Growth Suppression and Induction of Chemosensitivity in Human Gallbladder Epithelial Carcinoma Cells (GBCE) by Adenovirus-Mediated Transfer of the Wild-type p53 Gene

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Purpose: Mutations in the p53 gene are reported in 50~90% of gallbladder and bile duct cancer, and have been implicated in chemoresistance. We undertook this study to determine whether the introduction of the wild type p53 gene into GBCE (human gallbladder cancer cell line with a heterozygous p53 mutation) by an adenoviral vector could increase the sensitivity of the cell to 5-FU, a commonly used drug in the treatment of gallbladder cancer.

Materials and Methods: GBCE cells were transfected with either Ad/p53 or Ad/E1 in the presence of 5-FU. Gene expression was confirmed by western blotting. Nude mice were injected subcutaneously with GBCE cells. When tumors formed, intratumoral injection of Ad/p53 was performed. Reduction of tumor size was compared in two weeks of Ad/p53 gene transfection.

Results: Ad/p53 transfection induced a dose-dependent inhibition of tumor growth. Tumor colony formation was more inhibited with p53 gene transfection than with mock transfection in the presence of 5-FU. The reduction in tumor size was more pronounced with p53 transfection than with mock infection.

Conclusion: These treatment modalities could be utilized in the treatment of p53 mutant human gallbladder cancers. (Cancer Research and Treatment 2003;35:521-527)

Key Words: p53, Adenoviral vector, Chemosensitivity, Gallbladder cancer

INTRODUCTION

Mutations in the p53 gene, the most frequent genetic abnormality found in human tumors, are reported in 50~90% of gall bladder and bile duct cancer (1,2). p53 mutation appears to have an important role in the carcinogenesis because p53 expression is observed in early and advanced carcinoma, and is known to be related to poor prognosis (1). Also, p53 alterations have been implicated in the chemoresistance of most cancers. Consequently, the p53 gene has been a major candidate for somatic gene therapy approaches to human cancer, often with the goal of restoring chemosensitivity (3). GBCE is a well differentiated human gallbladder cancer cell line with a heterozygous p53 mutation (4). Transfer of a wild type p53 gene has been shown to result in growth arrest or apoptosis in tumor cells derived from various tissues (3). The adenoviral vector has emerged as a leading candidate for in vivo gene therapy in the past few years. An advantage over traditional DNA transfection and retroviral transfer in its high efficiency of transferring potentially therapeutic genes into a wide range of host cells has been well recognized (3).

Advanced stages of human gallbladder carcinoma showed frequent incidence of obstructive jaundice with cholangitis. Percutaneous transhepatic biliary drainage (PTBD) or endoscopic retrograde cholangiopancreatography (ERCP) is frequently performed to relieve obstructive jaundice. Since gallbladder and biliary cancer are repeatedly accessible for local injection via these routes and responses can be easily measured by computed tomography, tubogram, and bilirubin levels, they are good models for adenovirus-mediated gene therapy.

Therefore, we investigated the effects of adenoviral p53 transfection to see whether it inhibits colony formation on soft agar clonogenic assay, induces gross reduction of tumor size (in vivo), and has synergistic effects of chemosensitivity to 5-FU, a commonly used drug in the treatment of gallbladder cancer (in vitro).
MATERIALS AND METHODS

1) Cell lines

The p53 mutant well-differentiated human gallbladder cancer cell lines (GBCE) were kindly provided by Dr. Lee SP, University of Washington, Seattle. The cells were maintained in a RPMI 1640 ( Gibco BRL, Grand Island, NY) medium containing 10% heat-inactivated fetal bovine sera (Gibco BRL).

2) Construction and generation of recombinant adenovirus p53 vector

Three replication-defective recombinant adenoviruses, Ad/LacZ (harboring the β-galactosidase gene), Ad/ΔE1, and Ad/p53 (harboring the human p53 gene) were constructed from human adenovirus serotype 5 using standard homologous recombination techniques (5). The expression of these genes was driven by a CMV promoter. Quantities of Ad/p53 and Ad/LacZ suitable for in vitro and in vivo studies were produced by infecting 293 cells with validated viral stock and purified by cesium chloride gradient ultracentrifugation and dialysis. The recombinant adenoviruses were propagated with 293 cells and the titers of the adenoviral preparation were determined by a plaque-forming assay on 293 cells. The recombinant adenoviruses produced were diluted to a titer of 5×10⁷ PFU/ml in PBS and stored at -70°C.

3) Transduction efficiency with Ad/LacZ

The multiplicity of infection (MOI) was defined as the ratio of the total number of plaque-forming units used in a particular infection to the total number of cancer cells to be infected. Twenty-four hours after infection, Ad/LacZ and C293 cells were analyzed by β-gal staining for β-gal expression. C293 cells were used for control purposes.

4) Western blotting

After a 24-hour Ad/p53 transfection of GBCE cells at 10 MOI, cells were treated with RIPA buffer (50 mM TrisCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.1% SDS, 10 mM sodium deoxycholate). The cell lysate (20 μg) was electrophoresed in 12% SDS-polyacrylamide gel, followed by blotting onto a nitrocellulose membrane filter. In next step, anti-p53 antibody (DAKO, CA) was treated, horseradish peroxidase-conjugated anti-mouse antibody (Amersham, Buckinghamsire, England) was then added. Finally, the protein was detected by an enhanced chemiluminescence system (Amersham, Buckinghamsire, England).

5) In vitro chemosensitivity experiment

GBCE cells were transfected with either Ad/p53 or Ad/ΔE1 at various concentrations (0, 1, 10, 100 MOI) at day 0, and then, the solutions containing various concentrations (0, 1, 10, 100 μM) of 5-FU (Chongwae Ltd., Seoul, Korea) were added at day 1. On days 14 ~ 21, the number of colonies on soft agar were counted.

6) In vivo experiment of tumor reduction by Ad/p53 gene transfection

Nude mice were subcutaneously injected with GBCE cells, bilaterally. When tumors formed, intratumoral injection of Ad/p53 was performed. Reduction of tumor size was measured in two weeks of Ad/p53 gene transfection. The tumor sizes were observed every other day, Tumor volumes were estimated according to the formula. A×B², where A and B equal the long and short diameter of tumor xenografts, respectively. Six mice were used for each group.

Ad/p53-mediated apoptosis was measured by an apoptotic index. Apoptotic nuclei were defined as the dense red nuclear staining in nuclei with pyknotic or karyorrhectic histologic features. The number of nuclei was determined by counting the number of positively labeled nuclei over 1,000 tumor cells. The apoptotic index was expressed as the number of apoptotic nuclei per 100 cells.

7) Statistical analysis

Statistical analysis of calculated means was performed using the Kruskall Wallis test. 
P<0.05 was considered significant. The SPSS/PC+ software package (SPSS, Inc., Chicago, IL) was used.

RESULTS

1) Adenoviral infection of GBCE cells

The conditions for optimal adenovirus transduction of GBCE were determined by infecting these cells with adenovirus-expressing β-gal genes. The transduction efficiency was assessed by counting the number of blue cells after X-gal staining. There appeared to be a linear relationship between the number of infected cells and the number of adenovirus particles used. Cells inoculated with a single dose of 100 MOI of Ad/LacZ exhibited 60% blue cells (Fig. 1), and this was improved to 100% when C293 cells were targeted (data not shown).

2) Western blot analysis

MS-5 stromal cells were used for a western blot analysis as a negative control. GBCE cells transfected with Ad/LacZ or Ad/p53 at an MOI of 20 or 100 to detect wt-p53 were compared. GBCE cells transfected with Ad/LacZ showed weak bands of wt-p53, indistinguishable from that of GBCE alone, whereas the GBCE cells transfected with Ad/p53 showed thick bands of wt-p53 (Fig. 2). Cells transfected with Ad/p53 at 100 MOI showed thicker bands of wt-p53 relative to those with 20 MOI (data not shown).

3) Combined effect of adenovirus-mediated transfer of the wt-p53 gene with 5-FU (in vitro)

To determine whether wt-p53 could enhance drug cytotoxicity, we infected cells with Ad/p53 in the absence and presence of 5-FU. Infection with Ad/p53 rendered cells more sensitive to 5-FU. There was an inverse dose-response relationship between adenoviral titer and the number of colony formation on soft agar (Fig. 3A). Tumor colony formation of GBCE (1×10⁴) was more inhibited when the p53 gene and 5-FU were administered concomitantly rather than individually. A uniform feature was that colonies were hardly visible when 5-FU and Ad/p53 were co-administered (Fig. 3B).
4) Effect of adenovirus-mediated transfer of the wt-p53 gene in the absence of 5-FU (in vivo)

Tumor formation was prominent 5–6 weeks after subcutaneous injection of normal GBCE cells (1×10^6). The volume of tumors transfected with Ad/ŒE1 and Ad/p53 were compared 2–3 weeks after gene transfection. The size of tumors reduced more with p53 gene transfection compared with mock transfection (Fig. 4). Tumors obtained from either side of the nude mice were sectioned for in situ hybridization and H&E. While there was no significant difference in the apoptotic index between the GBCE cells transfected with Ad/ŒE1 and Ad/p53, the necrotic area was more prominent with the p53 gene transfection than with mock transfection (Fig. 5).

Fig. 1. GBCE cells (1×10^6) were transfected with variable MOI (multiplicity of infection). [(A) 0 MOI, (B) 1 MOI, (C) 10 MOI, and (D) 100 MOI] of Ad/LacZ, and stained with β-gal. Transduction efficiency of GBCE increased with a higher MOI (original magnification, ×40).

Fig. 2. Western blot analysis of p53 expression in lysates of MS-5 stromal cell line (a), GBCE alone (b), GBCE transfected with Ad/E1 (c) and GBCE transfected with Ad/p53 (d). Open arrow indicates the specific band of p53.
Fig. 3. (A) Dose-response relationship between adenoviral titer and the number of colony formation on soft agar. Data represent mean SD of triplicate dishes. (B) Tumor colony formation of GBCE (1×10^3) was inhibited more when the p53 gene and 5-FU were administered concomitantly rather than given individually. NS, not significant; *p < .05.

Fig. 4. Effect of intratumoral injections of Ad/p53 in the absence of 5-FU. (A, B, C) The size of tumor transfected with Ad/p53 (Rt, solid arrow) markedly decreased compared with Ad/ΔE1- mock transfected tumor (Lt, open arrow). (D) The size of tumors transfected with Ad/ΔE1 and Ad/p53 were compared with GBCE alone, 2 weeks after gene transfection in terms of tumor volume.
Fig. 5. Whereas in situ hybridization for apoptotic body (black arrow) in tumors obtained from a nude mouse showed no significant difference between GBCE cells transfected with Ad/§E1 (A) and Ad/p53 (B) [(original magnification ×100)], H&E staining for p53 showed marked difference of tumor necrosis between GBCE cells transfected with Ad/§E1 (C) and Ad/p53 (D) [original magnification ×100].

**DISCUSSION**

Gallbladder cancer and biliary cancer are extremely aggressive diseases: most of them are detected at an advanced stage and less than half of them have a chance of curative surgery. Patients with an advanced disease show less than 6 months of median survival and respond poorly to conventional chemotherapy (6). The goal of this study was to examine the growth suppression of cells by adenovirus-mediated transfer of the wt-p53 gene and induction of chemosensitivity in GBCE- a well- differentiated human gallbladder cancer cell line with heterozygous point mutations of p53 (codon 273: Arg to Leu). An advantage of this approach is that tumor cytotoxicity, induced by 5-FU may be enhanced by the addition of local production of p53. A number of studies have confirmed the potential of p53 as an agent for cancer gene therapy in various cancer cell lines, such as those of the colon, breast, and lung by the introduction of a wild-type p53 gene via a recombinant adenovirus (7), yet this has rarely been reported for biliary cancer cell lines. An adenovirus system has potential advantages for gene delivery in vivo, such as convenient production of high titer viruses and high transfection efficiency. Although adenoviruses bind efficiently to epithelial cells, and can efficiently transfer genes into both replicating and non-replicating cells, the transfection efficiency using adenovirus can vary depending on different cell types. In other words, susceptibility of tumor cell types to adenoviral vector infection is variable, thereby necessitating verification on an individual cell line basis. Considering transfection efficiency to be GBCE using Ad/LacZ reaching 60%, the adenoviral vector containing wt-p53 (Ad/p53) is an attractive delivery system for investigating the effect of exogenous wt-p53 on gallbladder cell lines both in vitro and in vivo.

Although the titer of adenoviral vector that we used was $5 \times 10^7$ PFU/ml in PBS, a titer up to $10^{11} \sim 10^{12}$ could easily be obtained with repeated CsCl-ultracentrifugation in the lab. For chemo-gene therapy, the levels of expression and the high infectivity may be more significant than the duration of expression because drugs can kill infected cells within several days.

One critical issue in viral-mediated gene therapy is how to efficiently deliver genes to the tumor mass. We directly measured the transfection efficiency using a reporter gene, and there was a dose-dependent relationship with increasing titer of the adenovirus. Western blot analysis demonstrated that production of p53 protein was significant in adenoviral mediated infected cells, whereas there was little p53 expression in mock infected cells, indicating that we successfully transfected wt-p53 into GBCE cells.

Potential mechanisms by which p53 may mediate antitumor
effects include direct effects on the transduced tumor cells (e.g., apoptosis or the induction of dormancy by down-regulation of VEGF), as well as indirect effects on neighboring non-transduced cells (i.e., bystander effects).

It was also shown that the growth suppressive function of certain p53 mutants could be recovered by a synthetic peptide, which restores the C-terminal domain of the gene responsible for DNA binding. The induction of apoptosis is one of the several documented functions of wt-p53. Tumor necrotic areas were prominent and broad in tumors transfected with Ad/p53 compared with those transfected with Ad/ifE1, whereas a difference of apoptotic index between GBCE cells transfected with Ad/ifE1 and Ad/p53 was modest in this study.

The human tumor clonogenic assay, a soft agar clonogenic assay first utilized in the 1970s, has a sensitivity of 60%–70% and 90% predictive value for resistance in a variety of tumor samples tested (8). Addition of 5-FU modestly decreased colony formation on soft agar. Addition of Ad/ifE1 decreased the colony formation on soft agar presumably due to random incorporation of the DNA into the housekeeping genes of GBCE. A striking and consistent feature was that colonies were hardly visible when 5-FU and p53 gene were administered concomitantly in contrast to when each were given individually, indicating that adenovirus-mediated transfer of p53 gene was more efficient compared with our previous results obtained using retroviral vector (9). Our present study clearly showed p53 to have a role in inducing chemosensitivity in cells.

Clinical trials utilizing the Ad-p53 gene have been performed in several tumors using different gene delivery methods (10–17). With the bolus injection method used in this trial, the distribution of Ad-p53 was limited to a short distance from the injection site (14). However, the narrow distribution was overcome by multiple injections (15).

Toxicities related to direct intratumoral injection of Ad-p53 during phase I or II of clinical trials in patients with lung, head and neck cancer have been shown to be minimal (10–13). As the intratumoral injection was designed to control only local masses, it is limited in treating the systemic spread of refractory tumor cells (18). Ad-p53 should be combined with DNA-damaging agents (such as radiation or chemotherapy) and the use of a conditional replication competent adenovirus controlled by a tumor-selective promoter, such as the human telomerase reverse transcriptase (hTERT) should be incorporated for efficacious systemic treatment (19–21). Substantial problems in systemic targeting of tumor cells with adenoviral vectors include adenovirus-neutralizing antibodies, heterogeneity or lack of expression of coxsackie-adenoviral receptors and integrin co-receptors in tumor cells (16).

CONCLUSION

We observed an increased in vitro 5-FU chemosensitivity and in vivo tumor size reduction after direct gene transfer of Ad/p53 into GBCE cells. These treatment modalities, therefore, may be beneficial in the treatment of p53 mutant human gallbladder cancer as well as biliary cancer.

REFERENCES


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