Cancer Research

Direct and Bystander Killing of Sarcomas by Novel Cytosine Deaminase Fusion Gene

Margaret Ramnaraine, Weihong Pan, Michael Goblirsch, et al.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/63/20/6847

Cited Articles	This article cites by 20 articles, 8 of which you can access for free at: http://cancerres.aacrjournals.org/content/63/20/6847.full.html#ref-list-1
Citing articles	This article has been cited by 6 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/63/20/6847.full.html#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Direct and Bystander Killing of Sarcomas by Novel Cytosine Deaminase Fusion Gene[1]

Margaret Ramnaraine, Weihong Pan, Michael Goblirsch, Christine Lynch, Victor Lewis, Paul Orchard, Patrick Mantyh, and Denis R. Clohisy[2]

Departments of Orthopedic Surgery [M. R., W. P., M. G., C. L., D. R. C.], Preventive Sciences [P. M.], and Pediatrics [P. O.], University of Minnesota, Minneapolis, Minnesota 55455, and Department of Pediatric Oncology, Alberta Children's Hospital, Calgary, Alberta, T2T 5C7 Canada [V. L.]

ABSTRACT

Soft tissue and bone sarcomas of the extremities can be difficult to eradicate, and standard treatment may require limb amputation. New therapies to decrease tumor size could improve the effectiveness of treatment and decrease the frequency of limb amputation. Cytosine deaminase (CD)-based gene therapy has been shown to be effective in decreasing growth of solid tumors when animals with CD-expressing tumor cells are treated with 5 fluorocytosine (5FC), an inert prodrug that is converted to 5-fluorouracil (5FU) by CD. In this investigation, we used a novel CD-containing fusion gene to determine whether CD-based gene therapy affected soft tissue or bone sarcomas. The novel fusion gene (NGFR-CD) encodes for a protein with extracellular and transmembrane domains of human nerve growth factor receptor (NGFR) and cytoplasmic CD. Murine 2472 (2) sarcoma cells were transduced with fusion genes containing either the bacterial (NGFR-$_b$CD) or yeast (NGFR-$_y$CD) CD gene. 5FC treatment killed NGFR-$_b$CD- and NGFR-$_y$CD-transduced sarcoma cells *in vitro* through direct and bystander effects ($P < 0.01$). In contrast, 5FC treatment of mice with s.c. 2NGFR-$_b$CD or 2NGFR-$_y$CD tumors affected only 2NGFR-$_y$CD tumors. 5FC had no effect on growth of NGFR-$_b$CD tumors but caused significant decrease in the size of 2NGFR-$_y$CD tumors (51 ± 60 *versus* 938 ± 767 mm^3, treated *versus* control, $P < 0.01$). Evaluation of bystander killing *in vivo* revealed significant tumor killing, with a 5-fold reduction in s.c. tumor volume evident in saline *versus* 5FC-treated mice when tumors were comprised of 90% 2472 cells and 10% 2NGFR-$_y$CD selected for fluorescence-activated cell sorting ($P < 0.01$). Bone sarcomas were eliminated in 9 of 10 5FC-treated mice, compared with 11.8 ± 6.0 mm^2 in saline-treated mice ($P < 0.002$). In addition, 5FC treatment of bone sarcomas caused a significant reduction in cancer-induced bone destruction ($P < 0.002$) and resulted in a reduction in the number of osteoclasts. Finally, 5FC treatment had no effect on animal weight or survival, whereas doses of 5FU providing equivalent tumor reduction as 5FC resulted in treatment-associated deaths and significant weight loss ($P < 0.001$).

INTRODUCTION

Enzyme/prodrug gene therapy can involve transducing tumor cells with a gene encoding nonmammalian enzyme converting a nontoxic prodrug to a cytotoxic agent (1). One enzyme/prodrug therapy uses the prodrug 5FC.[3] 5FC is deaminated by CD into the cytotoxic drug 5FU (2). Designing therapies where CD is expressed in localized areas of cancer is attractive because such treatments could deliver very high local concentrations of 5FU to tumor sites while avoiding toxic systemic levels. CD genes from *Escherichia coli* and *Saccharomyces*

cerevisiae have been evaluated in experimental animals as enzyme/prodrug therapies. Both the bacterial ($_b$) and yeast ($_y$) enzyme/prodrug systems have been shown to inhibit tumor growth. However, recent work suggests that therapies using $_y$CD are superior because $_y$CD has a 22-fold higher 5FC conversion efficiency than $_b$CD (3, 4).

The CD enzyme/prodrug system has advantages over other enzyme/prodrug systems. One advantage is that 5FU can function as a cytotoxic molecule and as a radiosensitizer (5). This makes the CD system particularly attractive for treating tumors that will receive radiation treatment such as colon cancer. It has been shown in experimental models that CD-based prodrug systems are effective in killing colon cancer cells (2, 3, 6, 7), and recently, a synergistic effect has been reported with combined 5FC treatment and radiation (4, 8). A second advantage of the CD enzyme prodrug system is 5FU-directed bystander killing (2, 4, 6). Bystander killing occurs when tumor cells not expressing CD are eliminated by passive diffusion of 5FU from adjacent CD-expressing, 5FU-producing cells. An advantage of the bystander effect is making it unnecessary to deliver the gene in all cells to obtain an effect *in vivo*.

Novel means for reducing the size of extremity sarcomas are needed to improve local treatment of these devastating cancers and to decrease the frequency of limb amputation. In this investigation, a novel fusion gene containing a truncated form of the human NGFR and either the bacterial or yeast CD gene was used to determine whether CD-expressing soft tissue or bone sarcoma cells would respond to 5FC treatment. Both direct and bystander tumor killing were evaluated. In addition, as osteolysis plays a pathological role in bone sarcoma progression, we examined the possibility that 5FC treatment of CD-transduced bone sarcomas decreased tumor-induced osteolysis.

MATERIALS AND METHODS

Cell Culture. 2472 cells, originally derived from a malignant tissue tumor (sarcoma) in a C3H mouse and PA317 amphotropic packaging cell line, were obtained from the American Type Culture Collection (Manassas, VA). Powdered media was purchased from Sigma/Aldrich Chemical (St. Louis, MO), and sera from Hyclone (Logan, UT). 2472 cells were maintained in NCTC-135 media containing 10% horse sera, passaged once, and fed twice/week. PA317 cells were maintained in DMEM containing 10% newborn calf sera and passaged twice weekly. dPBS was used for all washes and reconstituted from 10× stock (Mediatech, Inc., Herndon, VA).

Vector Design. The retrovirus vectors containing the fusion genes comprised of the truncated human NGFR gene and either the bacterial (*E. coli*) CD gene or the yeast (*S. cerevisiae*) CD gene, designated NGFR-$_b$CD and NGFR-$_y$CD, respectively, were provided by Dr. Paul Orchard (9). Briefly, the NGFR-$_b$CD and NGFR-$_y$CD were constructed using the PCR, eliminating the termination codon of the NGFR gene and incorporating a [gly$_4$ser]$_2$ linker before the CD genes that had the initial ATG sequence eliminated. The modified CD genes were subcloned into a pCR2.1 vector (Invitrogen Life Technologies, Inc., Carlsbad, CA) and sequenced to confirm its identity. The fusion genes were excised from pCR2.1 and subcloned into the LXSN vector (10). Clones were screened to confirm the correct orientation. The final constructs, termed LNG/$_b$CDSN and LNG$_y$CDSN, were transfected into the PA317 packaging line, and 0.4 mg/ml G418 (Life Technologies, Inc.) were used to select positive clones (11). High-titer clones were chosen to generate viral supernatants, which were used to transduce the 2472 tumor line.

[3] The abbreviations used are: 5FC, 5-fluorocytosine; CD, cytosine deaminase; 5FU, 5-fluorouracil; NGFR, nerve growth factor receptor; 2472, NCTC clone 2472 fibrosarcoma; dPBS, Dulbecco's PBS; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase.

Retrovirus Transduction. Tumor cells were plated at 10^5 cells in a 100-mm dish and were transduced with a 2-ml aliquot of viral supernatant containing 20 μg/ml DEAE-Dextran. Cells were cultured for 4 h with rocking before 6 ml media was added. Cells were grown for 48 h before changing to selection media, which contained 0.8 mg/ml G418. Transduced 2472 tumor cells were designated 2NGFR-$_b$CD and 2NGFR-$_y$CD, with 2 indicating 2472 cells. Transduced tumor cells were maintained as the parent lines, except for the addition of 0.8 mg/ml G418.

FACS Analysis. Transduced tumor cells were harvested at passage, and an aliquot of 10^6 cells was used for FACS analysis. Briefly, the cells were pelleted in 12×75-mm tubes, rinsed once in PBS containing 0.1% BSA, and divided into two 100-μl tubes containing either 100 ng/10^6 cells of a biotinylated monoclonal mouse antihuman NGFR (clone 20.4, provided Dr. P. Orchard) or an equal amount of a biotinylated isotype control immunoglobulin. Cells were incubated on ice for 30 min, washed once in 10-fold volume of dPBS containing 0.1% BSA, followed by addition of 100 ng/10^6 cells of the secondary antibody conjugated to Streptavadin-PE (BD Biosciences PharMingen, San Diego, CA). Cells were returned for an additional 30 min of incubation on ice, followed by a third wash and resuspension in 0.5 ml of 4% paraformaldehyde in dPBS. Analysis of the presence of the NGFR antigen on cells was performed using a FACScaliber (BD Biosciences Immunocytometry Systems, San Jose, CA) flow cytometer for acquisition and FLOJO software for quantitation, plotting the isotype control or nontransduced cell line against NGFR-expressing cells. For positive sorting, 20×10^6 cells were pelleted into a sterile 12×75-mm tube and labeled as above using 2 μg of antibody in a total volume of 1 ml. The brightest 20% NGFR-positive cells were recovered using a FACSVantage flow cytometer. Positive cells were subcultured, maintained as heterogeneous lines, and designated 2NGFR-$_y$CD$^+$.

Western Analysis. Cells from transduced and nontransduced cell lines (at least 10^7 total) were treated with trypsin, pelleted, and washed with PBS. Cell pellets were resuspended in 0.5 ml ice-cold lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.2% SDS, 1 mM NaF, and 1 mM Na_3PO_4] with protease inhibitor mixture tablets (Roche, Indianapolis, IN), then incubated on ice for 20 min and centrifuged 2 min at full speed in a microcentrifuge at 4°C. Supernatant was stored at -80°C. Two hundred μg of cell lysate/lane were separated on a 10% reducing SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween 20 (PBST) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. Polyclonal antihuman NGFR (p75) antibody (R&D Systems, Inc., Minneapolis, MN) and polyclonal anti-CD (Bio-Trend, Destin, FL) were applied at dilutions of 1:500 and 1:1000, respectively. Membranes were washed three times for 10 min in PBST before 1-h room temperature incubation with HRPs and conjugated secondary antibodies (bovine antigoat-HRP, 1:2000 or bovine antisheep-HRP, 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA). After six 5-min washes in PBST and one 20-min wash in PBS, the membranes were incubated with enhanced chemiluminescence (Pierce, Rockford, IL) for 1 min before exposure to Kodak X-Omit AR film. Membranes were reprobed with antitubulin as a loading control.

***In Vitro* Cytotoxicity Assay.** Cell counts and viability were assessed using a tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye conversion assay (Promega, Madison, WI). To assess drug cytotoxicity, 5 replicate wells containing 2.5×10^3 cells in 0.2 ml were cultured in 96-well plates with various dilutions of 5FU (1–20 μM) or 5FC (0.025–10.0 mM). Cells were demi-depleted at 3 days, a second dose of drug was added and cells returned to incubation for an additional 3 days. Dye was added on day 6 and allowed to react for 2 h at 37°C. Dye conversion was read using an ELISA plate reader at 490 nm against 610 nm. Cytotoxicity was determined by calculation of the number of viable cells as measured against wells containing no drug. Drug cytotoxicity experiments were performed on each transduced and nontransduced cell line in at least three distinct experiments, and average data were used to plot *in vitro* killing. Bystander killing was assessed, as above, using various ratios of parental and transduced cells.

***In Vivo* Tumor Killing.** A total of 12 C3H/HeJ (National Cancer Institute) or NOD.CB17-*Prkdc^{SCID}* (Jackson Labs, Bar Harbor, ME) mice were used for each tumor or treatment group. The Animal Care and Use Committee of the University of Minnesota approved all experimental protocols. Just before implantation, tumor cells were trypsinized, pelleted, rinsed in dPBS, and

resuspended at appropriate concentrations in dPBS containing 1% BSA. Tumor cells were implanted either s.c. or in the left femur intramedullary space.

s.c. injection of 5×10^6 tumor cells was performed over the left flank in a total volume of 200 μl. To facilitate tumor measurements, a small patch of fur was shaved over the implant site with tumor length and width measured every other day starting at day 6. Tumor volume was calculated using the formula for the volume of an ovoid: volume $= 4 \div 3 \times p \times$ (length $\div 2$) $\times$ (width $\div 2$) (2). Treatment with 5FC, 400 mg/kg/day (dissolved as 12.5 mg/ml in 0.9% sterile NaCl, injection grade), or 5FU (10, 25, or 50 mg/kg, diluted in 0.9% saline) was started when tumors reached ~100 mm^3. Mice were euthanized 11 days later. At tissue harvest, mice and tumors were weighed, tumors were measured, and s.c. tumors fixed in Z-fix (Anatech, Battle Creek, MI) for histology. Bystander effect was evaluated in s.c. tumors in C3H/HeJ mice, as described above, with a mixture of 90% (4.5×10^6) 2472 cells and 10% (0.5×10^6) nonsorted or sorted 2NGFR-$_y$CD cells.

Intraosseous injections of tumor cells were performed as described previously (12). In brief, injection of 10^5 2NGFR-$_y$CD cells in a 20-μl aliquot of dPBS containing 1% BSA was performed through a knee arthrotomy on mice anesthetized with a ketamine/xylazine mixture (100 and 7.5 mg/kg, respectively). Treatment with 5FC commenced 7 days after tumor cell injection, and the animals were sacrificed 9 days later. Radiographs (Faxitron MX-20 cabinet X-ray machine and Kodak Min-R 2000 film) of femora ($\times 5$) were performed and bone destruction scores were assessed as follows: 0, no bone loss; 1, intramedullary osteolysis without full thickness cortical osteolysis; 2, intramedullary osteolysis and full thickness cortical erosion through one cortex; and 3, intramedullary osteolysis and full thickness cortical erosion through both cortices (13). Femora were fixed in Z-fix for 24 h, decalcified in neutral 10% EDTA for 10 days, and processed for histology.

Histology, Histomorphometry, and Immunohistochemical Analysis. Tumors and decalcified femora were embedded in paraffin and cut at 5 μm. Routine H&E stain was performed on all specimens, and four replicate sections were used for histomorphometric measurements. Images of whole femora were acquired using a Spot 2 digital charge-coupled device (CCD) on a Nikon E-1000 microscope at $\times 1$ and ImagePro Plus (Media Cybernetics Inc., Silver Spring, MD) used to quantitate the area of bone containing tumor.

Immunohistochemistry for NGFR was performed on deparaffinized and hydrated sections of s.c. tumor and femora. Staining was a standard three-step avidin-biotin complex procedure. Briefly, monoclonal mouse antihuman NGFR (clone 20.4, provided by Dr. P. Orchard) was diluted 1:2000 and incubated at 4°C overnight. A biotinylated goat antimouse IgG F(ab')$_2$ (Rockland, Gilbertsville, PA) was used as the secondary antibody (1:1000 dilution), and the tertiary reagent was HRP avidin-biotin complex (HRP-avidin-biotin complex method; Vector Labs, Burlingame, CA). 3,3'-Diaminobenzidine (Zymed, San Francisco, CA) was used to develop the staining reactions, and sections were lightly counterstained with Mayer's hematoxylin, dehydrated, and coverslipped using Permount. Epitope retrieval involved 15-min rinses with first, 0.05% saponin, and second, 0.1% Triton X-100. All antibody incubations were followed by three rinses with dPBS. A nonspecific antibody (dPBS containing 10% horse sera), and avidin/biotin blocking steps were performed before addition of the primary antibody. Nonspecific peroxidase activity was blocked by incubation with 3% hydrogen peroxide (10 min) after the secondary antibody.

Statistics. *In vitro* data are presented as means $\pm$ SD and *in vivo* data as means $\times$ SE. Statistical significance was determined by Student's *t* test. *P* of <0.05 was considered statistically significant.

RESULTS

Characterization of NGFR-CD-Transduced 2472 Cells. The NGFR-$_b$CD and NGFR-$_y$CD fusion genes were designed with the extracellular and transmembrane domains of the NGFR protein to facilitate localization of the chimeric protein to the cell surface, with the cytosine deaminase portion of the fusion protein in the cytoplasm. This permits NGFR to be detected on the cell surface, allowing it to function as a positive selection marker and the cytoplasmic CD portion of the protein to function as a negative selectable element (Fig. 1).

2472 cells transduced with 2NGFR-$_b$CD or 2NGFR-$_y$CD expressed
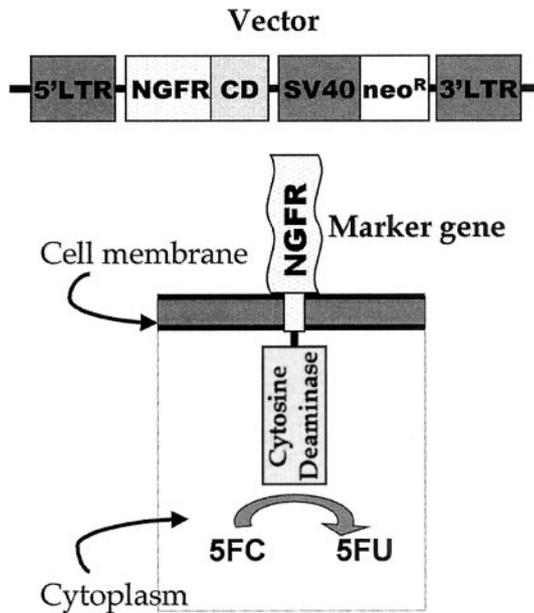
Vector

Fig. 1. Schematic of NGFR/CD fusion construct. Schematic of the retrovirus vector (*top*) and relative placement of the fusion protein across the cell membrane. Cytosine deaminase converts 5FC to 5FU.

abundant cell surface NGFR. FACS analysis indicated that parent 2472 cells did not express NGFR and determined that after selection in G418, ~60% of the transduced cells expressed surface NGFR (Fig. 2*A*). Comparison of 2NGFR-$_b$CD and 2NGFR-$_y$CD FACS expression profiles showed an approximate 4-fold greater expression in 2NGFR-$_y$CD cells compared with 2NGFR-$_b$CD cells. NGFR protein was detected in each transduced sarcoma and levels of protein expression paralleled FACS data. NGFR-$_b$CD was a single band at apparent molecular weight of M_r 95,000 and NGFR-$_y$CD had two bands with apparent molecular weights of M_r 67,000 and M_r 63,000. NGFR protein levels were higher in 2NGFR-$_y$CD-expressing cells than in 2NGFR-$_b$CD cells, and NGFR protein levels were higher in 2NGFR-$_y$CD cells selected by FACS for NGFR expression (2NGFR-$_y$CD$^+$) compared with cells selected by neomycin alone (Fig. 2*B*). Western analysis detected $_y$CD protein in 2NGFR-$_y$CD cells (Fig. 1*D*) and $_b$CD in 2NGFR-$_b$CD cells (Fig. 2*C*). Taken in total, these findings indicate that the NGFR-$_b$CD and NGFR-$_y$CD fusion genes produce intact NGFR-CD proteins of expected size and that cell surface expression of NGFR can be used for positive selection of cells transduced with the NGFR-$_y$CD or NGFR-$_b$CD fusion gene.

Effect of 5FU on Sarcoma Cells *in Vitro*. Similar concentrations of 5FU killed the parent sarcoma cell line (2472) and 2NGFR-CD cells. 2NGFR-$_b$CD were cultured in the presence of increasing doses of 5FU. After 5FU treatment, cell viability was assessed and findings indicated that the parent and transduced sarcoma cells were sensitive to 5FU with an estimated ED$_{50}$ of 2 μM (Fig. 3*A*).

Effect of 5FC on Sarcoma Cells *in Vitro*. 5FC treatment killed sarcoma cells transduced with the CD-NGFR genes. 5FC treatment had no effect on the parent 2472 cell line, but tumor cell killing was observed with both NGFR-CD-transduced cell lines. A decrease in cell survival was seen at 0.25 mM 5FC, and <10% of NGFR-CD cells were alive when exposed to 10 mM 5FC after 6 days in culture (Fig. 3*B*). Comparison of sarcoma cells transduced with NGFR-$_b$CD and 2NGFR-$_y$CD indicated that cells transduced with NGFR-$_y$CD were more sensitive to 5FC treatment. After exposure to 0.75, 1.0, and 1.25 mM 5FC, there were significantly increased numbers of 2NGFR-$_b$CD cells compared with 2NGFR-$_y$CD cells. After treatment with 0.75 mM

5FC, >50% of 2NGFR-$_b$CD cells were alive, compared with 15% of 2NGFR-$_y$CD (Fig. 3*B*).

Evaluation of Bystander Killing *in Vitro*. 2NGFR-$_y$CD cells killed untransduced 2472 cells through a bystander effect. Bystander killing was examined *in vitro* by culturing parent 2472 cells with 2NGFR-$_y$CD cells in various concentrations and exposing the cells to increasing doses of 5FC. Bystander killing occurred with as few as 10% 2NGFR-$_y$CD cells (Fig. 3*C*). ED$_{50}$ increased with decreasing percentages of 2NGFR-$_y$CD cells. Cultures containing 100, 50, and 10% 2NGFR-$_y$CD cells had ED$_{50}$ of 0.01, 0.05, and 0.25 mM 5FC, respectively. Finally, 2.5 mM 5FC eliminated >85% of tumor cells with each mixture of transduced and parent sarcoma cells (Fig. 3*C*).

Effect of 5FU on s.c. 2472 Tumors. 5FU treatment affected growth of s.c. sarcomas. Animals with s.c. 2472 tumors were treated with 10, 25, and 50 mg 5FU/day. Although 10 mg/kg/day 5FU had no effect on tumor volume, doses of 25 and 50 mg/kg/day affected tumor growth. Treatment with 25 mg/kg/day halted tumor growth and treatment with 50 mg/kg/day decreased tumor volume compared with initial values. When compared with saline treatment, significant decreases in tumor volume were present 7 days after 5FU treatment (25 and 50 mg/kg/day). At the end of each experiment, there was a >100% reduction in the tumor volume among mice treated with 25 or 50 mg 5FU/day compared with mice receiving no treatment (Fig. 4*A*). Corresponding reduction in tumor weights were observed. Final tumor weights for saline, 10, 25, and 50 mg/kg 5FU/day being 0.6 ± 0.4, 0.6 ± 0.1, 0.08 ± 0.06, and 0.04 ± 0.02, respectively (Table 1). When final tumor volumes were compared with tumor volumes before 5FU
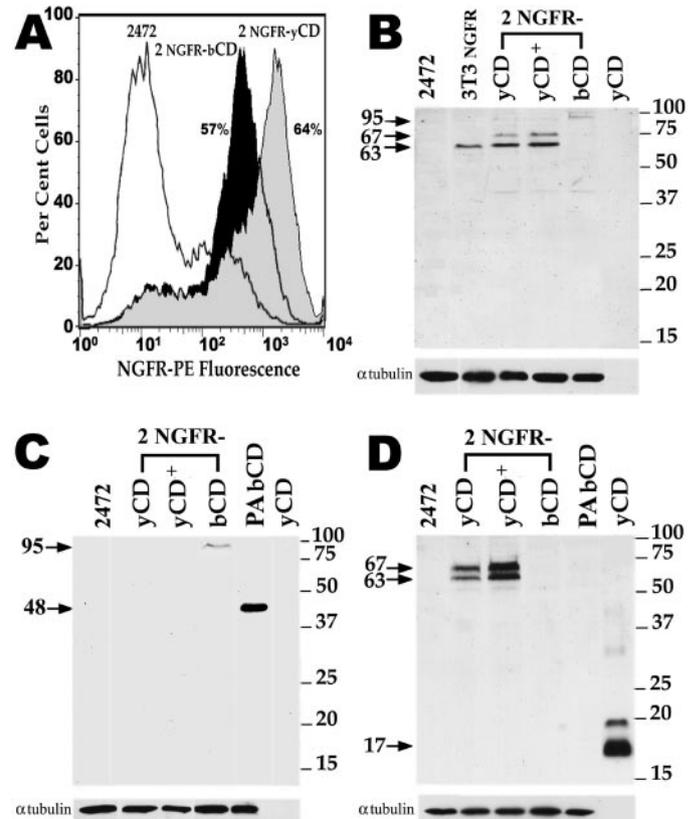

Fig. 2. Demonstration of NGFR/CD fusion protein. *A*, flow cytometric expression profiles showing relative NGFR expression for 2472, 2NGFR$_b$CD, and 2NGFR$_y$CD cells. Western analysis for NGFR (*B*), $_b$CD (*C*), and $_y$CD (*D*). 2472 = parent line; 3T3 NGFR = NIH 3T3 cells transduced with LNGFRSN; PA $_b$CD = PA317 cells transduced with L $_b$CDSN; 2NGFR-$_y$CD = 2472 cells transduced with NGFR $_y$CD; 2NGFR $_y$CD$^+$ = 2NGFR $_y$CD after positive selection for NGFR by flow cytometry; 2NGFR $_b$CD = 2472 cells transduced with NGFR $_b$CD; and $_y$CD = purified $_y$CD protein. α-Tubulin is shown as a loading control.
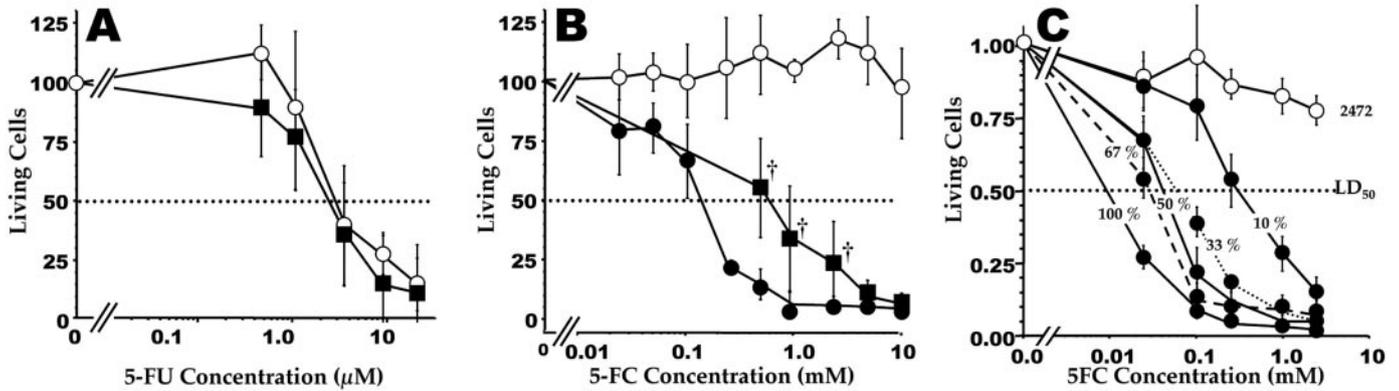
6849

Fig. 3. Effect of 5FU and 5FC on cancer cells *in vitro*. Sarcoma cells (2472, ○) and transduced sarcoma cells (2NGFR $_b$CD, ■; 2NGFR $_y$CD, ●) were cultured in the presence of increasing doses of (*A*) 5FU or (*B*) 5FC. Combinations of 2472 and 2NGFR $_y$CD cells were cultured with increasing doses of 5FC (*C*). Data in *A* and *B* represent mean ± SD of six separate experiments. Percent designation in (*C*) represents proportion of 2NGFR $_y$CD cells in 2NGFR $_y$CD:2472 mixture, mean ± SD of five replicates in a single experiment. †, significantly different ($P < 0.01$) compared with 2NGFR $_y$CD.

treatment, only the highest dose of 5FU (50 mg/kg/day) was effective at decreasing the initial tumor volume (Fig. 4*A*).

On the basis of body weights and treatment-related deaths, there was substantial morbidity associated with 5FU treatment. Toxicity of 5FU treatment was assessed by animal weights and treatment-associated deaths. Treatment with 10 mg/kg/day 5FU had no effect on weight gained during treatment but treatment with 25 or 50 mg/kg/day caused significant weight loss. At completion of the experiment, saline-treated and mice treated with 10 mg/kg/day 5FU had gained 3.6 ± 1.2 and 3.8 ± 1.4 g, respectively. In contrast, mice treated with 25 and 50 mg/kg 5FU/day had lost 4.0 ± 0.7 and 3.4 ± 1.0 g, respectively ($P < 0.001$; Table 1). 5FU treatment also caused animal deaths. Although no animals treated with saline, 10, or 25 mg/kg/day 5FU died, all mice treated with 50 mg/kg/day 5FU died before the experiment's end (17 days).

Effect of 5FC on s.c. 2NGFR-CD Tumors. 5FC treatment decreased growth of s.c. 2NGFR-$_y$CD tumors. Mice with 2472, 2NGFR-$_b$CD and 2NGFR-$_y$CD tumors were treated with 5FC, and tumor volume and weight were monitored. As expected, tumor volumes in saline-treated mice progressively increased over time. 5FC treatment had no effect on 2NGFR-$_b$CD tumors but showed profound effect on 2NGFR-$_y$CD tumors. 5FC treatment in animals with 2NGFR-$_y$CD tumors reduced tumor volumes below pretreatment size and dramatically decreased tumor volume compared with tumors in saline-treated mice (51 ± 60 *versus* 938 ± 767 mm³; Fig. 4*B*). Corresponding reduction in tumor weights was also seen. Tumor weights from 5FC

treated mice were significantly smaller than control (0.6 ± 0.4 *versus* 0.1 ± 0.09 g, $P < 0.001$) and were indistinguishable from tumor weights in mice treated with 25 or 50 mg/kg 5FU/day (Table 1). In addition, analysis of animal weights and treatment-associated deaths revealed that 5FC treatment had no effect on animal weight and caused no treatment-associated complications (Table 1).

To determine whether decreased tumor volume after 5FC treatment represented immunological killing, similar experiments were performed in NOD/SCID mice. Profound reduction in tumor volume was noted when NOD/SCID mice with 2NGFR-$_y$CD tumors were treated with 5FC ($n = 9$). Eight mice had no detectable tumor, and 1 mouse had a 4-mm³ tumor. In contrast, the mean tumor volume in saline-treated mice was 953 ± 349 mm³. This finding indicates that significant immunological killing was not occurring, and the primary mechanism for tumor killing was a direct cytotoxic effect of prodrug-generated 5FU.

Evaluation of Bystander Killing in s.c. Tumors. 2NGFR-$_y$CD cells killed untransduced 2472 cells *in vivo* through a bystander effect. The possibility that sarcoma cells transduced with the NGFR-$_y$CD fusion gene could direct bystander killing of nontransduced sarcoma cells was assessed by growing s.c. tumors composed of 10% 2NGFR-$_y$CD cells and 90% 2472 cells and treating tumor-bearing mice with saline or 5FC. As expected, tumor volumes in saline-treated mice progressively increased over time. 5FC treatment slowed tumor growth. After ≥7 days of treatment, there was a significant reduction in tumor volumes between saline and 5FC-treated mice (Fig. 4*C*). In
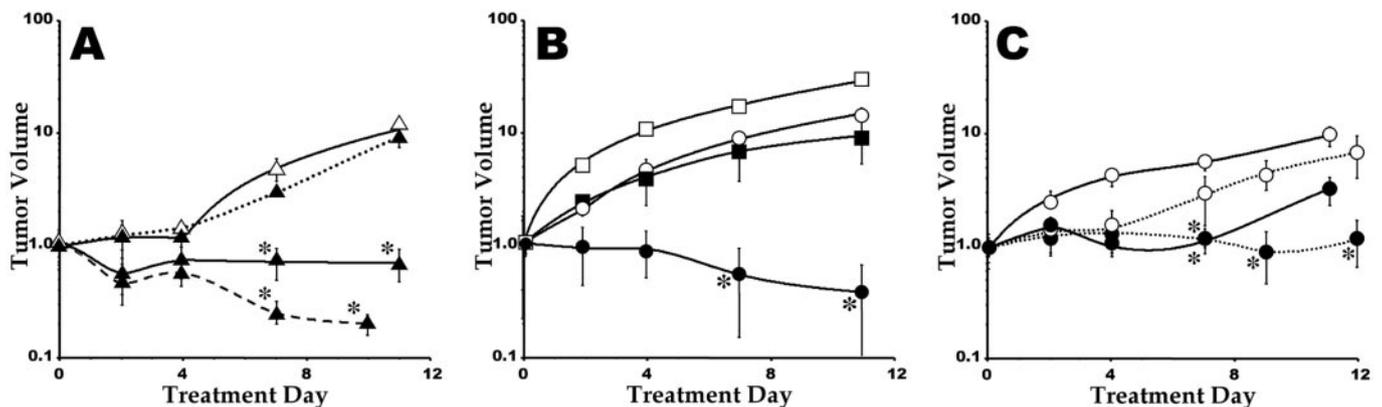

Fig. 4. Effect of 5FU and 5FC on cancer cells *in vivo*. *A*, treatment of 2472 tumors with saline (△) or increasing doses of 5FU (10 mg/kg/day, ····▲····; 25 mg/kg/day, ———▲———; 50 mg/kg/day, — — — ▲ — — —). *B*, treatment of 2NGFR $_y$CD (*circle*) and 2NGFR $_b$CD (*square*) tumors with saline (*open symbol*) or 400 mg/kg/day 5FC (*closed symbol*). *C*, treatment of tumors containing 10% 2NGFR$_y$CD:90% 2472 (*circle*) and 90% 2472:10% 2NGFR $_y$CD⁺ (*circle with dotted line*) with saline (*open symbol*) or 400 mg/kg/day 5FC (*closed symbol*). ∗, significantly less ($P < 0.01$) than saline treated.

6850

Table 1[a]

Treatment	Pretreatment weight Mean $\pm$ SD (n)	Final weight Mean $\pm$ SD (n)	Weight change Mean $\pm$ SD (n)	Tumor weight Mean $\pm$ SD (n)
Saline	22.6 $\pm$ 2.0 (30)	27.1 $\pm$ 1.9 (30)	4.00 $\pm$ 1.25 (30)	0.60 $\pm$ 0.42 (30)
10 mg/kg/d 5FU	22.4 $\pm$ 1.6 (8)	27.6 $\pm$ 1.1 (8)	4.68 $\pm$ 1.59 (8)	0.59 $\pm$ 0.13 (8)
25 mg/kg/d 5FU	23.0 $\pm$ 1.1 (8)	19.0 $\pm$ 1.3 (8)[b]	$-$4.06 $\pm$ 0.75 (8)[b]	0.08 $\pm$ 0.06 (8)[b]
50 mg/kg/d 5FU	21.0 $\pm$ 1.8 (12)	17.60 $\pm$ 1.7 (12)[b]	$-$3.47 $\pm$ 1.05 (12)[b]	0.04 $\pm$ 0.02 (12)[b]
400 mg/kg/d 5FC	22.7 $\pm$ 2.2 (18)	26.3 $\pm$ 2.0 (18)	3.83 $\pm$ 1.44 (18)	0.10 $\pm$ 0.09 (18)[b]

[a] Mice with subcutaneous tumors were treated with saline and increasing doses of 5FU or 5FC. Posttreatment animal weights and tumor weights were determined after 11 days of treatment.
[b] $P < 0.001$; saline *versus* drug.

addition, after 10 days of treatment, tumor volumes in saline-treated mice were two times greater than tumor volumes in 5FC-treated mice (Fig. 4*C*).

Bystander killing mediated by 2NGFR-$_y$CD cells was significant but incomplete, with residual tumor remaining at the completion of each experiment. We next examined the possibilities that increased NGFR-$_y$CD gene expression would enhance the bystander effect. 2NGFR-$_y$CD cells were sorted by FACS and the highest (20%) NGFR-expressing cells were expanded in culture. They showed a 2-fold increase in the level of 2NGFR-$_y$CD expression as determined by FACS analysis. These sorted cells, designated 2NGFR-$_y$CD$^+$, were mixed 1:9 with 2472 cells and injected as a s.c. tumor inoculum. Treatment of mice with these tumors revealed increased bystander killing (Fig. 4*C*). Specifically, after 12 days of treatment, there was a 5-fold reduction in tumor volume between saline and 5FC-treated mice. However, as with the unsorted 1:9 mix tumors, there was tumor growth despite treatment with 5FC.

We hypothesized that if 5FC treatment eliminated NGFR-$_y$CD-expressing cells and therefore limited the bystander effect. Findings indicated that 5FC treatment of mice with 10% 2NGFR-$_y$CD/90% 2472 sarcomas eliminated NGFR-expressing tumor cells. Immunohistochemical evaluation of tumors that grew from 10% 2NGFR-$_y$CD/90% 2472 cells showed different findings in saline-treated and 5FC-treated mice. As expected, tumors from saline-treated mice had NGFR-expressing cells (Fig. 5*B*). In contrast, NGFR-expressing cells could not be identified in tumors from 5FC-treated mice (Fig. 5*A*). This finding suggests that 5FC treatment was killing 2NGFR-$_y$CD cells before bystander killing could reduce the tumors below pretreatment size.

Effect of 5FC on Bone-Residing 2NGFR-yCD Tumors. 5FC treatment eliminated bone-residing 2NGFR-$_y$CD tumors. The effect of 5FC treatment on bone-residing 2NGFR-$_y$CD tumors was measured by evaluating tumor area in tumor-injected femora. Routine histological examination revealed extensive tumor in the femora of each saline-treated animal ($n = 10$) but identified tumor in only 1 of the 10 5FC-treated animals. This impression was confirmed by detection of

NGFR-expressing tumor cells in femora from saline-treated animals and only minimal positive NGFR staining cells in bones from 5FC-treated animals (Fig. 6, *A* and *B*). Bone tumor area in 5FC-treated mice was 1.3 $\pm$ 4.1 mm^2, a significant reduction compared with 11.8 $\pm$ 6.0 mm^2 in saline-treated mice ($P < 0.002$).

2472 sarcoma tumors are known to cause aggressive bone destruction by stimulating the formation and activation of osteoclasts, the body's principal bone resorbing cell (12). As experiments with s.c. sarcomas demonstrated 2NGFR-$_y$CD bystander killing of cancer cells, we sought to determine whether 2NGFR-$_y$CD tumors could direct bystander killing of normal host cells (osteoclasts), cells which contribute significantly to the morbidity and progression of bone cancer (14).

Bystander killing of osteoclasts by 2NGFR-$_y$CD bone cancers was profound and prevented cancer-induced bone destruction. Histological sections showed minimal or no bone destruction at sites of osseous tumor injection in mice treated with 5FC. This was a dramatic contrast to effects in bone of saline-treated mice where there was widespread bone destruction (Fig. 6, *C* and *D*). Radiographic bone destruction scores were assigned to tumor-injected bones from saline- and 5FC-treated mice. All tumor-injected femora from saline-treated mice had radiographic evidence of bone destruction with a mean bone destruction score of 2.8 $\pm$ 0.5. In contrast, only 1 of 10 tumor-injected femora from 5FC-treated mice had radiographic evidence of tumor osteolysis. The mean bone destruction score for femora from 5FC-treated mice was 0.2 $\pm$ 3 ($P < 0.002$; Fig. 7). Histological evaluation of bones showed that femora from saline-treated mice had areas of bone resorption with significant numbers of osteoclasts. Femora from 5FC-treated mice had occasional areas of bone resorption but no osteoclasts, indicating that 5FC treatment had eliminated osteoclasts that previously formed at sites of tumor (Fig. 8).

DISCUSSION

Our results show that the $_y$CD enzyme/prodrug system has significant cytotoxic effects on soft tissue and bone sarcomas. We show that
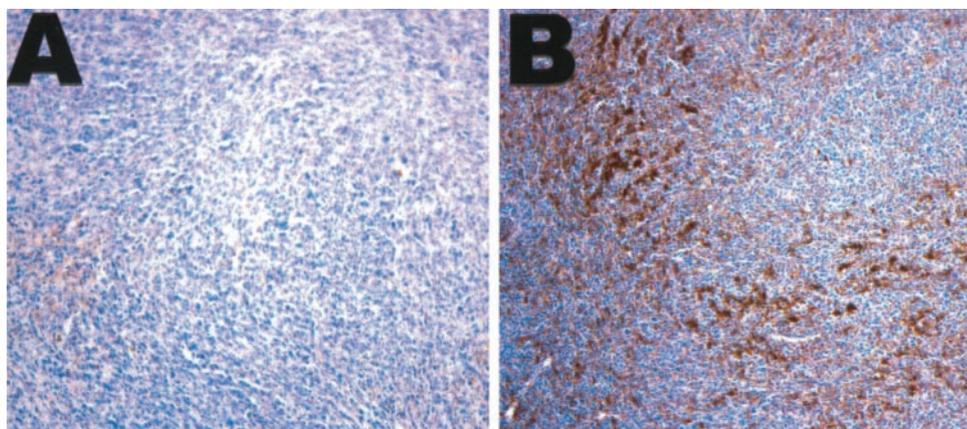

Fig. 5. Detection of NGFR/CD fusion protein *in vivo*. s.c. tumors containing 10% 2 NGFR/$_y$CD: 90% 2472 and treated with 400 mg/kg/day 5FC (*A*) or saline (*B*) were stained by immunohistochemistry for NGFR (*brown*). Serial sections using isotype control antibody were similar to (*A*; data not shown).
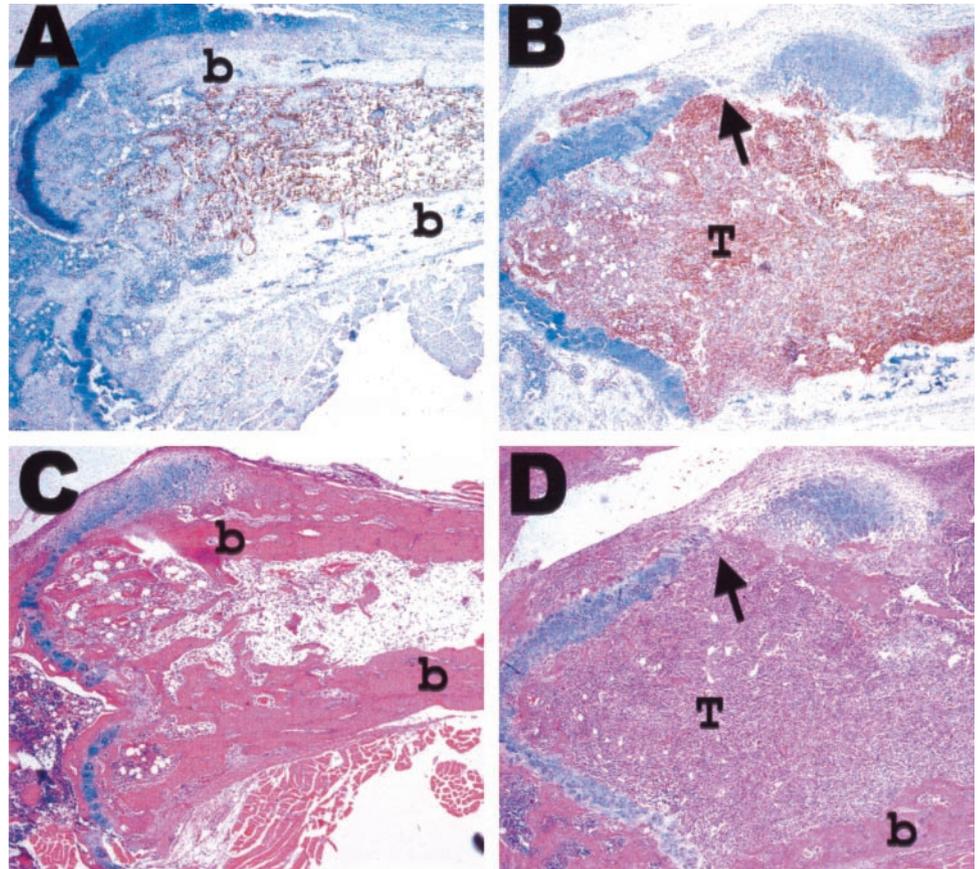
6851

Fig. 6. Effect of 5FC on bone-residing sarcomas. Histological sections of tumor-injected bones from mice treated with 400 mg/kg/day 5FC (*A* and *C*) or saline (*B* and *D*) for 9 days. Serial sections were stained for NGFR (*A* and *B*) or H&E (*C* and *D*). T = tumor, b = bone; *arrow* indicates where the tumor has completely eroded bone.

sarcoma cells transduced with NGFR-$_y$CD are sensitive to 5FC treatment in cell culture, in soft tissue, and in bone. s.c. sarcomas transduced with the NGFR-$_y$CD gene regressed after 5FC treatment, and bone sarcomas were eliminated after 5FC treatment in 9 of 10 animals. In contrast, sarcoma cells transduced with NGFR-$_b$CD were killed *in vitro* by 5FC treatment, but the growth of s.c. sarcomas transduced with NGFR-$_b$CD was not affected by 5FC treatment.

Previous direct comparison of bacterial and yeast CD in animal models report that the $_y$CD systems provide superior tumor killing compared with $_b$CD systems. Kievit *et al.* (3) studied the effect of $_b$CD and $_y$CD in HT29 human colon cancer cells, reporting that 5FC treatment had no effect on growth of $_b$CD-transduced tumors but caused regression of $_y$CD-transduced tumors. Decreased tumor killing by $_b$CD-transduced HT29 cells was attributed to a 22-fold higher K_m for $_b$CD compared with $_y$CD. Circumstances in this study do not permit direct comparison of NGFR-$_y$CD and NGFR-$_b$CD-transduced cells because CD protein was significantly higher in NGFR-$_y$CD-transduced cells.

Our evaluation of treatment-associated morbidity and mortality indicated that the administration of 5FC in association with CD expression had clear advantages over 5FU treatment. This is an important finding because it provides direct evidence supporting the concept that systemic toxicity is minimal, despite the capacity to eliminate CD-expressing enzyme prodrug system has a tumor killing effect. Furthermore, it shows that catastrophic systemic toxicity (death) results with 5FU doses that achieved the equivalent effect as 5FC.

When nontransduced sarcoma cells were mixed with 2NGFR-$_y$CD cells and treated with 5FC, significant killing occurred *in vitro* and *in vivo*. 5FC treatment of a mixture of nontransduced and NGFR-$_y$CD cells *in vitro* dramatically reduced the number of cancer cells. 5FC treatment of s.c. sarcomas grown from a mixture of 2472 and 2NGFR-$_y$CD cells significantly slowed tumor growth. Similarly, *in vivo* bystander killing and slowed tumor growth has been observed in an experimental s.c. model of HT29 colon cancer, s.c. WiDr colon tumors, and intrahepatic HT29 colon cancers (2, 8).

The 2472 sarcoma studied in this article has been shown previously to induce significant bone destruction (osteolysis; Ref. 12). The mechanism for this destruction is tumor stimulation of osteoclastic bone resorption caused by stimulation of osteoclast formation and activity. Previous work has defined the benefit of inhibiting tumor-induced osteoclastogenesis and has demonstrated that osteoclast-targeted ther-

Fig. 7. Effect of 5FC on bone-residing sarcomas. Radiographs of tumor-injected bones from mice treated with 400 mg/kg/day 5FC (*A*) or saline (*B*) for 9 days. *Arrows* indicate area of bone destruction in saline-treated femora.
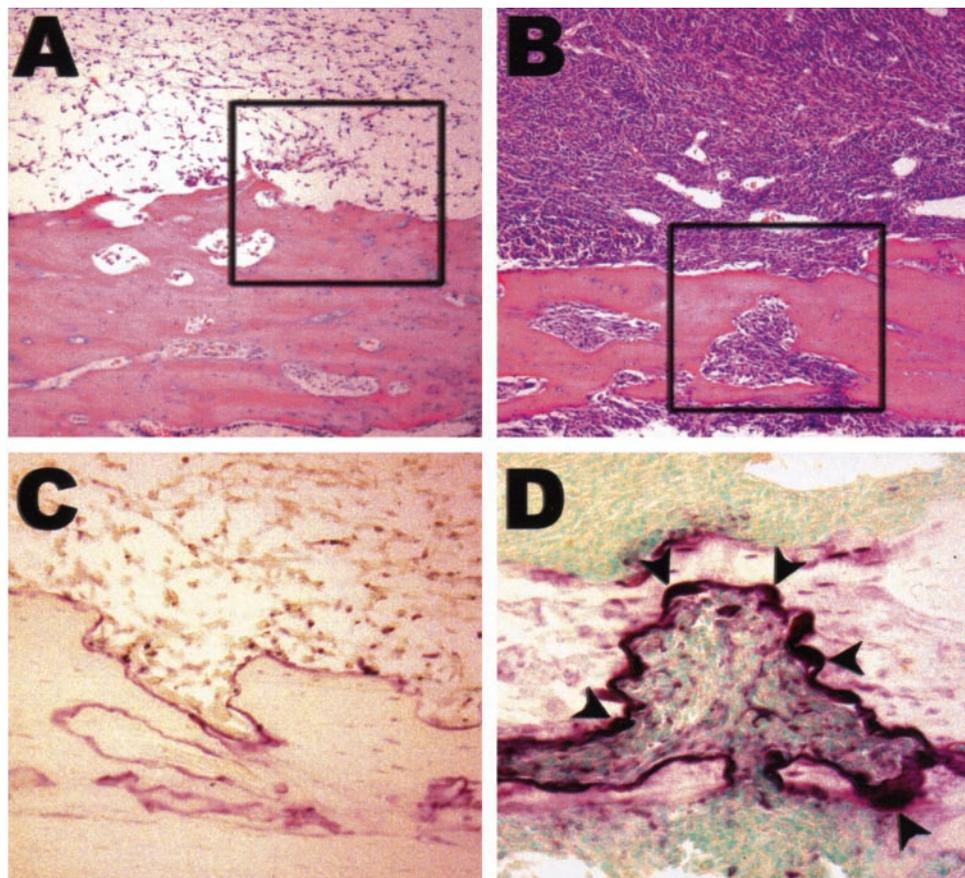
6852

Fig. 8. Bystander killing of osteoclasts by 5FC in bone-residing sarcomas. Histological sections of tumor-injected bones from mice treated for 9 days with 400 mg/kg/day 5FC (*A* and *C*) or saline (*B* and *D*). Serial sections were stained for H&E (*A* and *B*), shown at ×100 or telomeric repeat amplification protocol (*C* and *D*); outlined area in H& , shown at ×400. *Arrowheads* identify garnet-stained osteoclasts in bone from saline-treated animal.

apies decrease both cancer-induced bone destruction and cancer-induced pain (15, 16). In this investigation, 5FC treatment eliminated osteoclasts at sites of bone sarcomas. This resulted in a dramatic reduction in sarcoma-induced bone destruction and introduces the interesting possibility that tumor-generated 5FU may be exerting bystander killing effects on osteoclasts.

In conclusion, we report that $_y$CD-based enzyme/prodrug therapies can decrease the size of bone and soft tissue sarcomas in an animal model. These findings suggest that the $_y$CD-based therapies may be an effective means for decreasing the size of bone and soft tissue sarcomas, providing the gene can be delivered *in vivo*. Surgical management of large extremity sarcomas remains challenging and can necessitate limb amputation. Identification of the $_y$CD prodrug system as an experimental means for decreasing sarcoma tumor size provides impetus to explore the possibility that this enzyme prodrug system can be used as an adjuvant therapy for the management of sarcomas. In addition, as the 5FC/CD enzyme/prodrug system has shown efficacy as a radiosensitizer in both 5FU-sensitive and 5FU-resistant tumors (4, 17, 18), it will be important to determine whether the combination of 5FC/$_y$CD enzyme/prodrug system with radiation has a synergistic effect on sarcomas. Obstacles remain, however, and the most formidable of these is identification of an efficient gene delivery system. Concepts to enhance gene delivery that are currently under investigation include local intratumoral injection of nonselective retroviral or adenoviral vectors and viral targeting through engineering of the viral coat (19, 20).

ACKNOWLEDGMENTS

REFERENCES

1. Greco, O., and Dachs, G. U. Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives. J. Cell. Physiol., *187:* 22–36, 2001.
2. Huber, B. E., Austin, E. A., Richards, C. A., Davis, S. T., and Good, S. S. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the *cytosine deaminase* gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc. Natl. Acad. Sci. USA, *91:* 8302–8306, 1994.
3. Kievit, E., Bershad, E., Ng, E., Sethna, P., Dev, I., Lawrence, T. S., and Rehemtulla, A. Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. Cancer Res., *59:* 1417–1421, 1999.
4. Kievit, E., Nyati, M. K., Ng, E., Stegman, L. D., Parsels, J., Ross, B. D., Rehemtulla, A., and Lawrence, T. S. Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts. Cancer Res., *60:* 6649–6655, 2000.
5. Lawrence, T. S., Davis, M. A., and Maybaum, J. Dependence of 5-fluorouracil-mediated radiosensitization on DNA-directed effects. Int. J. Radiat. Oncol. Biol. Phys., *29:* 519–523, 1994.
6. Trinh, Q. T., Austin, E. A., Murray, D. M., Knick, V. C., and Huber, B. E. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine *versus* thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. Cancer Res., *55:* 4808–4812, 1995.
7. Chung-Faye, G. A., Chen, M. J., Green, N. K., Burton, A., Anderson, D., Mautner, V., Searle, P. F., and Kerr, D. J. *In vivo* gene therapy for colon cancer using adenovirus-mediated, transfer of the fusion gene cytosine deaminase and uracil phosphoribosyl-transferase. Gene Ther., *8:* 1547–1554, 2001.
8. Nyati, M. K., Symon, Z., Kievit, E., Dornfeld, K. J., Rynkiewicz, S. D., Ross, B. D., Rehemtulla, A., and Lawrence, T. S. The potential of 5-fluorocytosine/cytosine deaminase enzyme prodrug gene therapy in an intrahepatic colon cancer model. Gene Ther., *9:* 844–849, 2002.
9. Lewis, V. A., B. L., Blake, N., Lund, T. C., McIvor, R. S., and Orchard, P. J. Human nerve growth factor receptor and cytosine deaminase fusion genes. Hum. Gene Ther., *14:* 1009–1016, 2003.
10. Miller, A. D., and Rosman, G. J. Improved retroviral vectors for gene transfer and expression. Biotechniques, *7:* 980–982, 984–986, 989–990, 1989.

11. Miller, A. D., and Buttimore, C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol., *6:* 2895–2902, 1986.

12. Clohisy, D. R., Ogilvie, C. M., Carpenter, R. J., and Ramnaraine, M. L. Localized, tumor-associated osteolysis involves the recruitment and activation of osteoclasts. J. Orthop. Res., *14:* 2–6, 1996.

13. Schwei, M. J., Honore, P., Rogers, S. D., Salak-Johnson, J. L., Finke, M. P., Ramnaraine, M. L., Clohisy, D. R., and Mantyh, P. W. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. J. Neurosci., *19:* 10886–10897, 1999.

14. Mantyh, P. W., Clohisy, D. R., Koltzenburg, M., and Hunt, S. P. Molecular mechanism of cancer pain. Nat. Rev. Cancer, *2:* 201–209, 2002.

15. Clohisy, D. R., Ramnaraine, M. L., Scully, S., Qi, M., Van, G., Tan, H. L., and Lacey, D. L. Osteoprotegerin inhibits tumor-induced osteoclastogenesis and bone tumor growth in osteopetrotic mice. J. Orthop. Res., *18:* 967–976, 2000.

16. Honore, P., Luger, N. M., Sabino, M. A., Schwei, M. J., Rogers, S. D., Mach, D. B., O'Keefe, P. F., Ramnaraine, M. L., Clohisy, D. R., and Mantyh, P. W. Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. Nat. Med., *6:* 521–528, 2000.

17. Hanna, N. N., Mauceri, H. J., Wayne, J. D., Hallahan, D. E., Kufe, D. W., and Weichselbaum, R. R. Virally directed cytosine deaminase/5-fluorocytosine gene therapy enhances radiation response in human cancer xenografts. Cancer Res., *57:* 4205–4209, 1997.

18. Pederson, L. C., Buchsbaum, D. J., Vickers, S. M., Kancharla, S. R., Mayo, M. S., Curiel, D. T., and Stackhouse, M. A. Molecular chemotherapy combined with radiation therapy enhances killing of cholangiocarcinoma cells *in vitro* and *in vivo*. Cancer Res., *57:* 4325–4332, 1997.

19. McCormick, F. Cancer gene therapy: fringe or cutting edge? Nat. Rev. Cancer, *1:* 130–141, 2001.

20. Kirn, D., Niculescu-Duvaz, I., Hallden, G., and Springer, C. J. The emerging fields of suicide gene therapy and virotherapy. Trends Mol. Med., *8:* S68–S73, 2002.