

# Differential Effects of Adenovirus-p16 on Bladder Cancer Cell Lines Can Be Overcome by the Addition of Butyrate<sup>1</sup>

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## ABSTRACT

**High frequency of p16 alteration and high local recurrence rate of bladder cancer make this cancer an ideal target for p16 gene therapy. However, a low transduction rate of p16 via adenoviral vector causes an inconsistent result. In this study, we have tested adenovirus-p16 in several bladder cancer cell lines and investigated a way of improving the low transduction rate. Adenovirus-p16 showed a strong antitumor effect on bladder cancer cell lines (253J and T24) with strong Coxackie-adenoviral receptor (CAR) expression but little antitumor effect on bladder cancer cell lines (J82 and HT1376) with little CAR expression. In this study, we suggest a simple way of overcoming the differential effects of the adenovirus. The addition of butyrate to media was found to increase the transduction rate of adenovirus remarkably and increase the antitumor effect of adenovirus-p16 in bladder cancer cell lines with little CAR expression. Butyrate effects were related with increased CAR expression on the cell surface as well as increased transgene expression from adenoviral vector. From these observations, application of adenovirus-p16 gene therapy with butyrate can overcome the obstacle of low gene transfer and enhance the antitumor effect of adenovirus-p16 in bladder cancer.**

Received 5/1/00; revised 10/24/00; accepted 10/25/00.

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<sup>1</sup> Supported by Grant 01-98-006 from the Seoul National University Hospital Research Fund.

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## INTRODUCTION

Tumor suppressor gene therapy is one of the major types of cancer gene therapy (1). Losses of tumor suppressor genes are involved in many types of cancer, including bladder cancer. *p16*, located in 9p21, is one of the tumor suppressor genes that are involved in the G<sub>1</sub>-S regulation of the cell cycle. Loss of p16 activity by deletion, point mutation, or hypermethylation of the p16 promoter has been detected frequently in bladder cancer, resulting in a G<sub>1</sub> regulation defect (2–5). 9p deletion appears to be an early event in bladder carcinogenesis and has been found in >50% of superficial bladder cancer (6). Allelic loss of 9p could be a marker for the early detection of bladder cancer. Seventy-five % of bladder tumors present as superficial lesions. Superficial tumors can be treated by transurethral resection, but the high reported rates of local recurrence require another treatment modality (7). Furthermore, deletions of the *p16*<sup>INK4a</sup> gene are related to high recurrence in superficial bladder cancer (8). Gene therapy with tumor suppressor genes, such as *p16*, could be an ideal treatment modality for superficial recurrent bladder tumor, which has an associated high incidence of genetic abnormalities and would be easy to apply by cystoscopy.

Adenovirus is a very useful method of gene transfer with high efficiency and a high level of expression. Unfortunately, recent studies have revealed that expression of CAR,<sup>3</sup> an important receptor of adenoviral entry, is frequently lost in bladder cancer cells (9). This could be a new obstacle for adenoviral gene therapy in bladder cancer.

In this study, we investigated CAR expression in several human bladder cancer cell lines and the antitumor effects of adenovirus-p16 according to the CAR level and p16 status and also found a way to overcome the low transduction rate in bladder cancer.

## MATERIALS AND METHODS

**Cell Lines.** Four human bladder cancer cell lines (J82, HT1376, T24, and 253J) were purchased from the Korean Cell Line Bank, Seoul National University College of Medicine (Seoul, Korea). All cell lines were maintained in RPMI 1640 + 8% FBS + penicillin/streptomycin. RmcB (CRL-2379) for CAR antibody was purchased from American Type Culture Collection (Manassas, VA).

**Adenovirus-p16.** We had already constructed adenovirus-p16 and tested its efficacy in human lung cancer cell lines (10, 11). Briefly, the p16<sup>INK4A</sup> cDNA in pGEX-2TK (provided by Frederic Kaye, National Cancer Institute, Bethesda, MD) was cloned into an adenoviral shuttle vector,

<sup>3</sup> The abbreviations used are: CAR, Coxackie and adenovirus receptor; CMV, cytomegalovirus; β-gal, β-galactosidase; moi, multiplicity of infection; ad-p16, adenovirus-p16; Rb, retinoblastoma; FACS, fluorescence-activated cell sorter.

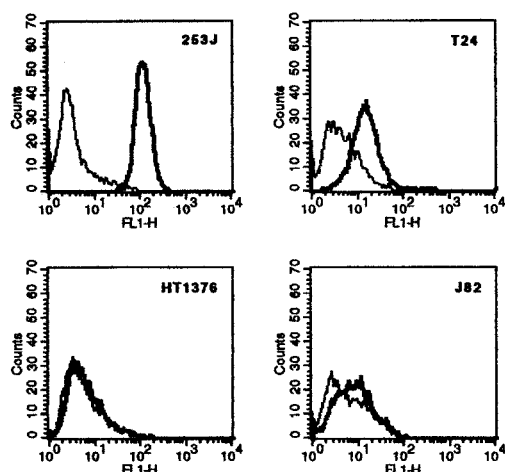


Fig. 1 CAR expression status in human bladder cancer cell lines. Moderate to high levels of CAR expression were found in 253J and T24. In contrast, little CAR expression was found in J82 or HT 1376.

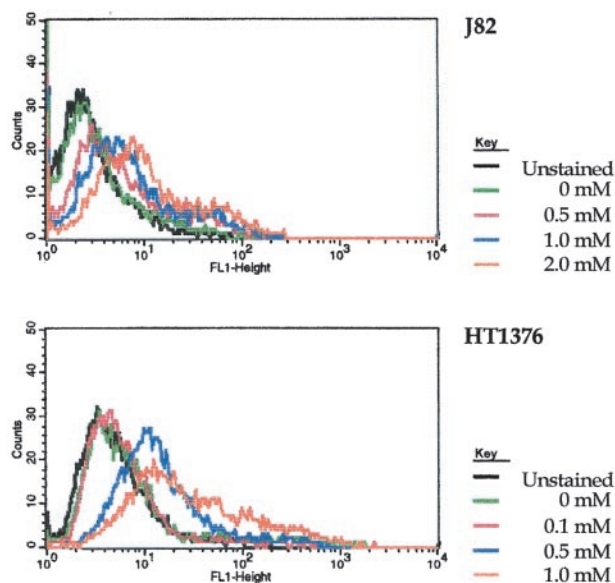


Fig. 2 Addition of butyrate increased the expression of CAR in J82 and HT1376. CAR expressions were increased progressively, according to the increasing concentrations of butyrate.

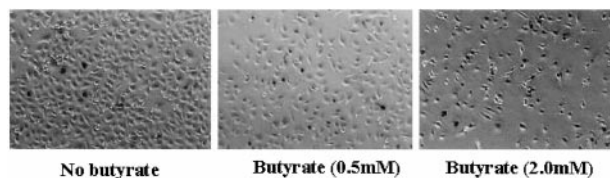


Fig. 3 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside staining showed that addition of butyrate during transduction with ad- $\beta$ -gal increased the transduction efficiency in J82 (a, no butyrate: 8.5%; b, 0.5 mM butyrate, 24.3%; c, 2.0 mM butyrate, 76.2%).

Table 1 Transduction efficiency of ad- $\beta$ -gal in human bladder cancer cell lines

Transduction efficiencies were consistent with CAR status. Butyrate (0.5 and 2 mM) addition improved the transduction efficiency of ad- $\beta$ -gal remarkably in cells expressing small amounts of CAR.

Cell lines	-butyrate (%)	+butyrate (0.5 mM, %)	+butyrate (2.0 mM, %)
253J	95.2 $\pm$ 5.6	ND <sup>a</sup>	ND
T24	28.3 $\pm$ 5.6	66.1 $\pm$ 5.1	98.1 $\pm$ 2.6
J82	8.5 $\pm$ 2.0	24.3 $\pm$ 5.9	76.2 $\pm$ 4.1
HT1376	5.1 $\pm$ 1.5	20.1 $\pm$ 3.7	60.0 $\pm$ 6.2

<sup>a</sup> ND, not done.

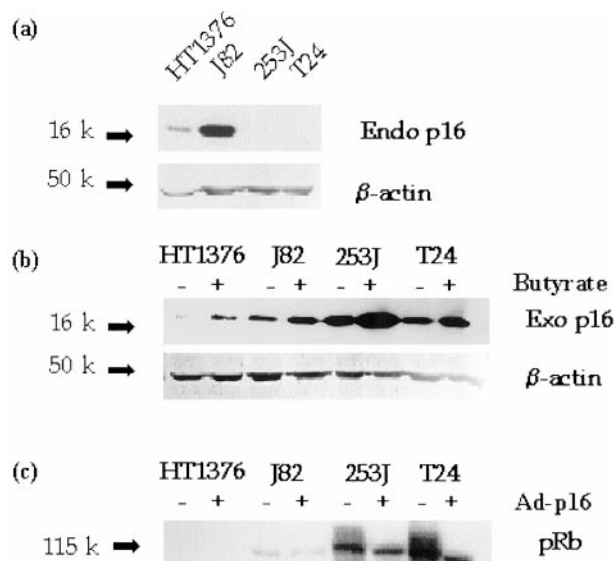


Fig. 4 Western blot analysis of p16 protein from ad-p16 transduction and the change of pRb after adenovirus transduction. a, endogenous p16 production was found in J82 and HT 1376. b, butyrate increased expression of p16 from four ad-p16-transduced bladder cancer cells, especially butyrate increased p16 expression remarkably in HT1376, in which a little p16 expression was found in normal medium (butyrate: 0.5 mM in HT1376; 2.0 mM in J82, 253J, and T24). c, ad-p16 transduction induced dephosphorylation of pRb in 253J and T24.

pAC CMVpLpA (provided by Robert D. Gerard, University of Texas Southwestern Medical Center, Dallas, TX). The pAC CMV-p16 and pJM17 were cotransfected into 293 cells using the calcium phosphate method. Adenovirus-p16 with CMV promoter was generated by homologous recombination (11). For the experiment, adenovirus-null (recombinant adenovirus without any therapeutic gene) or ad- $\beta$ -gal was used as a control virus.

**Transduction with ad-p16.** Exponentially growing human bladder cancer cell lines were transduced with 100 moi of ad-p16 for 1 h in serum-free media and incubated with complete media (RPMI 1640 + 8% FBS + penicillin/streptomycin) until use. When we used butyrate for the experiment, cell culture before and after transduction was conducted in sodium butyrate (0.1 to 2 mM, Sigma)-containing media.

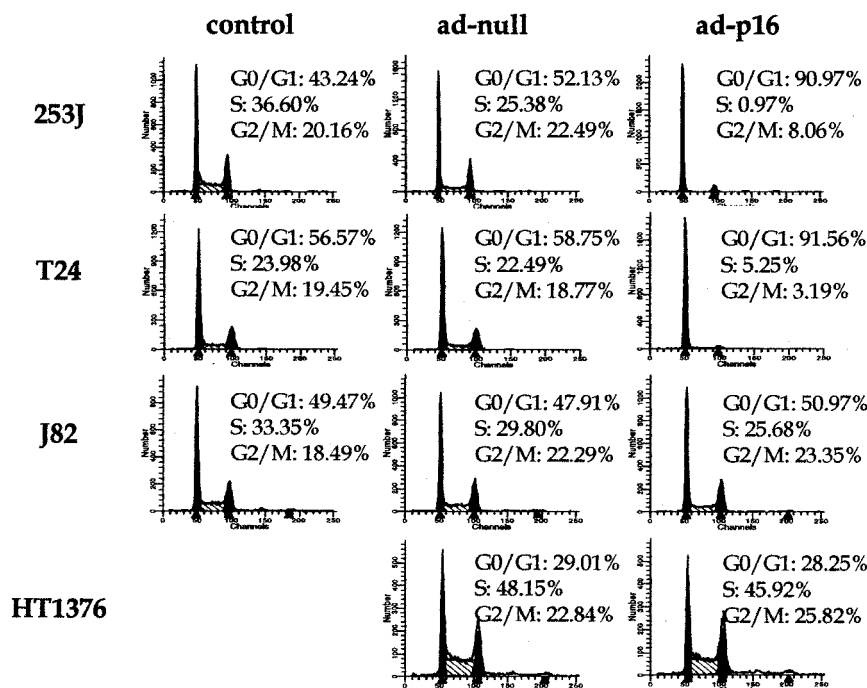


Fig. 5 Strong G<sub>1</sub>-S arrest in human bladder cancer cell lines with CAR expression (253J and T24) but minimal G<sub>1</sub>-S arrest in cancer cell lines with little CAR expression (J82 and HT1376). control, untransduced; null, transduced with adenovirus-null; p16, transduced with ad-p16.

**Determination of CAR Expression on the Cell Surface by Flow Cytometry.** Flow cytometric analysis for CAR was used to measure CAR expression on human bladder cancer cells. Briefly, cells were detached with a scraper and were incubated with a saturating quantity of primary antibody [mouse monoclonal antibody against human CAR from the RmCB (CRL-2379) cell line] for 30 min at 4°C and washed with PBS. After incubation with secondary antibody [FITC-conjugated F(ab')<sub>2</sub> of antimouse IgG] for 30 min at 4°C, cells were analyzed immediately on a flow cytometer (FACSCalibre; Becton Dickinson, San Jose, CA). To measure the effect of butyrate on CAR expression, the same experiments were done with cells incubated in media containing butyrate of various concentrations (0.1 to 2 mM) for 24 h.

**Transduction Efficiency by ad-β-gal with or without Butyrate.** The effect of butyrate on the transduction rate was investigated. Bladder cells were transduced with 100 moi of ad-β-gal and maintained with butyrate (0–2 mM)-containing media for 48 h. Ad-β-gal transduction rates were compared by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining.

**Western Blot for p16 and Rb Proteins.** Four bladder cancer cell lines were transduced with ad-p16 (100 moi). After protein extraction at 24 h of incubation, Western blot assays were performed using the ECL Western blotting detection system and protocol (Amersham). The primary antibodies for p16 and for Rb were polyclonal rabbit anti-p16 antibody (PharMingen, San Diego, CA) and monoclonal mouse antihuman-Rb antibody (PharMingen), respectively.

**Cell Cycle Analysis by FACS.** To check cell cycle change attributable to p16 gene transfer, exponentially growing cancer cells were transduced with ad-p16 (100 moi), ad-null, and media as a mock infection. After 48-h incubation, cell cycle

proportion was measured by DNA histogram by the protocol of the CycleTest™ plus DNA reagent kit (Becton Dickinson, San Jose, CA).

**Analysis of Cell Growth Inhibition.** Four bladder cancer cell lines were transduced with ad-p16 (100 moi), ad-null (100 moi), or media as a mock infection in a six-well plate. Cell numbers were counted daily by hemocytometer from days 1 to 6. In two cell lines showing little growth inhibition by ad-p16 in normal media, we transduced and kept the cells in media with butyrate (0.5 mM in HT1376 and 2 mM in J82). Cell growth was measured by the same method.

## RESULTS

**Determination of CAR Expression on the Cell Surface by Flow Cytometry and Effect of Butyrate on CAR Expression.** FACS analysis for CAR expression revealed high expression of CAR in 253J and moderate expression in T24. However, little expression of CAR in J82 was found and almost no expression in HT1376 (Fig. 1). However, the addition of butyrate of various concentrations from 0.1 to 2.0 mM increased the expression of CAR in J82 and HT1376 (Fig. 2). CAR levels were increased progressively according to increasing butyrate concentration.

**Transduction Efficiency of ad-β-gal Was Dependent on CAR Expression and Improved by Butyrate.** High transduction rates were observed in two bladder cancer cell lines with moderate to high CAR expression; however, low transduction rates were observed in two cancer cell lines with low CAR expression. In cancer cell lines with low CAR expression, butyrate treatment improved the transduction rate dramatically up to 60–76% (Fig. 3; Table 1).

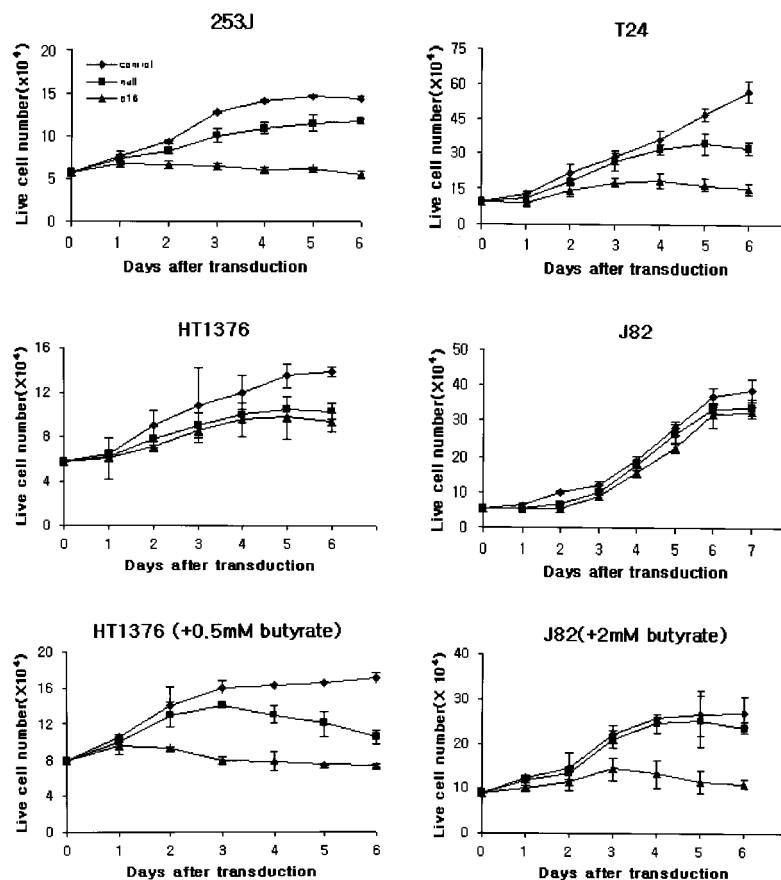


Fig. 6 Growth suppression of bladder cancer cell lines by ad-p16 transduction. ad-p16 transduction suppressed the cell growth of 253J and T24. However, in HT1376 and J82, ad-p16 transduction could not suppress the cell growth in normal media. In butyrate-containing media, HT1376 and J82 became very sensitive to ad-p16 transduction (Fig. 6, e and f). Bars, SD.

**Western Blot Assay for p16 and Rb Protein.** Among four bladder cancer cell lines, endogenous p16 production was found in J82 and HT1376 but not in T24 or 253J (Fig. 4a). ad-p16 transduction induced high expression of exogenous p16 in 253J and T24. In HT1376, ad-p16 transduction in normal media induced very little expression of exogenous p16; however, addition of butyrate increased p16 expression. In J82, exogenous p16 production was also increased by butyrate (Fig. 4b). These data were also consistent with increased transduction rate of adenovirus by butyrate as shown in Fig. 3 and Table 1. Endogenous expression of Rb protein was found in 253J and T24 in both the unphosphorylated and phosphorylated forms. In J82, only small amounts of unphosphorylated pRb were found. ad-p16 transduction in 253J and T24 induced the dephosphorylation of pRb (Fig. 4c).

**Cell Cycle Analysis by FACS.** Strong G<sub>1</sub>-S arrest was found in 253J and T24. In 253J, G<sub>1</sub>-S arrest was more profound (S-phase fraction: 0.97% in ad-p16, 25.38% in ad-null, and 36.60% in untransduced cells). In T24, the S-phase fraction in ad-p16 transduced cells was 5.25% compared with 23.98% in untransduced cells and 22.49% in ad-null transduced cells. However, minimal G<sub>1</sub>-S arrest was found in J82 and HT1376 (Fig. 5).

**Analysis of Cell Growth Inhibition.** Adenovirus-mediated p16 transfer to cancer cell lines with CAR expression and no p16 expression (253J and T24) strongly inhibited the growth of cancer cell lines. However, in bladder cancer cell lines

with little to no CAR expression and with p16 expression (J82 and HT1376), adenovirus-mediated p16 did not inhibit the growth of cells. However, addition of butyrate to the media restored the growth-inhibitory activity of ad-p16 in J82 and HT1376 compared with control and ad-null transduced cells in butyrate-containing medium (Fig. 6, e and f). These findings suggest that the increased transduction efficiency of p16, even in p16-positive cell lines, induced growth suppression.

## DISCUSSION

The endogenous status of p16 and pRb are known as determinants of the effect of ad-p16 gene therapy. Craig *et al.* (12) showed that antitumor effects of ad-p16 were strong in cancer cells with mutant or null-type for *p16* and wild type for *pRb*. Antitumor effects of ad-p16 were weak in cancer cells with wild type for p16 and nonfunctional pRb (12). This study also showed the same results. Antitumor effects (cell cycle arrest and growth suppression) were profound in T24 and 253J, which have no p16 expression but intact pRb expression. In J82 and HT1376 that have p16 expression and no pRb expression, the antitumor effects of ad-p16 were minimal.

In addition to p16 and pRb status, the expression level of CAR is another determinant for the effect of ad-p16 gene therapy (9). FACS showed high expression of CAR in 253J and moderate expression in T24. In these two cell lines, ad-p16

showed strong G<sub>1</sub>-S arrest and cell growth inhibition. Little expression of CAR was noted in J82 and HT1376 in that ad-p16 showed little G<sub>1</sub>-S arrest and weak growth inhibition. In contrast to a previous report (9), T24 expressed moderate numbers of CAR on the cell surface. Unfortunately, these results did not allow us to determine which determinant is the major factor, because the presence of p16 expression in bladder cancer cells coexists with little expression of CAR.

Li *et al.* (13) confirmed the role of CAR in adenovirus entry and suggested adenovirus-CAR for increasing CAR density and improving adenovirus gene transfer efficiency. Hidaka *et al.* (14) also suggested that CAR deficiency on adenovirus targets could be circumvented either by supplying CAR by adenovirus or by modifying adenovirus fiber to bind to another cell surface receptor.

In this study, we suggest a simple method of overcoming the low transduction rate of adenovirus into CAR-deficient bladder cancer cell lines, which involves the use of sodium butyrate that has already been used to improve viral gene expression at the transcriptional level (15–17). We have previously used butyrate to increase the expression of transgene from adenoviral vector (ad-mB7; Ref. 18). However, we had some reservations as to whether butyrate could improve the transduction rate in cancer cell lines with little CAR expression.

In this experiment, we revealed that addition of butyrate to the media increased CAR expression in cancer cell lines with little CAR expression (J82 and HT1376). CAR expression was dependent of the concentration of butyrate. Higher butyrate concentration induced higher CAR expression. Furthermore, increased CAR expression by butyrate was correlated with increased transduction rate by ad- $\beta$ -gal and increased exogenous p16 production. These could be direct evidence of the role of butyrate on adenoviral transduction rate.

Although increased transgene expression by butyrate could be still an important factor to overcome the low transduction rate of adenovirus to CAR-deficient cell lines, this study revealed increase in CAR expression by butyrate is another important mechanism. The observation that butyrate could improve the expression of adenoviral transgene, even in CAR-deficient cell lines with induction of CAR expression, has a very important impact upon the problem of the low transduction rate of adenoviral vector in some tumor cell lines. This increased expression leads to marked growth suppression of ad-p16 in J82 and HT1376 (p16 positive and little CAR expression); those are resistant to ad-p16 in normal media. This finding suggests that increased production of exogenous p16 could suppress the growth of even p16-positive cancer cells.

Thus, we suggest that ad-p16 gene therapy with butyrate could overcome the differential effect of adenoviral gene therapy and become a powerful new strategy for bladder cancer.

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