Osteocalcin Promoter-based Toxic Gene Therapy for the Treatment of Osteosarcoma in Experimental Models

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Abstract

Osteocalcin (OC), a noncollagenous bone matrix protein, is expressed in high levels by osteoblasts. To determine whether the OC promoter mediates cell-specific gene expression in cells of osteoblast lineage, we constructed a recombinant adenovirus, Ad-OC-TK, which contains the OC promoter that drives the expression of herpes simplex virus thymidine kinase (TK). We tested the expression of TK by this virus in osteoblast cell lines as well as in non-osteoblastic cell lines by assessing the enzyme activity of TK in vitro. Whereas the OC promoter failed to drive the expression of the TK gene in several non-osteoblastic cell lines such as WH, a human bladder transitional carcinoma, and NIH 3T3, an embryonic mouse fibroblast cell line, the OC promoter mediated high levels of expression in osteoblast cell lines including murine ROS and human MG-63 cells. The addition of acyclovir (ACV), a pro-drug for the inhibition of cell proliferation, resulted in the induction of osteoblast-specific cell death in vitro. Intratumoral injection of Ad-OC-TK into murine ROS osteosarcoma abolished tumor growth in a host treated with subsequent i.p. ACV injection in vivo. The Ad-OC-TK virus plus ACV treatment appears to be highly selective in blocking the growth of both murine and human osteosarcoma cell lines in vitro and murine osteosarcoma in vivo.

Introduction

Osteosarcoma, a bone cancer occurring primarily in teenagers and young adults, affects approximately 2100 individuals yearly in the United States. This malignancy accounts for as many as 5% of all childhood malignancies and 60% of all malignant childhood bone tumors. Despite radical surgical resection of the primary tumor and aggressive adjuvant chemotherapy, the overall 2-year metastasis-free survival rate approaches only 66%. More than 30% of patients with this disease develop lung metastasis within the first year. The survival rate approaches only 66% for the past 10 years, despite changes in adjuvant chemotherapy. Because of the poor response rate of previously treated patients with relapsed osteosarcoma to second-line chemotherapy, it is important to develop new therapeutic approaches that can be applied either separately or in conjunction with current treatment modalities. The goals of this study are: (a) to design a new gene therapy treatment modality by demonstrating effective delivery and expression of a therapeutic toxic TK gene in a tissue-specific manner (e.g., osteoblast-like osteosarcoma cells) using an OC promoter; and (b) to determine if the Ad-OC-TK (a recombinant Ad vector containing the TK gene driven by the OC promoter) plus acyclovir treatment will exhibit a cytotoxic effect on the growth of osteosarcoma cells in vitro and tumors in vivo.

The concept of delivery and expression of therapeutic toxic genes to tumor cells through the use of tissue-specific promoters has been well recognized. This approach could decrease the toxic effect of therapeutic genes on neighboring normal cells when virus-mediated gene delivery results in the infection of the normal cells. Examples include the use of the albumin or α-fetoprotein promoter to target hepatoma cells, the bone morphogenetic protein promoter for brain to target glioma cells, the tyrosinase promoter to kill melanoma cells, and the carcinoembryonic antigen promoter for gastric carcinoma cells. To date, the best studied therapeutic gene is herpes simplex virus TK gene. Herpes simplex virus-TK converts the pro-drug ACV to a phosphorylated form that is cytotoxic to dividing cells. Critical to successful results is the “bystander” effect, which confers cytotoxicity on neighboring nontransduced cells; effective tumor cell kill can be achieved without the delivery to and expression of suicide genes in every tumor cell in vivo. This approach has been demonstrated recently to be efficacious in causing regression of many solid tumors, including metastatic colon carcinoma in the rat liver, gastric carcinoma, and malignant mesothelioma.

OC, a noncollagenous Glα protein produced specifically in osteoblasts, is synthesized, secreted, and deposited at the time of bone mineralization. A recent study showed that immunohistochemical staining of OC was positive in primary osteoblastic osteosarcoma and chondroblastic osteosarcoma specimens as well as in five of seven fibroblastic osteosarcomas. In an effort to develop a new treatment modality for osteosarcoma, we used an osteoblast-like osteosarcoma-specific OC promoter to drive toxic TK gene expression in the form of a recombinant Ad, Ad-OC-TK. Our results demonstrate that Ad-OC-TK, when delivered concurrently with ACV, is highly effective in inhibiting murine and human osteoblast tumor cell proliferation in vitro and murine osteosarcoma growth in vivo.

Materials and Methods

Cells and Cell Culture. ROS 17/2.8, a rat osteoblastic osteosarcoma cell line, was generously provided by Dr. Cindy Farrach-Carson (University of Texas Dental Branch, Houston, TX). MG-63, a human osteoblast-derived osteosarcoma cell line; 293, a transformed human embryonic kidney cell line; and NIH 3T3, an embryonic mouse fibroblast cell line, were purchased from American Type Culture Collection (Rockville, MD). WH, a human bladder transitional cell carcinoma, was established by our laboratory (15). The ROS 17/2.8 and MG-63 cell lines are considered the osteoblast lineage because of their morphological, biochemical, and molecular characteristics; they were incubated in DMEM (Life Technologies, Inc., Grand Island, NY) and 20% F12K (Irvine Scientific, Santa Ana, CA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (Sigma Chemical Co., St. Louis, MO). The WH and NIH 3T3 cell lines were maintained in T medium (18) containing 5% FBS. The 293 cells were maintained in MEM (Life
Technologies, Inc.) with 10% FBS and 1% tryptose phosphate broth (Life Technologies, Inc.). The cells were fed three times a week with fresh growth medium unless otherwise indicated.

Construction and Large-scale Production of a Recombinant Ad Vector Containing the OC Promoter. Construction of the recombinant Ad-OC-TK virus was accomplished as shown in Fig. 1. All plasmids were constructed according to standard protocols. Briefly, pΔE1SP1 (a gift from Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada) was digested with HindIII (New England Biolabs, Beverly, MA) and treated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the supplier's protocols. The HindIII-digested 3.1-kb fragment containing the murine OC promoter (1.3 kb)-driven TK (1.8 kb) construct was ligated into pΔE1SP1 Ad vector using T4 ligase (New England Biolabs). An expression clone, pΔE1SP1-OC-TK, was obtained with TK gene expression driven in a 3' to 5' orientation of an adenoviral DNA. Five μg of CsCl2-purified pΔE1SP1-OC-TK and pJM17 were cotransfected into 293 cells by the N-[l-(2,3-dioleoyloxyl)propyll-N,N,N-trimethylammoniummethyl sulfate (Boehringer Mannheim Biochemicals)-mediated transfection method (19). The culture medium of the 293 cells showing the completed cytopathic effect was collected and centrifuged at 1000 x 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-TK virus were obtained by plaque purification according to the method of Graham and Prevec (20). One of the viral clones was propagated in 293 cells; cells were harvested 36 to 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 CsCl2 gradient centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. The viral titer was determined by plaque assay. The control viruses used in this study, Ad-RSV-β-Gal and Ad-CMV-β-Gal, were constructed in a similar manner (18).

X-Gal Staining of Ad-RSV-β-Gal Virus-infected Cells. ROS, MG-63, WH, and NIH 3T3 cells, after infected with various doses of recombinant Ad-RSV-β-Gal, were grown in 60-mm dishes to about 80–90% confluency for 2 days, then the medium was discarded and fixed with 0.05% glutaraldehyde. After discarding the fixative solution, cells were rinsed thoroughly three times in PBS at room temperature. An X-Gal solution mixture [0.5 ml; 35 mM K3Fe(CN)6, 35 mM K4Fe(CN)6·3H2O, 1 mM MgSO4, and 1 mg/mL X-Gal (Sigma Chemical Co.)] was added to cover cells. Cells were then incubated 1 h to overnight at 37°C. Positive cells were stained blue, and the percentage of blue cells was calculated.

Determination of TK Activity in Target Cells Infected with Ad-OC-TK. Crude cell extracts containing TK were prepared from Ad-OC-TK-infected ROS, MG-63, WH, and NIH 3T3 cells. Briefly, cells were cultured in 100-mm dishes to 60 to 70% confluency, at which point they were exposed to 20 MOI of Ad-OC-TK overnight. The culture medium was then replaced with fresh medium after overnight infection, and the replaced culture medium was removed after an additional 48 h incubation. The infected cells were washed with PBS, trypsinized, and then counted; the expressed TK enzymatic activity was recovered from cells by the repeated freeze and thaw method. The resultant cell suspension was centrifuged at 300 x g for 10 min, and the supernatant fraction was frozen at −80°C for TK activity assay. TK activity was assayed by phosphorylation of [3H]GCV, as described previously (20), with modification. Briefly, the supernatant fraction harvested from approximately 1 x 106 cells containing TK was mixed with an equal volume of TK assay buffer containing

![Diagram](image-url)
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To assess whether Ad-OC-TK may drive the expression of the TK gene in a cell type-specific manner, ROS, MG-63, WH, and NIH 3T3 cells were exposed to Ad-OC-TK (20 MOI/target cell), and their crude cellular lysates were obtained and assayed for TK activity. TK activity was indirectly determined by measuring the amount of phosphorylated [3H]GCV mediated by TK enzyme present in cell lysate. The mean TK-mediated [3H]GCV phosphorylation (i.e., TK activity) per 10^6 cells was significantly higher in osteosarcoma ROS and MG-63 cells than in the WH and NIH 3T3 cell lines (Fig. 2). TK activity in ROS cells following infection with 20 MOI of Ad-CMV-β-Gal (18) serves as a negative control.

Cytotoxicity of ACV in Ad-OC-TK-transduced ROS and MG-63 Cells in Vitro. To determine whether Ad-mediated transduction with the OC-TK gene would render ROS or MG-63 cells sensitive to cell killing by ACV, we first tested the toxicity of ACV (range, 0–1000 μg/ml) in noninfected ROS, MG-63, WH, and NIH 3T3 cells and observed that ACV at doses below 40 μg/ml did not appreciably inhibit the growth of all cell lines tested (data not shown). The growth of ROS (Fig. 3A) and MG-63 (Fig. 3B) cells, infected with 20 MOI of Ad-OC-TK, was significantly inhibited by the addition of ACV (10 μg/ml); cells infected with Ad-OC-TK (20 MOI/target cell) or treated with ACV (10 μg/ml) alone did not exhibit altered growth or morphology during a 7–9 day observation period. Consistent with the low levels of TK activity, the growth of WH (Fig. 3C) and NIH 3T3 (Fig. 3D) cells after Ad-OC-TK infection was not affected by the addition of pro-drug ACV in the cell culture medium. Similarly, Ad-OC-TK or ACV alone did not affect proliferation in these cells.

Cytotoxicity of ACV in Ad-OC-TK-infected ROS Tumor Growth in Vivo. ROS sarcoma xenografts were induced by s.c. injection of ROS cells (1 × 10^6 cells/site) in athymic mice. After tumor formation, animals were treated with either ACV alone, Ad-OC-TK alone, or Ad-OC-TK plus daily ACV i.p. injection. After

Adenoviral Transduction Efficiency to ROS and MG-63 Cells and TK Expression in Vitro. To test the efficiency of Ad infection in ROS, a rat osteogenic sarcoma cell line, and in MG-63, a human osteosarcoma cell line, we used a recombinant adenoviral vector containing the RSV-β-Gal expression cassette (Ad-RSV-β-Gal). Based upon X-Gal staining of β-Gal activity, a dose-dependent increase of Ad infection was noted in ROS and MG-63 cells, with the percentage of cells infected increasing gradually 60, 75, and 100% as the adenoviral MOI increased 20, 40, and 60 MOI per target cell (data not shown). A similar efficiency of adenoviral infection was observed in WH and NIH 3T3 cells. In comparison to uninfected control cells, mild cytotoxicity was noted through the application of Ad up to 60 MOI (data not shown).

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Ad-OC-TK and ACV-induced Inhibition of Tumor Growth in Vivo. Congenitally athymic nude (nu/nu) mice (Harlan Co., Houston, TX), 5 to 6 weeks of age, were inoculated s.c. with ROS (3 × 10^6) cells, a rat osteosarcoma cell line. When the tumor became palpable (4–5 mm in diameter), the animals were randomly assigned to three experimental groups: group 1, ACV only; group 2, Ad-OC-TK only; and group 3, Ad-OC-TK plus ACV. For Ad-OC-TK injection, a microliter syringe fitted with a 28-gauge needle was used to deliver 75 μl of Ad-OC-TK (1 × 10^8 plaque-forming units). The Ad-OC-TK was injected intratumorally along both the long and short axes of the tumor: one injection parallel to the long axis, and one perpendicular to the axis. The needle point was then rotated within the tumor to maximize the area of Ad delivery. Ad-OC-TK was injected every other day for a total of three doses. Tumor volume was calculated by the following formula: volume (a rotational ellipsoid) M1 × M22 × 0.5236 (M1, long axis; M2, short axis; Ref. 18). Acyclovir treatment only or Ad-OC-TK plus ACV experimental groups were treated with an i.p. injection of ACV at a dose of 40 mg/kg body weight daily for 7 days. Ad-OC-TK and/or ACV treatment did not adversely affect the body weight of experimental animals. Tumor volume was calculated every other day for the first 8 days on day 20 and day 32.

Results

Ad-OC-TK and ACV-induced Inhibition of Tumor Growth in Vivo. To test the efficiency of Ad infection in ROS, a rat osteogenic sarcoma cell line, and in MG-63, a human osteosarcoma cell line, we used a recombinant adenoviral vector containing the RSV-β-Gal expression cassette (Ad-RSV-β-Gal). Based upon X-Gal staining of β-Gal activity, a dose-dependent increase of Ad infection was noted in ROS and MG-63 cells, with the percentage of cells infected increasing gradually 60, 75, and 100% as the adenoviral MOI increased 20, 40, and 60 MOI per target cell (data not shown). A similar efficiency of adenoviral infection was observed in WH and NIH 3T3 cells. In comparison to uninfected control cells, mild cytotoxicity was noted through the application of Ad up to 60 MOI (data not shown).

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Fig. 2. Determination of TK activity in target cells infected with Ad-OC-TK. Cells were exposed to Ad-OC-TK (20 MOI/target cell), and their crude cellular lysates were obtained and assayed for TK activity. The mean TK-mediated [3H]GCV phosphorylation (i.e., TK activity) per 10^6 cells was significantly higher in osteosarcoma ROS and MG-63 cells than in the WH and NIH 3T3 cell lines. TK activity in ROS cells following infection with Ad-CMV-β-Gal was used as a negative control.
Osteocalcin Promoter-Based Toxic Gene Therapy

In this study, we have demonstrated that: (a) Ad-OC-TK-infected osteosarcoma cell lines of the osteoblast cell lineage (e.g., ROS and MG-63 cells) expressed high levels of TK activity, and cell lines not of the osteoblast lineage, such as WH and NIH 3T3, expressed low TK activity; and (b) consistent with measured TK activity, the addition of ACV, a pro-drug substrate for TK, followed by Ad-OC-TK infection inhibited the growth of osteosarcoma cell lines in vitro and osteosarcoma xenografts in vivo. The growth of WH and NIH 3T3 cells after Ad-OC-TK infection was not affected by the addition of ACV in the tissue culture medium. Acyclovir treatment or Ad-OC-TK infection alone did not affect proliferation in any cell lines tested.

Fig. 3. Cytotoxicity of ACV in Ad-OC-TK transduced osteoblastic ROS and MG-63 cells in vitro. The growth of murine osteogenic ROS (A) and human osteosarcoma MG-63 (B) cells, infected with 20 MOI of Ad-OC-TK, was significantly inhibited by the addition of ACV (10 μg/ml). Consistent with low levels of TK activity, the growth of WH (C) and NIH 3T3 (D) cells after Ad-OC-TK infection was not affected by the addition of ACV in the tissue culture medium. Acyclovir treatment or Ad-OC-TK infection alone did not affect proliferation in any cell lines tested.

Discussion

Osteosarcomas, of all pediatric tumors, continue to challenge pediatric oncologists. Despite aggressive therapy, a significant number of osteosarcoma patients relapse locally and systemically. For these patients, second-line chemotherapy is of limited efficacy (2). To develop new therapeutic modalities for osteosarcoma, we have explored the possibility of targeting this tumor with toxic gene therapy mediated through the osteosarcoma-specific OC promoter. Since OC is the protein most commonly secreted by osteosarcoma cells of the osteoblastic lineage and is also a marker of osteoblastic differentiation (21), we have chosen to use the OC enhancer element to achieve tissue-specific expression of the toxic TK gene in rat and human osteogenic sarcoma cell lines. Because sarcoma cells are normally very resistant to conventional DNA transfection, we have used a highly infectious Ad as the gene delivery vehicle for OC-TK. In this study, we have demonstrated that: (a) Ad-OC-TK-infected osteosarcoma cell lines of the osteoblast cell lineage (e.g., ROS and MG-63 cells) expressed high levels of TK activity, and cell lines not of the osteoblast lineage, such as WH and NIH 3T3, expressed low TK activity; and (b) consistent with measured TK activity, the addition of ACV, a pro-drug substrate for TK, followed by Ad-OC-TK infection inhibited the growth of osteosarcoma cell lines in vitro and osteosarcoma xenografts in vivo. The growth of WH and NIH 3T3 cells was not affected by Ad-OC-TK either with or without ACV administration. Therefore, Ad-OC-TK constitutes a tumor-specific toxic gene therapy that inhibits the growth of proliferating osteosarcoma cells and spares significant tissue damage in the surrounding nonproliferating normal tissues and cells that are of nonosteoblastic lineage. The OC promoter-mediated gene delivery system is superior to conventional gene therapy in which TK expression is driven by universal promoters such as the CMV promoter (22) and the long terminal repeat promoters from RSV or Moloney murine leukemia virus (23). These promoters could potentially lead to the expression of nonspecific TK activation in all cells, resulting in the eradication of proliferating normal and neoplastic cells.
Historically, the most challenging osteosarcoma patients are the ones who develop unresectable pulmonary metastases after a curative resection; these patients have a poor prognosis and die within 1 year of the development of metastatic disease (24). When clinicians encounter such patients, new therapeutic options may be considered. In this study, we report, for the first time to our knowledge, the use of Ad-OC-TK gene therapy agent for osteosarcoma patients as well as for patients with melanoma, breast cancer, or prostate cancer who have symptomatic osseous metastases.

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References

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