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E1A, E1B Double-restricted Adenovirus for Oncolytic Gene Therapy of Gallbladder Cancer[1]

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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 *in vivo* 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, cytotoxicity 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[3] 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_r 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 cells from G_1 phase to S phase and thus enables Ad to replicate. However, as E2F is activated, p53 is activated through the $p14^{ARF}$-p53 pathway, and the replication of Ad stops because of apoptosis induced by p53 or cell cycle arrest induced by its downstream factor, p21. Ad E1B-55kD inhibits the function of p53 and enables Ad to continue proliferating actively in the host cells. In many cancers, the pRb pathway is disrupted, causing deregulation of the cell cycle, and the p53 pathway is abnormal, causing an escape from apoptotic response (23). Therefore, the functions of E1A and/or E1B-55kD protein are dispensable for Ad to replicate in many cancer cells, but they are not dispensable in normal cells that have no abnormalities in the pRb pathway or p53 pathway.

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

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[3] The abbreviations used are: GBC, gallbladder cancer; Ad, adenovirus; Ad5-wt, wild-type adenovirus type 5; CPE, cytopathic effect; 5-FU, 5-fluorouracil; i.t., intratumoral; MOI, multiplicity of infection; pRb, retinoblastoma protein; PFU, plaque-forming unit(s); HEK, human embryonic kidney; FBS, fetal bovine serum; Rb, retinoblastoma; CDK, cyclin-dependent kinase.

our new E1 double-restricted Ad might enhance the safety of E1B-55kD-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 endothelial cells were maintained in a CS-C 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 STGHE (S*X*G*X*E) mutation at the LTCHE (L*X*C*X*E) Rb-binding pocket motif in Ad5 E1A exon 1 was obtained by reverse transcription-PCR using primers 1003 (GCGAATTCACCATGAGACATATTATCTGCCACGGAGGTGTT), 1024 (GGCGGATCCAGCCTCGTGGCCAGTACTATCGATCACCTCCG-GTACAAG), and plasmid pSKd+[A-P](TGA55K) (31) as a template. The PCR fragment was digested with *Eco*RI and *Bam*HI, and the resultant 396-bp fragment was subcloned into the *Eco*RI/*Bam*HI sites of pBluescript SKII+, resulting in plasmid pSKII+E1AmSxG (3336 bp). The *Bst*XI fragment (23 bp) from pSKII+E1AmSxG was subcloned into the *Bst*XI sites of the pSKd+[A-P](TGA55K) (31), resulting in plasmid pSKd+dAdB-3 containing the S*X*G*X*E (STGHE) mutation instead of the L*X*C*X*E (LTCHE) Rb-binding pocket motif in E1A exon 1. The *Cla*I fragment (2276 bp) from plasmid pSKd+dAdB-3 containing E1A with the S*X*G*X*E (STGHE) mutation together with the E1B-55kD mutation (dB; Ref. 31) was subcloned into the *Cla*I sites of pAx1cw (31), resulting in cosmid pAxdAdB-3. The recombinant Ad, AxdAdB-3, was generated by cotransfection of pAxdAdB-3 cosmid DNA with the Ad genomic DNA-terminal protein complex into HEK293 host cells, as described previously (31). Generation of AxE1AdB has been described previously (28, 31). Ad5-wt was purchased from the American Type Culture Collection, and Ax1w1: mock (RDB 1746) was provided by the RIKEN DNA Bank (Ibaraki, Japan). All Ad vectors were purified by CsCl gradient centrifugation.

Immunoprecipitation and Western Blotting Analysis. WI-38 cells (5×10^5 cells) were seeded in 90-mm culture dishes; infected with Ad5-wt, AxE1AdB, or AxdAdB-3 at a MOI of 100; and then incubated for 4 days. After incubation, the cells were harvested and lysed with radioimmunoprecipitation assay buffer [150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, and 50 mM Tris (pH 7.4)]. The cell lysate was incubated with anti-Ad5-E1A mouse monoclonal antibody (Ab-1; NeoMarkers, Fremont, CA) for 60 min, with rotation at 4°C. After incubation, the lysate was further incubated with protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. The bound proteins were eluted with sample buffer from protein A/G plus agarose and analyzed by Western blotting. pRb was detected with anti-pRb mouse monoclonal antibody (Ab-1; Oncogene, Boston, MA). Signals were visualized with the enhanced chemiluminescence plus system (Amersham, Buckinghamshire, England) using horseradish peroxidase-conjugated antimouse IgG sheep polyclonal antibody.

Viral Replication. Cells were seeded in 12-well plates with appropriate medium at a density of 1×10^5 cells/well, and cells were infected with Ad5-wt, AxE1AdB, or AxdAdB-3 at a MOI of 10. The medium was replaced with fresh medium 6 h after infection. Cells and culture supernatant were harvested 4, 6, and 8 days after infection. Cells were sonicated, and virus in lysates was quantitated. The titer of Ad was evaluated by standard plaque-forming assay with HEK293 cells. Viral replication was also examined by electron microscope (H7000; Hitachi Co., Tokyo, Japan). Cells were fixed in PBS (10.9 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, and 8.2 g/liter NaCl) containing 2% glutaraldehyde, and ultrathin sections were stained with uranium acetate and lead nitrate before examination.

Cell Viability and Growth *in Vitro*. All cells but the WI-38 cells were seeded in 96-well plates at a density of 2×10^3 cells/well with appropriate medium, and the WI-38 cells were seeded at a density of 5×10^3 cells/well in DMEM containing 0.2% FBS. GBC cells were mock infected or infected with Ad5-wt, AxE1AdB, or AxdAdB-3 at a MOI of 1, 10, or 100. Primary cultures of epithelial cells, endothelial cell hepatocytes, and WI-38 cells were similarly infected at a MOI of 10 or 100. Numbers of viable cells were evaluated by a colorimetric WST-1 assay. WST-1 (Takara, Shiga, Japan), a tetrazolium salt, is cleaved to a formazan product by enzymes in metabolically active cells, and the reaction is quantitated with an automatic plate reader at 450 nm with a reference wavelength of 650 nm. To translate the assay results to the numbers of viable cells, standard curves were made in each experiment, and the sample dose for the assay was adjusted, if necessary, to use the linear part of each curve.

Animal Studies. Female, 4-week-old, BALB-c *nu*/*nu* athymic mice (CLEA Japan, Tokyo, Japan) were quarantined for 1 week. A s.c. GBC xenograft model was prepared by injecting 1×10^7 TGBC-44TKB cells in 100 μl of DMEM without serum into the left flank of each mouse. Tumors were measured with calipers, and the volume was calculated as 0.4 × longest diameter × width^2. When the tumor volume had reached approximately 200–250 mm^3 (a diameter of 8–10 mm), animals were randomly assigned to one of the following five groups: (*a*) i.t. injection of AxE1AdB (2×10^8 PFU) in PBS (100 μl; $n = 8$); (*b*) i.t. injection of AxdAdB-3 (2×10^8 PFU) in PBS (100 μl; $n = 8$); (*c*) i.t. injection of PBS (100 μl) alone ($n = 6$) on days 1–3 and 8–10; (*d*) i.t. injection of AxdAdB-3 (2×10^8 PFU) in PBS (100 μl; $n = 6$) on days 1–3 and 8–10 plus i.p. injection of 5-FU (30 mg/kg; $n = 8$); or (*e*) i.p. injection of 5-FU (30 mg/kg) alone on days 4–6 and 11–13. An i.p. dissemination model was prepared by i.p. injection of 1×10^7 TGBC-44TKB cells in DMEM (200 μl) without serum, with subsequent growth of tumors for 14 days. After i.p. dissemination, tumors were recognized in one mouse by sacrifice, and the other mice were treated according to one of the following regimens: (*a*) i.p. injection of AxdAdB-3 (2×10^8 PFU) in PBS (200 μl; $n = 6$); or (*b*) i.p. injection of PBS (200 μl) alone ($n = 6$) on days 1–3 and 8–10. For histological analysis, tumors were fixed in 4% formalin, and the tissue sections (4 μm) were stained with H&E. The presence of Ad was immunohistochemically tested by using anti-E1A rabbit polyclonal antibodies (13S-5; Santa Cruz Biotechnology) with a VECTASTAIN ABC-PO (rabbit IgG) kit (Vector Laboratories, Burlingame, CA) and 3–3′ diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with hematoxylin. These experiments were approved by the Animal Studies Ethics Committee of the University of Tsukuba.

Statistical Analysis. The significance of differences between groups was assessed by Student's unpaired two-tailed *t* test. Survival was analyzed by the Kaplan-Meier method. Results were compared for statistical significance by applying the generalized Wilcoxon test.

RESULTS

The scheme for construction of the two E1 mutant Ad genomes is shown in Fig. 1*a*. The two E1 mutant Ads, AxE1AdB and AxdAdB-3, both had a deletion in the gene for E1B-55kD, 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. 1*b*).

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–

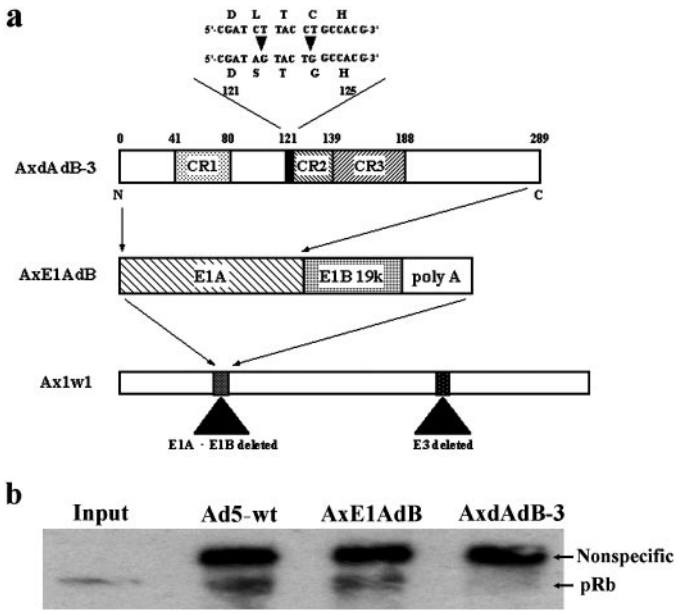

Fig. 1. Schematic representation of the E1 mutant Ads. *a*, the schematic of two E1 mutant Ads. AxE1AdB is an E1 construct that lacks the E1B-55kD region and has a SV40 polyadenylate sequence at the end of the construct. AxdAdB-3 is identical to AxE1AdB, except for a mutation in E1A. The amino acid sequence L*X*C*X*E of CR2 was changed to S*X*G*X*E to eliminate the ability to bind to pRb. *b*, detection of pRb by Western blotting in immunoprecipitates of E1A/host protein complexes. 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. 2*a*). 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. 2*b*): 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. 4*a*). 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. 4*b*). 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. 4*c*).

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 Xenografts 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 mm^3, 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. 7*a*). Histochemical analysis on day 15 revealed diffuse and extensive necrosis (Fig. 7*b*, *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. 7*c*).

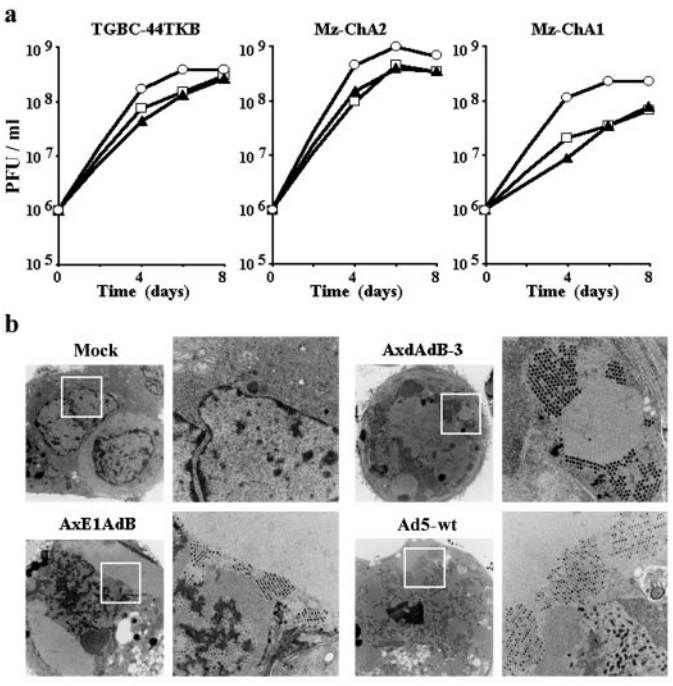

Fig. 2. Replication of mutant and wild-type Ads in GBC cells. *a*, replication of the two E1 mutant Ads was compared with that of Ad5-wt in three GBC cell lines (TGBC-44TKB, Mz-ChA2, and Mz-ChA1). The GBC cells were infected with Ad5-wt (○), AxE1AdB (▲), and AxdAdB-3 (□) at a MOI of 10, and replication of virus was determined 4, 6, and 8 days after infection. *b*, electron micrographs of TGBC-44TKB cells 4 days after infection with the indicated viruses at a MOI of 10. Original magnification: ×2,000 (*left panels*); ×10,000 (*right panels*; magnification of *squares* in *left panels*).

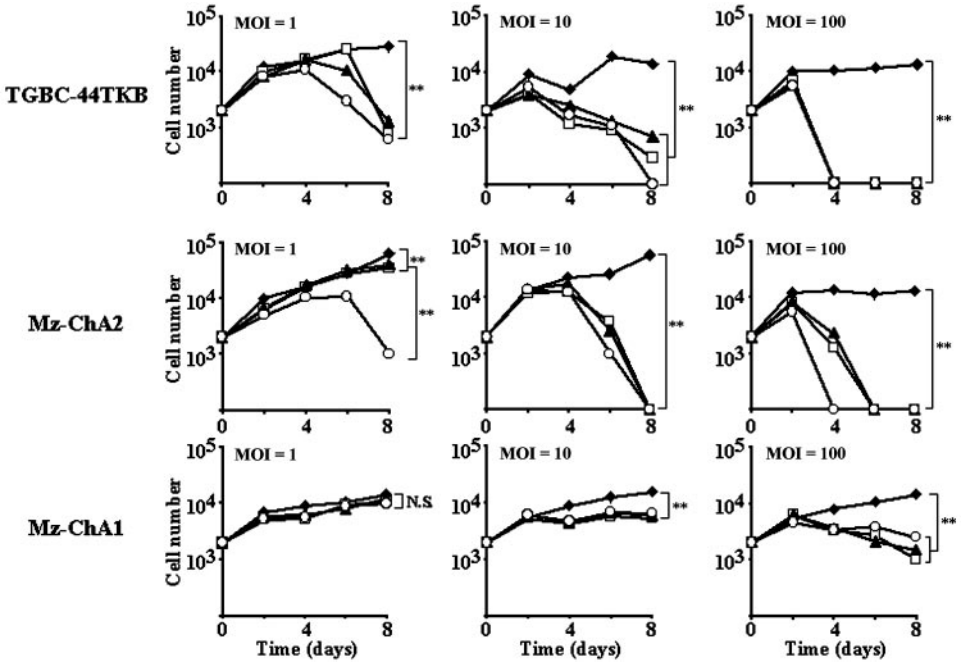

Fig. 3. CPEs of mutant and wild-type Ads on GBC cells. Three GBC cell lines (TGBC-44TKB, Mz-ChA1, and Mz-ChA2) were mock-infected (♦) or infected with either Ad5-wt (○), AxE1AdB (▲), or AxdAdB-3 (□) at MOIs of 1, 10, and 100. Numbers of viable cells were determined by WST-1 assay 2, 4, 6, and 8 days after infection. Differences, as compared with results after mock infection, are indicated as ** (significant; $P < 0.01$) or *N.S.* (not significant).

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. 8*a*, *arrows*) revealed that among many viable cells, some tumor cells already became necrotic (Fig. 8*b*). Immunohistochemical staining detected diffuse and numerous staining of E1A in the remaining viable cells in the tumor (Fig. 8*c*). 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. 8*d*, *left panel*). By contrast, in all mice that had received i.p. AxdAdB-3, there was no such abdominal distension (Fig. 8*d*, *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. 8*e*). 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.

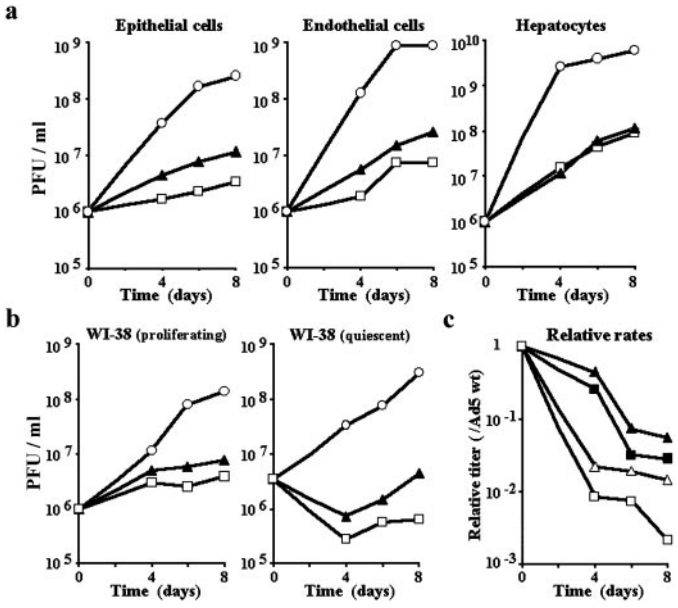

Fig. 4. Replication of mutant and wild-type Ads in normal human cells. *a*, replication of the two E1 mutant Ads (AxE1AdB and AxdAdB-3) in primary cultured human epithelial cells, endothelial cells, and hepatocytes was compared with that of Ad5-wt at a MOI of 10. The replication of Ad5-wt (○), AxE1AdB (▲), and AxdAdB-3 (□) was determined 4, 6, and 8 days after infection. *b*, replication of Ads in proliferating and quiescent WI-38 cells. WI-38 cells proliferating in 10% FBS or quiescent in 0.2% FBS were infected with Ad5-wt, AxE1AdB, and AxdAdB-3 at a MOI of 10, and the rate of replication was determined. *c*, relative rates of replication of the two E1 mutant Ads, as compared with that of Ad5-wt in quiescent and proliferating WI-38 cells. Relative rates of replication were assessed in terms of the ratio of the viral titer of the E1 mutant Ad to that of Ad5-wt. The relative rates of replication of AxE1AdB (▲) and AxdAdB-3 (■) in proliferating WI-38 cells and those of AxE1AdB (△) and AxdAdB-3 (□) in quiescent WI-38 cells are shown.

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-

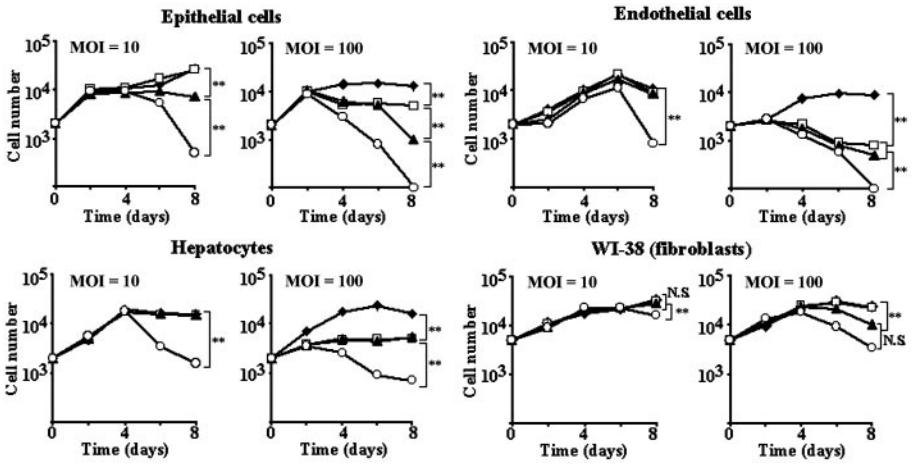

Fig. 5. CPEs of mutant and wild-type Ads on human normal cells. *a*, primary cultured cells (epithelial cells, endothelial cells, and hepatocytes) and WI-38 cells were mock-infected (♦) or infected with Ad5-wt (○), AxE1AdB (▲), and AxdAdB-3 (□) at a MOI of 10 and a MOI of 100. Numbers of viable cells were determined by WST-1 assay 2, 4, 6, and 8 days after infection. Differences, as compared with results for Ad5-wt, are indicated as ** (significant; $P < 0.01$) or *N.S.* (not significant).

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

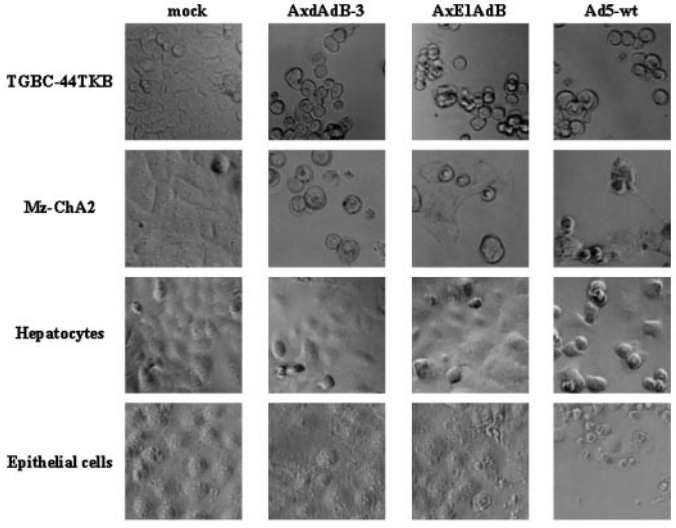

Fig. 6. Light micrographs of GBC cells and normal cells 6 days after infection. The GBC cells (TGBC-44TKB and Mz-ChA2 cells) and normal cells (hepatocytes and epithelial cells) were infected with the indicated virus at a MOI of 10. Original magnification, ×400.

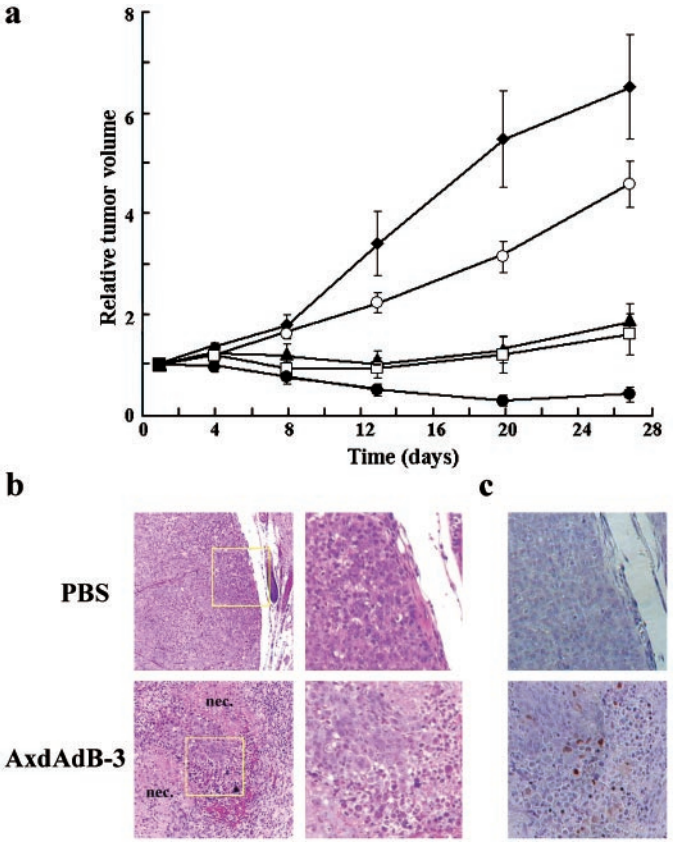

Fig. 7. Antitumor effects of i.t. injected E1 mutant Ads on s.c. implanted GBC in nude mice. TGBC-44TKB cells (1×10^7) were injected into the left flank and allowed to grow until tumors reached a volume of 200–250 mm^3. The tumors were treated by one of the following regimens: i.t. injection of AxE1AdB (2×10^8 PFU; ▲), AxdAdB-3 (2×10^8 PFU; □), or PBS only (♦) on days 1–3 and 8–10 or i.t. injection of AxdAdB-3 (2×10^8 PFU) with i.p. 5-FU (30 mg/kg; ●) or 5-FU alone (○) on days 4–6 and 11–13. *a*, time course of changes in relative tumor volume (relative to the volume at initial injection). *b*, histological analysis (H&E staining) on day 15 of an AxdAdB-3-treated tumor and a PBS-treated tumor. In the AxdAdB-3-treated tumor, extensive tumor necrosis (*nec.*) was detected. Original magnification: ×100 (*left panels*); ×200 (*right panels*; magnification of *squares* in *left panels*). *c*, immunohistochemical detection of E1A. Tumor cells that expressed E1A protein were detected as *brown spots* (sections were counterstained with hematoxylin). Original magnification, ×200.

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

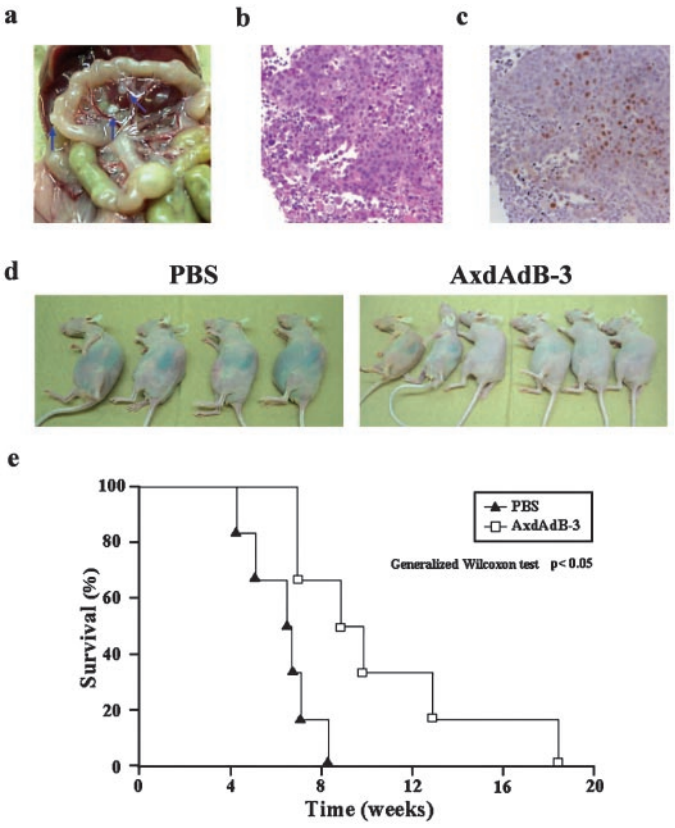

Fig. 8. Antitumoral effects of i.p. injected AxdAdB-3 on peritoneally disseminated GBC in nude mice. TGBC-44TKB cells (1×10^7) were i.p. injected into mice and allowed to proliferate for 2 weeks. The tumors were treated with i.p. injection of 2×10^8 PFU of AxdAdB-3 or PBS alone on days 1–3 and 8–10. *a*, macroscopic view of disseminated tumor nodules in an AxdAdB-3-treated mouse on day 11 (indicated by *arrows*). Histological appearance (*b*; H&E staining) and detection of Ad E1A (*c*; immunohistochemical staining) in a tumor in an AxdAdB-3-treated mouse. Original magnification, ×200 (*b* and *c*). *d*, appearance of AxdAdB-3-treated and PBS-treated mice on day 35. *e*, survival curves for AxdAdB-3-treated mice (□) and PBS-treated mice (▲). The median survival was 7 weeks and 10 weeks for PBS-treated mice and AxdAdB-3-treated mice, respectively.

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. 7*a*), 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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