/10^3 cells)</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>HuH-7</td>
<td>4204.5 ± 2460.0</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>475.4 ± 10</td>
</tr>
<tr>
<td>HLF</td>
<td>571.5 ± 49</td>
</tr>
</tbody>
</table>

* Results are represented as means ± SD (n = 6).

<table>
<thead>
<tr>
<th>CD activity (pmol/min/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>HLF</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
</tr>
<tr>
<td>HLF</td>
</tr>
</tbody>
</table>

BT, below threshold, not detectable.
Table 2  IC50 of 5FC or 5FU in parental, AdAFPCD-infected, or AdCACD-infected human hepatoma cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 of 5FU (μM)</th>
<th>Parental</th>
<th>AdAFPCD</th>
<th>AdCACD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuH-7</td>
<td>23.5</td>
<td>15,990</td>
<td>136.6</td>
<td>3.4</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>3.8</td>
<td>20,048</td>
<td>56.7</td>
<td>7.6</td>
</tr>
<tr>
<td>HLF</td>
<td>4.5</td>
<td>28,644</td>
<td>24,651</td>
<td>3.4</td>
</tr>
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</table>

Ratio of IC50

<table>
<thead>
<tr>
<th></th>
<th>AdAFPCD</th>
<th>AdCACD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuH-7</td>
<td>117.1</td>
<td>4702.9</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>353.6</td>
<td>2637.9</td>
</tr>
<tr>
<td>HLF</td>
<td>1.2</td>
<td>7956.7</td>
</tr>
</tbody>
</table>

a These results are representative of three independent experiments.
b Values obtained from IC50 of 5FC-treated parental/infected cells.

AdAFPCD, AdAFPlacZ, AdCACD, or AdCAIacZ at a moi of 3 and then exposed to 5FC at various concentrations for 6 subsequent days. The 5FC concentration yielding IC50 in HCC cell lines was determined by counting the number of viable cells in each group. As shown in Table 2, all parental cell lines were sensitive to 5FU at low micromolar concentrations. After infection with AdAFPCD, AFP-producing cell lines (HuH-7 and PLC/PRF/5) became sensitive to 5FC and the IC50 to 5FC shifted from >10,000 μM to 56.7–136.6 μM. By contrast, the viability of HLF, the AFP-nonproducing cell line, was not affected by 5FC even when the cells were infected with AdAFPCD. Additionally, the IC50 ratio value calculated from the difference between parental and AdAFPCD-infected AFP-producing cells was much higher than that of AFP-nonproducing HLF cells. All hepatoma cell lines infected with AdCACD became sensitive to 5FC (the IC50 values of HuH-7, PLC/PRF/5, and HLF were 3.4 μM, 7.6 μM, and 3.6 μM, respectively), indicating that HLF cells were also susceptible to the CD/5FC system. The MTT assay revealed that the viability of AdAFPCD-, AdAFPlacZ-, AdCACD-, or AdCAIacZ-infected cells at mois used in the absence of 5FC were similar to those of uninfected cells. 5FC sensitivities of HCC cell lines infected with AdAFPlacZ or AdCAIacZ were similar to those of uninfected control cells (data not shown).

In Vivo Treatment of Transplanted Human HCC Cells. Adenovirus-mediated gene transfer to PLC/PRF/5-derived s.c.tumors in vivo was evaluated using an adenovirus carrying the lacZ reporter gene. The lacZ gene was expressed in the tumors injected with AdAFPlacZ (Fig. 1A) or AdCAIacZ (Fig. 1B), but not in those injected with vehicle only (Fig. 1C). These data demonstrate that the lacZ gene can be efficiently delivered to solid tumors derived from AFP-producing HCC cells in vivo using an adenovirus vector containing the AFP promoter/enhancer. The lacZ gene was also expressed in the tumors that were injected with an adenovirus carrying the lacZ gene driven by an ubiquitously strong CAG promoter. Consistent with the in vitro studies, administration of AdAFPCD or AdCACD into the tumor foci of PLC/PRF/5 cells along with systemic 5FC administration resulted in a suppression of tumor growth compared to that in the control groups (Fig. 2). Tumors in the control groups—AdAFPCD treatment without 5FC administration, AdCACD treatment without 5FC administration, and vehicle treatment with or without systemic 5FC administration, showed almost similar growth curves. In contrast, tumors injected with AdAFPCD or AdCACD with concomitant 5FC administration resulted in suppression of tumor growth, with about 70–85% reduction in growth compared to controls at day 35. No associated systemic toxicity (reflected as animal death) from adenovirus infection nor from 5FC administration was demonstrated.

DISCUSSION

In the present study, we capitalized on the metabolic function of E. coli CD to inhibit tumor growth in vivo by using a replication-deficient recombinant adenovirus to transfer the CD gene selectively to HCC tumor xenografts in nude mice. Although transfer and expression of the CD gene has no cytotoxicity, the systemically administered nontoxic prodrug 5FC is converted into an antitumor chemo-

![Fig. 1. Adenovirus-mediated β-galactosidase expression in AFP-producing PLC/PRF/5 solid tumors in vivo. PLC/PRF/5 xenografts were grown s.c. in athymic nude mice. Histological feature of lacZ gene expression (blue) in s.c. tumor following injection with 1 × 10⁹ pfs of AdAFPlacZ (A) or AdCAIacZ (B) intratumorally and stained with X-gal and eosin. C, histological feature of s.c. tumor injected with vehicle only and stained with X-gal and eosin as control. Bar, 100 μm.](image-url)
therapeutic agent 5FU by CD in target tissues, thus effectively suppressing local tumor growth without systemic toxicity.

Currently available in vivo gene delivery systems generally lack the specificity of target cells. Although nonviral approaches, such as the DNA-liposome complex, ligand-DNA complex, and direct injection of DNA, have been reported for in vivo gene transfer, relative low rates of gene transduction are demonstrated and target tissues are restricted (35). Retrovirus vectors that contain transcriptional control elements for preferential expression of suicide genes within tumor cells are referred to as virus-directed enzyme/prodrug therapy (33). However, application of the retrovirus vector for in vivo gene therapy may be limited due to low viral titers (35).

Instead, recombinant adenoviruses have been used as highly efficient vectors for in vitro and in vivo gene deliveries (35). Adenovirus-mediated gene transduction is demonstrated in a broad spectrum of eukaryotic cells independent of cell replication. However, this vector presents two problems. One may be the nonspecific transduction of eukaryotic cells independent of cell replication. Although nonviral approaches, such as the DNA-liposome complex, ligand-DNA complex, and direct injection of DNA, have been reported for in vivo gene transfer, relative low rates of gene transduction are demonstrated and target tissues are restricted (35). Retrovirus vectors that contain transcriptional control elements for preferential expression of suicide genes within tumor cells are referred to as virus-directed enzyme/prodrug therapy (33). However, application of the retrovirus vector for in vivo gene therapy may be limited due to low viral titers (35).

The concentration of 5FC yielding 50% growth inhibition in AdAFP CD-infected HuH-7 and PLC/PRF/5 was 136.6 μM and 56.7 μM, respectively. These concentrations of 5FC could be acceptable, since nontoxic peak levels of 5FC in serum among the patients receiving 5FC therapy for antifungal diseases reach between 25 and 100 μg/ml (194–775 μM; Refs. 38 and 39).

The correlation between baseline AFP secretion and induction of CD activity and 5FC/FU sensitivity is not straightforward. In Table 1, the HuH-7 and PLC/PRF/5 cell lines differed in AFP secretion by nearly three orders of magnitude and yet had comparable inductions of CD with the AdAFP CD vector. This was true for 5FC/5FU sensitization. In the previous study using the chloramphenicol transference assay, we found that promoter activities of the AFP enhancer/promoter fragment were not always in parallel to the amount of AFP produced by human hepatoma cells, indicating that AFP production by these cells might be regulated by posttranscriptional levels (23).

Hepatic delivery should optimize vector delivery to the tumor efficiently and minimize the risk of exposure to normal tissues outside at the extrahaepatic organs. Since HCC patients often have varying sizes of multiple tumors in the liver without extrahepatic metastasis, gene delivery to the liver tumor in vivo should be performed via hepatic artery or portal vein. Since toxicity was observed in experimental animal studies at high doses of recombinant adenovirus, it was important to determine whether we could use optimal amounts of the virus without hepatic damage.

The elevated serum levels of AFP among these patients are mainly produced by tumors. However, surrounding hepatocytes may often

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4 Unpublished observation.
express AFP to some degree. After AdAFPCD infection with these nontumorous tissues, cytotoxicity may be induced by SFC systemic administration. In view of the clinical aspects of HCC, safe studies of these vectors are a necessity.

Higher expression of the CD gene by an ubiquitously strong CAG promoter of AdCACD was found in all three cell lines. AdCACD-mediated gene therapy also has the potential for the treatment of hepatocellular carcinoma; however, the toxicity of the nontumorous tissue must be considered. Localized delivery to the tumor such as a direct injection of AdCACD into the tumor would be preferable to minimize toxicity to normal cells.

The current study indicates that the CD gene can be efficiently transferred to human AFP-producing hepatoma cells using recombinant adenovirus in vivo. These results suggest that recombinant adenosine transmioter of the CD gene under control of a cell type-specific promoter could theoretically be a useful vector for the systemic treatment of metastasis.

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REFERENCES

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