Tumor-Selective Replication of an Oncolytic Adenovirus Carrying Oct-3/4 Response Elements in Murine Metastatic Bladder Cancer Models

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Abstract

Purpose: Oncolytic adenoviruses are attractive therapeutics for cancer because they selectively replicate in tumors. However, targeting tumor metastasis remains a major challenge for current virotherapy for cancer. Oct-3/4 is specifically expressed in embryonic stem cells and tumor cells. Oct-3/4 highly expressed in cancer cells may be a potential target for cancer therapy. We developed an E1B-55 kDa–deleted adenovirus, designated Ad.9OC, driven by nine copies of Oct-3/4 response element for treating Oct-3/4–expressing metastatic bladder cancer.

Experimental Design: We examined the expression of Oct-3/4 in human bladder tumor tissues and bladder cancer cell lines. We also evaluated the cytolytic and antitumor effects of Ad.9OC on bladder cancer cells in vitro and in vivo.

Results: Oct-3/4 expression was detected in bladder cancer cell lines, as well as in human bladder tumor tissues. Notably, Oct-3/4 expression was higher in metastatic compared with nonmetastatic bladder cancer cells. Ad.9OC induced higher cytolytic activity in metastatic bladder cancer cells than in their nonmetastatic counterparts, whereas it did not cause cytotoxicity in normal cells. Pharmacologic and short hairpin RNA–mediated Oct-3/4 inhibition rendered bladder cancer cells more resistant to Ad.9OC-induced cytolysis. Replication of Ad.9OC was detected in murine bladder cancer cells and bladder tumor tissues. We also showed the effectiveness of Ad.9OC for treating bladder cancer in subcutaneous, as well as metastatic, bladder tumor models.

Conclusions: Ad.9OC may have therapeutic potential for treating Oct-3/4–expressing tumors. Especially, metastatic bladder tumors are good target for Ad.9OC treatment. Because Oct-3/4 is expressed in a broad spectrum of cancers, Ad.9OC may be broadly applicable.

Oct-3/4 (also known as Oct-4, Oct-3, and POU5f1), a member of the POU family, is an octamer-binding transcription factor that is a key regulator in undifferentiated pluripotent cells, including human embryonic stem cells and germ cells (1–3). Oct-3/4 was shown as a sensitive and specific immunohistochemical marker of gonadal germ cell tumors, specifically seminoma and embryonal carcinoma (4). Oct-3/4 is expressed in several human cancer cells, but not in normal somatic tissues (5–9). It is also believed to play a critical role in tumorigenesis (5–9). Although only a few targets of Oct-3/4 transcriptional regulation are known (10, 11), it was revealed that Oct-3/4 can function as a homodimer or heterodimer on palindromic octamer DNA sequences (classic consensus ATTTGCAT) to repress or activate transcription according to flanking sequence or chromatin structure (for review, see ref. 12).

Oncolytic viruses have been used as a cancer treatment option because they selectively replicate in and kill cancer cells. The results of clinical trials of oncolytic viruses for head and neck, ovarian, brain, and prostate cancers have been encouraging in terms of efficacy with minimal, if any, toxicity (for review, see ref. 13). However, current therapies for metastatic tumor fail to show efficacy. Successful treatment of metastasis requires systemic effects, which still remains a major challenge for cancer therapy. Consequently, targeting of oncolytic adenoviruses to tumors aiming at increasing their efficacy and safety profile after systemic application has become an important issue for virotherapy.

In this study, we constructed an E1B-55 kDa–deleted adenovirus, designated Ad.9OC [Ad5–9×Oct-3/4 response element (ORE)–cytomegalovirus minimal (CMVmini)], under the control of nine copies of the ORE ligated to CMVmini promoter.

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We tested the feasibility of using ORE to transcriptionally regulate adenoviral replication in Oct-3/4-expressing cancer cells and thereby to enhance tumor-selective viral replication, oncology, antitumor efficacy, and survival advantage in a syngeneic model of murine MBT-2 bladder tumor. Our results indicate that Ad.9OC represents a potentially applicable anticancer agent for the treatment of primary and metastatic cancers that express Oct-3/4.

Materials and Methods

Cells and mice. Murine MBT-2 bladder tumor cells were obtained from C.R. Yang (Taichung Veterans General Hospital). Human bladder cancer cell lines (J82, T24, and TCC-SUP) and human Hep3B hepatocellular carcinoma and murine NIH3T3 cell lines were originally obtained from American Type Culture Collection. Human TSGH-8301 bladder cancer cell line, normal human uroepithelial (SV-HUC-1), and normal murine mammary gland epithelial (NMuMG) cell lines were obtained from Bioresource Collection and Research Center. HM-1 murine embryonic stem cell line (14) was a kind gift from D.W. Melton (Edinburgh University, Western General Hospital). MBT-2/LM7 (lung-metastatic MBT-2 clone 7) was derived from a lung metastatic nodule after s.c. transplantation of MBT-2 tumors to C3H/HeN mice. All cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum. Male C3H/HeN mice (6-8 weeks old) were used for the in vivo studies. All animal experiments were done following the guidelines approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University.

Construction of multiple copies of ORE by template repeated-PCR. Multiple copies of the ORE were obtained by template repeated-PCR (15). The oligonucleotides 5'-ATTTGAAATGCAAATGG-3' and 5'-CTCATTGTGACATCACTCA...AAATGG-3' were used as both primers and templates in the template repeated-PCR. The resulting DNA fragments with seven, eight, and nine copies of ORE (7-9-ORE) were cloned into the vector pGEM T-Easy (Promega) and subsequently sequenced to verify their correctness.

Semiquantitative reverse transcription–PCR. Total RNA was isolated from frozen tissues or cell lines using Trizol reagent (Invitrogen). The REVERSE-IT first-strand synthesis kit (Ambion) was used for cDNA synthesis. The PCR primers used for human and murine Oct-3/4 were 5'-GTCGAGTCTGGTTCTTG-3' and 5'-ATTTGAAATGCAAATGG-3'. PCR amplification was done using standard protocols. The PCR products were run on agarose gels containing ethidium bromide and photographed under UV light. In some experiments, the intensity of amplicons was quantified by a densitometric analysis using Image-Pro plus software (Media Cybernetics), and the signals were normalized to the corresponding β-actin signals.

Analysis of the effect of ORE on CMVmini promoter activity. DNA fragments containing seven to nine copies of tandemly repeated sequences of ORE (7-9×ORE) and the CMVmini promoter derived from pTRE vector (Clontech) were placed upstream of the luciferase gene of pRL2, a single dual-luciferase reporter vector (16). Briefly, the CMVmini promoter was digested with NotI/BglII from pGL3-CMVmini and cloned into pRL2, generating pRL2-CMVmini. Meanwhile, seven to nine copies of ORE were digested with EcoRI from pGEM T-Easy containing 7-9×ORE and then cloned into pRL2-CMVmini. Ad.9OC was then generated by micrographic examination. Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for MBT-2 and MBT-2/LM7 cells and with WST-1 assay for NMuMG and SV-HUC-1 cells, as described previously (21). In a separate experiment, MBT-2/LM7 cells seeded in six-well plates were infected with Ad.9OC or Ad.5WS1 at an MOI of 1. Cell survival was monitored by MTT assay at 48 and 72 h postinfection.

To further confirm the role of Oct-3/4 in regulation of the 9×ORE-CMVmini promoter and hence affecting Ad.9OC-induced cytolytic activity, MBT-2/LM7 cells were treated with retinoic acid (all-trans-retinoic acid; Sigma), which is known to suppress Oct-3/4 expression (22), and the level of Oct-3/4 expression was detected after 24, 48, and 72 h by RT-PCR analysis. Furthermore, pSUPEREGFP vector (Oligoengine) was used for expression of short hairpin RNA (shRNA) targeted to mouse Oct-3/4 (23) in MBT-2 and MBT-2/LM7 cells. Cells grown in six-well plates were transfected with 2 μg of psUPEREGFP-mOct-3/4-shRNA, psUPEREGFP as control shRNA, or pIIVC-Lac as control plasmid by LipofectAMINE 2000 (Invitrogen), and the levels of RNA knockdown were detected by RT-PCR 48 h posttransfection. In separate experiments, cells grown in six-well plates were treated with 2.5 μmol/l of...
retinoic acid or transected with 2 μg of Oct-3/4 shRNA or control shRNA. After 24 h, cells were infected with Ad.9OC at an MOI of 1.5. Cell viability was assessed 3 days later by MTT assay.

To investigate cell death induced by Ad.9OC, MBT-2/LM7 cells were infected with Ad.9OC or Ad.LacZ at an MOI of 1 or 10 or left untreated. The CytoTox 96 nonradioactive cytotoxicity assay (Promega) based on the release of lactate dehydrogenase was used to measure the cytotoxicity induced by adenoviruses.

**Immunoblot analysis.** Total cell lysates were harvested for detection of Oct-3/4 expression by immunoblot analysis with Oct-3/4 monoclonal antibody (Santa Cruz Biotechnology). The blot reprobed with anti-β-actin monoclonal antibody (AC-15, Sigma) served as the loading control. Horseradish peroxidase–conjugated goat anti-mouse IgG (Santa Cruz) was used as the secondary antibody, and protein-antibody complexes were visualized by the enhanced chemiluminescence system (Amersham Biosciences).

To assess productive replication of Ad.9OC in murine and human tumor cells, cells infected with Ad.9OC at an MOI of 10 were harvested after 48 h for detecting adenoviral E1A and after 72 h for detecting adenoviral fiber and hexon proteins by immunoblot analysis using antibodies against adenovirus E1A (1:5,000, M58, BD Biosciences), hexon (1:3,000, Abcam), and fiber (1:2000, 4D2, Abcam), as well as β-actin (AC-15, Sigma). After incubation with horseradish peroxidase–conjugated secondary antibodies, the protein bands were detected using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore) and visualized with a Biospectrum AC imaging system (UVP).

To detect light chain 3 II (LC3-II) formation, MBT-2/LM7 cells were infected with Ad.9OC or Ad.LacZ at an MOI of 1 or 10 for 24 h, and their lysates were subjected to immunoblot analysis. The membrane was incubated overnight with primary antibody against microtubule-associated protein 1 LC3 antibody (1:1,000, MBL) at 4°C and was sequentially incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibody. The protein bands were detected as described above.

**Animal studies.** MBT-2 or MBT-2/LM7 cells (2 × 10⁶) were inoculated s.c. into the dorsal flank of C3H/HeN mice at day 0. Three groups of six or seven mice were injected i.t. with 5 × 10⁶ plaque-forming units of Ad.9OC or Ad.YH in 100 μL of saline or with saline at days 9, 11, and 13. All mice were monitored for tumor growth after inoculation. Tumors were measured twice a week in two perpendicular axes with a tissue caliper, and the tumor volume was calculated as (length of tumor) × (width of tumor)² × 0.45. The mean tumor volume was calculated while all the mice were still alive. The time of animal death was recorded. All moribund animals, which were killed, were also recorded as dead.

We also used experimental metastatic MBT-2/LM7 tumor model to test whether Ad.9OC could systemically target metastatic bladder tumor cells in vivo. To enhance the ability to quantify tumor burden in the lungs, pulmonary metastasis was induced by inoculation of 5 × 10⁵ of MBT-2/LM7-Luc cells, which expressed luciferase, via the tail vein into C3H/HeN mice at day 0. Mice were then treated with 5 × 10⁶ plaque-forming units of Ad.9OC or Ad.YH or with saline via the tail vein at days 3 and 5 and sacrificed at day 20. Lung tissue were excised, fixed in 10% neutral formalin overnight, dehydrated, and embedded in paraffin. Tissue sections of 4 μm thickness were stained with Mayer's H&E for assessment of tumor nodules under microscopy with an Olympus DP1T digital camera system (Olympus Optical). Luciferase activity of the lungs was also measured as described previously (24). Growth index (mean area of metastasis / total area) of tumor nodules in the lung was quantified at the microscopic level as previously described (25).

**Immunohistochemical analysis.** Frozen sections of tumor and normal tissues from patients with bladder superficial transitional cell carcinoma (TCC) were immunostained with Oct-3/4 monoclonal antibody (Santa Cruz), followed by detection with the DAKO LSAB 2 System (DAKO) according to the manufacturer’s instructions. After sequential incubation with biotinylated secondary antibody, horseradish peroxidase–conjugated streptavidin, and aminoethyl carbazole as substrate chromogen, the slides were counterstained with hematoxylin.

For detection of adenoviral replication in subcutaneous MBT-2 and MBT-2/LM7 tumors, C3H/HeN mice that had been inoculated s.c. with 2 × 10⁶ of MBT-2 or MBT-2/LM7 cells at day 0 were injected i.t. with Ad.9OC, Ad.YH or saline at day 15. They were killed at day 17, and tumors were excised for frozen sections. Cryostat sections (5 μm) were prepared and incubated with antibodies against adenovirus hexon (1:100, Abcam) and fiber (1:100, 4D2, Abcam).

**Analysis of intracellular autophagic vacuoles.** Autophagy can be characterized by the development of acidic vesicular organelles (AVO; ref. 26). MBT-2/LM7 cells that had been infected with Ad.9OC or Ad.LacZ at an MOI of 1 or 10 or left untreated for 24 h were harvested, fixed, and permeabilized in chilled 70% ethanol overnight at -20°C. After centrifugation, cells were washed and resuspended in PBS containing 0.1% Triton X-100 (Sigma), 0.2 mg/mL RNase (Invitrogen), and 20 μg/mL propidium iodide (Sigma) for determining cell cycle profiles. Samples were analyzed for DNA content with the FACS caliber flow cytometer (BD Biosciences), and relative cell cycle distribution was analyzed using the CellQuest software (Verity Software House).

**Statistical analysis.** All values represent the mean ± SE. Statistical significance between groups, unless otherwise stated, was assessed with Student’s t test. Tumor volumes were compared using two-way ANOVA. The survival analysis was done using the Kaplan-Meier survival curve and the log-rank test. Tumor burden in the lung presented as relative light units was analyzed using Student's t test with Welch’s correction. Any P value of <0.05 is regarded statistically significant.

**Results**

Oct-3/4 expressions in human bladder tumor tissues and bladder cancer cell lines of human and murine origins. To determine whether Oct-3/4 was expressed in bladder tumors, we used RT-PCR and immunohistochemical approaches to examine Oct-3/4 expression in clinical bladder TCC and normal tissues. Tumors of patient 1 and patient 4 belonged to stage pT1 grade 3 TCC. Tumors of patient 2 and patient 3 were stage pT1 grade 2 TCC. Figure 1A shows that Oct-3/4 transcripts were detected in these four clinical bladder tumor specimens, but not in normal human tissue. Accordingly, Oct-3/4 protein was also detected immunohistochemically in bladder tumor tissues, but not in normal tissues (Fig. 1B). We then systemically studied more tumor specimens from patients with superficial bladder TCC and nontumor bladder tissues. Our data revealed that bladder tumors with intense Oct-3/4 expression are associated with further disease progression, greater metastasis, and shorter cancer-related survival compared with those with moderate and low expressions.5 In addition, Oct-3/4 expression was also detected in bladder cancer cell lines of human and murine origins by RT-PCR analysis (Fig. 1C). Of note, Oct-3/4 expression was not detected in normal human SV-HUC-1 urothelial cell line and murine NIH3T3 fibroblasts. Next, we examined the expression levels of Oct-3/4 protein in murine cells by immunoblot analysis (Fig. 1D). As expected,

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Oct-3/4 Response Element–Driven Oncolytic Adenovirus

Oct-3/4 was highly expressed in murine HM-1 embryonic stem cell line. Furthermore, the expression level of Oct-3/4 in MBT-2/LM7 cells, which exhibited higher metastatic potential, was higher than that in their parental MBT-2 cells. In marked contrast, NIH3T3 cells did not express Oct-3/4.

Responsiveness of ORE to Oct-3/4 transactivation in metastatic and nonmetastatic bladder cancer cells. We constructed pFRL2-(7–9 × ORE)-CMVmini and pFRL2-CMVmini plasmids and used them to examine the responsiveness of ORE to Oct-3/4 transactivation in different cells by a dual-luciferase reporter assay. As shown in Fig. 2A, significant increases in firefly luciferase activity were observed in MBT-2 and MBT-2/LM7 cells transfected with pFRL2-(7–9 × ORE)-CMVmini compared with those transfected with pFRL2-CMVmini, whereas the levels of luciferase gene expression remained relatively low in NIH3T3 cells transfected with the same reporter constructs. Notably, reporter constructs carrying eight or nine copies of ORE conferred higher responsiveness to endogenous Oct-3/4 in MBT-2/LM7 cells than those carrying seven copies of ORE. However, a saturation effect was observed for reporter constructs with seven copies of ORE in MBT-2 cells. These results suggest that the ORE-CMVmini promoter activity was detected at a level much higher in metastatic than nonmetastatic bladder cancer cells, but was relatively low in NIH3T3 fibroblasts.

To further determine whether the ORE was regulated by the expression level of Oct-3/4 transcription factor, we cotransfected NIH3T3 cells, which do not express endogenous Oct-3/4 protein, with pTCY-Oct-3/4 expression vector and pFRL2-9 × ORE-CMVmini reporter plasmid and examined Oct-3/4–mediated firefly luciferase expression by the dual-luciferase reporter assay.

Fig. 2B shows that Oct-3/4 dose-dependently enhanced the transcriptional activity of 9 × ORE-CMVmini promoter. Collectively, Oct-3/4 transcription factor regulated transcriptional activity via the ORE in bladder cancer cells, especially in metastatic MBT-2/LM7 cells.

Cytolytic effects of Ad.9OC on bladder cancer cells. Because MBT-2/LM7 cells expressed higher Oct-3/4 than MBT-2 cells (Fig. 1D), we next used these two cell lines to investigate whether difference in the Oct-3/4 expression level influenced the cytolytic effect of Ad.9OC. Figure 2C indicates that Ad.9OC exerted higher cytolytic effects on metastatic MBT-2/LM7 cells than on nonmetastatic MBT-2 cells. Furthermore, the viability of either MBT-2 or MBT-2/LM7 cells infected with Ad.YH did not differ significantly from that of their mock-infected counterparts. In marked contrast, normal murine NMuMG and human SV-HUC-1 cells were resistant to cytolysis induced by either Ad.9OC or Ad.YH. These results suggest that in Oct-3/4–overexpressing bladder cancer cells, Ad.9OC exerted much greater cytolytic activity than Ad.YH. Moreover, tumor-specific cytolytic effect of Ad.9OC was positively correlated with the expression level of Oct-3/4 in bladder cancer cells.

To examine whether Ad.9OC exerted higher cytolytic effects than Ad5WS1, an oncolytic adenovirus driven by its E1A promoter, in Oct-3/4–overexpressing bladder cancer cells, cell survival was determined in MBT-2/LM7 cells 48 and 72 h after infection with either Ad.9OC or Ad5WS1 at an MOI of 1. Figure 2D shows that the viability of the cells infected with Ad.9OC was significantly lower than that infected with Ad5W1. More dramatic cytolytic effect of Ad.9OC on MBT-2/LM7 cells was observed at 72 h postinfection.

Correlation between down-regulation of Oct-3/4 expression and Ad.9OC–induced cytolysis in bladder cancer cells. Because Ad.9OC replication is under the transcriptional control of the 9 × ORE-CMVmini promoter, which is responsive to Oct-3/4, we next investigated whether pharmacologic and shRNA–mediated Oct-3/4 inhibition decreased the cytolytic effect of Ad.9OC on bladder cancer cells and hence increased the survival of the infected cells. As retinoid acid can down-regulate the expression of the Oct-3/4 gene via its upstream enhancer (22), we treated MBT-2/LM7 cells with retinoid acid and examined Ad.9OC–induced cytolytic activity. Figure 3A shows that retinoid acid suppressed Oct-3/4 expression in a time-dependent manner as determined by RT-PCR analysis. Accordingly, treatment of MBT-2/LM7 cells with retinoid acid increased cell survival after Ad.9OC infection (Fig. 3B). Notably, retinoid acid (2.5 μmol/L) alone did not cause cytotoxicity. To further confirm these results, we also determined whether knockdown of Oct-3/4 by shRNA reduced Ad.9OC–induced cytolytic effects on Oct-3/4–expressing bladder cancer cells. RT-PCR analysis revealed that Oct-3/4 shRNA knocked down Oct-3/4 expression in MBT-2/LM7 and MBT-2 cells, whereas control shRNA had no effects (Fig. 3C). In addition, because Oct-3/4 expression was higher in MBT-2/LM7 than in...
MBT-2 cells, the efficiency of shRNA-mediated knockdown of Oct-3/4 was ~50% in MBT-2/LM7 cells, whereas almost complete inhibition of Oct-3/4 expression was achieved in MBT-2 cells. Remarkably, shRNA-mediated knockdown of Oct-3/4 rendered bladder cancer cells more resistant to Ad.9OC-induced cytolytic effects, which was more evident in MBT-2 cells (Fig. 3D). Taken together, these results showed that cancer-specific cytolytic effects of Ad.9OC were attenuated in cancer cells when their Oct-3/4 levels were down-regulated.

**Productive replication of Ad.9OC in bladder cancer cells in vitro and in vivo.** Because Ad.9OC lysed Oct-3/4-expressing bladder cancer cells, we next identified productive replication of Ad.9OC in these cells. After Ad.9OC infection at an MOI of 10, E1A protein was detected abundantly in MBT-2/LM7 cells, whereas it was less in MBT-2 cells as revealed by immunoblot analysis (Fig. 4A). More importantly, fiber and hexon proteins, which are two adenoviral late proteins, were also identified in MBT-2/LM7 cells and, to a lesser extent, in MBT-2 cells. In human Hep3B cancer cells, which were shown to be permissive for replication of oncolytic adenovirus, such as Ad5WS1 (18), E1A, fiber, and hexon proteins were readily detectable. However, these three adenoviral proteins were hardly detectable in normal murine NMuMG cells. To assess viral replication in MBT-2 and particularly in MBT-2/LM7 tumors in vivo, expressions of adenoviral fiber and hexon proteins were determined in the tumors inoculated with Ad.9OC, Ad.YH, or saline. These two viral late proteins were detected immunohistochemically in MBT-2/LM7 and MBT-2 tumors after intratumoral injection.
infection with Ad.9OC, whereas only background expressions of Oct-3/4 were observed in the tumors injected with Ad.YH or saline treatment. Furthermore, increased survival of Ad.9OC-infected cells by retinoid acid treatment was also observed in Ad.9OC-treated mice in comparison with saline-treated mice (P = 0.0093) in the MBT-2/LM7 tumor model (Fig. 4C), whereas Ad.9OC only slightly prolonged mouse survival in the MBT-2 tumor-bearing mice (Fig. 4D). However, the median survival time for each treatment between the two tumor models did not differ significantly. In the MBT-2/LM7 model, the median survival time for Ad.9OC-treated group was 45 days compared with 35 and 30 days for Ad.YH and saline treatment groups, respectively. Similarly, in the MBT-2 model, the median survival time was 46, 31, and 27 days for Ad.9OC, Ad.YH, and saline treatment groups, respectively.

**Reduction of metastatic lung nodules by Ad.9OC treatment in an experimental metastatic cancer model.** Because inhibition of metastatic tumor growth is still a major challenge for cancer treatment and the antitumor efficacy of Ad.9OC is more evident in the metastatic MBT-2/LM7 tumor than the non-metastatic MBT-2 tumor, we next investigated whether Ad.9OC could inhibit established pulmonary tumor nodules. Mice that had been inoculated i.v. with MBT-2/LM7-luc cells were treated i.v. with Ad.9OC or Ad.YH or with saline at days 3 and 5. Gross appearances of pulmonary colonies produced by MBT-2/LM7 cells 20 days after tumor cell inoculation are shown in Fig. 5A. Numbers and sizes of lung tumor nodules were reduced in mice treated with Ad.9OC compared with those treated with either Ad.YH or saline. Because MBT-2/LM7 cells used in this experiment expressed luciferase, which facilitated quantification of the metastatic tumor burden in the lungs, we determined luciferase activity as a marker of tumor burden. Figure 5B shows that the level of luciferase expression in the lungs from Ad.9OC-treated mice was much lower than that from mice treated with either Ad.YH or saline. Furthermore, histologic sections also revealed that Ad.9OC-treated animals had minor tumor lesions compared with their Ad.YH-treated counterparts (Fig. 5C). Quantitative analysis at the microscopic level also shows that the growth index (mean area of metastasis / total area) of tumor nodules was significantly lower in the Ad.9OC treatment group than in the saline treatment group (Fig. 5D). The number and size of lung tumor nodules were reduced in mice treated with Ad.9OC compared with those treated with either Ad.YH or saline. Because MBT-2/LM7 cells used in this experiment expressed luciferase, which facilitated quantification of the metastatic tumor burden in the lungs, we determined luciferase activity as a marker of tumor burden. Figure 5B shows that the level of luciferase expression in the lungs from Ad.9OC-treated mice was much lower than that from mice treated with either Ad.YH or saline. Furthermore, histologic sections also revealed that Ad.9OC-treated animals had minor tumor lesions compared with their Ad.YH-treated counterparts (Fig. 5C). Quantitative analysis at the microscopic level also shows that the growth index (mean area of metastasis / total area) of tumor nodules was significantly lower in the Ad.9OC treatment group than in the saline treatment group (Fig. 5D).

**Induction of autophagy-related nonapoptotic cell death in MBT-2/LM7 cells by Ad.9OC.** To gain insight into the mechanism through which Ad.9OC caused cell death in Oct-3/4-expressing bladder cancer cells, we examined Ad.9OC-induced autophagic and/or apoptotic cell death in the cancer cells. Giving that during autophagy LC3-I is cleaved and conjugated to phosphatidylethanolamine to form LC3-II (27), we analyzed the conversion of LC3-I to LC3-II with immuno-blot analysis using anti-LC3 antibody. Elevated levels of LC3-II were observed in MBT-2/LM7 cells infected with 10 MOI of Ad.9OC for 24 h, whereas the levels of LC3-II did not increase after infection with either 1 or 10 MOI of Ad.YH or with 1 MOI of Ad.9OC (Fig. 6A). Furthermore, serum starvation for 24 h also moderately increased the content of LC3-II, which served as the positive control for induction of autophagy. Because autophagy can be characterized by the development of AVOs (26), we further detected AVOs to confirm that the number of MBT-2/LM7 cells containing autophagic vacuoles was increased by Ad.9OC treatment. The amounts of cells containing AVOs were increased after treatment with increasing MOI of Ad.9OC, whereas Ad.LacZ treatment did not significantly enhance the formation of AVOs in MBT-2/LM7 cells (Fig. 6B).

To elucidate the role of autophagy in Ad.9OC-induced cytolyis in bladder cancer cells, we next used the lactate dehydrogenase...
release assay to examine cell death in MBT-2/LM7 cells after Ad.9OC or Ad.LacZ infection at an MOI of 1 or 10 for 24 h (Fig. 6C). As expected, Ad.LacZ did not cause cell death. Notably, Ad.9OC induced cell death in MBT-2/LM7 cells in a dose-dependent fashion. However, neither Ad.9OC nor Ad.LacZ induced apoptosis in MBT-2/LM7 cells, as revealed by analysis of cell population in the sub-G₁ phase (Fig. 6D). Notably, Ad.9OC arrested cells in the G2-M phase, whereas Ad.LacZ did not have such effect (Fig. 6D). Taken together, these results indicate that MBT-2/LM7 cells underwent nonapoptotic cell death by Ad.9OC infection. Furthermore, nonapoptotic cell death in bladder cancer cells caused by Ad.9OC infection was associated with induction of autophagy in the infected cells.

Discussion

There are two theories to explain the origin of cancer, namely, dedifferentiation theory and stem cell theory (for review, see ref. 28). Because cancer cells have many phenotypic traits similar to undifferentiated embryonic cells, it is still unclear whether cancer cells are derived from differentiated cells that have dedifferentiated or from adult stem cells in the tissue. The Oct-3/4 gene is specifically expressed in embryonic stem cells and in tumor cells but not in cells from differentiated tissues. Oct-3/4 expression in some tumors has been described as being reexpressed or restored by the transformation process (6, 7), which favored the dedifferentiation theory of carcinogenesis. However, a more recent study reported by Tai et al. (29) found that expression of Oct-3/4 gene was detected in adult stem cells and immortalized nontumorigenic cells, as well as tumor cells and cell lines, but not in differentiated cells. These results provided evidence to support the stem cell theory of carcinogenesis. In our study, bladder tumor cells also express Oct-3/4. To clarify whether Oct-3/4 is reexpressed or remains expressed during the carcinogenic process, the origin of Oct-3/4–expressing bladder tumor cells still needs to be identified. In this study, we used a therapeutic strategy by using an oncolytic adenovirus carrying the ORE for targeting Oct-3/4–expressing bladder cancer cells, especially high Oct-3/4–expressing metastatic bladder cancer.

In this study, we determined Oct-3/4 expression in murine and human bladder cancer cell lines, in addition to tissues...
from human patients with bladder superficial TCC. Various levels of Oct-3/4 were detected in the clinical samples, which corresponded with observations in a recent report (9). More interestingly, we found that Oct-3/4 expression was higher in metastatic compared with nonmetastatic bladder cancer cells, whereas it was not detectable in normal cells (Fig. 1D). Our study also showed that Oct-3/4 overexpression enhances migration and invasion of bladder cancer cells. Most importantly, our data also revealed that, in clinical samples, bladder tumors with intense Oct-3/4 expression are associated with further disease progression and greater metastasis compared with those with moderate and low expressions. These observations indicated that high Oct-3/4-expressing bladder cancer cells may have high metastatic ability.

In the present study, 9OC was derived from Ad5WS1 (18) by replacing its internal E1A promoter with nine copies of ORE combined with CMVmini promoter. Our results show that Ad.9OC exerted cytolytic effects on bladder cancer cells expressing Oct-3/4 but spared normal cells, including epithelial cells that express cossackievirus and adenovirus receptor (30), the major receptor for adenovirus serotype 5. Furthermore, we show a positive correlation between Ad.9OC-induced cytolytic effects and the expression level of Oct-3/4 in cells. Thus, tumor cells with higher Oct-3/4 expression may allow more Ad.9OC replication and thereby induce more potent cytotoxicity. Because normal cells do not express Oct-3/4 and are resistant to Ad.9OC-induced cytolysis, from a safety standpoint, Ad.9OC may be an ideal oncolytic agent for killing Oct-3/4-overexpressing tumors with a more metastatic phenotype. Furthermore, the use of Ad.9OC may be safer than Onyx-015 (31) or Ad5WS1 (18), the prototype of oncolytic adenovirus driven by the E1A promoter that is constitutively active in normal and cancer cells. Tumor specificity is an important factor in the application of oncolytic virus for cancer therapy. Clinical data with Onyx-015 point to the need of more potent and selective oncolytic viruses. Despite intratumoral treatment of patients with oncolytic adenoviruses demonstrated selective replication in tumors and safety, oncolytic adenoviruses have
not shown systemic efficacy through i.v. administration to date (32, 33). For metastatic cancer, systemic efficacy is required for effective treatment. Intravascular application of oncolytic viruses is a requirement to reach cells within tumors and all metastases. In this regard, Ad.9OC may represent as a novel therapeutic strategy specific for Oct-3/4–expressing primary and metastatic cancer cells.

We also show that inhibition of Oct-3/4 expression by retinoid acid in bladder cancer cells rendered cells less susceptible to Ad.9OC-induced cytolytic effects (Fig. 3B and D), further confirming that the replication and oncolytic activity of Ad.9OC were governed by Oct-3/4. Retinoid acid exerts pleiotropic effects on vertebrate development, cell differentiation, and homoeostasis (34, 35). It is commonly used for the induction of embryonic stem cell differentiation. Retinoic acid–induced differentiation of embryonic stem cells or embryonic carcinoma cells is accompanied by reduced Oct-3/4 expression. Previous reports showed that retinoic acid represses Oct-3/4 gene expression through several retinoic acid–responsive elements located in the promoter-enhancer region (36). We speculated that suppression of Oct-3/4 expression by retinoid acid in bladder cancer cells may also be mediated by the same molecular mechanism.

Because most murine cells express low levels of coxsackievirus and adenovirus receptor, it is generally believe that human adenovirus serotype 5 does not efficiently infect murine cells nor does it replicates well in these cells. However, we reported previously that the susceptibility of murine MBT-2 cells and human J82 bladder cancer cells to adenoviral vector was similar, especially when higher viral dose was applied (21). Furthermore, infection efficiency by adenovirus could be enhanced in both cells to a similar degree by etoposide

![Fig. 6. Ad.9OC induces autophagic cell death in MBT-2/LM7 cells. MBT-2/LM7 cells were infected with Ad.9OC or Ad.LacZ at an MOI of 1 or 10 for 24 h and then subjected to various assays. A, levels of LC3-II and LC3-I in cells infected with Ad.9OC or Ad.LacZ determined by immunoblot analysis using anti-LC3 antibody. Note that higher levels of LC3-II were observed in cells infected with 10 MOI of Ad.9OC, whereas the contents of LC3-II remained constant in Ad.LacZ-infected or untreated cells. Serum starvation, which induced autophagy, served as the positive control for accumulation of LC3-II. The expression of β-actin served as the loading control. Similar results were obtained in two independent experiments. B, detection of AVOs by acridine orange staining in cells infected with Ad.9OC or Ad.LacZ and quantified by flow cytometry. Note that the amounts of cells containing AVOs were increased in cells after Ad.9OC treatment but remained relatively constant after Ad.LacZ treatment. Similar results were observed in two independent experiments. C, cell death in MBT-2/LM7 cells infected with Ad.9OC or Ad.LacZ determined by the lactate dehydrogenase release assay. Cell death is presented as the ratio of the lactate dehydrogenase released from virus-infected cells to that from the untreated control cells (n = 3). The results were consistent in two independent experiments. **, P < 0.01; ***, P < 0.001. D, representative flow histograms depicted subdiploid fraction in MBT-2/LM7 cells infected with Ad.9OC or Ad.LacZ for 24 h. The values shown in each histogram are the percentages of cells in sub-G1 (left) and G2-M (right) phases. Note that Ad.9OC (P < 0.05), but not Ad.LacZ, significantly arrested the cells in the G2-M phase and that neither Ad.9OC nor Ad.LacZ induced apoptosis in MBT-2/LM7 cells, as revealed by relatively constant sub-G1 fraction compared with that in mock-infected cells.](http://www.aacrjournals.org/clinican/2008/14/4/236/ClinCancerRes2008144February1520081236.png)
treatment through up-regulation of coxsackievirus and adenovirus receptor expression (21). In this study, immunoblot analysis revealed that human cancer cells, such as Hep3B hepatocellular carcinoma cells, produced more adenoviral late proteins (fiber and hexon) than murine bladder cancer cells, suggesting that human cancer cells are still more permissive to adenoviral infection. Although Ad.9OC may more efficiently infect and lyse human bladder cancer cells than MBT-2 cells, MBT-2, and MBT-2/LM7 tumor models allow us to study the therapeutic efficacy of Ad.9OC in immunocompetent mice. Along the same line, some novel immunocompetent murine tumor models have been reported (37).

In our in vitro results (Figs. 2C and 4A), Ad.9OC replicated more efficiently and thus induced higher cytolysis in MBT-2/LM7 cells than in MBT-2 cells due to higher Oct-3/4 expression in MBT-2/LM7 cells. It would be expected that Ad.9OC was more effective for treating MBT-2/LM7 tumors than MBT-2 tumors in mice. Ad.9OC was effective in enhancing the survival time in MBT-2/LM7 tumor-bearing mice (Fig. 4C), but not in MBT-2 tumor-bearing mice (Fig. 4D). Nevertheless, in terms of tumor growth, Ad.9OC generated similar antitumor activity for MBT-2/LM7 and MBT-2 tumors (Fig. 4C and D). As in vivo environment may be more complex than in vitro condition, this inconsistency between in vitro and in vivo results can be expected. Furthermore, in vivo antitumor mechanisms in immunocompetent mice may be contributed by not only direct tumor cell death caused by adenovirus infection but also by host factors, such as inflammatory and immune responses against adenoviral antigens and tumor antigens released from dead tumor cells. Therefore, the degree of cytolytic activity of Ad.9OC in vitro may not translate directly into antitumor effect in tumor-bearing mice.

Although oncolytic adenovirus has emerged as a powerful anticancer agent, the precise mechanism by which oncolytic adenovirus kills tumor cells is still unclear. It was shown that oncolytic adenovirus—induced lung cancer cell death was mediated through a basic apoptotic machinery—dependent mechanism that resembled necrosis-like programmed cell death (38). A recent study also reported that an oncolytic adenovirus regulated by the human telomerase reverse transcriptase promoter could inhibit mammalian target of rapamycin signal pathway and induce autophagic cell death of malignant glioma cells (39). In the present study, we show that bladder cancer cells underwent nonapoptotic cell death by Ad.9OC. Moreover, nonapoptotic cell death caused by Ad.9OC infection was associated with induction of autophagy in infected cells. This is in agreement with the finding reported by Ito and colleagues (39). Therefore, oncolytic adenovirus induces autophagic cell death in cancer cells. Because in cancer cells, apoptotic machinery is frequently deregulated; oncolytic adenovirus still can kill these cancer cells through an autophagic mechanism, which is apoptosis-independent. However, the role of autophagy-induced cell death in the antitumor activity of oncolytic adenovirus in vivo still needs to be elucidated.

In conclusion, we reported high expression of Oct-3/4 in metastatic bladder cancer cells compared with nonmetastatic bladder cancer cells. Our results also provide new insights into the design of a novel oncolytic adenovirus driven by the ORE, which is regulated by Oct-3/4 that is highly expressed in bladder cancer cells. Effective antitumor efficacy on Oct-3/4-overexpressing metastatic tumors could be achieved by i.t. or i.v. administration of Ad.9OC in syngeneic murine tumor models. Taken together, our work indicates that Ad.9OC may have therapeutic potential for the treatment of Oct-3/4—expressing tumors. Most importantly, our results indicate that metastatic bladder tumors are good target for Ad.9OC treatment. Because Oct-3/4 is expressed in a broad spectrum of cancers, this oncolytic adenovirus may be broadly applicable.

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References


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