Abstract

Purpose: The purpose of this study was to examine the tumor specificity, cytotoxicity, and granulocyte macrophage colony-stimulating factor expression of CG0070, a conditionally replicating oncolytic adenovirus, in human bladder transitional cell carcinoma (TCC) cell lines and determine its antitumor efficacy in bladder TCC tumor models.

Experimental Design: Virus yield and cytotoxicity assays were used to determine tumor specificity and virus replication-mediated cytotoxicity of CG0070 in a panel of human bladder TCC cell lines and primary cells in vitro. Two s.c. and one orthotopic bladder TCC xenograft tumor models were used to assess antitumor activity of CG0070.

Results: In a matched isogenic pair of cell lines with differing retinoblastoma (Rb) pathway status, CG0070 showed selective E1a and granulocyte macrophage colony-stimulating factor (GM-CSF) expression in Rb pathway-defective cells. CG0070 replicated in Rb-defective bladder TCC cell lines as efficiently as wild-type adenovirus but produced 100-fold less virus in normal human cells. CG0070 was up to 1,000-fold more cytotoxic in Rb pathway-defective bladder TCC cells in comparison with normal human cells. Antitumor activity of CG0070 was shown in two bladder TCC s.c. xenograft tumor models following intratumoral injections and intravesical treatment in an orthotopic xenograft tumor model when compared with PBS treatment.

Conclusions: In vitro and in vivo studies showed the selective replication, cytotoxicity, GM-CSF production, and antitumor efficacy of CG0070 in several bladder TCC models, suggesting a potential utility of this oncolytic agent for the treatment of bladder cancer. Further studies are warranted to show the role of human GM-CSF in the antitumor efficacy of CG0070.

Approximately 60,000 cases of urinary bladder cancer were diagnosed and ~12,000 patients died of the disease in 2004. Roughly 75% of patients are male, making bladder cancer the fourth most common cancer affecting U.S. men (8th in women). More than 90% of bladder cancer cases are in the transitional urothelium and thus referred to as transitional cell carcinoma (TCC). Superficial bladder cancer has traditionally been treated by transurethral resection. However, patients with less differentiated large or multilocular bladder tumors and patients with carcinoma in situ or stage T2 and T3 bladder cancer are at high risk for tumor recurrence and development of muscle-invasive disease or distant metastasis. In spite of early intervention with Bacillus Calmette-Guerin and transurethral resection, the high recurrence rate of bladder cancer warrants the development of new therapeutic modalities (1).

Viruses that replicate selectively in tumor cells, leading to cell lysis and release of oncolytic virions, are being developed as biological anticancer agents (2–5). The advantage of conditionally replicating selective oncolytic adenoviruses is that the virus replication in the tumor will amplify the input virus potentially leading to spread of the virus throughout the tumor mass. Additionally, tumor antigens could be released from lysed tumor cells and systemic antitumor immune response may be induced to augment the oncolytic activity. Viruses are either inherently capable of tumor-specific replication or tumor specificity can be generated through genetic manipulation of the viral genome. Of the many approaches to restrict viral replication to tumor cells, the use of human tumor-selective transcriptional regulatory elements in the control of key essential viral genes has gained immense attention (2). For adenoviruses, the early essential genes have been placed under the control of tumor-selective promoters to restrict the virus replication to target cells resulting in several tissue- or tumor-specific oncolytic agents (6–9).

Retinoblastoma (Rb) pathway defects are found in the cells of most cancers, including bladder, head and neck, colon, prostate, and melanoma (10). CG0070 is a conditionally armed oncolytic adenovirus.
replicating oncolytic serotype 5 adenovirus (Ad5) in which the human E2F-1 promoter, a Rb pathway–defective tumor-specific transcription regulatory element (11, 12), drives expression of the essential E1a viral genes to restrict viral replication and cytotoxicity to Rb pathway–defective tumor cells. In addition, CG0070 encodes the cDNA for human granulocyte macrophage-colony stimulating factor (GM-CSF), a cytokine known to be a potent inducer of specific, long-lasting antitumor immunity in animal models (13), under the control of the endogenous viral E3 promoter. Because the E3 promoter is activated by E1a gene product, both viral replication and GM-CSF expression may be ultimately under the control of the tumor-selective E2F-1 promoter. Due to in situ expression of GM-CSF, in addition to its direct oncolytic effect after local treatment, CG0070 may also induce systemic, tumor-specific immunity such that uninfected local tumor as well as distant tumor metastases may be affected.

In the current study, the tumor selectivity, cytotoxicity, and GM-CSF production and the antitumor efficacy of CG0070 have been evaluated in several bladder TCC models. In addition, the antitumor synergy of CG0070 was evaluated in combination with docetaxel in a bladder xenograft tumor model.

**Materials and Methods**

**Cells and culture methods.** Bladder TCC cell lines SW780 and RT4, human cervical carcinoma cell line HeLa-S3, human embryo lung fibroblast cell line W138, and the SV40 transformed, Rb-defective variant W138-VA13 were obtained from American Type Culture Collection (Manassas, VA). AE1-2A is an adenovirus-complementing cell line (14) and was cultivated in Richter’s medium containing 5% heat-inactivated fetal bovine serum. Bladder TCC cell lines 253J B-V and UC14 were kindly provided by Colin Dinney (M.D. Anderson Cancer Center, Houston, TX). The primary human cells included human aortic endothelial cells (hAEC; Cambrex BioWhittaker, Walkersville, MD) and normal human embryo lung fibroblasts (MRC-5, American Type Culture Collection). A clonal cell line, SW780-Luc, was generated by stably transducing the human bladder TCC cell line SW780 with a lentiviral vector encoding for the luciferase gene product under the control of the cytomegalovirus early gene promoter/enhancer.

**Viruses**

**Viral replication and amplification.** The large plasmid containing the CG0070 adenoviral genome with the designed alterations was constructed as follows: a shuttle plasmid pDr20hGmF carrying the human GM-CSF gene with the left end packaging site (I) was generated from recombination between plasmid pDR1F and pAr15pAE2fhGmF (15, 16). The donor plasmid pDr20hGmF was digested with SpeI/SpeI. The large fragment containing the human GM-CSF cDNA was cotransformed into E. coli B15183 competent cells along with PacI/ SpeI–digested pAr5pAE2fhGmF plasmid DNA to generate full-length clone by homologous recombination (17, 18). SauI-digested full-length plasmid was transfected into AE1 2a cells using the LipofectAMINE-Plus reagent system (Life Technologies, Rockville, MD). The virus was amplified in roller bottles and purified by CsCl gradient centrifugation. Ar20-1004 is identical to CG0070 except for the presence of murine GM-CSF cDNA in the E3 region instead of the human GM-CSF cDNA. Ar20-1061 is identical to CG0070 except for the absence of GM-CSF cDNA in the E3 region. For large-scale preparations, the virus was amplified in HeLa-S3 cells and purified by chromatography. Virus particle titers were determined either spectrophotometrically or by high-performance liquid chromatography as previously described (19). The infectious titers (plaque-forming units) were determined in 293 cells by plaque assay (7).

**Cell viability assay and virus yield assay.** Cytotoxicity assays were done on bladder TCC cell lines and nontumor cells using the Promega CellTiter aqueous nonradioactive cell proliferation kit (Promega, Inc., Madison, WI). The data were analyzed using GraphPad Prism 4 (San Diego, CA) analysis software. The virus yield assay was done as previously described (20).

**E1a mRNA.** E1a expression was quantitated by reverse transcription-PCR on RNA isolated using RNAzol B (Tel-TEST) as described earlier (21). The expression level of E1a for each vector was normalized to viral DNA copy number (hexon DNA copy number) determined 4 hours postinfection as described earlier (22).

**GM-CSF expression.** Duplicate wells of human bladder TCC cell lines were infected at the indicated particle/cell ratios for 24 hours. Cell supernatants were collected and human GM-CSF protein was quantitated by ELISA (R&D Systems, Minneapolis, MN) following the protocol of the vendor. The sensitivity of the assay was 7.8 pg/mL. Biological activity of GM-CSF was measured in TF-1 cells as previously described (15).

**Efficacy studies in tumor models in nude mice.** Female NCR (nu/nu) mice (4-6 weeks of age; body weight of 18-20 g) were purchased from Simonsen Labs (Gilroy, CA). Mice were injected s.c. in the right flank with 2 × 10⁶ SW780 or 253J B-V cells in Matrigel (injection volume of 200 µL). When tumors reached a mean tumor volume of ~150 mm³ [volume = (W² × L) / 2; W, width; L, length, in cubic millimeters], animals (n = 10 per group) were randomly distributed into several treatment groups.

The antitumor synergy of CG0070 in combination with docetaxel was evaluated in SW780 s.c. xenograft tumor model. Monotherapy and combination group received CG0070 and docetaxel as indicated in the figure.

Tumor volume was measured twice weekly for the duration of the study beginning on day 1. Body weight was measured once per week for the duration of the study. Mice were euthanized if the tumor volume exceeded 2,000 mm³ or if they lost >15% of their initial body weight.

Orthotopic tumors were generated on the luminal surface of the bladder by intravesically instilling human bladder TCC cell line SW780-Luc. Female NCR nude mice were anesthetized with isoflurane and a 24-gauge catheter was introduced through the urethra into the bladder. The residual urine was emptied and the bladder was flushed with PBS. One-hundred microliters of a 0.1% solution of dodecyl α-D-maltoside were then instilled into the bladder intravesically and retained for 5 minutes; after which, the bladder was washed with PBS. Dodecyl α-D-maltoside treatment was followed by an intravesical treatment with 100 µL of 0.25% solution of trypsin for 10 minutes. SW780-Luc cells (1 × 10⁶ in 80 µL) were then administered intravesically and a purse string suture was placed around the urethral opening. The purse string was removed after 1 to 3 hours and the cells were allowed to drain.

Two weeks following cell implantation, tumor-bearing mice were anesthetized with isoflurane and a 24-gauge catheter was introduced through the urethra into the bladder. The residual urine was emptied and the bladder was flushed with PBS. Seventy microliters of a 0.1% solution of dodecyl α-D-maltoside were then instilled into the bladder intravesically and retained for 5 minutes; after which, the bladder was washed with PBS. Dodecyl α-D-maltoside treatment was followed by an intravesical treatment with 100 µL of 0.25% solution of trypsin for 10 minutes. SW780-Luc cells (1 × 10⁶ in 80 µL) were then administered intravesically and a purse string suture was placed around the urethral opening. The purse string was removed after 1 to 3 hours and the cells were allowed to drain.
Statistical analysis. Statistical tests were done using the GraphPad Prism software (La Jolla, CA). Tukey’s test was done on log10-transformed data to test for significance between the CG0070-treated group and the PBS-injected control in tumor model studies.

Results

Virus structure. The genomic structure of the oncolytic adenoviral vector CG0070 is shown schematically in Fig. 1A. The human E2F-1 promoter, which provides tumor specificity to any downstream gene products, was cloned in place of the endogenous E1a promoter in the Ad5 backbone. To protect from transcriptional read-through activating E1a expression, a SV40 polyadenylation signal was inserted 5’ of the E2F-1 promoter. CG0070 includes the entire wild-type E3 region except for the gp19kD-coding region. In place of the gp19kD gene, CG0070 carries the cDNA for human GM-CSF under the control of the E3 promoter. The rest of the viral vector backbone, including packaging signal, E2, E4, late protein regions, and inverted terminal repeats, is identical to the wild-type Ad5 genome. An alternate oncolytic adenovirus constructed, Ar20-1004, is identical to CG0070 except that it carries the cDNA for murine rather than human GM-CSF.

Rb status–dependent gene expression and virus replication. Measuring gene expression of CG0070 in cells with normal and defective Rb pathways provides a good measure of the Rb status dependence of the E2F-1 promoter and the selectivity of CG0070. A matched isogenic pair of cell lines, Wi38 and Wi38-VA13, which differed in their Rb pathway status, was compared for the ability to support CG0070 function as measured by expression levels of E1a and GM-CSF. Wi38-VA13 is an SV40-transformed cell derived from Wi38 in which the SV40 T antigen has disrupted the pRb-E2F pathway, resulting in high levels of free E2F transcription factor. The deregulation of the Rb pathway and up-regulation of the endogenous E2F-1 gene expression have been shown earlier in Wi38-VA13 cell line (21). The E1a mRNA level 24 hours after infection was assessed by quantitative PCR and the data were normalized to the number of Ad genome copies determined by hexon DNA levels 4 hours postinfection (Fig. 1B). Following infection with 1,000 particles per cell, an average of 2.19 × 10^5 copies of E1A mRNA was observed in W138 cells in comparison with 911 copies in W138-VA13 cells. A separate experiment in this cell line pair showed the dependence of GM-CSF production on a defective Rb pathway (Fig. 1B). One-thousand-fold more GM-CSF was measured in CG0070-infected Wi38-VA13 cells than in W138 cells. These results showed that the human E2F-1 promoter in CG0070 is capable of selectively regulating adenoviral E1a gene transcription and downstream E3 promoter–controlled human GM-CSF expression in Rb pathway–defective cells.

Following adenovirus infection of the target cells, the amount of virus produced reflects numerous processes, including the ability of a particular cell type to be infected, to transactivate promoters, to replicate the virus, and to carry out other complex activities. The production of CG0070 virus in human bladder TCC cell lines (Rb pathway defectve) and human normal cells
(Rb positive) was compared with that of wild-type adenovirus Ad5. CG0070 replicated in the Rb pathway–defective human bladder TCC cell lines RT4, SW780, UC14, and 253J B-V cells as efficiently as wild-type adenovirus Ad5, producing similar levels of progeny virus (3,000-9,000 plaque-forming units per cell), but was highly attenuated (~100-fold) in Rb-positive normal human cells including MRC-5 fibroblasts and hAEC primary aortic endothelial cells (Fig. 2A). CG0070 produced >2 log more progeny virus in the bladder TCC cell lines in comparison with primary cells. Under similar conditions, wild-type Ad5 produced similar levels of progeny virus in both tumor and primary cells.

**Cytotoxicity in vitro.** Another measure of the effectiveness of an oncolytic virus such as CG0070 is the ability to lyse tumor cells preferentially compared with normal or primary cells. Because CG0070 is designed to replicate preferentially in tumor cells that have a defective Rb pathway, tumor and normal cell lines were chosen based on this characteristic, and the cytotoxic effects of CG0070 in these cells were quantified. A panel of human Rb pathway–defective bladder TCC cell lines and normal human cells were infected with CG0070 at various multiplicities of infection (MOI). The cytotoxicity results, as assessed by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (a quantitative cell viability assay), showed that CG0070 is cytotoxic in the human bladder TCC cell lines (RT4, SW780, and UC14) but is highly attenuated in normal human cells (MRC-5 and hAEC; Fig. 2B). By day 10 following infection with 1 MOI of CG0070, for example, the viability of bladder TCC cell lines were reduced to 10% to 20% in comparison with >95% viability of normal cells. The viability of the cell lines was reduced to similar levels when infected with 100- to 1,000-fold lower levels of wild-type Ad5 (9).

**GM-CSF expression in bladder TCC cells.** To measure the amount of human GM-CSF expression by CG0070, bladder TCC cell lines and primary cells were infected with the virus at three different MOIs (viral particles per cell). Supernatants were collected 24 hours later and the total amount of GM-CSF protein expressed was quantified by ELISA. A general dose-response for GM-CSF expression was seen in all of the cell types tested (Table 1). The level of GM-CSF expression varied <3-fold among the CG0070-infected bladder tumor cell lines at each MOI. However, significant differences in the level of GM-CSF expression were seen between the TCC cell lines and the normal lung fibroblast cell MRC-5. For example, at an MOI of 1,000, the amount of GM-CSF protein expressed in MRC-5 cell was 17- to 45-fold lower than that expressed in TCC cell lines. The difference in GM-CSF expression was even greater at MOI of 10, where 30- to 140-fold more protein was expressed in TCC cell lines than in MRC-5. A bioassay for GM-CSF showed that the GM-CSF expressed in the bladder TCC cells was biologically active as judged by its effect on proliferating TF-1 cells (data not shown).

**Antitumor efficacy in xenograft tumor models.** The antitumor efficacy of CG0070 as a monotherapy was evaluated in two s.c. xenograft bladder TCC tumor models. S.c. tumors of bladder

### Table 1. Production of GM-CSF in CG0070-infected bladder TCC cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Viral particles/cell</th>
<th>ELISA (ng/10⁶ cells/24 h)</th>
</tr>
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<tbody>
<tr>
<td>RT4</td>
<td>1,000</td>
<td>1,457 ± 60</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>361 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>UC14</td>
<td>1,000</td>
<td>2,807 ± 145</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>992 ± 11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>114 ± 3</td>
</tr>
<tr>
<td>SW780</td>
<td>1,000</td>
<td>3,853 ± 245</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>707 ± 16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>214 ± 7</td>
</tr>
<tr>
<td>253J B-V</td>
<td>1,000</td>
<td>1,492 ± 101</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>934 ± 67</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>153 ± 2</td>
</tr>
<tr>
<td>MRC-5</td>
<td>1,000</td>
<td>85 ± 18</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>24 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.5 ± 0.02</td>
</tr>
</tbody>
</table>

**NOTE:** ELISA data represent the mean ± SD of replicate wells in the units of ng/10⁶ cells/24 h.
TCC cell line 253J-B-V were established in nude mice and were injected with three different dose levels of CG0070 as indicated in the legend to Fig. 3A. Significant antitumor efficacy was observed following intratumoral injection of five doses of CG0070 at a concentration of $3 \times 10^{10}$ viral particles per dose ($P < 0.0001$) as well as the two lower doses of $3 \times 10^{9}$ and $3 \times 10^{8}$ viral particles per dose ($P < 0.0001$) compared with PBS-injected tumors. By day 60, the saline-treated tumors had increased 10.6-fold in volume whereas the CG0070-treated tumors at a concentration of $3 \times 10^{10}$ viral particles per dose decreased in tumor size to 85% of the day 1 volume. At doses of $3 \times 10^{8}$ and $3 \times 10^{9}$ viral particles per injection, the mean tumor volume remained at the day 1 level until approximately day 43, then slowly increased to ~2-fold in volume by day 60. Significant differences in efficacy could not be observed between the three concentrations of virus that were employed in this study. No significant difference in body weight gain was evident between the control and treatment groups (data not shown). Mice treated with CG0070 at the highest dose showed complete tumor regressions in half of the treated animals (5 of 10) compared with mice treated with PBS (0 of 10).

The antitumor efficacy of CG0070 was also evaluated in s.c. SW780 bladder TCC tumor model. Significant inhibition in tumor growth was observed following five intratumoral injections of $3 \times 10^{10}$ viral particles per injection of CG0070 (Fig. 3B). By day 28, on average the PBS-treated tumors increased 12-fold in volume from day 1 whereas the CG0070-treated tumors increased only 1.4-fold. The PBS-treated tumors showed an average tumor growth rate of 59.0 mm$^3$/d at day 28 whereas the CG0070-treated tumors showed an average tumor growth rate of only 1.9 mm$^3$/d at the same time point, representing a 96% inhibition of tumor growth rate ($P < 0.005$). Body weight and weight gain were generally unaffected in all the treatment groups (data not shown).

Virus infection and virus-mediated apoptosis within the tumor following intratumoral injection of CG0070 were monitored by immunohistochemical staining (Fig. 3C). The human SW780 tumor cells in the xenograft were identified presenting a 96% inhibition of tumor growth rate ($P < 0.005$). Body weight and weight gain were generally unaffected in all the treatment groups (data not shown). 

Antitumor efficacy of CG0070 in bladder TCC xenograft models. A, 253J-B-V tumor model. NCR nu/nu mice bearing s.c. 253J-B-V tumors were injected intratumorally with PBS or CG0070 five times at a dose volume of 50 μL, on the days indicated by the arrows (days 1, 3, 5, 8, and 10). During each dosing, CG0070 was injected intratumorally at multiple locations within the tumor. The group average tumor volumes ± SD ($n = 10$ per group) are shown for mice that received PBS, $3 \times 10^{9}$, $3 \times 10^{8}$, or $3 \times 10^{7}$ viral particles per dose. All of the CG0070-treated groups were statistically different from the PBS-treated group ($P < 0.001$) on day 60. The dip in the tumor volume curve was due to the removal of four animals with large tumor in the PBS control group (one at day 43 and three at day 47) and in two other groups, one animal each with large tumor in the $3 \times 10^{8}$ viral particles (day 50) and $3 \times 10^{9}$ viral particles (day 57) virus treatment groups. B, SW780 tumor model. NCR nu/nu mice bearing s.c. SW780 tumors were injected intratumorally with either PBS or CG0070 on the days, 1, 3, 5, 8, and 10 (arrows). The group mean tumor volume ($n = 10$ per group) is shown for mice that received PBS or CG0070 ($3 \times 10^{10}$ viral particles per injection). Points, mean; bars, SE, $P < 0.005$, CG0070 versus PBS treatment. C, intratumoral replication of CG0070 and apoptotic cell staining. Representative tumor sections ($n = 2$). Tumors were collected on day 22 following intratumoral administration of either PBS (a-e) or CG0070 (d-f). Serial sections of the paraffin-embedded bladder tissue were used for staining. Human bladder SW780 cells were stained with antihuman cytokeratin 20 AE1 antibody (a and d). Virus replication was monitored by staining for hexon protein (b and e) in tumor sections. Apoptotic cell staining was used to locate the cells that were undergoing apoptosis within the tumor mass (c and f). Final color development in all the immunohistochemical protocols involved the use of chromogen substrate Diaminobenzidine that results in a positive staining pattern as indicated by the arrow showing the regions of specific antibody interaction (magnification, >20).
Substantial staining for hexon protein could be observed 12 days following the final virus injection (day 22) within the tumor mass. Less extensive but still demonstrable hexon staining was observed as late as day 29, which suggested that virus replication persisted for a prolonged period after virus injection (data not shown). Apoptosis associated with viral replication within the tumor mass was visualized as described in Materials and Methods. Apoptotic cell staining was observed in successive sections of tumors collected on day 22, overlapping with hexon staining, suggesting that apoptotic activity was centered in regions of viral replication.

The antitumor activity of CG0070 was further evaluated in an orthotopic bladder TCC model that more closely resembles the actual treatment setting in patients. Female NCR nude mice bearing orthotopic SW780-Luc bladder tumors received six intravesical doses of CG0070 (3 × 10^{10} viral particles per dose) either once weekly for 6 consecutive weeks or twice weekly for 3 consecutive weeks. Based on tumor imaging in situ, all of the tumors in the PBS-treated group (n = 8) increased in size compared with the baseline except one animal in which the tumor did not grow (Fig. 4A). In the CG0070 treatment group (one dose/wk), in situ tumor imaging showed that four of nine animals were tumor-free by day 42 following the initiation of treatment (Fig. 4B). The tumor in one animal increased in size, three animals were euthanized with a large tumor burden, and one animal was found dead in
the cage due, presumably to the large tumor as recorded by prior imaging data. In contrast, five of eight animals in the CG0070 treatment group (2 doses/wk) were tumor-free by day 32 following the initiation of treatment (Fig. 4C). The tumor of one animal had decreased in size (dropping from 1.45 × 10^6 to 5.71 × 10^5 photon count); that of another was stable in size; and one animal was euthanized with a large tumor burden on day 23. Immunohistochemical evaluation of the bladder sections confirmed the absence of tumor cells in CG0070-treated mice (five of eight in 2 doses/wk group and four of nine in 1 dose/wk group) deemed tumor-free by in vivo imaging (data not shown). Replication of virus in orthotopic tumor following intravesical treatment with CG0070 was shown by staining for hexon in histologic sections of bladder that were explanted 24 hours following virus treatment (Fig. 4D).

Several studies have shown the utility of docetaxel as a monotherapy and in combination with other chemotherapeutic agents in the treatment of metastatic bladder cancer (23, 24). Hence, the antitumor efficacy of Ar20-1004 in combination with docetaxel was assessed in the SW780 xenograft tumor model. Ar20-1004, which encodes murine GM-CSF but is otherwise identical to CG0070, was used in this study and is considered to be a murine homologue of CG0070. Animals with s.c. SW780 tumors were treated with Ar20-1004 alone, docetaxel alone, or both agents. A significant decrease in tumor volume between control and all of the treatment groups (P < 0.0001) was evident (Fig. 5). The PBS control group showed an average tumor growth rate of 25 ± 2.8 mm^3/d. Significant differences in efficacy were observed between the combination and monotherapy treatment groups (P < 0.03) with the highest antitumor efficacy obtained in the combination treatment in comparison with the monotherapy groups. The Ar20-1004 and docetaxel monotherapy groups showed an average growth rate of 7.0 ± 1.6 and 5.3 ± 0.79 mm^3/d, respectively, by day 37. In contrast, tumor volumes in mice treated with Ar20-1004 and docetaxel combination had regressed by the same time point, exhibiting an average tumor growth rate of −0.78 ± 0.35 mm^3/d. Table 2 summarizes relative tumor volume of control and treated groups on four different time points. Analysis on fractionated tumor volume indicated a synergistic effect between Ar20-1004 and docetaxel in this study (Table 2). On day 29, there was 1.35-fold improvement in antitumor activity in the combination group when compared with the expected additive effect. By day 37, the average tumor volume in Ar20-1004- or docetaxel-treated mice increased further compared with the tumor volumes in mice treated with both agents. The tumor growth inhibition in the combination treatment group was 3.3-fold higher over an additive effect following treatment with either agent alone.

**Discussion**

Rb pathway defects are found in a majority of tumors, including TCC of bladder. Altered pRb (absent pRb or mutated pRb) expression has been associated with bladder cancer progression and patients with bladder tumors that have altered Rb protein products are at a high risk of recurrence and death (25). In some patients, overexpression of pRb has been observed in bladder tumors and has been suggested to be indicative of dysfunctional Rb status through upstream changes in the cell cycle pathway involving Rb phosphorylation leading to functional inactivation of pRb (26). Deletion of p16, a key protein in the Rb pathway, has been reported in 30% to 70% of TCC and is found in all tumor stages and grades. Furthermore, abnormalities of p16 are reported to be a poor prognostic marker associated with a high rate of recurrence in patients with superficial TCC of the bladder (27). Because tumor cells do not usually develop both Rb and p16 mutations, the additive prevalence of Rb and p16 gene mutations results in nearly uniform inactivation of the Rb pathway in TCC of the bladder (28).

CG0070 is a conditionally replicating oncolytic Ad5 adenovirus designed to preferentially replicate in and kill cancer cells. Tumor selectivity of CG0070 is based on the use of the E2F-1 promoter to control viral replication. The E2F-1 promoter mediated transgene expression has been previously shown to be selective in Rb pathway–defective tumor cells (15, 16). In this communication, we have examined the Rb status dependence of gene expression of CG0070 and its potential application in the treatment of bladder TCC. In vitro studies showed that following infection with CG0070, 100 times more E1a mRNA and 1,000 times more GM-CSF were detected in the Rb pathway–defective cells compared with normal cells. This suggested that the expression of E1a gene in CG0070, which is under the control of the E2F-1 promotor, as well as the transgene in the E3 region is highly dependent on the Rb status of the cells. Further, viral replication and cytotoxicity of CG0070 were significantly enhanced in Rb pathway–defective cells. In Rb pathway–defective bladder TCC cells, CG0070 produces 1,000-fold more viruses in tumor cells in comparison with the normal cells. CG0070 is highly cytotoxic to the bladder tumor cells with minimal cytolysis of normal cells. Even when the tumor cells are infected with CG0070 at an MOI
of 1, almost all the cells are lysed by day 10 following infection, compared with minimal cell killing observed with CG0070 in primary cells such as MRC5 and hAEC even at an MOI of 10. In vivo studies with CG0070 showed the strong antitumor activity of the virus in bladder TCC xenograft tumor models and showed significant antitumor synergy when combined with the chemotherapeutic agent docetaxel. Previous studies with E2F-1 promoter–controlled oncolytic adenoviruses have shown similar tumor cell specificity and cytoxicity in vitro in Rb pathway–defective cells in comparison with normal cells (15, 16, 21, 29, 30). In addition, these viruses showed significant antitumor efficacy in several s.c. xenograft tumor models in mice. Several genomic structural features distinguish CG0070 from these E2F-1 controlled viruses including the absence of an endogenous E1a promoter, inclusion of a SV40 Poly(A) sequence 5′ to the packaging signal to avoid any nonspecific transcriptional initiation, which may increase specificity, unchanged location of the packaging signal with respect to the wild-type Ad5 virus to avoid potential recombinants, and inclusion of the GM-CSF transgene in the E3 gp19kd position under the control of the E3 promoter.

CG0070 is also intended to selectively produce GM-CSF in Rb pathway–defective tumor cells due to the dependence of the E3 promoter that drives GM-CSF expression on transactivation by E1A. CG0070-mediated GM-CSF expression was observed in all bladder TCC cell lines examined. At 100 viral particles per cell or higher, production of biologically active GM-CSF exceeded 40 ng/mL/106 cells/24 h (Table 1), a level that all bladder TCC cell lines examined. At 100 viral particles per E3 promoter that drives GM-CSF expression on transactivation Rb pathway–defective tumor cells due to the dependence of the GM-CSF transgene under the control of the E3 gp19kd position. Specific transcriptional initiation, which may increase specificity, unchanged location of the packaging signal with respect to the wild-type Ad5 virus to avoid potential recombinants, and inclusion of the GM-CSF transgene under the control of the E3 promoter.

Table 2. Combination treatment with Ar20-1004 and docetaxel

<table>
<thead>
<tr>
<th>SD*</th>
<th>Ar20-1004</th>
<th>Docetaxel</th>
<th>FTV relative to untreated controls</th>
<th>combination treatment</th>
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<tr>
<td></td>
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<td>15</td>
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<td>0.205</td>
<td>0.055</td>
<td>0.105</td>
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</tr>
<tr>
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<td>0.069</td>
<td>0.051</td>
<td>1.353</td>
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<tr>
<td>37</td>
<td>0.446</td>
<td>0.371</td>
<td>0.165</td>
<td>0.050</td>
<td>3.300</td>
</tr>
</tbody>
</table>

*Study day after first treatment with the virus.
†Fractional tumor volume (FTV) calculated as mean tumor volume experimental/mean tumor volume control.
‡Mean FTV of Ar20-1004 × mean FTV of docetaxel
§Obtained by dividing the expected FTV by the observed FTV. A ratio of >1 indicates a synergistic effect and a ratio of <1 indicates a less than additive effect.

The antitumor effects observed in the present study are likely entirely due to the oncolytic activity of CG0070 because the human GM-CSF encoded by this virus is not biologically active in mice (31). Optimal demonstration of enhancement of antitumor responses by treatment with CG0070 would necessitate testing the vectors in immunocompetent animal models with Ar20-1004, which carries the mouse GM-CSF transgene. Nevertheless, in the absence of a robust immunocompetent murine animal model, studies in immune-deficient nude mice have shown that an oncolytic adenovirus similar to CG0070, but expressing murine GM-CSF instead of human GM-CSF, enhances antitumor activity in comparison with the parental non-GM-CSF-expressing vector, presumably through the activation of innate immune responses that are maintained in nude mice (15, 16). Histochemical examination of the virus-injected tumors showed that both vectors induced necrosis and mononuclear cell infiltration into the tumor but only the murine GM-CSF-expressing adenovirus resulted in eosinophil infiltration. Similar mononuclear cell infiltrations were observed following intravesical treatment of bladder of immunocompetent mice only with Ar20-1004 but with an identical virus to Ar20-1004 that does not encode the GM-CSF transgene (data not shown). It is anticipated that CG0070 will have greater efficacy in immunocompetent humans wherein the GM-CSF expressed by the virus has the potential to activate an immunologically specific antitumor immune response.

Intravesical administration of a therapeutic agent would greatly assist in maximizing the drug delivery to the target tumor tissue with minimum distribution to the vital organs outside of the bladder. Hence, bladder cancer has been treated traditionally through the intravesicle instillation of therapeutic agents such as Bacillus Calmette-Guerin, chemotherapeutic agents, and gene therapeutic agents. Local delivery of an armed oncolytic adenovirus in the bladder would allow for efficient infection of the superficial tumors by adenovirus without significant systemic exposure and thus restrict the transgene expression from the infected tumor cells wherein the virus replicates with minimal overall circulating levels of the transgene. In addition to the antitumor efficacy observed in s.c. xenograft tumor models with CG0070 either as a single agent or in combination with docetaxel, results from the studies done in the orthotopic tumor model in nude mice showed the antitumor potential of CG0070 in a model treatment setting. Two phase I trials have been done with nonreplicating adenovirus-based vectors that show the relative safety and feasibility of intravesicle instillation of virus (32, 33). One of the limiting factors in these phase I trials for the treatment of bladder cancer has been the low infectivity of the bladder epithelium with adenovirus vectors due to the barrier imposed by the polyanionic glycosaminoglycan layer (34, 35). Pretreatment of the bladder with the transduction enhancement agents such as Syn3 and dodecyl β-D-maltoside may enhance the infectivity of adenovirus in tumors with consequently improved clinical efficacy (36, 37).

Immunotherapy with Bacillus Calmette-Guerin, delivered intravesically into the bladder, results in a massive local immune response characterized by the induction of proinflammatory cytokines in the urine and in bladder tissue (38).
Studies in immunocompetent murine models have shown the role of different lymphocyte subpopulations in the antitumor activity of Bacillus Calmette-Guerin. Under normal conditions, very few leukocytes can be detected in the suburothelial stroma of the bladder following Bacillus Calmette-Guerin instillation; significant influx of various leukocyte subpopulations and chemokines is thought to probably contribute to the antitumor effect. An oncolytic virus expressing GM-CSF may be particularly effective in eliciting such a response because the cell death resulting from virus replication will release tumor antigens that will then induce a GM-CSF-mediated immune response.

In summary, we have shown that the E2F-1 promoter in CG0070 tightly controls the expression of viral E1A gene and GM-CSF, resulting in high tumor selectivity of CG0070 towards Rb pathway–defective bladder TCC cells. Production of biologically active GM-CSF is induced in a dose-related fashion at levels known to stimulate antitumor immunity in the tumor vaccine setting. Significant antitumor efficacy has been shown in two s.c. xenograft bladder TCC tumor models and an orthotopic TCC tumor model in mice as a monotherapy and in combination with doxetaxel in one xenograft tumor model. These in vitro and in vivo findings show the strong antitumor activity of armed oncolytic adenovirus CG0070 and support its evaluation for the treatment of bladder cancer in humans.

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CG0070, a Conditionally Replicating Granulocyte Macrophage Colony-Stimulating Factor–Armed Oncolytic Adenovirus for the Treatment of Bladder Cancer

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