E1A, E1B Double-restricted Adenovirus for Oncolytic Gene Therapy of Gallbladder Cancer

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ABSTRACT

New treatments, such as gene therapy, are necessary for advanced gallbladder cancer (GBC), but little has been studied. Recent studies have introduced mutant adenoviruses (Ads) with either defective E1B-55kD or mutated E1A, focusing on tumor-specific replication, and the results have been promising. To enhance the safety of this approach, we constructed AxdAdB-3, a double-restricted Ad with a mutant E1A and E1B-55kD deletion. We studied the effects of this Ad in vitro and in vivo on GBC, as well as its safety for normal human cells. We compared the replication and cytopathic effects of AxdAdB-3 in several lines of GBC and primary normal cells with those of wild-type Ad or of AxE1AdB, an E1B-55kD-deleted Ad. The efficacy of AxdAdB-3 was examined in nude mice with s.c. implanted or i.p. disseminated GBC. AxdAdB-3 replicated in and caused oncolysis of GBC cell lines (TGBC-44TKB and Mz-ChA2) as efficiently as wild-type Ad or AxE1AdB in vitro. By contrast, AxdAdB-3 replicated much less effectively in primary normal cells (e.g., epithelial cells, endothelial cells, and hepatocytes) than in GBC cells and had only mild cytopathic effects, unlike wild-type Ad. Furthermore, cytopathicity of AxdAdB-3 in normal cells was milder than that of AxE1AdB. AxdAdB-3 significantly (P < 0.01) suppressed the growth of GBC (TGBC-44TKB) xenografts. AxdAdB-3 was also effective in the treatment of mice with peritoneally disseminated GBC (TGBC-44TKB), demonstrating tumor-selective replication and oncolysis that resulted in significantly (P < 0.05) prolonged survival. The present study shows that the E1 double-restricted Ad effectively and selectively replicates in and causes oncolysis of GBC in vitro and in vivo with reduced negative effects on normal cells, suggesting that this approach could be a promising tool for gene therapy of GBC.

INTRODUCTION

GBC is the fifth most common gastrointestinal malignancy, with an incidence of 2.5 and 10 per 100,000 persons in the United States and Japan, respectively (1). The disease is only curable at its early stages (2), but only about 35% of the patients could receive curative surgery, and other currently available treatments also have little effect on advanced GBC. The 5-year survival rate of such patients is only about 5% (2, 3). Clearly, a new treatment modality, such as gene therapy, is necessary to extend patient survival. Mutations in p53 have been found in more than 50% of patients with GBC (4–6), and a deficiency in p16, which leads to the loss of pRb function, has been reported in approximately 80% of patients with GBC (7, 8). However, few studies of gene therapy have been conducted thus far (9).

Initial gene therapy trials for cancer have used predominantly nonreplicating viruses as vectors but have not been very successful, primarily because of the limited efficiency of gene transfer. By contrast, oncolytic gene therapies using mutant viruses, directed toward tumor-specific replication, have recently emerged as potentially ideal treatments for solid tumors (10–22; for reviews see Refs. 10–14). Indeed, clinical trials with dl1520 (ONYX-015) have shown remarkably good results when used in combination with chemotherapy (10, 17). dl1520 (ONYX-015) is a mutant Ad lacking a p53-binding protein of $M_t$ 55,000 that is encoded by E1B, and it replicates efficiently in tumor cells that lack p53 (10, 14–18). More recently, another type of selectively replicating Ad with a mutation in the pRb-binding site of E1A has been reported by several investigators (19–22). These viruses propagate selectively in tumor cells and subsequently destroy the cancerous tissue, leaving the surrounding normal tissue unaffected.

Theoretical backgrounds of the tumor-specific replication of these mutant Ads are as follows (10, 14, 15). After infecting host human cells, Ad produces an early expression protein, E1A, which binds to pRb to activate E2F. The activated E2F induces a transition of host tissue unaffected. dl1520 (ONYX-015) is a mutant Ad lacking a p53-binding protein of $M_t$ 55,000 that is encoded by E1B, and it replicates efficiently in tumor cells that lack p53 (10, 14–18). More recently, another type of selectively replicating Ad with a mutation in the pRb-binding site of E1A has been reported by several investigators (19–22). These viruses propagate selectively in tumor cells and subsequently destroy the cancerous tissue, leaving the surrounding normal tissue unaffected.

One of the problems associated with E1B-55kD-deleted Ad is that this virus does replicate in and cause some CPE in normal cells (24–27). Similarly, it has been shown that E1A mutant Ad not only replicates in cancer cells but can also actively propagate in normal proliferating cells (19).

We have developed a new type of recombinant Ad, AxdAdB-3, which carries mutations in both the E1A and E1B regions of Ad5 (mutated E1A and defective in E1B-55kD) to enhance the likelihood of tumor-specific replication of the Ad and enhance the safety of its use. Because of the frequent abnormalities in pRb pathways and p53 pathways in GBC, we expected this E1 double-restricted Ad to be especially effective for GBC. In the present study, we attempted to clarify the efficacy of this double-restricted Ad, as well as that of an E1B-55kD-deficient Ad (single-restricted Ad), AxE1AdB (28), both in vitro and in vivo, as a possible tool for the treatment of GBC. Our results suggest that gene therapy with oncolytic E1 mutant Ads is a potentially useful approach for treatment of advanced GBC and that...
our new E1 double-restricted Ad might enhance the safety of E1B-55K-deficient Ad in cancer therapy.

**MATERIALS AND METHODS**

**Cell Lines and Culture.** TGBC-44TKB, which was established by Dr. T. Todoroki from a poorly differentiated adenosquamous GBC, has a deficient p53 and a mutated p16 (data not shown). Two other GBC cell lines, Mz-ChA1 and Mz-ChA2, which were provided by Dr. A. Knuth (Johannes-Gutenberg University, Mainz, Germany; Ref. 29), both have a mutated p53 and a deficient p16 (6, 30). HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA). Primary cultures of human hepatocytes, human intestinal epithelial cells (referred to as epithelial cells), and human adult microvascular endothelial cells (referred to as endothelial cells) were purchased from the Applied Cell Biology Research Institute (Kirkland, WA). A human fibroblast cell line, WI-38 (RCB 702), was purchased from RIKEN Cell Bank (Ibaraki, Japan). All GBC cell lines and WI-38 cells were maintained in DMEM supplemented with 10% FBS. The primary hepatocytes and primary epithelial cells were maintained in CS-2 complete serum-free medium (Cell Systems, Kirkland, WA), and the primary epithelial cells were maintained in CS-2.0 complete serum-free medium (Cell Systems). Culture plates coated with type I collagen were used for the primary culture of these human normal cells. For preparation of growth-arrested (quiescent) fibroblasts, the medium of confluent monolayer WI-38 cells was replaced by DMEM that contained 0.2% FBS to starve the cells.

**Construction of Viruses.** A mutant Ad5 E1A gene fragment containing a SXTGE (SXXGX) mutation at the LTCHX (LXXCX) Rb-binding pocket motif in Ad5 E1A exon 1 was obtained by reverse transcription-PCR using primers 1003 (GGCGATTCCCGACTGACATATCTGCGCAGGATGTG), 1024 (GGCGGTTTCACCGGGTGTGAGAATATCAATGATGACTCCCGGTTGCAAG), and plasmid pSKd/A-P/(TGAA5K) (31) as a template. The PCR fragment was digested with EcoRI and BamHI, and the resultant 396-bp fragment was subcloned into the EcoRI/BamHI sites of pBluescript SKII (+), resulting in plasmid pSKII/E1AmSXG (3336 bp). The BstXI fragment (23 bp) from pSKII/E1AmSXG was subcloned into the BstXI sites of the pSKd/A-P/(TGAA5K) (31), resulting in plasmid pSKd/E1AmBd-3 containing the SXTGE (STGXE) mutation instead of the LTCHX (LTXCX) Rb-binding pocket motif in E1A exon 1. The CIIaf fragment (2276 bp) from plasmid pSKd/E1AmBd-3 containing E1A with the SXTGE (STGXE) mutation together with the E1B-55K mutation (db; Ref. 31) was subcloned into the CIIaf sites of pAxC1w (31), resulting in plasmid pAxDAdB-3. The recombinant Ad, AxDAdB-3, was generated by cotransfection of pAxDAdB-3 cosmid DNA with the Ad genomic DNA. The construction of the two E1 mutant Ad genomes is shown in Fig. 1. The scheme for construction of the two E1 mutant Ad genomes is shown in Fig. 1. The two E1 mutant Ads, AxE1AdB and AxdAdB-3, both had a deletion in the gene for E1B-55K, which binds to p53 and inhibits its function. AxdAdB-3 also had a mutation in the CR2 region of E1A, which eliminated the ability of E1A to bind to and inactivate the function of pRb. Indeed, no pRb could be detected by anti-E1A antibody in the immunoprecipitates of WI-38 cells lysates after AxdAdB-3 infection, whereas after infection with Ad5-wt or AxE1AdB, pRb was detected (Fig. 1b).

**RESULTS**

The scheme for construction of the two E1 mutant Ad genomes is shown in Fig. 1a. The two E1 mutant Ads, AxE1AdB and AxdAdB-3, both had a deletion in the gene for E1B-55K, which binds to p53 and inhibits its function. AxdAdB-3 also had a mutation in the CR2 region of E1A, which eliminated the ability of E1A to bind to and inactivate the function of pRb. Indeed, no pRb could be detected by anti-E1A antibody in the immunoprecipitates of WI-38 cells lysates after AxdAdB-3 infection, whereas after infection with Ad5-wt or AxE1AdB, pRb was detected (Fig. 1b).

**Replication and CPEs of E1 Mutant Ads in GBC Cells.** First, we compared the replication and cytopathy of the two E1 mutant Ads (AxE1AdB and AxdAdB-3) in GBC cells with those of Ad5-wt. The two E1 mutant Ads proliferated as effectively (300–500-fold increase compared with the initially administered doses) as did Ad5-wt (400–
Ad5-wt in TGBC-44TKB cells (Fig. 2f) confirmed the replication of the two E1 mutant Ads as well as that of Ad5-wt effectively in Mz-ChA1 cells. Electron microscopic examination confirmed the replication of E1 mutant Ads using mouse anti-E1A antibody.

Input lane infected with Ad5-wt, AxE1AdB, or AxdAdB-3 (MOI) immunoprecipitates of E1A/host protein complexes. Total lysates from WI-38 cells were used for immunoprecipitation. Total lysates from WI-38 cells infected with Ad5-wt, AxE1AdB, or AxdAdB-3 (MOI = 100) were immunoprecipitated using mouse anti-E1A antibody. Input lane is the total lysates from WI-38 cells.

600-fold) in TGBC-44TKB and Mz-ChA2 cells (Fig. 2a). The two mutant Ads (70–80-fold) and Ad5-wt (200-fold) replicated less effectively in Mz-ChA1 cells. Electron microscopic examination confirmed the replication of the two E1 mutant Ads as well as that of Ad5-wt in TGBC-44TKB cells (Fig. 2b): numerous viral particles could be seen in the cytoplasm of infected cells 5 days after infection (10 MOI), which caused nuclear destruction and cell death. By contrast, no viral particles were found in mock-infected cells.

On WST-1 assay, both AxdAdB-3 and AxE1AdB showed significant dose-dependent and time-dependent CPE to TGBC-44TKB and Mz-ChA2 cells, as well as Ad5-wt (Fig. 3). In TGBC-44TKB cells, the E1 mutant Ads caused cytolysis even at a low MOI of 1. The sensitivity of Mz-ChA1 cells to the replicating Ads was lower than that of TGBC-44TKB and Mz-ChA2 cells, but these cells were also killed by the mutant Ads at a MOI of >10. The CPEs of the two E1 mutant Ads on GBC cells were similar to those of the wild-type Ad.

Replication and Cytotoxicity of E1 Mutant Ads in Human Normal Cells. Next, we compared the replication and CPE of the two E1 mutant Ads with those of Ad5-wt in several primary cultured normal cells. Ad5-wt replicated well in these normal cells; its titer increased approximately 250-fold in epithelial cells and 870-fold in endothelial cells within 6 or 8 days after infection. By contrast, the replication of the E1-mutated Ads (AxdAdB-3 in particular) was markedly suppressed in these normal cells (Fig. 4a). Whereas the titer of AxE1AdB increased approximately 12-fold in the epithelial cells and 25-fold in the endothelial cells, the titer of AxdAdB-3 increased only 3.4-fold in the epithelial cells and only 7-fold in the endothelial cells. In primary hepatocytes, the titer of Ad5-wt increased approximately 6400-fold, whereas the titer of the two E1 mutant Ads had only increased 90-fold. Because most normal cells are usually in a quiescent state, we next compared the replication of different Ads in the proliferative state and in the state in a normal fibroblast cell line, WI-38 (Fig. 4b). Whereas Ad5-wt replicated efficiently in both proliferating and quiescent WI-38 cells, the replication of the E1 mutant Ads was greatly suppressed in both cells with a greater extent of suppression in quiescent cells. The replication of AxdAdB-3 was inhibited more strongly than that of AxE1AdB in both proliferating and quiescent cells; the replication of AxE1AdB was one-eighteenth and one-sixty-eighth that of Ad5-wt, whereas that of AxdAdB-3 was one-thirty-fifth and one-four hundred and sixtieth that of Ad5-wt in proliferating cells and quiescent cells, respectively, and in particular, it was approximately one-seventh of AxE1AdB in quiescent cells (Fig. 4c).

The two E1 mutant Ads, at a MOI of 10, had almost no CPEs on the primary epithelial cells, endothelial cells, and hepatocytes on WST-1 assay (Fig. 5). At a MOI of 100, they had mild CPEs on these cells, but such effects were clearly and significantly (P < 0.01) weaker than those of Ad5-wt. AxdAdB-3 demonstrated significantly (P < 0.01) weaker CPE than AxE1AdB in epithelial cells and fibroblasts. These findings were also confirmed by light microscopy (Fig. 6).

In Vivo Antitumor Effects of E1 Mutant Ads in GBC Xeno-grafts and i.p. Disseminated GBC. We next examined the antitumor effects of the two E1 mutant Ads in vivo. In s.c. GBC xenografts of more than 200 mm3, the tumors of PBS-injected mice grew rapidly and reached approximately 7× the initial volume within 28 days. Tumor growth in mice treated with AxE1AdB or AxdAdB-3 was significantly (P < 0.01) suppressed, and the volumes had increased less than 2-fold at 28 days. Tumor was regressed in the mice given the combination of AxdAdB-3 plus i.p. 5-FU, and the tumors in three of eight mice disappeared completely as a result of this combination (Figs. 7a). Histochemical analysis on day 15 revealed diffuse and extensive necrosis (Fig. 7b, nec.) in tumors treated with AxdAdB-3, but not in those treated with PBS. Immunohistochemical staining of the AxdAdB-3-treated tumors detected Ad E1A protein in the remaining viable cells around necrotic areas, suggesting that the virus was still spreading in the tumor tissues (Fig. 7c).
Finally, we examined the antitumor effects of AxdAdB-3 in a peritoneal dissemination model. Histological analysis (day 11) of the disseminated tumor nodules on mesentery of AxdAdB-3-treated mice (Fig. 8a, arrows) revealed that among many viable cells, some tumor cells already became necrotic (Fig. 8b). Immunohistochemical staining detected diffuse and numerous staining of E1A in the remaining viable cells in the tumor (Fig. 8c). No immunostaining was detected in other normal tissues, such as the peritoneum, serous membrane, and intestinal wall. In all mice treated with PBS, the cancer cells continued to spread diffusely, causing extensive abdominal distension with formation of bloody ascites by day 35 (Fig. 8d, left panel). By contrast, in all mice that had received i.p. AxdAdB-3, there was no such abdominal distension (Fig. 8d, right panel). This difference was associated with a significant difference in survival; mice treated with AxdAdB-3 survived significantly (P < 0.05) longer than mice treated with PBS (median survival, 7 weeks in PBS-treated mice versus 10 weeks in AxdAdB-3-treated mice; Fig. 8e). The cause of death in all mice injected with PBS was considered to be peritoneal dissemination. By contrast, among the six mice treated with AxdAdB-3, only two died with bloody ascites; extensive metastasis to the liver was considered to be the cause of death in the others.

**DISCUSSION**

In the present study, we evaluated the efficacy and safety of a novel E1A, E1B double-restricted Ad, AxdAdB-3. We found that this Ad replicates less efficiently in and is less toxic to normal cells than the E1B-55kD single-restricted Ad, AxE1AdB, while retaining similar potent oncolytic effects against several GBC cell lines in vitro and in vivo.

Various reports have demonstrated the efficacy of E1B-55kD-deleted Ads (including ONYX015) and the E1A mutant Ad (16–22). However, one of the problems associated with E1B-55kD-deleted Ad is that the proposed mechanism of its attenuation in normal cells depends on the inability of this virus to inactivate cellular p53 and its downstream factor, p21, which functions upstream of pRb to induce cell cycle arrest. Thus, if E1B-55kD-deleted Ad infects a normal cell, the intact E1A of this virus can bind to pRb, thereby bypassing the p21 checkpoint, and cell arrest does not occur (15). Such mutant Ads do, in fact, replicate in and are somewhat toxic to normal cells (24–27). In the case of the E1A mutant Ad, the proposed mechanism of its attenuation in normal cells depends on its inability to bind to pRb and to activate E2F. Thus, the E1A-mutant Ad not only replicates in cancer cells but is also likely to proliferate actively in normal prolif-
erating cells in which E2F has already been activated (19). The intact E1B-55kD protein of the virus would inhibit the function of p53, and the virus would then continue to proliferate until it had caused significant damage to the normal proliferating cells. We postulated that the E1 double-mutant Ad, with mutations in genes for both E1A and E1B, would be able to inactivate neither pRb nor p53 and thus would only multiply in cells with abnormalities in both the pRb and p53 pathways, and not in cells with normally functioning pRb or p53 pathways. Indeed, in the present study, AxdAdB-3 showed more attenuated replication (Fig. 4) and had a significantly lower CPE in normal cells than did AxE1AdB (Fig. 5). These results support our hypothesis that our new E1A, E1B double-restricted Ad should be safer than the E1B single-restricted Ad.

AxdAdB-3 had a dose-dependent and potent CPE, as did Ad5-wt and AxE1AdB, on several lines of GBC cells in vitro (Fig. 2). In s.c. GBC xenografts, i.t. injected E1 mutant Ads caused extensive tumor necrosis and significantly inhibited tumor growth (Fig. 7). AxdAdB-3 could also efficiently infect disseminated GBC, decrease the incidence of massive bloody ascites, and prolong survival (Fig. 8). These results suggest that i.p. injection of the E1 double-restricted Ad might be a potentially effective therapy for GBC with peritoneal dissemination. This is of particular clinical significance in view of the high incidence of peritoneal dissemination in patients with advanced GBC, which is the result of both the thin wall of the gallbladder and the tendency of malignant cells to invade lymphatic vessels.

One may think that the major problem of oncolytic virotherapy is...
its limited anticancer activity rather than its safety. However, these viruses have demonstrated significant efficacy in clinical trials when combined with chemotherapy (16, 17, 32, 33) and in preclinical studies when combined with additional therapeutic genes [e.g., suicide genes, genes for cytokines (28, 34, 35)]. Indeed, combination therapy with 5-FU showed enhanced efficacy (Fig. 7a), which may be due to chemosensitizing effects of E1A protein (36, 37). We are currently studying the combination of AxdAdB-3 with herpes simplex virus-thymidine kinase to increase the efficacy of AxdAdB-3. We believe that the enhanced safety profile of our E1A, E1B double-restricted Ad, compared with the E1B single-restricted Ad, would make a significant difference in efficacy, especially when the virus is armed with additional cytotoxic genes or used in combination with other therapeutic agents. An increased width of the therapeutic window by reducing toxicity would eventually be a benefit to the patients because the dose of the agent could be increased or the treatment could be combined with additional potent modalities.

All of the GBC cell lines tested had abnormalities in both p53 and p16, which probably explains why AxdAdB-3 was able to replicate in and lyse these cells as effectively as the E1 single-restricted (E1B-55kD-deleted) Ad or wild-type Ad (Figs. 2 and 3). One might assume that the types of cancer responsive to AxdAdB-3 are limited because the mutant Ad requires abnormalities in both the pRB pathways and p53 pathways. E1B-55kD single-restricted Ad was originally expected to multiply only in cells with a mutant p53 (19), but it was subsequently found to also replicate in and kill cancer cells with wild-type p53 (15, 24–26). A proposed explanation for this latter phenomenon is mutant p14 or an abnormality in the p53 signaling pathway (38). Currently, it is assumed that abnormalities in p53 or its upstream signals (p14, mdm2, and ATM) are present in most cancers (23). Similarly, abnormalities in components of the pRb signaling pathway (pRb, p16, CDK2, and CDK4/6) are also expected in nearly all cancer cells (23). It has been reported that approximately 80% of patients with GBC have some abnormalities in p16 (7, 8), and more than 50% of patients with GBC have mutations in p53 (4–6). Thus, we might reasonably expect the E1 double-restricted Ad to be very effective in many patients with GBC, as well as in patients with other malignancies. Additional studies regarding the effects of this mutant Ad in many other cancers might prove that both pRb pathways and p53 pathways are simultaneously impaired in many cancer cells, as has recently been suggested in a review by Sherr and McCormick (23).

The minimal effective dose of the E1 mutant Ad differed among GBC cell lines. Mz-ChA1 cells have a mutated p53 and a deleted p16, but they were barely sensitive to any of the Ads at a MOI of 10. This insensitivity was due to the low Ad infection rate of Mz-ChA1 cells, which was one-tenth that of other GBC cell lines (data not shown). Recently, expression of the Coxsackie Ad receptor, the primary cellular receptor for Ad, was shown to affect the oncolytic potential of replicating Ad (39). Approaches designed to achieve a more selective mode of infection and improve the rate of infection (31, 40, 41) will be required to kill GBC cells with low Ad infectivity, and such studies are under way in our laboratory.

In conclusion, we evaluated the efficacy and safety of an E1A, E1B double-restricted Ad, AxdAdB-3, for use in the treatment of GBC. Our data suggest that this mutant Ad is less toxic than E1B-55kD-defective Ad to normal cells but has a similar potent dose-dependent oncolytic effect on GBC cells. The double mutant Ad suppressed tumor growth in vivo to a significant extent, and its effects were enhanced in combination with 5-FU. Furthermore, it significantly prolonged the survival of mice with diffuse peritoneal dissemination of GBC. An increased safety profile of this Ad would allow its combined use with genes of potent antitumor activity to further increase its efficacy.

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