**Featured Article**

**A Cox-2 Promoter-Based Replication-Selective Adenoviral Vector to Target the Cox-2-Expressing Human Bladder Cancer Cells**

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**ABSTRACT**

**Purpose:** Cyclooxygenase-2 (Cox-2), an enzyme that catalyzes the synthesis of prostaglandins, is overexpressed in a variety of premalignant and malignant conditions, including urinary bladder cancer. In the present study, we examined the feasibility of using Cox-2 promoter-based replication-selective adenovirus for targeting bladder cancer cells that express Cox-2 transcriptionsal activity.

**Experimental Design:** A series of human cancer cell lines, including three bladder cancer cell lines (KK47, T24, and 5637), were evaluated for their Cox-2 and CAR (the Coxsackievirus and adenovirus receptor) mRNA expression levels by quantitative real-time PCR. AdE3-cox2–327, a replication-selective adenovirus in which the expression of E1a is controlled by the Cox-2 promoter, was generated, and its tissue-specific activity was tested in vitro and in vivo.

**Results:** Three bladder cancer cell lines express higher levels of Cox-2 mRNA than does the human prostate cancer cell line PC3, the primary cultured human benign prostatic fibroblast, PF cells, and the human colon cancer cell line Colo320. Relatively higher expression of CAR mRNA was detected in the KK47, 5637, respectively, and Colo320 than in the T24, PC-3, and PF cells. In vitro assays revealed significant growth suppression of both Cox-2- and CAR-expressing bladder cancer cells KK47 and 5637 in comparison with the other cells that lack Cox-2 expression and/or CAR expression.

**Conclusions:** The present study demonstrated both specificity and efficacy of AdE3-cox2–327, a selectively replicated adenovirus, toward the Cox-2-expressing bladder cancer cells in vitro and in vivo. We also found that CAR expression in the target cancer cells is an important factor for the efficacy of selectively replicated adenovirus-based gene therapy.

**INTRODUCTION**

A number of strategies using genetically engineered viruses have been proposed to treat cancerous diseases. Recently, cancer therapy with selectively replicative viruses is being intensively investigated both in the laboratory and in clinical trials (1, 2). Cancer therapy using replication-selective viruses offers two major advantages: the viruses kill tumor cells in a tumor-specific manner, and their selective replications amplify their oncolytic effects (2). In the present study, we present a novel strategy for the treatment of bladder cancer using a replication-selective adenoviral vector.

Cyclooxygenase-2 (Cox-2), which is primarily responsible for prostaglandins produced in inflammatory sites (3), is virtually undetectable in most tissues under physiological conditions (4). In contrast, recent studies demonstrated that Cox-2 is expressed in several cancer tissues and may have an important role in carcinogenesis (5, 6). The up-regulation of Cox-2 has been well established in breast, colon, and lung cancers, as well as in bladder cancer (7). This tumor-specific expression of Cox-2 suggested to us the potential utility of this Cox-2 promoter for the construction of a novel replication-selective adenovirus for the treatment of bladder cancer.

The development of a replication-selective adenoviral vector designed to replicate exclusively in tumor cells has the potential to significantly advance the treatment of cancers (1, 2). In several replication-selective recombinant adenoviral vectors, the transcription of essential viral genes, such as an E1a region, is controlled by replacing the native viral promoters with tumor-specific promoters to optimize tumor-selective replication (8–11). Thus, the regulatory regions of the Cox-2 gene were a reasonable choice for such an approach to treat Cox-2 up-regulated cancers including bladder cancer. Other investigators also have constructed a Cox-2 promoter-based replication-selective adenoviral vector for the treatment of pancreatic cancer (12) or ovarian cancer (13).

In the present study, we explored the possibility of developing a new therapeutic agent that can selectively replicate and amplify the oncolytic effect in Cox-2-expressing bladder cancer cells by using a recombinant replication-selective adenoviral vector containing E1a, an adenoviral early gene required for viral replication, controlled by tissue-specific Cox-2 promoter.

**MATERIALS AND METHODS**

**Cells and Cell Culture.** The established cell lines derived from human transitional cell carcinoma of the bladder, S637 (14, 15) and T24 (16), were purchased from the American...
Type Culture Collection (Manassas, VA); KK47 (17) was generously provided by Dr. Seiji Naito (Department of Urology, Kyushu University, Fukuoka, Japan). The lung cancer cell lines A549 (18) and H358 (19), the human prostate cancer cell line PC-3 (20), the human colon cancer cell line Colo320 (21), and the transformed human embryonic kidney cell line 293 (22) were also obtained from the American Type Culture Collection. PF cells, the primary cultured human benign prostatic fibroblast (23), were also used in this study. In the present study, we maintained 293 cells in MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum. The other cell lines were all maintained in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 unit/ml penicillin, and 100 unit/ml streptomycin (Sigma Chemical Co., St. Louis, MO). The cells were fed three times a week with fresh growth medium.

Real-Time Quantitative Reverse Transcription-PCR Assay. The cells were trypsinized, and the cell pellets were collected by centrifugation at 1000 \times g for 5 min. Isogen (Nippon Gene, Tokyo, Japan) was used to extract total RNA and genomic DNA from the cells. All of the RNA samples were treated with DNase to remove the contaminating genomic DNA, and all of the DNA samples were also treated with RNase to remove the total RNA. Quantitative real-time PCR using the TaqMan fluorogenic detection system was performed according to previously described methods (24). The primers and TaqMan probe for Cox-2, Coxsackievirus and adenovirus receptor (CAR), and E1a gene of adenovirus type 5, were designed using the primer design software Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA). Table 1 shows the sequences of the TaqMan probes and primers for Cox-2, CAR, and E1a. The primers and the TaqMan probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; TaqMan GAPDH control reagent kit) were purchased from Perkin-Elmer Applied Biosystems. RNA and DNA for GAPDH were used as an endogenous control.

Transfection of Plasmids and Luciferase Assay. The 386-bp fragment of the human cyclooxygenase-2 (Cox-2) promoter, the start site of which is at 327 relative to the start of transcription (25), was cloned out by PCR and was inserted into pGL3-basic vector (Promega, Madison, WI) for the construction of Cox2-327-Luciferase vector. For transient expression of the transfected genes, all of the cell lines, seeded at a density of 3 \times 10^5 cells/60-mm dish, were transfected by DOTAP (N-C1,2-Dimyristoyl-dihydroxypropyl)-NNN-trimethylammonium

**Table 1**  Primers and probes

<table>
<thead>
<tr>
<th>Source</th>
<th>Primer (Forward)</th>
<th>Primer (Reverse)</th>
<th>Probe</th>
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<tbody>
<tr>
<td>Cox-2</td>
<td>5′-GATACTCCAGCAGATGATCTACCC-3′</td>
<td>5′-AGACCAGGCACAGACACAAAGA-3′</td>
<td>5′-TCTACCTGAGGATACCTACGGTTGCT-3′</td>
</tr>
<tr>
<td>CAR</td>
<td>5′-CAGAAGCTACATCAGGCAATATCA-3′</td>
<td>5′-CTCTGAGGGAGCTGTTCAAGTC-3′</td>
<td>5′-TCTCATGCTTCTCCACATGGGAGA-3′</td>
</tr>
<tr>
<td>E1a</td>
<td>5′-GCTGGCCCTATTTACCA-3′</td>
<td>5′-CATCCACAGCTGGCG-3′</td>
<td>5′-TGCGGTGAGTTGTTGCCG-3′</td>
</tr>
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![Fig. 1](https://example.com) Construction of the replication-selective adenoviral vector AdE3-cux2-327. Recombinant AdE3-cux2-327 virus was generated by homologous recombination between pXC1-cux2-327 and pBHGE3. The pBHGE3 DNA is noninfectious, whereas cotransfection of pXC1 and pBHGE3 creates an infectious virus by homologous recombination. ITR, inverted terminal repeat; Ψ, package signal; 16mu, 16 minute unit; 100/0, nature fusion; ΔΨ, deleted package signal; Ap r, ampicillin-resistant; ori, replication origin; Ad5, adenovirus type 5; Ψ−Ad5, Ad5 without package signal.

![Fig. 2](https://example.com) Cotransfection of 293 cells intracellular recombination

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mesylate) (Boehringer Mannheim Corporation, Indianapolis, IN) in accordance with the manufacturer’s protocol. After an additional 24-h incubation, cells were washed twice with PBS and were lysed with lysis buffer provided by Promega as a part of the luciferase assay system. A 50-μl aliquot of cell extract was then mixed with 100 μl of reagent solution containing luciferin and ATP (Luciferase Assay System; Promega). The interaction of luciferase with luciferin in the presence of ATP generates light at 562 nm. The emission of light, which peaks within 10 s, was recorded with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The transduction efficacy was controlled by cotransfected CMV-β-gal plasmid. The data presented represent the average of three different dishes in the same experiment.

**Construction and Production of the Replication-Selective Adenoviral Vector, AdE3-cox2-327.** The pXC1 plasmid has adenovirus 5 (Ad5) sequences from bp 22 to 5790 containing the E1 gene (Microbix Biosystems Inc., Toronto, Ontario, Canada; Ref. 26). A unique AgeI site was introduced at nucleotide position 552, essentially as described by Rodriguez et al. (8) to generate the plasmid pXC1-AgeI. The Cox2-327 promoter was ligated to pXC1-AgeI plasmid to obtain pXC1-cox2-327. The pHGE3 (27) plasmid contains Ad5 sequences with a wild-type E3 region and an E1 deletion of bp 188-1339. The pHGE3 DNA is noninfectious, whereas cotransfection of pXC1 and pHGE3 creates an infectious virus by homologous recombination. Recombinant AdE3-cox2-327 virus was prepared by homologous recombination (28) using 10 μg of pXC1-cox2-327 mixed with 20 μg of pHGE3 (Fig. 1); the resulting recombinant virus was precipitated with CaCl₂, and was then used to transfect 293 cells, as described elsewhere (29). AdE3-cox2-327 was demonstrated to replicate, in a restricted manner, –cox2 used to transfect 293 cells, as described elsewhere (29). AdE3-cox2-327 virus was obtained by plaque purification

**In Vitro Experiment.** The cells were seeded at a density of 1000/well in 24-well tissue-culture plates. After a 12-h incubation, each cell line was infected for 3 h with AdE3-cox2-327 or the Ad5CMV-LacZ viral control. The viable cell numbers of triplicate cultures of all treatment groups were measured at day 10 by the Alamar Blue method according to the manufacturer’s instructions (Alamar Biosciences, Inc., Sacramento, CA). Briefly, the cultures were aseptically added to 40 μl of Alamar Blue and were returned to the incubator for 3 h; the fluorescence was measured by a fluoroscan with excitation at a wavelength of 560 nm and emission at 590 nm (Titertek Fluoroscan II; Labosystems, Tokyo, Japan). The survival rate was calculated assuming the rate of untreated cells to be 100%. To evaluate the Cox2-327 promoter activity in each of the cell lines, the expression level of E1α mRNA in early time points before the start of viral replication was measured by a TaqMan real-time PCR. The total RNA samples were isolated from the cells infected with 1, 6, and 12 h after the 3-h infection. To assess the AdE3-cox2-327 viral replication in the cell lines, the expression of E1α mRNA was also quantified by a TaqMan real-time PCR assay. Genomic DNA samples were isolated from the cells infected with 1 multiplicity of infection (MOI) of Ad-cox2-327 at 1, 3, 5, and 7 days after the infection. PCR products of E1α gene were cloned into pGEM T easy Vector (Promega, Tokyo, Japan) and sequenced. The standard curves were generated from serially diluted solutions of the plasmid clone as templates. The expression level of E1α DNA was calculated as copies per microgram of genomic DNA.

**Animal Studies.** Athymic BALB/c (nu/nu) mice (Charles River Japan, Yokohama, Japan), 5–6 weeks of age, were inoculated s.c. with KK-47 cells (1.0 × 10⁶ cells/50 μl of medium). When tumors with diameters of 3–5 mm developed, 18 animals were randomly assigned to three experimental groups as follows: group 1, no treatment (6 animals); group 2, Ad5CMV-LacZ viral control (6 animals); group 3, AdE3-cox2-327 treatment (6 animals). Ad5CMV-LacZ or AdE3-cox2-327 (1 × 10⁹ plaque-forming units/50 μl of PBS) was intratumorally injected on day 1 and day 3 with a microliter syringe fitted with a 28-gauge needle. Tumor volume was measured every other day.

![Fig. 2](image-url)  
**Fig. 2** Relative mRNA levels of Cox-2 (A) and CAR (B) by quantitative reverse transcription-PCR of cell lines. A, the expressions of Cox-2 mRNA were markedly increased in KK47, T24, and 5637. AS49 and H538, were used as Cox-2-positive and -negative controls, respectively. B, relatively higher expressions of CAR mRNA were observed in KK47, 5637, Colo320, AS49, and H538 as compared with that of T24, PC-3, and PF cells. Values represent relative levels of mRNA normalized to the PC-3 cell line (A, mRNA levels of Cox-2) or T24 cell line (B, mRNA levels of CAR) with triplicate determinations of each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an endogenous RNA control to normalize for differences in the amount of total RNA.

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day and was calculated using the following formula: volume (a rotational ellipsoid) = \( \frac{4}{3} \pi M_1 M_2^2 \times 0.5236 \), where \( M_1 \) = the long axis and \( M_2 \) = the short axis (31). The relative tumor volume was calculated assuming the rate of each tumor volume at day 0 to be 100%. All aspects of the experimental design and procedure were reviewed and were approved by the institutional ethics and animal welfare committees of the Kobe University School of Medicine.

**Statistical Analysis.** Statistical significance was determined by an ANOVA, with \( P < 0.05 \) considered to be statistically significant.

**RESULTS**

**Relative Quantification of mRNA Expressions of Cox-2 and CAR.** Fig. 2 shows the mean relative quantifications of the Cox-2 and CAR mRNA expressions found in the cell lines used. To normalize for differences in the amount of total RNA, we selected GAPDH as an endogenous RNA control. The relative quantification was calculated assuming the rate of each mRNA expression in KK47, T24, and 5637, and A549, as compared with those of the other cell lines. In this experiment, we used A549 as Cox-2-positive cells and H358 as Cox-2-negative cells (32). Recent studies have indicated that CAR is a common receptor for both Coxsackievirus and group C adenovirus (33, 34). The loss of adeno-receptor expression may, therefore, impact the therapeutic response. The KK47, 5637, Colo320, A549, and H358 cell lines had relatively higher levels of CAR mRNA expression as compared with the other cell lines. The KK47, 5637, and A549 cell lines possessed relatively higher levels of both Cox-2 and CAR mRNA expressions.

**Luciferase Activities of the Cox2–327 Promoter.** To assess whether the Cox2–327 promoter may have a cell type-specific activity, we performed the transient expression assay. Consistent with the expressions of Cox-2 mRNA, the luciferase activity of the Cox2–327 promoter was markedly increased in KK47, T24, 5637, and A549, as compared with the other cell lines (Fig. 3).

**Adenoviral E1a mRNA Expressions in Early Time Point.** To examine the tissue specificity of Cox2–327 promoter in adenoviral construct, the expression level of E1a mRNA in early time points before the start of viral replication was measured by a TaqMan real-time reverse transcription-PCR. Consistent with the promoter activities in the plasmid construct, relatively higher E1a mRNA expression was observed in KK47, T24, 5637, and A549 cells, as compared with the other cells (Fig. 4).

**In Vitro Cytotoxicity of AdE3-coc2–327.** Whereas AdE3-coc2–327 induced no cell growth inhibition in the T24, PC3, Colo320, PC3, and H358 cells at 0–1 MOI, AdE3-coc2–327 induced significant cell growth inhibition at 0.1–1 MOI in the KK47, 5637, and A549 cells, which expressed both Cox-2 and CAR (Fig. 5). The 293 cell line, which expressed the transforming E1 gene of adenovirus type 5, had a significant sensitivity to the Ad5CMV-LacZ control adenoviral vector at 0–1 MOI, whereas the other cell lines had no significant sensitivity to the Ad5CMV-LacZ (data not shown).

**Cell-Type-Specific Replication of AdE3-coc2–327.** To assess whether the AdE3-coc2–327 could selectively replicate in the specific cell line, we quantified the replicated DNA copy numbers of E1a in the cell lines infected with 1 MOI of Ad-coc2–327 at days 1, 2, 3, 5, and 7. TheKK47, 5637 and A549
cells at day 1–7, and T24 cells at day 3–7 contained significantly higher levels of E1a DNA as compared with the H358 cell (Fig. 6).

Antitumor Effect of AdE3-cox2–327 in the KK47 Subcutaneous Tumor. The animals with KK47 s.c. tumor were treated with either AdE3-cox2–327 or Ad5CMV-LacZ, twice via intratumoral injections. After completion of the treatment, the group treated with AdE3-cox2–327 demonstrated a significant growth inhibition of the KK47 tumor for up to 24 days (Fig. 7).

DISCUSSION

Nearly 50% of patients with muscle-invasive bladder cancer already have occult distant metastasis and will develop clinically evident manifestations within 1 year. Despite recent advances in combination chemotherapy, nearly all patients with metastatic bladder cancer will die within 2 years (35). There is increasing recognition that the development of novel targeted molecular therapies with or without present treatment modalities could improve survival in this cancer. In the present study, we developed a genetically engineered replication-selective adenovirus and explored the feasibility of this agent for the treatment of bladder cancer.

Cancer gene therapy using a replication-deficient viral vector has a major limitation in the therapeutic gene transfer to every cancer cell in the tumor site, whereas a replication-selective virus, designed to replicate selectively in the targeted cancer cells, allows efficient tumor penetration. There are several strategies for the tumor selective-replication of the viral vector: (a) to delete the viral genes required for the replication in normal cells but not in tumor cells, such as E1B55K gene (36) or Rb-binding gene of E1A (37) in the adenoviral vector; (b) to use tissue- or tumor-specific promoter to control the expression of viral genes essential for viral replication (8–13). Our adenoviral vector, AdE3-cox2–327, is also based on this strategy; and
(c) to use wild-type viruses that naturally replicate selectively in tumor cells, such as a reovirus (38).

The urinary bladder is uniquely suited for adenoviral gene therapy for several reasons: it is a hollow organ and is accessible for endoscopy and for intravesical or intratumoral administration of an adenoviral vector. The intravesical approach has the practical advantage of allowing simple instillation of a vector into the bladder lumen, a delivery method that gives easy access to the superficial bladder tumors. On the other hand, the direct intratumoral injection of adenoviral vector using endoscopy might achieve high-concentration vector exposure and might be suitable for muscle-invasive bladder tumor. A previous study showed that Cox-2 is highly expressed in malignant bladder tissue. A previous study might achieve high-concentration vector exposure and might be suitable for muscle-invasive bladder tumor. A previous study showed that Cox-2 is highly expressed in malignant bladder tumor but not in benign bladder tissues (7). Therefore, the intravesical injection of the AdE3-cox2–327 vector into the muscle-invasive tumor site might be able to achieve the efficient tumor penetration while sparing the surrounding normal bladder tissue.

Our data suggested that the cell-specific cytotoxic effect and adenoviral replication of AdE3-cox2–327 required the expressions of both CAR and Cox-2 in the cells. AdE3-cox2–327 demonstrated the cytotoxic effect and viral replication in KK47, 5637, and A549 cells, which express both CAR and Cox-2. By contrast, AdE3-cox2–327 did not replicate in either T24, which expressed Cox-2 but not CAR, nor in Colo320 and H358, which expressed CAR but not Cox-2.

The variability of the CAR expression level was observed in several bladder cancer cells, and it strongly correlated with adenoviral infectivity to the bladder cancer cells (39). Recently, adenoviral gene therapy using an Ad5/35 chimeric vector (40) or a RGD-4C modified adenoviral vector (12, 13) was suggested to circumvent CAR deficiency. Thus, those strategies may have a potential to improve the therapeutic efficacy of AdE3-cox2–327 in the bladder cancer cells lacking CAR expression.

AdE3-cox2–327, similar to some other replication-selective adenoviral vectors (41, 42), has an intact Ela promoter upstream of the Cox-2–327 promoter. Although we confirmed the in vitro cell-specificity of AdE3-cox2–327, further investigation of the in vivo tumor specificity is warranted for clinical application. This molecular therapeutic approach using AdE3-cox2–327 may lead to the development of a novel efficient modality for the treatment of bladder cancer and other Cox-2-expressing cancers such as breast, ovarian, lung, and gastrointestinal cancers.

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A Cox-2 Promoter-Based Replication-Selective Adenoviral Vector to Target the Cox-2-Expressing Human Bladder Cancer Cells

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