Identification of Human Uroplakin II Promoter and Its Use in the Construction of CG8840, a Urothelium-specific Adenovirus Variant That Eliminates Established Bladder Tumors in Combination with Docetaxel

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ABSTRACT

Uroplakins (UPs) are a group of integral membrane proteins that are synthesized as the major differentiation products of mammalian urothelium. UPII gene expression is bladder specific and differentiation dependent, but very little is known about its transcription response elements. To identify the promoter elements, a DNA fragment of 2239 bp upstream of the UPII gene was amplified by PCR and linked to a promotorless firefly luciferase reporter gene. Transient transfection experiments showed that the DNA segment located between −1809 and +1 bp resulted in preferential expression in bladder carcinoma cells with negligible expression in nonurothelial cells. This promoter was engineered into adenovirus (Ad) type 5 to drive the expression of the E1A and E1B genes and to create an attenuated replication-competent Ad variant, termed CG8840. Viral replication and the cytopathic effect of CG8840 were evaluated by virus yield and −3(−4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in bladder transitional cell carcinoma (TCC) cell lines RT4 and SW780; nonbladder cancer cell lines G361 (melanoma), LNCaP (prostate cancer), PA-1 (ovarian cancer), and U118 (brain cancer); and human primary cells including lung fibroblasts, bladder smooth muscle cells, and mammary epithelial cells. CG8840 replicated in and eliminated bladder TCC efficiently, with high specificity (10,000:1) in comparison with nonbladder cell lines. The antitumor activity of CG8840 was examined in BALB/c nu/nu mice carrying s.c. human TCC xenografts. Intratumoral and i.v. administration of CG8840 in RT4 human bladder cancer xenografts caused significant (P < 0.01) inhibition of tumor growth. Synergistic antitumor efficacy was observed when CG8840 was combined with docetaxel, resulting in significant regression of RT4 bladder cancer xenograft tumors within 6 weeks after i.v. administration of CG8840 (3.33 × 109 plaque-forming units/animal on day 1) and docetaxel (20 mg/kg on days 2, 6, and 9). These results demonstrate the utility of the UPII promoter in the generation of urothelium-specific adenoviral vectors and provide a potential foundation for the development of bladder tumor-specific oncolytic viral therapies.

INTRODUCTION

UPs are a group of integral membrane proteins that have recently been identified as the major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and prevent the urothelial luminal surface from rupturing during bladder distention (1, 2). Furthermore, it has been shown that mammalian urothelial plaques contain four major integral membrane proteins: UP-Ia (27 kDa), UP-Ib (28 kDa), UPII (15 kDa), and UPIII (47 kDa (3−5)). Immunohistochemical survey of various tissues found UPs to be urothelium specific. Antibodies to UPs stained 88% of papillary noninvasive TCCs, 53% of invasive TCCs, and 66% of TCC metastases, without significant binding to any of the nonurothelial carcinomas (6). These findings suggest that UPs may serve as a unique marker for the positive identification of urothelial-derived TCCs (6, 7).

Bladder cancer is a commonly occurring cancer, and more than 50,000 new cases are diagnosed every year. Bladder cancer is a superficial disease confined to the mucosa in the majority of patients. Of the various therapeutic modalities, transurethral resectioning of the tumor is considered to be the most effective treatment for the management of superficial bladder cancer. However, 70% of these superficial bladder tumors will recur after endoscopic resectioning, and 20% progress to life-threatening invasive disease within 2 years of cystectomy (8). Intravesical chemotherapy or immunotherapy is a promising approach, but studies to date have shown limited efficacy, particularly in preventing tumor progression, and significant side effects. In the search for a more effective treatment, we have explored the possibility of developing a tumor-specific Ad for the treatment of bladder cancer. Previously, we demonstrated the use of tumor-specific Ad in the treatment of prostate cancer (9, 10) both as a single agent and in synergy with radiation or chemotherapy agents (11, 12) and in hepatocellular carcinoma (13). A completed Phase I/II clinical study has demonstrated that the prostate tumor-specific virus CG7060 is well tolerated with significant antitumor activity in patients with locally recurrent prostate cancer after only a single intraprostatic injection (14).

In this study, we will describe the identification of a human urothelium-specific promoter and the construction of a bladder tumor-specific Ad. This Ad variant, CG8840, was found to be very effective in controlling bladder tumor growth in experimental animal studies. Furthermore, it showed significant synergy with chemotherapeutic agents in achieving complete tumor regression.

MATERIALS AND METHODS

Cells and Culture Methods. SW780 and RT4 (bladder TCC) cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Irvine Scientific), 100 units/ml penicillin, and 100 μg/ml streptomycin. The G361 (skin carcinoma), HepG2 (liver carcinoma), LoVo (colon carcinoma), and PA-1 (ovarian carcinoma) cell lines were also obtained from the American Type Culture Collection. The 293 cell line (human embryonic kidney containing the E1 region of Ad) was obtained from Microbix Biosystems, Inc. (Toronto, Ontario, Canada). Normal hBMSCs and normal hLFs were purchased from Clonetics (San Diego, CA) and cultured in defined medium provided by the supplier (Clonetics).

Chemotherapeutic Agents. Doxorubicin (Pharmacia, Bridgewater, NJ) and docetaxel (Taxotere; Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, PA) were purchased from the Stanford Pharmacy (Stanford Medical Center, Palo Alto, CA).

Cloning of the hUPII Gene 5′-Flanking Region and Plasmid Construction. The DNA region 5′ upstream of the transcription site was amplified from human genomic DNA with Genome Walker Kit (Clontech, Palo Alto, CA) by PCR with two synthetic primers, 100.84.1 and 100.84.2 (Table 1). 100.84.1 and 100.84.2 are complementary to the 5′-untranslated region of the first exon.
in the hUPII gene from nucleotide +24 to +47 and +3 to −22, respectively (15). The resulting PCR fragment was ligated into pGL3-Basic vector (Promega Corp., Madison, WI) at KpnI and SacI sites to create the plasmid pCP657. This plasmid provides the hUPII TREs for all of the constructs reported here. pGL3-Basic, pGL3-Promoter, and pGEM-T vectors were purchased from Promega Corp., Madison, WI) at KpnI and ligated into a similarly cut CP1199 to create CP1131. The resulting PCR fragment was ligated into pGL3-Basic vector (Promega Corp., Madison, WI). Four experiments were conducted to characterize the growth properties of the Ad variants. For the virus yield assay, CV802 (wild-type Ad type 5), CV876 (hUPII/E1A and IRES/E1B), and CG8840 (same as CV876, except for an additional deletion in E1B-19kDa) were used to infect monolayers (5 × 10⁵ cells) of 293 cells, RT4 cells, SW780 cells, G361 cells, LNCaP cells, normal hLFs, and normal hBSMCs at a MOI of 2 (2 PFU/cell). After a 4-h incubation at 37°C with 5% CO₂ cells were lyzed by three freeze-thaw cycles and plaqueed on 293 cells for a week. Potential recombinants were screened by PCR, plaque-purified, and expanded in monolayers of 293 cells as described previously (16). Viral constructs of CV876 and CG8840 were confirmed by PCR and restriction enzyme mapping. Wild-type Ad CV802 was constructed as described previously (16).

**In Vitro Characterization.** Four experiments were conducted to characterize the growth properties of the Ad variants. For the virus yield assay, CV876 (wild-type Ad type 5), CV876 (hUPII/E1A and IRES/E1B), and CG8840 (same as CV876, except for an additional deletion in E1B-19kDa) were used to infect monolayers (5 × 10⁵ cells) of 293 cells, RT4 cells, SW780 cells, G361 cells, LNCaP cells, normal hLFs, and normal hBSMCs at a MOI of 2 (2 PFU/cell). After a 4-h incubation at 37°C with 5% CO₂, cells were lyzed by three freeze-thaw cycles and plaqueed on 293 cells for a week. Potential recombinants were screened by PCR, plaque-purified, and expanded in monolayers of 293 cells as described previously (16). Viral constructs of CV876 and CG8840 were confirmed by PCR and restriction enzyme mapping. Wild-type Ad CV802 was constructed as described previously (16).

**DNA Transfection and Luciferase Assays.** For transfection, SW780 and RT4 cells were plated at 5 × 10⁵ cells/6-cm culture dish in complete medium. Reporter constructs were introduced into the cells as described previously (10). Generally, when more than one construct was used, 5 μg of the smallest construct and proportionally larger amounts of DNA were used for each of the other constructs to keep the plasmid molar concentrations equal and constant. After a 72-h incubation at 37°C with 5% CO₂, cells were harvested in 500 μl of this lysate buffer (Analytical Luminescence Laboratories). Fifty μl of this lysate were used to measure luc activity in a microtiter plate luminometer (ML3000; Dynatech Laboratories). Luciferase activities were corrected for variation in protein concentrations of the cell extracts (Bio-Rad) or normalized to β-galactosidase activity. Luciferase activities and relative induction factors are expressed as a mean from at least three independent experiments.

**Virus Construction.** pBHGE3 was purchased from Microbix Biosystems, Inc. CP306 and CP686 were described previously (13, 16). A 2.2-kb DNA fragment from the hUPII 5’-flanking region was amplified with pCP657 as template and two synthetic oligonucleotides (127.2.1 and 127.2.2) as primers. The PCR fragment was digested with PinAI and ligated into a similarly cut CP686 (13) to create CP1088. To create CP1131, the E1B-19kDa coding region was deleted by digesting CP1088 with BssHII and BglII. A 1.8-kb DNA fragment from the hUPII gene was amplified with CP1131 as template and two oligonucleotides (127.2.1 and 127.2.2) as primers. The PCR product was digested with PinAI and ligated into a similarly cut CP1199 to create CP1131. CP1088 and CP1131 were cotransfected with pBHGE3 by LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) in 293 cells. After day 12, the cells were lyzed by three freeze-thaw cycles and plated on 293 cells for a week. Potential recombinants were screened by PCR, plaque-purified, and expanded in monolayers of 293 cells as described previously (16).

**Materials and Methods.** After 72 h, luc activity (in relative light units; normalized to micrograms of protein) in RT4 cells was calculated as the mean of three independent experiments, which were done in duplicate.

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**Table 1: Oligonucleotides**

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**Fig. 1.** Identification of the transcriptional regulatory sequences of the proximal promoter of the hUPII gene. A, the structure of the reporter constructs. The thick lines represent the hUPII upstream regions that were retained in the reporter constructs (not drawn to scale). Positions are given relative to the transcription start site. B, RT4 and SW780 cells were transfected with 5 μg of the deleted promoter fragments linked to luc. All transfections were done in the presence of 1 μg of pCMV-Gal vector. Relative luc activities are expressed as values compared with β-galactosidase activity and different constructs as described in “Materials and Methods.” After 72 h, luc activity (in relative light units; normalized to micrograms of protein) in RT4 cells was calculated as the mean of three independent experiments, which were done in duplicate.
washed twice with prewarmed PBS, and then 2 ml of complete RPMI 1640 containing the indicated chemotherapeutic agents were added into each well. After an additional 72 h, the cells were scraped into the culture medium and then lysed by three freeze-thaw cycles. The supernatant of each duplicate point was tested for virus production by triplicate plaque assay for 12 days under semisolid agarose on 293 cells (16). For the CPE assay (17), cells were grown to 80% confluency and infected with either CG8840 or CV802 for 2 h at increasing MOIs ranging from 0.001 to 10. Plates were monitored daily for CPE. The assay was terminated when essentially total cytolysis was observed at a MOI of 0.1 with CV802. For the one-step growth curve, replicate monolayers of RT4 cells, G361 cells, or normal hLFs were infected at a MOI of 2 to obtain a synchronous infection of all of the cells. Duplicate cultures were harvested at various times after infection, and infectious virus production was determined by plaque assay on 293 cells (16). MTT assays were performed to determine cell viability. LNCaP, HBL-100, OVCAR-3, HepG2, and 293 cells were seeded at 5000 cells/well in a 96-well plate (Falcon) 48 h before infection as described previously (17) with modifications. Cells were infected with either CV802 or CG8840 at a MOI of 0.005 PFU/cell in the presence or absence of docetaxel. Cell viability was measured at the times indicated by MTT as described previously (11, 18). Twelve replicate samples were taken for each time point, and each experiment was repeated at least three times.

In Vivo Antitumor Efficacy. Eight-week-old athymic BALB/c nude mice were obtained from Simonson Laboratories (Gilroy, CA) and acclimatized to laboratory conditions 1 week before tumor implantation. Xenografts were established by injecting 1 × 10^6 RT4 cells suspended in 100 μl of RPMI 1640 and 100 μl of Matrigel s.c. near the small of the back. When tumor volume reached between 300 and 500 mm^3, mice were randomized into groups of two. The first group received 3.33 × 10^7 PFUs of this virus preparation lot is 30) CG8840/μl tumor volume on days 1, 5, and 8 via intratumoral administration. As a control, a second group was treated with normal saline (i.e., control) via intratumoral administration at day 1. Tumors were measured weekly in two dimensions by external caliper, and volume was estimated by the formula \[ V = \frac{4}{3} \pi r^3 \], where \( r \) equals length, and \( W \) equals width (in mm; Ref. 16). For combination treatment, tumor-bearing mice were randomized into groups of two. The first group received 3.33 × 10^7 PFUs of CG8840 at day 1 via the tail vein. The second group was given docetaxel alone. Docetaxel was administered i.v. at a dose of 20 mg/kg on days 2, 6, and 9. The third group was given CG8840 (i.v.) at day 1 and docetaxel at the same dose and schedule as the second group. As a control, a fourth group was treated with 0.1 ml of normal saline (i.e., control) i.v. at day 1 and then treated i.v. with 0.1 ml of normal saline for 3 days. The dose and route of administration of docetaxel were selected based on the results of previous studies in nude mice (11). Animals were humanely killed when their tumor burden became excessive. The difference in mean tumor volume between treatment groups was compared for statistical significance using the unpaired, two-tailed t test. Federal and institutional guidelines for animal care were followed.

RESULTS

Identification of a Transcriptional Regulatory Element for the hUPII Gene. To identify the promoter region, we first created one construct, CP657, in which the 2.2-kb sequence upstream of the hUPII gene (from −2226 to +1 of the translation initiation site) was cloned upstream of the Luc gene (Fig. 1A). This construct was used to transfect bladder transient carcinoma RT4 and SW780 cells. Cells transfected with CP657 produced a significantly higher Luc activity in human bladder carcinoma cells when compared with cells transfected with CMV-Luc, in which the Luc gene was under the transcriptional control of human CMV promoter or pGL3-Basic (Fig. 1B). These data suggest that the 2.2-kb 5’-flanking sequence contains a promoter that is active in bladder cells.

The 2.2-kb 5’-flanking region was sequenced by fluorescent dye terminator labeling using AmpliTaq DNA polymerase. Computer analysis of the sequence found a number of matches to consensus binding sites for various transcription factors. In particular, the sequence between −81 and −77 matches the consensus TATA box sequence for mammalian RNA polymerase II promoters (19, 20).

There is high homology (about 66%) between the hUPII 5’-flanking region from −2226 to +1 (relative to the translation initiation site) and that of the mUPIII promoter (21). Alignment analysis of the sequence found approximately 85% homology between the hUPII (−209 to −1) and the mUPIII promoter (−231 to −1 relative to the translation initiation site; data not shown).

To define the minimal functional size of this promoter that would direct high expression, various 5’ and 3’ deletions were created (Fig. 1). The deletion of the sequences between −2226 and −1809 (CP1151) did not lead to a significant loss of promoter activity compared with CP657. The same was true when the sequence from −2225 to −1115 was deleted (CP1152 and CP656). Removal of the sequence from −1115 to −595 (CP663) or from −1115 to −213 (CP662) resulted in a 2–12-fold reduction of Luc activity. When the deletion from the 3’ border was extended to include the TATA box sequence (CP1153), the level of Luc activity was approximately the same as that seen with CP1154, a background level of Luc expression. Constructs containing the DNA sequence from −2226 to +1 but with deletions from −1025 to −600 (CP1155), −1453 to −1008 (CP1157), or −1781 to −1574 (CP1156) showed promoter activity similar to that observed in CP663. The combination of these results indicates that the putative TATA box sequence and its upstream region are indispensable for a high level expression of linked gene.

Tissue Specificity of the hUPII Gene Promoter. The hUPII protein is recognized to be a potentially useful biomarker of bladder cancer and is expressed exclusively in the bladder urothelium (15, 22, 23). It was therefore important to determine whether the promoter described above retained the high level of tissue specificity characteristic of the hUPII gene.

To test its tissue specificity, a variety of cell lines were transfected with four reporter constructs: (a) CP652 (−213 to +1); (b) CP656 (−1115 to +1); (c) CP1151 (−1809 to +1); and (d) CP657 (−2226 to +1). The cell lines used represent several tissues including human liver carcinoma (HepG2), human colon carcinoma (LoVo), human rectal carcinoma (SW1463), human ovarian carcinoma (PA-1), and human bladder transitional carcinoma (SW780 and RT4) and bladder nontransitional carcinoma (UM-UC3). The cell lines were transfected with reporter constructs and an internal control plasmid, pCMV-β-gal (with the CMV promoter driving the β-galactosidase gene). The summary of the results is shown in Fig. 2. In RT4 and SW780 cells, CP656, CP657, and CP1151 produced approximately 80–100-fold higher Luc activity than pGL3-Basic. In no other cell line did CP656, CP657, or CP1151 lead to more than a 10-fold induction of Luc synthesis. The highest levels of activity outside of bladder transitional
cells were observed in PA-1 cells, where CP656 and CP1151 reached approximately 10- and 1.5-fold higher Luc activity than pGL3-Basic. No significant difference in cell specificity was observed between CP1151 and CP657, whereas CP656 produced detectable Luc activity in UM-UC3 and PA-1 cells. These results indicate that the sequence CP1151 and CP657, whereas CP656 produced detectable Luc activity approximately 10- and 1.5-fold higher Luc activity than pGL3-Basic.

No significant difference in cell specificity was observed between approximately 10- and 1.5-fold higher Luc activity than pGL3-Basic. cells were observed in PA-1 cells, where CP656 and CP1151 reached approximately 10- and 1.5-fold higher Luc activity than pGL3-Basic. No significant difference in cell specificity was observed between CP1151 and CP657, whereas CP656 produced detectable Luc activity in UM-UC3 and PA-1 cells. These results indicate that the sequence CP1151 and CP657, whereas CP656 produced detectable Luc activity approximately 10- and 1.5-fold higher Luc activity than pGL3-Basic.

The UPII Promoter Can Restrict Ad Replication to Bladder TCC. A novel approach for targeting therapy is to use a tissue-specific regulator to drive therapeutic gene expression in target cells. To test whether the hUPII promoter can restrict Ad replication to bladder cancer cells, two Ad variants were constructed by transcriptional targeting of the E1A and E1B genes (Fig. 3A). CV876 contains the hUPII TRE from −2225 to +1 cloned upstream of the E1A transcription start site to drive E1 expression and also the E1B gene, which is connected by an IRES (13). CG8840 is similar to CV876, except that the coding sequence of the E1B-19kDa protein from nucleotide 1714 to 1975 was deleted. CV802, an Ad that has a wild-type E1 region, was used as a wild-type control in this study (16).

To determine whether the Ad variants described above replicate preferentially in bladder cancer cells, a virus yield assay for burst size was performed. Virus replication efficiency was evaluated in bladder cancer cell lines (RT4 and SW780), a melanoma cell line (G361), an ovarian tumor cell line (PA-1), a human embryonic kidney cell line (293), normal hLFs, and normal hBSMCs. Both RT4 and SW780 cells are human bladder TCCs. 293 cells serve as a positive control for replication efficiency because this cell line expresses Ad type 5 E1A and E1B proteins constitutively. Results shown in Fig. 3B indicate that CV876 and CG8840 replicated well on RT4 and SW780 cells. However, these variants replicated poorly on nonbladder cells. For example, both CV876 and CG8840 replicated 10,000-fold less efficiently in G361 cells, LNCaP cells, hLFs, and hBSMCs than CV802 (Fig. 3B). Thus, the hUPII TRE-engineered Ad preferentially replicates in bladder tumor cells, suggesting that the hUPII promoter in Ad remains tissue specific and restricts virus replication to bladder carcinoma cells.

Effect of E1B-19kDa Deletion. Previous studies have shown that E1B-19kDa mutant viruses can cause enhanced and unusual CPEs along with the induction of host cell and viral DNA degradation (24, 25). In addition, it has been observed that an E1B-19kDa-deleted Ad showed higher efficiency in inducing apoptosis and tumor cell killing compared with wild-type virus (26, 27). To take advantage of this effect, an E1B-19kDa-deleted virus, CG8840, was constructed to improve the cytotoxicity of the replicating Ad in bladder carcinoma cells. Compared with CV876, CG8840 exhibited an increased virus burst size in bladder tumor cells, but there was similar replication efficiency in nonbladder tumor cells (Fig. 3B). Cell viability studies further demonstrated a superior cytotoxicity of CG8840 to CV876 in the target bladder carcinoma cells (data not shown). Due to the higher cytolysis observed with CG8840, subsequent studies were done only with CG8840.

Growth Kinetics of CG8840. One-step growth kinetics of CG8840 was evaluated in RT4 cells, G361 cells, and hLFs (Fig. 4A). Progeny virus titers in total cell lysates at the times indicated were determined in 293 cells. The data presented in Fig. 4A suggest that CG8840 grows efficiently and generates virus yields similar to those of CV802 in RT4 cells (Fig. 4A). However, its growth is attenuated in G361 cells and hLF. CG8840 exhibited a greater change in growth kinetics in G361 and hLF cells and produced approximately 10,000-fold less infectious virus at 120 h than CV802 (Fig. 4A). There was no increase in the number of infectious CG8840 viral particles during the eclipse period that lasted approximately 24 h in fibroblasts and 72 h in G361, whereas the eclipse period for CV802 was 12 h. In agreement with the results presented above, even at 120 h after infection, the burst size of CG8840 in nonbladder cancer cells failed to reach 1. In contrast, the wild-type Ad5, CV802, replicated 105-fold and reached a burst size of 103.

CPE in Various Cell Lines. To characterize the differential viral CPEs in various cells, CPE and MTT assays were performed. Bladder cancer cells (RT4 and SW780) and nonbladder cells (G361, PA-1, and nHLF) were tested for their sensitivity to CG8840 and wild-type Ad (CV802) infection. As shown in Fig. 4B, CG8840 caused complete monolayer cytolysis of RT4 and SW780 cells within 8 days, similar to CV802, at a MOI of 0.1. For nonbladder cells, in contrast, cell monolayers infected with CG8840 did not show significant CPEs at the same time points with MOIs of 10, 1.0, 0.1, and 0.01, whereas CV802 caused monolayer cytolysis at MOIs as low as 0.1 within 8
Fig. 4. Characterization of CG8840. A, growth curve of CG8840. RT4, G361, and human primary fibroblasts were infected at a MOI of 2 PFUs/cell with either CG8840 or CV802, and virus production was measured by plaque assay on 293 cells. Data are the means of duplicate determinations. B, reduced CPEs of CG8840 in nontargeting cells. CPE assays were performed as described in “Materials and Methods.” The assay was terminated when complete cytolysis of the monolayers was observed at a MOI of 0.1 with CV802. C, viability of cells treated with either CG8840 or CV802.
Fig. 5. In vivo efficacy of CG8840. The BALB/c nude mice with preplanted bladder RT4 TCC tumors were grouped (n = 6) when tumor volume reached 300–500 mm³ and injected intratumorally with CG8840 (2 × 10⁷ PFUs on days 1, 5, and 8; ▲) or vehicle (control group; ●). Tumor size was measured weekly, and the tumor volume was normalized as 100% at the day of the treatment. Error bars, SE.

Fig. 6. In vitro synergistic effect of CG8840 with docetaxel. A, cell viability measured by MTT at different times. RT4 cells were treated with CG8840 at a MOI of 0.005, docetaxel at 1 ng/ml, or a combination of CG8840 and docetaxel. Cell viability was determined at various time points by MTT assay. Data presented in Fig. 4C show that CG8840 was toxic to bladder cancer cells (RT4 and SW780), but not to nonbladder cells including U118 (human glioblastoma cells) and primary human cells (hLFs and hBSCMs). There were no surviving RT4 and SW780 cells 7 days after infection. In contrast, the viability of U118, hLFs, and hBSCMs was nearly 100% through the course of the experiment. Wild-type Ad CV802 killed the six cell types tested, regardless of origin.

These results show that CG8840 replicates efficiently in and destroys the target bladder transitional carcinoma cells but is highly attenuated in nonbladder cells, including primary human cells. Furthermore, it was demonstrated that the hUPII TRE allows for tissue-specific replication of oncolytic Ad and thus targets the virus to kill bladder cancer cells.

Antitumor Efficacy of CG8840 in Vivo. The in vivo antitumor efficacy of CG8840 was assessed in the RT4 mouse xenograft model. Established human bladder RT4 tumors were treated with either vehicle or CG8840 (2 × 10⁷ PFUs/μl tumor). The tumor volume data presented in Fig. 5 show that there was a statistically significant decrease in tumor volume in the CG8840-treated group as compared with the control group (P < 0.01). In this study, intratumorally delivered CG8840 appeared to be effective in producing partial tumor regression in the RT4 mouse model. Five weeks after treatment, relative tumor volume decreased to 50% of baseline (from 418.6 to 167.7 mm³) for the CG8840-treated group but increased to 310% of baseline for the vehicle-treated group. Significant regression of tumor volume was also observed in animal xenografts treated with a single i.v. administration of CG8840 (data not shown).

Synergistic Antitumor Efficacy of CG8840 in Combination with Docetaxel. Previously, we have demonstrated in vitro and in vivo that a combination of CV787 with paclitaxel or docetaxel leads to synergistic antitumor efficacy (11). In the current study, different chemotherapeutic agents were tested in combination with CG8840 for their in vitro cell killing effect in SW780 or RT4 bladder carcinoma cells. We optimized drug concentration to the extent that it would not generate an extensive cytotoxic effect by itself. Under such conditions, some chemotherapeutic agents showed higher cell killing effect in combination with CG8840 (data not shown). Fig. 6A shows the synergistic cell killing effect of combining docetaxel with CG8840. SW780 cells treated with 1.0 ng/ml docetaxel retained 50% of cell viability 10 days after treatment, whereas CG8840 at a MOI of 0.005 PFU/cell destroyed 60% of the cells by day 10. However, when both docetaxel (1.0 ng/ml) and CG8840 (MOI = 0.005) were applied together, 100% of the cells were killed 10 days after treatment. To determine the potential synergistic effect from the combination treatment, the MTT cell viability data were subjected to further statistical analysis. Fig. 6B shows a representative IC₅₀ isobologram of docetaxel and CG8840 on SW780 cells at day 10. First, the dose-response curves of docetaxel alone or CG8840 alone were made (results not shown). Based on the original theory of Steel and Peckham (28) and the method of Aoe et al. (29), three isoeffect curves (mode I, IIa, and IIb) were constructed. According to this theory, a combination that gives data points to the left of the envelope of the mode IIb line can be described as supra-additive (synergism; Ref. 29). The isobologram showed several data points that were in the synergy area (Fig. 6B), indicating that a combination of CG8840 and docetaxel provides a synergistic effect on the killing of SW780 cells. Similar in vitro synergy in the killing of SW780 or RT4 cells could be observed when CG8840 (MOI = 0.005) was combined with other chemotherapeutic agents such as doxorubicin (50 ng/ml), mitomycin C (2.5 μg/ml), thiotepa (2.5 μg/ml), mitoxantrone (10 ng/ml), and vinblastine (5 ng/ml; data not shown).

Although CG8840 alone has significant in vivo antitumor activity, we tested the combination therapy with docetaxel for in vivo evaluation of synergy. Animals harboring 300 mm³ RT4 xenografts were grouped (n = 6) and injected with vehicle alone (control group), CG8840 alone (3.33 × 10⁹ PFUs/animal), docetaxel alone (20 mg/kg on days 2, 6, and 9), or CG8840 in combination with docetaxel. Fig. 7 shows weekly changes in the relative tumor size normalized to 100% at day 1. By week 7, animals in the control group experienced tumor growth of 320% of baseline, whereas in the CG8840 group and the combination group, animals experienced tumor reduction or were...
DISCUSSION

Expression of the UP gene was found exclusively in urothelium and also in 88% of noninvasive papillary TCCs, 53% of invasive TCCs, and 66% of TCC metastases, but not in any of the nonurothelial carcinomas, suggesting a high tissue specificity (6). The hUPII gene was previously mapped to chromosome 11q23 (15), but its regulatory elements were unknown. In this communication, we report the cloning of a urothelium-specific promoter from the hUPII gene. This region in the hUPII gene and its application in the generation of the bladder TCC-specific Ad variant, CG8840. Furthermore, we demonstrate that this bladder-specific Ad variant, per se, was able to bring about a significant reduction in the size of bladder tumors and completely eradicate tumors in combination with doxetaxel in preclinical models.

Previously, a 3.6-kb flanking sequence of the mUPII gene was shown to confer tissue specificity to reporter gene expression in the urothelium of transgenic mice (21). Sequence alignment indicated an 80% similarity in the promoter region between the hUPII gene and the mUPII gene. This region in the hUPII gene contains motifs that are typically found in the promoter region. The deletion analysis has further revealed that even 0.6 kb of the UP gene promoter was sufficient to exhibit a high bladder cancer cell specificity.

The strong tissue specificity of the hUPII promoter makes it an ideal candidate in the strategic development of tissue-directed delivery of therapeutic genes as well as in the design of targeted viruses useful in the treatment of bladder cancer. Bladder tumors are appealing targets for virotherapy because of the ease of access to them via the urethra. We demonstrate here that an attenuated replication-competent Ad variant whose replication is restricted to bladder tumor cells can be generated by engineering the hUPII promoter into Ad to transcriptionally target essential early viral genes. Experiments that examined the growth properties of these variants indicate that CG8840’s propagation and replication efficiency are attenuated in nonbladder cells but are similar to those of wild-type Ad in bladder tumor cells. Whereas it grows efficiently and generates virus yields similar to those of wild-type Ad in RT4 bladder carcinoma cells, CG8840 replicates poorly in melanoma, prostate cancer cells, and human primary cells (10,000-fold less efficiently when compared with wild-type Ad). The CG8840-induced CPE is also significantly attenuated relative to that of wild-type Ad in primary culture of normal cells. These findings suggested that the hUPII promoter could be used to target gene expression in bladder tumor cells.

CG8840 has a strong antitumor activity in the human TCC xenografts. Tumor growth was completely inhibited when animals received a single dose of CG8840 through either intratumoral or i.v. administration. CG8840-mediated antitumor efficacy was further enhanced when the virus was combined with chemotherapeutic agents such as doxetaxel. In vitro, CG8840 produced synergistic cytotoxicity in human TCC cells when the cells were treated with CG8840 and doxetaxel. In vivo, a single dose of CG8840 in combination with doxetaxel caused a significant regression of pre-existing HCC tumors within 7 weeks. This finding has further extended our previous observation that a tumor-specific Ad variant in combination with a chemotherapeutic agent produced a synergistic antitumor efficacy (11).

Adenoviral E1B-19kDa protein has been shown to exert an antiapoptotic function through multiple mechanisms (25, 30). The presence of the E1B-19kDa gene product may allow for greater accumulation of viral particles in the cell and prevent the premature lysis of the cell. This may be counterproductive to oncolysis, requiring accelerated cell lysis and rapid cell-to-cell spread of the replicating virus within the tumor mass. Previous studies have shown that the removal of the E1B-19kDa gene greatly enhances the apoptotic and tumor cell killing activity of the resulting virus (26, 27). In the present report, we provide evidence that the E1B-19kDa-deleted virus CG8840 produces greater viral burst size in bladder tumor cells in comparison with the non-E1B-19kDa-deleted virus. Although a greater viral burst size and greater oncolysis are achieved with a E1B-19kDa-deleted virus, the level of apoptosis is still not adequate, which may limit the spread of the virus within the tumor mass (31). The connective tissue and tumor matrix are also thought to have a negative impact on the spread of virus within the tumor mass (27). It may be essential to add additional genes that would allow for apoptosis after viral replication and thus provide a higher propensity to spread to the neighboring tumor cells.

Our study indicates that the promoter of the hUPII gene is similar to that of the mUPII gene and is able to confer preferential expression of genes in the bladder urothelium. The development of the bladder tumor-specific oncolytic virus with enhanced lytic property via E1B-19kDa deletion should add to the long list of strategies that are currently under clinical investigation for cancer treatment. The high therapeutic index and synergy observed in combination with chemotherapeutic agents may enhance the interest of clinicians who are searching for therapeutic modalities with high efficacy and low toxicity.

Fig. 7. In vivo synergistic effect of CG8840 with doxetaxel. RT4 nude mouse xenografts were grouped (n = 6) when tumor volume reached 300–500 mm³ and treated with CG8840 alone (3.33 × 10⁹ PFUs/animal on day 1, i.v. administration; ●), doxetaxel alone (20 mg/kg i.v.; administration on days 2, 6, and 9; ●), or vehicle control (○). Tumor size was measured weekly, and the tumor volume was normalized to 100% at the day of the treatment. Error bars, SE of mean.
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REFERENCES

Identification of Human Uroplakin II Promoter and Its Use in the Construction of CG8840, a Urothelium-specific Adenovirus Variant That Eliminates Established Bladder Tumors in Combination with Docetaxel


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