

A Conditional Replication-competent Adenoviral Vector, Ad-OC-E1a, to Cotarget Prostate Cancer and Bone Stroma in an Experimental Model of Androgen-independent Prostate Cancer Bone Metastasis¹

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Abstract

Prostate cancer has a high propensity to metastasize to bone, which often resists hormone, radiation, and chemotherapies. Because of the reciprocal nature of the prostate cancer and bone stroma interaction, we designed a cotargeting strategy using a conditional replication-competent adenovirus to target the growth of tumor cells and their associated osteoblasts. The recombinant Ad-OC-E1a was constructed using a noncollagenous bone matrix protein osteocalcin (OC) promoter to drive the viral early E1a gene with restricted replication in cells that express OC transcriptional activity. Unlike Ad-PSE-E1a, Ad-OC-E1a was highly efficient in inhibiting the growth of PSA-producing (LNCaP, C4-2, and ARCaP) and nonproducing (PC-3 and DU145) human prostate cancer cell lines. This virus was also found to effectively inhibit the growth of human osteoblasts and human prostate stromal cells *in vitro*. Athymic mice bearing s.c. androgen receptor-negative and PSA-negative PC-3 xenografts responded to a single intratumoral administration of 2×10^9 plaque-forming unit(s) of Ad-OC-E1a. In SCID/hg mice, intraosseous growth of androgen receptor-positive and PSA-producing C4-2 xenografts responded markedly to i.v. administrations of a single dose of Ad-OC-E1a. One hundred percent of the treated mice responded to this systemic Ad-OC-E1a therapy with a decline of serum PSA to an undetectable level, and 80% of the mice with PSA rebound responded to the second dose of systemic Ad-OC-E1a. Forty percent of the mice were found to be cured by systemic Ad-OC-E1a without subsequent PSA rebound or tumor cells found in the skeleton. This cotargeting strategy shows a broader spectrum and appears to be more effective than systemic Ad-PSE-E1a in preclinical models of human prostate cancer skeletal metastasis.

Introduction

Genetic therapy for prostate cancer has been applied in preclinical animal models and in patients with localized and metastatic diseases (1–16). The prevailing approach is to target a single cell compartment such as the tumor epithelial or associated endothelial compartment (1, 2, 5–7). Examples of transgenes delivered to tumor cells include suicide genes (2–5), tumor suppressor (6, 8), antiangiogenesis (7), and immune modulators (9–13). Recently, Henderson *et al.* (1, 14, 15) demonstrated the efficacy of conditional replication-competent adenovirus with viral replication driven by a tissue-specific promoter,

PSE,³ for the treatment of prostate cancer. In this article, we present a novel strategy for cotargeting both tumor epithelial and bone stromal cells using a conditionally replicating adenovirus driven by a tissue-specific but tumor-restrictive promoter, OC (4, 16). This strategy was based on the well-established reciprocal cellular interaction that occurs between prostate cancer and prostate or bone stromal cells (17, 18). Evidence suggests that permanent phenotypic and genotypic alterations are induced in prostate cancer and bone stromal cells subsequent to tumor-stromal interaction (17, 19). The cotargeting strategy accomplishes maximal cell kill by eliminating not only the growth of tumor epithelium but also by interrupting the intercellular communication and reciprocal induction between prostate tumor and bone or prostate stromal cells (17–19).

OC, a noncollagenous Gla protein, was found to be produced exclusively by differentiated osteoblasts and is deposited onto bone matrices at the time of bone mineralization (4, 20, 21). The OC promoter contains several species-specific and overlapping regulatory elements (22–29). The “osteocalcin box” contains sites to bind factors such as homeobox MSX proteins, AP-1, AP-2, NF-1, viral core enhancer, c-AMP, vitamin-D, and glucocorticoid receptors (22–29). The osteoblast-specific *cis*-acting element OSE2 binds to the transcription activator of osteoblast differentiation, Osf2/Cbfa1 (28). Mouse OC promoter contains an additional OSE1 *cis*-acting DNA element (29) but has a nonfunctional vitamin D responsive element (22). The current study used mouse OC promoter to drive viral replication through the regulation of E1a, an adenoviral early gene required for viral replication (30). We described this cotargeting strategy by demonstrating: (1) Ad-OC-E1a is a highly efficient inhibitor of the growth of prostate cancer and bone and prostate stromal cells *in vitro*; (2) Ad-OC-E1a has a broad spectrum of cell kill activity that caused lysis in PSA-producing and -nonproducing prostate tumor, bone, and prostate stromal cells *in vitro*; (3) systemic administration of Ad-OC-E1a inhibited the growth of human prostate tumor established previously in the skeleton; and (4) the cotargeting strategy is superior to targeting a single cell compartment in which only the growth of prostate cancer cells is affected. Results of this study demonstrate for the first time that systemically administered Ad-OC-E1a induced regression of preexisting human prostate cancer growth in the skeleton irrespective of their prior PSA and AR status.

Materials and Methods

Cells and Cell Culture. LNCaP, an androgen-responsive, AR-positive, PSA-secreting human prostate cancer cell line, was derived from a cervical lymph node metastasis by Horoszewicz *et al.* (31). From this parental cell line,

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³The abbreviations used are: PSE, prostate-specific antigen enhancer; OC, osteocalcin; PSA, prostate-specific antigen; AR, androgen receptor; pfu, plaque-forming unit(s); FBS, fetal bovine serum; MOI, multiplicity of infection.

we derived a series of androgen-independent (defined as cells that are capable of forming PSA-secreting solid tumors when inoculated in castrated athymic male mice without the supporting stromal cells or extracellular matrices) and lineage-related LNCaP sublines (32, 33). One of the sublines, C4-2, remains AR- and PSA-positive and acquires osseous metastatic potential when inoculated either s.c. or orthotopically (32, 33). ARCaP is an androgen-repressed, low AR- and PSA-expressing human prostate cancer cell line established by our laboratory (34). This cell line is highly tumorigenic and metastatic and is a model to study advanced human prostate cancer (34). PC-3 is an androgen-independent, AR- and PSA-negative human prostate cancer cell line established by Kaighn *et al.* (35) from the bone marrow aspirates of a patient with confirmed metastatic disease. DU-145 is an androgen-independent, AR- and PSA-negative human prostate cancer cell line established by Stone *et al.* (36) from a patient with prostate cancer brain metastasis. Lovo, a colon cancer cell line, was established by Drewinko *et al.* (37) from a localized colon tumor tissue specimen and was kindly provided by Dr. L. Y. Yang, University of Texas M. D. Anderson Cancer Center, Houston, TX. WH, a cell line derived from a human bladder transitional cell carcinoma specimen, was established by Zhou *et al.* (38). 293 is a transformed human embryonic kidney cell line established by Graham *et al.* (39) with the cells expressing a complementing adenoviral E1 region that supports adenoviral replication. A human prostate fibroblast cell line, 9096F, was established by our laboratory from a surgical prostate biopsy specimen (40). A human bone stromal cell line, MG-63, was established from an osteosarcoma specimen and was obtained from the American Type Culture Collection (Rockville, MD). The PC-3, DU-145, and 293 cell lines were also obtained from American Type Culture Collection. In this study, C4-2 and 9096F cells were maintained in T medium (Life Technologies, Inc.) containing 10% FBS as described previously (32, 33). LNCaP, PC-3, DU-145, ARCaP, WH, and MG-63 cells were all maintained in T medium (Life Technologies, Inc.) containing 5% FBS. Lovo cells were maintained in F-12 Nutrient Mixture (Life Technologies, Inc.) containing 10% FBS. 293 cells were maintained in MEM (Life Technologies, Inc.) containing 10% FBS. The cells were fed three times per week with fresh growth medium and maintained at 37°C in 5% CO₂.

Construction and Production of the Replication-competent Ad-OC-E1a. All of the plasmids were constructed according to standard published protocols (41). Briefly, a *Bam*HI-XcaI fragment containing the backbone of an Ad5 vector from 549 bp to 5792 bp was digested from pXC 548C, a derivative of plasmid pXC1 (42), and inserted into pΔE1sp1B (obtained as a gift from Dr. Frank Graham, MacMaster University, Hamilton, Ontario, Canada) between the *Bam*HI and XcaI site to create a pΔBPΔEII shuttle vector. A pOCE1a was constructed by inserting a 1370-bp fragment of murine OC promoter, which was cut from pIII.5 TK using *Xho*I and *Sal*I enzymes, into the *Xho*I site of pΔBPΔEII to drive the Ad5 *E1a* gene. The shuttle pOCE1a vector was cotransfected with a replication-defective recombinant Ad vector, pJM17, into 293 cells by the *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium-methyl sulfate (Boehringer Mannheim Biochemicals)-mediated transfection method (43) to generate a partially E3-deleted replication-competent adenovirus, Ad-OC-E1a. The resulting Ad-OC-E1a was demonstrated to replicate in a restricted manner only in cells that expressed OC promoter activity. The culture medium of the 293 cells showing complete cytopathic effect was collected and centrifuged at 1000 × *g* for 10 min. The pooled supernatants were aliquoted and stored at -80°C as primary viral stock. Viral stocks were propagated in 293 cells, and selected clones of Ad-OC-E1a virus were obtained by plaque purification according to the method of Graham and Prevec (44). One of the viral clones was selected, propagated in 293 cells, harvested 36–40 h after infection, pelleted, resuspended in PBS, and lysed. Cell debris was removed by subjecting the cells to centrifugation, and the virus in the cell lysate was purified by CsCl gradient centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at -80°C. The viral titer was determined by plaque assay as described previously (2, 8, 45). Other control viruses used in this study, Ad-CMV-pA and Ad-CMV-β-gal, were constructed, plaque purified, and propagated in 293 cells using a similar procedure. The specificity of Ad-OC-E1a replication in cells was assessed by determining the titer of the virus after infecting 293 (7.7 ± 3.2 pfu/cell), C4-2 (15 ± 8 pfu/cell), and WH (0.047 ± 0.021 pfu/cell) cells (*n* = 3).

Immunohistochemical Staining of Primary and Metastatic Human Prostate Tumor Specimens. Deparaffinized primary human prostate cancer specimens and lymph node and bone metastatic specimens were obtained from

the Department of Urology and Pathology, University of Virginia School of Medicine, Charlottesville, VA and McGill University, Montreal, Quebec, Canada. Tissues were treated with 3% H₂O₂, blocked with SuperBlock (Scytek Laboratories, Logan, UT), and reacted with a monoclonal IgG OC antibody (5–16 μg/ml; OC 4–30 antibody purchased from Takara Shuzo, Otsu, Japan). The antibody staining signals were amplified by a biotinylated peroxidase-conjugated streptavidin system (Bio-Genex Laboratories, San Ramon, CA). Background and negative control staining were routinely obtained by the use of a purified control mouse IgG and a clinical human colon cancer specimen, respectively. OC stain was visualized after reacting the conjugated peroxidase with either an AEC Chromogen, 3-amino-9-ethylcarbazole, or a diaminobenzidine as described previously (2, 8, 34, 38, 45). Positive OC is defined by ≥15% of the cell populations reacted positively with the OC antibody.

In Vitro Cell Growth Assay. LNCaP, C4-2, PC-3, DU-145, ARCaP, 293, WH, Lovo, MG-63 or 9096F cells (5 × 10³) were plated in 24-well plates. After 24 h, the cells were infected with Ad-OC-E1a with a range of concentrations from 0.01 to 5 MOI (or pfu/cell, which was estimated to be 0.2–100 virus particles/cell) for 2 h. Cells infected with Ad-CMV-pA or Ad-CMV-β-gal served as negative controls. Cell numbers were measured 3 days later by the crystal violet assay using an automated E max spectrophotometric plate reader (Molecular Devices Corp., Sunnyvale, CA) as described previously (2, 8, 45).

Assessment of Adenoviral Infectivity in Mouse and Human Bones. To determine whether normal mouse or healthy human bones are susceptible to Ad infection, we performed two studies. An Ad-CMV-β-gal (1 × 10⁹ pfu) was injected into the femur of an adult mouse, and the bone was harvested 3 days later for histochemical analysis of β-gal activity using a method established previously (2, 8, 44). Additionally, a normal bone specimen harvested from a 69-year-old man with bone fracture was cultured in T medium containing 0.6% soft agar. A human prostate cancer PC-3 xenograft cultured similarly in 0.6% soft agar was also infected and secured as a control. The tissue specimens were exposed to Ad-CMV-β-gal (1 × 10⁹ pfu) and were processed 3 days after infection. After harvesting bone and prostate tumor specimens, tissues were first washed in PBS and fixed in 0.05% glutaraldehyde at 4°C for 24 h. Bone specimens were put in PBS for 24 h after fixing and decalcified with 0.25 M EDTA in PBS (pH 7.4) at 4°C for 5 days. After decalcification, the specimens were stained overnight in a solution of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 2 mM MgCl₂ in PBS. Prostate tumor specimens were processed as described previously and were stained similarly as described above for β-galactosidase activity (2, 8, 44).

In Vivo Animal Experiment. To demonstrate oncolytic activity and tumor specificity of Ad-OC-E1a, athymic mice (20–25 g) were inoculated s.c. with 1 × 10⁶ PC-3 or Lovo cells suspended in 100 μl T medium containing 5% FBS. When the tumor became palpable (4–5 mm in diameter), the animals were randomly assigned to two experimental groups: group 1, Ad-OC-E1a; and group 2, Ad-CMV-β-gal. A single dose of virus (2 × 10⁹ pfu) was injected intratumorally in mice. After administration of the test viruses, tumor sizes were measured and recorded.

To evaluate the effect of systemic Ad-OC-E1a in the intraosseous prostate tumor model, 1 × 10⁶ C4-2 cells were injected into the bone marrow space of the right tibial bone in castrated male SCID/bg mice according to procedures published previously (46). Blood specimens (~100 μl) were obtained from the tail vein for PSA assay once per week. Serum PSA was determined by microparticle ELISA using an Abbott IMx machine (Abbott Laboratories, Abbott Park, IL). After the detection of serum PSA elevation, a single dose of 25 μl Ad-OC-E1a, 2 × 10⁹ pfu (or 4 × 10¹⁰ virus particles)/animal, was administered i.v. to mice. When serum PSA rebound had occurred, animals were treated with the second or third i.v. injection of the same dose of the test virus at the specified time points as indicated. Serum PSA was monitored weekly, and histopathology and X-ray of the tumors were routinely assessed when the animals were sacrificed.

Results

Immunohistochemical Staining of Primary and Metastatic Human Prostate Tumor Specimens. Whereas OC has been shown to be a specific marker expressed exclusively in osteoblast-lineage cells, OC was also found to be expressed in calcified normal smooth muscle tissues, vascular endothelial pericytes, and benign tumors

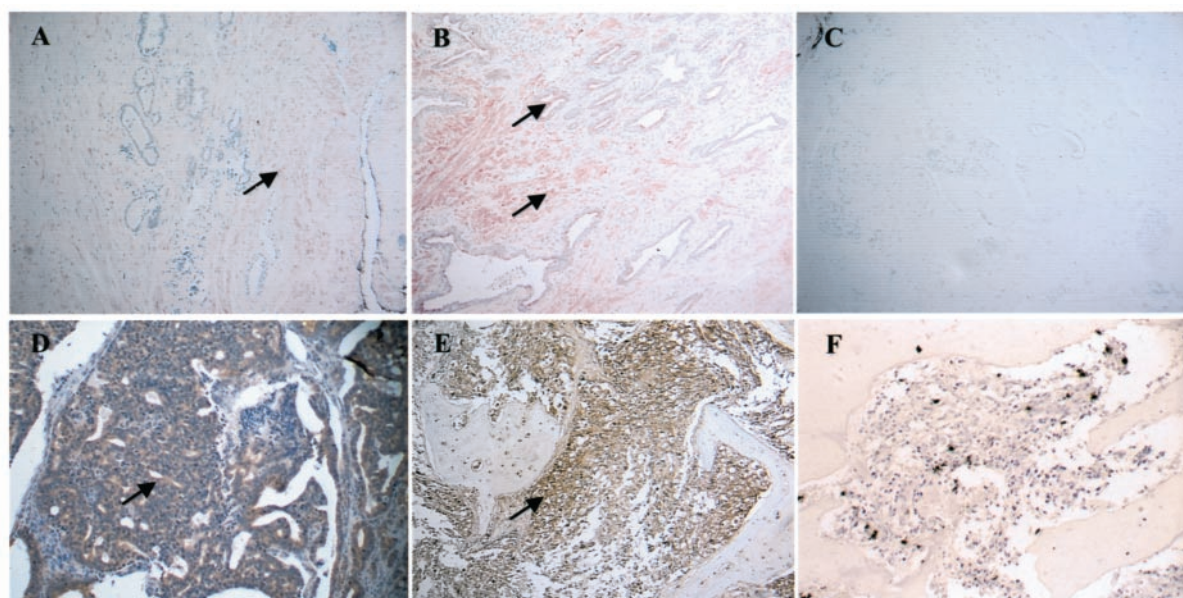
(47–50). We demonstrated OC expression (see *arrows*) by immunohistochemistry in a primary prostate tumor stroma (Fig. 1A), primary prostate tumor epithelium and stroma (Fig. 1B), a prostate tumor lymph node (Fig. 1D), and a bone (Fig. 1E) metastatic specimen. Positive OC stain was found in 85% (23/27) of the primary prostate tumor specimens and in 100% of the prostate tumor lymph node (12/12) and bone (10/10) metastatic specimens. OC stains are generally less intense in the primary prostate cancer but are very intense in metastatic prostate cancer (lymph node and bone) specimens. Background immunohistochemical staining of OC was demonstrated by the use of a control mouse IgG in a primary human prostate tumor (Fig. 1C), a human colon cancer (data not shown), and human prostate tumor bone metastatic specimens (Fig. 1F). Positive immunostaining of OC was also demonstrated in normal human bone specimens (data not shown) and tumor-associated osteoblasts (Fig. 1E).

Cytotoxicity of Ad-OC-E1a to Prostate Cancer Cell Lines *in Vitro*: Independent of Endogenous PSA and AR Status. To assess the cytotoxicity of Ad-OC-E1a, we exposed a number of human prostate cancer cell lines, LNCaP, C4–2, PC-3, DU-145, and ARCaP, *in vitro* to a wide range (0.01–5 MOI) of Ad-OC-E1a vector. We used 293, or WH and Lovo cells as positive or negative controls, respectively. We observed that whereas exposure of LNCaP and C4–2 cells to 5 MOI of Ad-OC-E1a inhibited the growth of these cells by 70% (Fig. 2a), this same dose of Ad-OC-E1a was ineffective in blocking the growth of WH and Lovo cells, which exhibit barely detectable or nondetectable OC promoter activity (data not shown). Cells infected similarly by the control viruses, either without an insert (Ad-CMV-pA) or with β -gal insert (Ad-CMV- β -gal), were also unaffected even when exposed to 5 MOI of the virus (Fig. 2a). Next, we evaluated the

efficacy of Ad-OC-E1a in several other human prostate cancer cell lines that either expressed a very low level (*e.g.*, ARCaP) or nondetectable (*e.g.*, PC-3 and DU-145) level of PSA and AR. Fig. 2b shows that all of the tested human prostate cancer cell lines were sensitive to Ad-OC-E1a-induced cell lysis *in vitro* irrespective of their intrinsic levels of PSA and AR expression. In addition, we also evaluated the effects of Ad-OC-E1a on the growth of a human prostate fibroblast and a human osteosarcoma cell line *in vitro*. As demonstrated in Fig. 2c, Ad-OC-E1a infection induced significant cell lysis in both cultured human prostate fibroblast (*e.g.*, 9096F) and osteoblast (MG-63) cell lines.

Abolishing s.c. PC-3 Tumor Growth *in Vivo* with Intratumoral Ad-OC-E1a. To establish the specificity of Ad-OC-E1a in inhibiting prostate tumor growth *in vivo*, we compared the activity of this virus on the growth of s.c. human prostate PC-3 tumors established previously with that of human colon Lovo tumors (serve as a negative control) *in vivo*. Fig. 3 shows that Ad-OC-E1a effectively inhibited the growth of PC-3 but not Lovo tumors when injected intratumorally. PC-3 tumors injected similarly with Ad-CMV- β -gal (data not shown) and Lovo tumors with Ad-OC-E1a exhibited a slight inhibitory effect on tumor volumes. These data are consistent with the observation that OC promoter activity is present in PC-3 but not in Lovo cells and that Ad-OC-E1a induced marked cytotoxicity in PC-3 but not Lovo cells *in vitro* (Fig. 2, a and b).

Systemic Ad-OC-E1a Eliminated C4–2 Human Prostate Tumors Established Previously in the Skeleton. A PSA-secreting and androgen-independent human LNCaP prostate cancer subline, C4–2, was chosen to evaluate the efficacy of systemic Ad-OC-E1a. Prostate tumors established previously in the skeleton with increased



Prostate Cancer Specimens

Primary Prostate Cancer
Lymph Node Metastasis
Bone Metastasis

Positive/Total (%)

23/27 (85%)
12/12 (100%)
10/10 (100%)

Fig. 1. Immunohistochemical demonstration of the presence of OC in primary and metastatic human prostate cancer specimens. Positive OC stain (*arrows*) was detected in primary cancer-associated stroma (A) and both prostate stroma and tumor epithelium (B). Positive immunostaining of OC was also found in lymph node (D) and bone (E) metastasis. Background immunostaining was found in control primary (C) and bone metastatic (F) prostate cancer when these specimens were stained by mouse IgG. Accumulated incidences of OC-positive specimens (positive/total) are expressed on *bottom panel*.

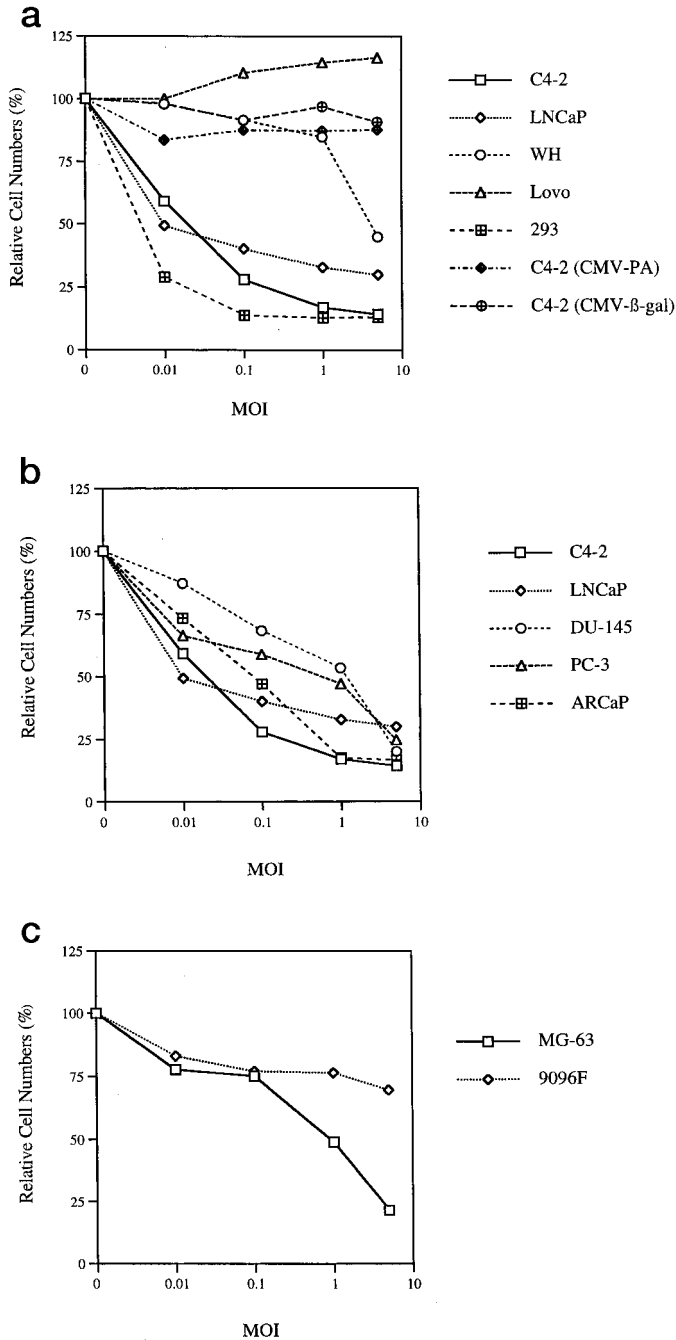


Fig. 2. Inhibition of human prostate cancer and bone and prostate stromal cell growth *in vitro* by the replication-competent Ad-OC-E1a. Cell growth was assessed *in vitro* in the presence of Ad-OC-E1a, Ad-CMV- β -gal, or Ad-CMV-pA. The percentage of cell viability was measured on day 3 after infection of the test virus (range, 0.01–5 MOI or pfu/cell). Results showed that although Ad-CMV-pA and Ad-CMV- β -gal did not affect the growth of C4-2, Ad-OC-E1a inhibited cell growth of C4-2 and 293 in a viral concentration-dependent manner. Ad-OC-E1a was not effective in inhibiting cell growth of WH and Lovo cells, because these cells lack OC promoter activity and OC expression. Results also showed the comparative aspect of efficacy of Ad-OC-E1a-induced cell lysis in C4-2, LNCaP, PC-3, ARCaP, and DU-145 human prostate cancer cell lines. Note PSA- and AR-positive (LNCaP, C4-2), PSA-negative (PC-3, DU-145), and marginally PSA- and AR-positive (ARCaP) human prostate cancer cell lines are all susceptible to Ad-OC-E1a-induced cell lysis. Additionally, results showed both human prostate stromal cells and osteoblasts are also sensitive to Ad-OC-E1a-induced cell lysis. Data represent the average of triplicate experiments determined with SD within 15% of the mean.

serum PSA were subjected to systemic Ad-OC-E1a administration. Serum PSA was followed weekly, and upon PSA rebound, Ad-OC-E1a treatment was repeated on animals. Six animals were evaluated in this study. Fig. 4a shows that in one control untreated mouse, serum

PSA underwent marked elevation from a basal 10 ng/ml at 6 weeks to 630 ng/ml at 15 weeks (Panel A). This profile of rapid PSA rise is consistent with our previous reports (2, 33, 46). Serum PSA profiles of Ad-OC-E1a-treated mice are shown in Fig. 4a from Panel B to F. Several variants of the PSA responses were noted. Mice 2 and 3 responded to systemic Ad-OC-E1a treatment with a complete regression of the skeletal tumors (Fig. 4, b and c), and a PSA nadir (*i.e.*, nondetectable PSA) was achieved for >15 weeks (see Panels B and C of Fig. 4a). These two mice are considered as cured by systemic Ad-OC-E1a treatment, because there was no subsequent PSA rebound, and no tumor was detected in the skeleton. Mice 4 and 5 responded to systemic Ad-OC-E1a with a marked and rapid PSA decline. PSA nadir in these mice was maintained for a variable period ranging from 1 to 6 weeks (see Panels D and E of Fig. 4a). These mice appear to have variable rebound of PSA during subsequent observation. Mouse 6 responded favorably to systemic Ad-OC-E1a initially with a PSA nadir lasting for 5 weeks. However, this mouse gradually escaped from systemic Ad-OC-E1a growth inhibition and appeared less responsive to the second and third dose of Ad-OC-E1a treatment (Fig. 4a, Panel F).

Gross Morphology, Histopathology, and Immunohistochemistry of Prostate Tumor Xenografts Harvested from the Skeleton in Mice Treated with Systemic Ad-OC-E1a. Marked gross anatomical differences were found between the control mice and mice responsive to Ad-OC-E1a (Fig. 4b). As shown by X-ray and gross anatomy, systemic Ad-OC-E1a induced marked regression of prostate tumors in

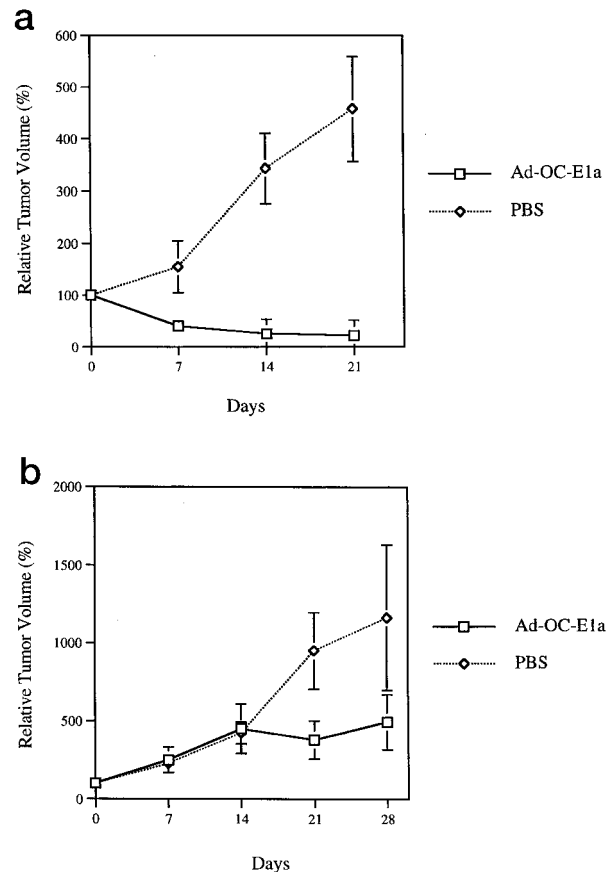


Fig. 3. Treatment of tumor xenografts with recombinant adenoviruses. Tumor xenografts were grown s.c. in athymic nude mice. Tumors were treated with recombinant viruses and PBS by intratumoral injection on day 0 and measured weekly. The data represent mean; bars, \pm SD. a, PC-3 xenograft tumors were treated with Ad-OC-E1a and PBS ($n = 4$). b, Lovo xenograft tumors were also treated with Ad-OC-E1a and PBS ($n = 4$).

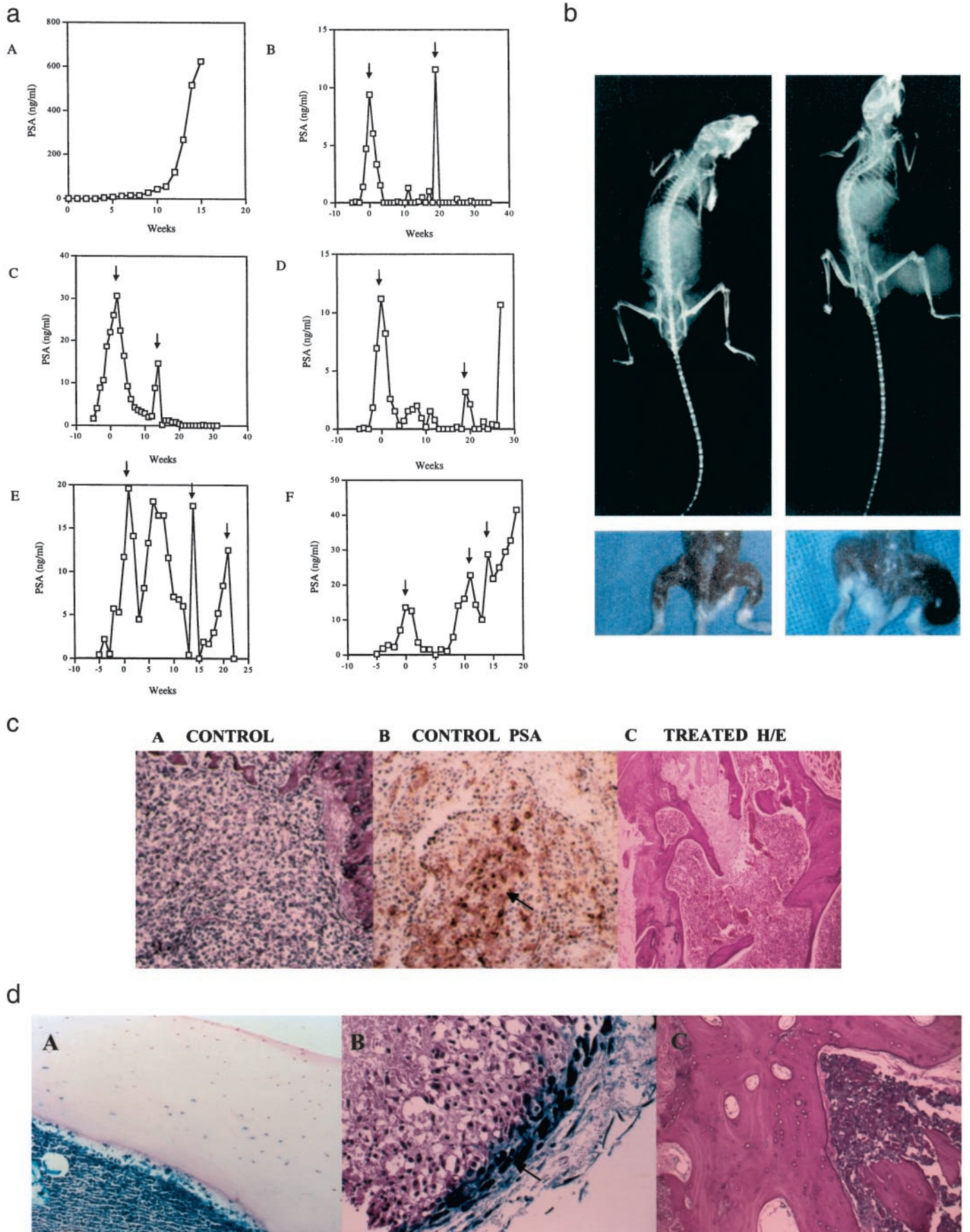


Fig. 4. a, demonstration of i.v. Ad-OC-E1a on serum PSA levels in SCID/bg mice injected intrasosseously with C4-2 cells. Panel A, serum PSA level of an untreated control mouse after intrasosseous injection of 1×10^6 C4-2 cells. Note exponential rise of serum PSA in the untreated mouse (mouse 1). Panels B-F, mouse 2-6 serum PSA levels of animals injected

the tibia. This marked improvement was confirmed by examining the histopathological section of the tumors obtained from the control and Ad-OC-E1a treated animals. Fig. 4c shows that in comparison to the systemic Ad-OC-E1a, the untreated mice have large tumors and stained positively by PSA antibody in the skeletal specimens (Fig. 4c, Panels A and B), whereas the prostate tumors cured by systemic Ad-OC-E1a failed to yield positive histopathology in the skeleton (Fig. 4c, Panel C) or positive immunohistochemical staining of PSA in the representative specimens (data not shown). We also compared the adenoviral infectivity in mouse bone *in situ*, human prostate PC-3 xenografts, and human bone maintained *in vitro* in soft agar. Results of this study showed that a single intraosseous administration of Ad-CMV- β -gal infected effectively the mouse bone cells without affecting the cortical bone (Fig. 4d, Panel A). *In vitro* Ad-CMV- β -gal efficiently infected upper layers of PC-3 tumor cells (Fig. 4d, Panel B) but not human bone cells (Fig. 4d, Panel C) maintained as explants in soft agar.

Discussion

Cancer therapies using adenoviral vectors can be divided into two broad categories, replication-defective and replication-competent (51). Because of the difficulties in infecting all of the cancer cells with adenoviral vectors, numerous laboratories have designed various versions of viral constructs with the primary goal of increasing the efficiency of viral infectivity (or transgene expression) or viral replication in competent tumor cells without damaging the normal tissues. One such approach relies on the ability of “bystander” genes such as hsv-TK or cytosine deaminase incorporated into replication-defective adenoviral vectors to convert prodrugs into biologically active growth-inhibitory products that elicit efficient cell kill even in cells that were not transduced with virus-bearing genes (2–5, 52, 53). The construction of replication-competent ONYX-015 lacking E1b, a MR 55,000 protein, can conceptually replicate in tumor cells that lack functional p53 protein (54). Conditional activation of viral gene expression and replication have also been achieved using tissue-specific promoters such as PSA or PSE for prostate cancer (1, 14, 15), α -fetal protein for liver cancer (55), and tyrosinase for melanoma (56, 57). Modification of adenoviral gene structure by introducing adenoviral death protein has achieved higher efficiency of viral replication (58). In the present study, we explored the possibility of using a novel tissue-specific (*i.e.*, osteoblast-specific) and tumor-restrictive (*i.e.*, restricted to calcified benign and malignant tumors) OC promoter to drive adenovirus replication in cells that contain OC promoter activity. This version of adenoviral vector allows the virus to replicate in both tumor epithelium (Fig. 2, *a* and *b*) and its supporting stromal cells including a human prostate stromal cell line (Fig. 2c) and a human osteoblast cell line (Fig. 2c). Thus, Ad-OC-E1a could potentially inflict maximal cell kill through, primarily, viral replication in tumor epithelium, and secondarily, by destruction of intercellular communication between tumor and stroma, inducing cell lysis in prostate fibromuscular stromal cells, osteoblasts, and potentially vascular endothelial pericytes (47–49). In experimental coculture studies both *in vitro* and *in vivo*, induction of osteoblast cell death by

hsv-TK/acyclovir or ganciclovir also markedly inhibited the growth of prostate tumor cells.⁴

Because OC expression is highly restricted to maturing osteoblasts (20–29, 47), Ad-OC-E1a may potentially damage bone and alter the balance between rate of bone resorption and formation. This concern has been addressed, and our study is summarized below. First, the cortical bone of both mouse and human restricts adenoviral infection. We observed that whereas mouse bone marrow is highly susceptible to adenoviral infection, human bone marrow including maturing osteoblasts appeared to be more resistant to adenoviral infection (Fig. 4d). Therefore, it is possible that in humans, Ad-OC-E1a replication may be limited to proliferating and maturing osteoblasts, which express OC promoter activity. Second, intraosseous administration of Ad-OC-hsvTK plus *i.p.* ganciclovir in intact adult mice did not induce any abnormal histopathology of the skeleton (4, 59). In fact, OC has been shown as an inhibitor of bone mineralization by preventing the growth of mineral crystals in an *in vitro* assay (60). This role of OC is consistent with the transgenic OC knockout mouse model where the destruction of OC-expressing cells by hsv-TK resulted in increased bone mass and bone formation (61, 62).

The tissue-specific and tumor-restrictive OC promoter potentially has several advantages over other prostate-specific promoters such as PSA or PSE enhancer (1, 15), human kallikrein 2 (hK2; 14), or prostate-specific membrane antigen (63). One advantage is that OC is expressed prevalently by human primary and metastatic prostate cancers with expression found in both tumor epithelium and/or surrounding stromal compartment (see Fig. 1). Another advantage is that OC expression is not limited to prostate tumors and was also found expressed by other calcified benign and malignant tissues such as smooth muscle plaques associated with heart valve and blood vessels (48, 49); benign tumors (50); and malignant osteosarcoma, brain, thyroid, breast, lung, and ovarian tumors (unpublished results) irrespective of their basal PSA and AR status. This is significant because it was estimated that ~20% of prostate cancer patients do not have elevated PSA despite the detection and progression of the disease (64). In addition, although AR gene amplification and overexpression were detected in almost 30% of the clinical prostate cancer specimens (65), AR-mutant or AR-null prostate cancer cells and tissues were nevertheless commonly observed (64–67). On the basis of the above observations, it is possible that PSA and/or AR-negative tumors may be responsive to Ad-OC-E1a but not to Ad-PSA-E1a-induced cell lysis.

Several previous publications demonstrated that an Ad vector-mediated toxic gene, hsv-TK, expression driven by OC promoter, inhibited the growth of osteosarcoma (16, 45, 68) and its metastasis (69) and inhibited prostate tumor growth both *in vitro* and *in vivo* (4, 16). Although intratumoral administration of Ad-OC-TK was used in most of these earlier studies, we observed significant remission of osteosarcoma lung metastasis and improvement of survival by systemic administration of Ad-OC-TK (69). The ability of systemic Ad-OC-TK to exert antitumor effects on osteosarcoma pulmonary

⁴ C-L. Hsieh, *et al.*, unpublished observation.

with Ad-OC-E1a (arrows, 2×10^9 pfu of Ad vector was administered via tail vein after detecting a rising serum PSA in animals that received intraosseous injection of 1×10^6 C4-2 cells). Systemic Ad-OC-E1a administration induced rapid decline of serum PSA, and 4/5 (80%) animals responded markedly to repeated Ad-OC-E1a treatment. *b*, gross morphology and X-ray evidence indicating the capability of Ad-OC-E1a in eradicating the growth of intraosseous human prostate tumor xenografts. Administration of Ad-OC-E1a via tail vein induced marked tumor regression in the treated mouse (*left panels*). *c*, regression of prostate tumors by Ad-OC-E1a is supported by histopathological evidence of the absence of prostate tumor cells in the skeleton (*Panel C*). In the untreated animals, both tumor cells (*Panel A*) and PSA (*Panel B*) were observed in the skeletal specimen. *d*, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining of normal mouse and human bone infected with Ad-CMV- β -gal (1×10^9 pfu). Note whereas the mouse bone marrow cells are susceptible to Ad-CMV- β -gal infection, the cortical bone of the mouse is resistant to Ad infection (*Panel A*). *Panel B*, human PC-3 prostate tumor is susceptible to Ad-CMV- β -gal infection when maintained as explants on soft agar. Normal human bone exposed to this virus appears to exhibit background β -gal activity suggesting that normal human bone cells may be resistant to Ad vector infection (*Panel C*).

metastasis without causing liver toxicity (59, 69) indicated the importance of considering the selection of tumor- or tissue-specific promoters to drive the expression of therapeutic genes or viral replications for cancer therapy. In this context, it is clear that conditional replication-competent adenovirus may have the advantage of amplifying the input of oncolytic virus and help the spread of agents to adjacent cells in a highly promoter- and cell-dependent manner. (1, 5, 14, 15, 51, 54, 58). In this study, we demonstrated substantial efficacy of systemic Ad-OC-E1a for the treatment of androgen-independent prostate cancer skeletal xenografts. However, we demonstrated that to eliminate the preexisting human prostate tumor xenografts in the bone, Ad-OC-E1a administration must be repeated. We obtained evidence that all of the mice responded initially to Ad-OC-E1a therapy (as judged by serum PSA response) and only one mouse (20%) escaped Ad-OC-E1a effects gradually and became an Ad-OC-E1a nonresponder. Forty percent (2/5) of the Ad-OC-E1a-treated mice have undergone complete tumor regression and are considered "cured" in the present protocol. Reasons why mice may lose their response to Ad-OC-E1a are presently unclear, but it is reasonable to suggest that Ad-OC-E1a infectivity may be reduced in the resistant tumors through a decreased coxsackie adenoviral receptor on tumor cell surface or a rapid clearance of Ad vectors at tumor sites from systemic circulation. Whereas the current protocol may be applicable to the treatment of clinical prostate cancer skeletal metastasis, some precautions need to be observed: (a) Ad-OC-E1a replication in normal human tissues requires more extensive testing, and human bone and human prostate cancer chimeric xenografts grown s.c. may be ideal for this evaluation (70); and (b) serum PSA response may be an indication but not the proof of tumor regression (71). Even if there is a potential pitfall in using altered serum PSA as the indicator for an antitumor effect, it is firmly established that serum PSA response does correlate with improved survival, pain relief, increased hemoglobin level, normalization of bone-derived alkaline phosphatase, weight gain, or improved performance status of prostate cancer patients (72). Smith *et al.* (73) found that a decrease in the serum PSA level of $\geq 50\%$ at 8 weeks was correlated with significantly increased survival. Such data validate the use of changes in the serum PSA level as a response parameter in trials of therapy in prostate cancer. In this study, we have shown that the PSA response correlated well with the histopathologies of prostate tumors in the skeleton and demonstrated the efficacy of systemic OC promoter-driven conditional replication-competent adenovirus in abolishing the growth of preexisting prostate tumors in bone.

In summary, we have established a novel replication-competent adenoviral therapy using a tissue-specific and tumor-restrictive OC promoter to drive the replication of adenovirus for the treatment of prostate cancer metastasis in an experimental human prostate cancer skeletal xenograft model. Ad-OC-E1a was shown to be effective in eliminating preexisting androgen-independent prostate tumors in the bone without adverse effects on mouse bone. This study establishes for the first time that cotargeting prostate cancer and bone stroma may be an effective strategy for destroying human prostate tumor skeletal metastasis.

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