c-Jun knockdown sensitizes osteosarcoma to doxorubicin

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Abstract

The oncogene c-Jun has been found to be up-regulated in a variety of cancers, including osteosarcoma. Doxorubicin is a frontline chemotherapeutic against osteosarcoma, but is limited by toxicity. DNAzymes are oligonucleotides capable of specific catalysis of target mRNA. A biocompatible c-Jun DNAzyme nanoparticle formulated from chitosan regressed the growth and metastasis of pre-established tumors, especially in combination with doxorubicin. *In vitro* data confirmed that c-Jun knockdown chemosensitized these cells to doxorubicin treatment. c-Jun down-regulation–mediated tumor inhibition also led to concomitant decreased osteolysis. Clinically, knockdown of c-Jun with chitosan nanobiotechnology may proffer an improved treatment outcome for osteosarcoma. [Mol Cancer Ther 2008;7(7):1909–12]

Introduction

Osteosarcoma growth originates from the bone, and commonly metastasizes to the lungs (1). This disease, which causes debilitation, if not fatality, mainly affects adolescents. Modern treatment includes limb-sparing surgery and chemotherapy with the 5-year survival rate approaching 70% (2). Despite this, patients still succumb to metastatic disease and it is often difficult to predict who will develop recurrent disease. Successful chemotherapy is associated with significant toxicity and patients >40 years are often regarded as unsuitable for conventional dose and intensity of treatment.

Thus, better management options are being explored, combined with better understanding of the molecular events that initiate and maintain tumor progression. One such molecule is c-Jun, a member of the basic region-leucine zipper (bZIP) protein family that homodimerizes and heterodimerizes with other bZIP proteins to form the transcription factor activating protein-1 (3). c-Jun has been found to be increased in higher-grade osteosarcoma (4, 5). Because no report exists of c-Jun mRNA silencing as a potential therapy modality for osteosarcoma, we embarked on this investigation.

Gene modulation may be used to enhance the efficacy of conventional frontline cytotoxics such as doxorubicin (6). One class of such a modulator, DNA enzymes or DNAzymes, are molecules capable of target-specific cleavage of mRNA (7), and to date, have been employed against a variety of gene targets (8). This study seminally establishes that a DNAzyme targeting the c-Jun transcript (9, 10) is capable of inducing significant inhibition of growth of an osteosarcoma cell line in culture.

It also shows the efficacy of the c-Jun DNAzyme against pre-established osteosarcoma in a clinically relevant orthotopic model for osteosarcoma, especially in combination with a frontline cytotoxic in doxorubicin. This is the first study of its kind showing the utility of nanoencapsulated DNAzymes, formulated using complex coercavation (11, 12). Also, it is the first demonstration of DNAzyme-mediated gene modulation in a metastasizing model of neoplasia.

Materials and Methods

Human osteosarcoma SaOS-2 cells were obtained from the American Type Culture Collection and used within 10 passages. Nanoparticles were formulated using complex coercavation (11, 12). The c-Jun plasmid and empty vector were obtained from Dr. Stephen Lye (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Ontario, Canada). Proteins were extracted and analyzed from *p*-formaldehyde–fixed sections (13). Immunohistochemistry and Western blots were done using antibodies from Santa Cruz Biotechnology (14). Briefly, for Western blotting, a primary rabbit anti-human antibody dilution of 1:1,000 (1 h) and a secondary goat anti-rabbit horseradish peroxidase antibody dilution of 1:1,000 (room temperature, 30 min) was used. Membranes were blocked with 5% skimmed milk in TBS/0.05% Tween 20. The c-Jun (Dz13) and scrambled DNAzyme (Scr) oligonucleotides were synthesized and prepared as described previously (9). Ethical approval for the use of human tissue and mice were obtained from St. Vincent's Health Human and Animal Ethics Committees, respectively. For treatment of pre-established tumors, treatment agents (250 ng of Dz13, 10 μL volume) were administered into the proximal tibial region with minimum disturbance of resident tumor at day 21 after tumor cell seeding. Doxorubicin (Sigma-Aldrich) was given i.p. at a

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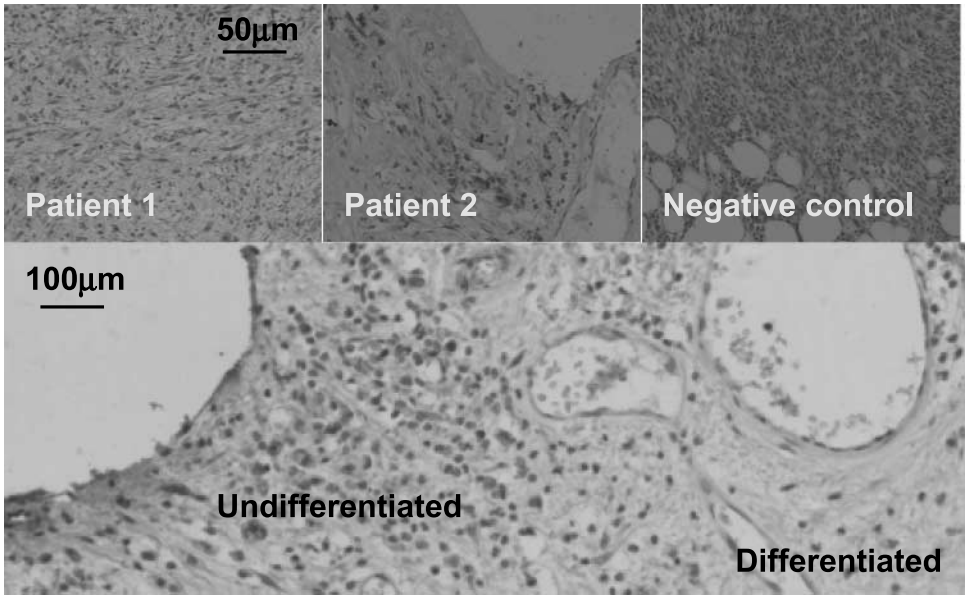

Figure 1. c-Jun protein present in clinical specimens with medium- to high-intensity staining. Immunohistochemical staining of intranuclear c-Jun protein is present in primary human osteosarcoma tissue. Negative control section is stained with secondary antibody (no primary) only.

dose of 3 mg/kg/injection. All other animal procedures were done as described previously (15). All data were analyzed using a one-way Student's *t* test with unequal variances.

Results and Discussion

We initially examined clinical specimens of primary osteosarcoma and used immunohistochemistry to reveal medium to intense staining of cancer cell nuclei for c-Jun

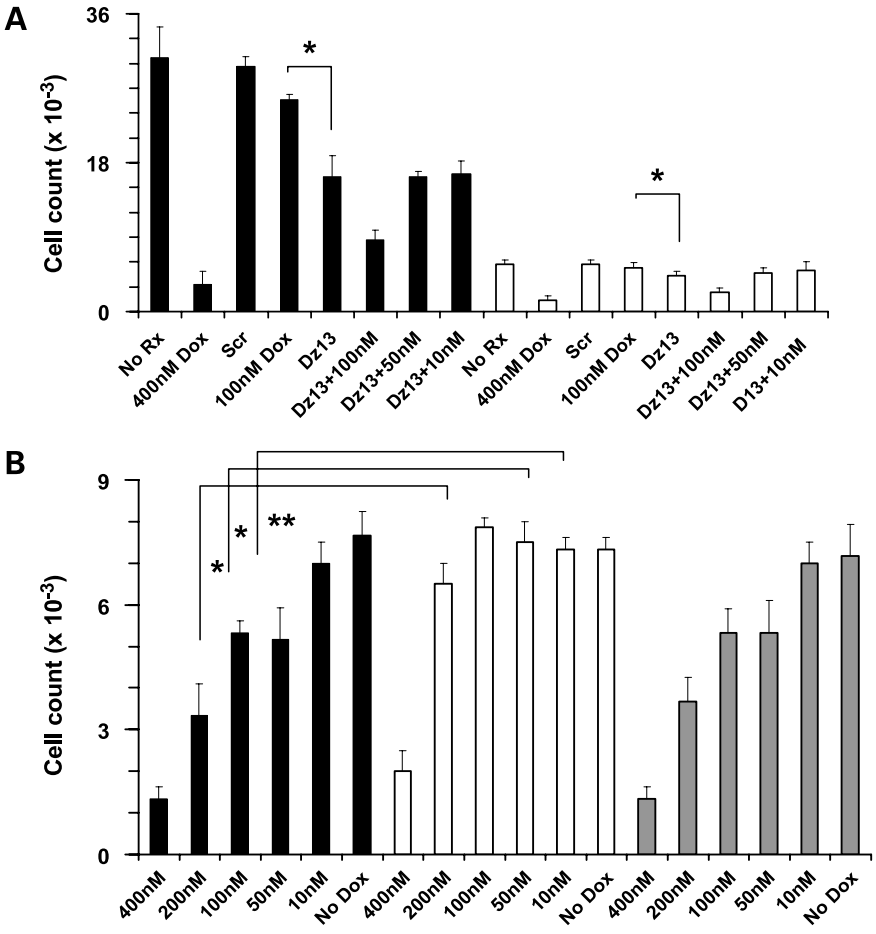

Figure 2. Activity of doxorubicin against SaOS-2 cells is dependent on c-Jun expression. **A,** Dz13 (200 nmol/L) sensitizes SaOS-2 cells to doxorubicin-mediated death after 24 h at 100 nmol/L at high (10%, *white columns*) and low (0.5%, *black columns*) fetal calf serum. *, $P < 0.01$ ($n = 4$). **B,** overexpression (2-fold) of c-Jun protects SaOS-2 cells from doxorubicin-mediated cell death at 24 h in medium with 10% FCS. *Black columns,* untransfected cells; *white columns,* cells overexpressing c-Jun; *gray columns,* cells transfected with empty vector. *, $P < 0.005$; **, $P < 0.01$ ($n = 4$).

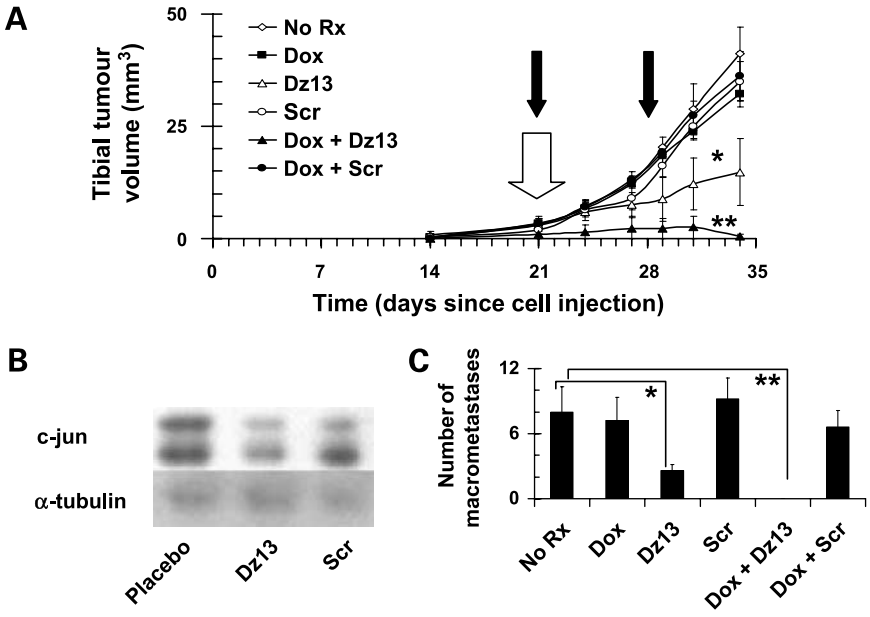

Figure 3. Efficacious effects of Dz13 + doxorubicin therapy. **A,** tumor growth curve showing efficacy due to 250 ng of Dz13 and the synergy of combined Dz13 + doxorubicin (3 mg/kg/injection/wk) therapy on SaOS-2 primary tumor growth. SaOS-2 cells were injected into the tibiae of mice, and 21 days later, treatment was initiated. *Black arrows,* date of injection of doxorubicin; *white arrow,* date of injection of nanoparticles. *, $P < 0.001$; **, $P < 0.0001$ ($n = 5$). **B,** confirmation of specificity of action of DNAzyme by Western blot of tumors showing down-regulation of c-Jun in the Dz13 group at the end of the study (5 weeks post–cell injection). **C,** Dz13 significantly reduces the formation of metastases, which is prevented in the Dz13 + doxorubicin group. *, $P < 0.005$; **, $P < 0.001$ ($n = 5$). *Columns,* mean; *bars,* SD.

(Fig. 1), akin to findings in an earlier study (4). Areas within the tumor that had a less differentiated pattern, that is a more aggressive growth pattern, were more intensely stained than those with a greater differentiation status. This suggests that c-Jun is clinically associated with tumor progression.

Doxorubicin is a preferred chemotherapeutic agent of choice for osteosarcoma at our center and in others (1); therefore, we combined Dz13 treatment with that of doxorubicin. Dz13 (200 nmol/L) sensitized SaOS-2 cells to a doxorubicin dose of 100 nmol/L under either high (10%) or low (0.5%) serum conditions (Fig. 2A). Thus, even under reduced serum conditions, Dz13 is able to reduce osteosarcoma cell growth, although slightly but significantly. However, it does sensitize these cells to doxorubicin at 100 nmol/L. Conversely, when c-Jun was overexpressed in these cells, doxorubicin-induced growth inhibition decreased (Fig. 2B). Even at a high dose of 200 nmol/L, doxorubicin was statistically ineffective.

The Dz13 nanoparticle was tested *in vivo*, in an osteosarcoma model closely resembling the clinical presentation of the disease (15). Treatments were given 3 weeks after cell inoculation as this serves as a genuine test of the ability of a therapy to be able to hinder tumor growth once it has established and is within the exponential and aggressive growth phase. This, also very relevantly, is the

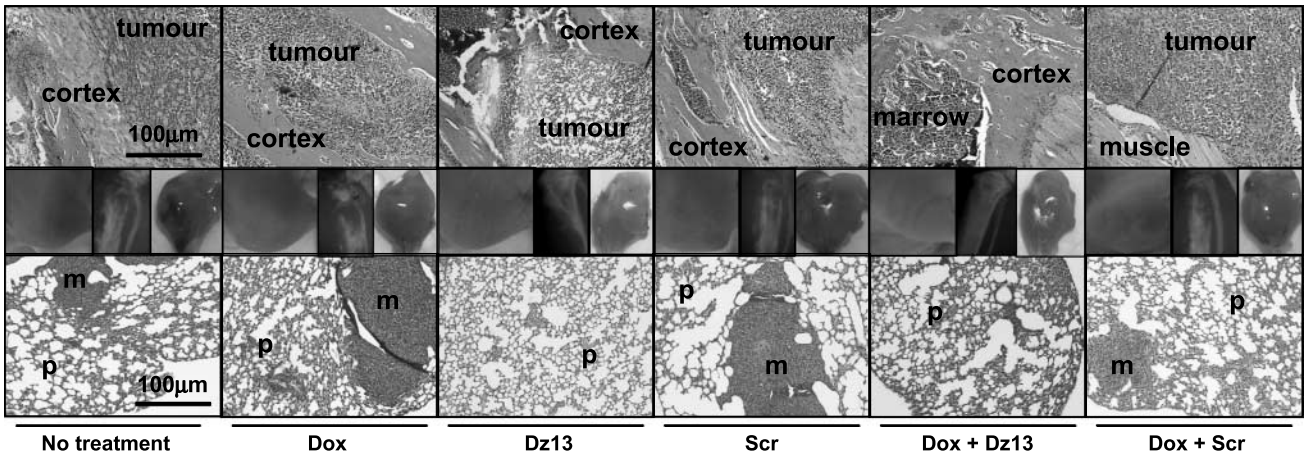

Figure 4. Antiosteolytic effects of Dz13 + doxorubicin therapy. Dz13 is efficacious against pre-established SaOS-2 growth at 250 ng Dz13/injection, especially when combined with 2 wk of doxorubicin therapy (3 mg/kg/injection/wk). *Top,* H&E of tumor section of the proximal tibia. Dz13 inhibits tumor growth, and in the combination group with doxorubicin, tumor growth is undetectable even at 5 wk post–cell injection. *Middle,* image of knee (*left*), radiograph of tibia (*middle*), and image of exposed knee (*right*). Dz13 reduces osteolysis which is further inhibited in the Dz13 + doxorubicin cohort. *Bottom,* H&E of lung sections. Dz13 inhibits the establishment of metastases in the lungs of mice.

period in which patients present to the clinic with initial complaints such as joint swelling and tenderness, and undergo initial diagnosis. We chose the intratibial route for nanoparticle administration as it is amenable to clinical exploitation in patients with osteosarcoma. As such, we administered the treatments at a distance from the palpable tumor toward the proximal end of the tibia.

This study confirmed that Dz13 is efficacious against pre-established SaOS-2 growth, especially when combined with doxorubicin therapy, with less bone damage in Dz13-treated cohorts (Fig. 3A). Combination therapy led to the best efficacy, with distinct regression of tumors in bone (Fig. 3A). However, there was a significant reduction in primary growth in mice receiving Dz13 alone, which confirms the ability of this molecule to halt osteosarcoma growth. These results are linked to c-Jun inhibition as Western blotting of Dz13-treated tumors confirmed c-Jun knockdown with no effect on housekeeping α-tubulin (Fig. 3B).

In addition to primary osteosarcoma growth, Dz13 inhibited the establishment of metastases in the lungs of mice, again complemented by co-therapy with doxorubicin to the extent of total inhibition (Fig. 3C). Once again, Dz13 was quite efficient per se in reducing secondary tumor establishment. This is the first time in which a DNAzyme has been shown to have activity against tumor spreading to a secondary site. The Scr oligonucleotide had no effect *in vivo*.

Thus, combining c-Jun down-regulation and doxorubicin leads to the regression of osteosarcoma growth in the SaOS-2 tumor model, and Dz13 sensitizes these cells to doxorubicin treatment. Figure 4 reveals a significant reduction in osteolysis when Dz13 was given alone and more so when it was combined with doxorubicin therapy. Both the tibial cortex and the growth plate cartilage of the combined therapy group of mice were more patent than in the other groups. The presence of metastases was also scarcer histologically in the co-therapy cohort of animals. The Scr oligonucleotide had no effect on metastasis.

These findings have important clinical implications because c-Jun levels are elevated with increasing grade of osteosarcoma (4, 5). The efficacy of the DNAzyme was heightened when combined with doxorubicin, a frontline chemotherapeutic for osteosarcoma. Dz13 was bioactive at concentrations as low as 100 nmol/L in culture and at 250 ng *in vivo*. These levels are comparable to small interfering RNA potency *in vitro*, and far lower than that required for small interfering RNA efficacy *in vivo* (usually in mg/kg doses given systemically; refs. 16, 17).

Part of the success in the present studies with Dz13 is due to the utility of chitosan nanoparticles in allowing controlled delivery. Quintessential to drug efficacy is efficient delivery to the target tissue, usually a major stumbling block in drug development (18). The chitosan nanoparticle, formulated using the technique of Roy and colleagues (11), provides a practical option for *in vivo* DNAzyme delivery because naked (free) DNAzymes do not enter cells efficiently and those complexed to cationic lipofection reagents are usually cytotoxic (19).

Down-regulation of c-Jun may therefore be beneficial in perturbing the growth of various types of cancers in the bone via stimulation of apoptosis in cancer cells. This may be useful clinically pending further preclinical evaluation of Dz13.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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