

**c-Jun Is Critical for the Progression of Osteosarcoma: Proof in an Orthotopic Spontaneously Metastasizing Model**

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**Abstract**

The oncogene c-Jun has been found to be up-regulated in a variety of cancers including osteosarcoma. DNA enzymes (DNAzymes) are oligonucleotides capable of specific catalysis of target mRNA. A c-Jun DNAzyme inhibited the growth and metastasis of osteosarcoma in an orthotopic spontaneously metastasizing model of the disease. c-Jun down-regulation–mediated apoptosis in osteosarcoma cells involved caspase-1, caspase-2, and caspase-8, but not the Fas/FasL pathway. Clinically, knockdown of c-Jun with DNAzymes may proffer an improved treatment outcome for these tumors originating in bone.

**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 in the prime years of their life. Osteosarcoma afflicts adolescents and young adults and is fatal unless treated. 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 of age are often regarded as unsuitable for conventional dose and intensity of treatment. Identifying the checkpoints that regulate the metastatic cascade is crucial for understanding the vulnerabilities of osteosarcoma and for isolating effector molecules that may disrupt the tumor machinery whereas preserving the integrity and function of normal tissue.

c-Jun has been found to be increased in high-grade osteosarcoma (3, 4). For osteosarcoma and other cancers afflicting bone, better management options are being explored. Hitherto, no report exists of c-Jun mRNA silencing for potential therapy of such cancers in bone. Similarly, the effects of c-Jun down-regulation on osteosarcoma cell biology have not been documented. Our article specifically addresses these deficits using DNA enzymes, or DNAzymes, gene shears capable of target-specific cleavage of mRNA (5), and have been used against a variety of gene targets to date (6). This study establishes that a DNAzyme targeting the c-Jun transcript (7, 8) is capable of inducing significant apoptosis in human cells of osteosarcoma. This is also the first demonstration of DNAzyme-mediated oncogene modulation in an orthotopic spontaneously metastasizing model of neoplasia.

**Results and Discussion**

We initially examined clinical specimens of primary osteosarcoma and used immunohistochemistry to reveal intense staining of cancer cell nuclei for c-Jun (Fig. 1A), akin to findings in an earlier study (3). Interestingly, as cells of a human line of osteosarcoma, SaOS-2, were transferred from culture into the bone, the expression of c-Jun increased significantly in both orthotopic growth and in pulmonary metastases (Fig. 1B). These results are in line with the literature in which c-Jun levels are increased in higher grade osteosarcoma (3, 4) and highlights the importance of the biological milieu on c-Jun expression.

Dz13, a DNAzyme that degrades c-Jun mRNA (7, 8) caused a dose-dependent increase in apoptosis in a panel of three human osteosarcoma cell lines (Fig. 2A). Specificity of cleavage activity in these cells was shown by Western blotting, with no effect on another closely associated transcription factor, c-fos, or on housekeeping α-tubulin expression levels (Fig. 2B). Incubation of Dz13-treated cells with a host of caspase inhibitors revealed that caspase-1, caspase-2, and caspase-8 were responsible for the apoptosis of SaOS-2 cells caused by c-Jun knockdown (Fig. 2C). However, both Fas and FasL antibodies failed to decrease apoptosis induction by Dz13 (Fig. 2C).

Although apoptosis is significantly induced by Dz13, incubation of SaOS-2 cells with this DNAzyme does not reduce the subsequent ability of these osteoblast-like surviving cells to synthesize bone mineral–like deposits in culture (Fig. 3A). The deleterious effects of the DNAzyme against normal cells resident in bone lumen (site of cancer growth in later studies) were ruled out when it was revealed that human bone marrow mononuclear cell proliferation was not inhibited by this construct (Fig. 3B). Thus, Dz13 did not perturb the normal physiologic function of these cells, ruling out a general cytotoxic effect.

Combining Dz13 with SaOS-2 cells prior to orthotopic implantation of the cells into the proximal tibiae of mice
resulted in significant inhibition of primary tumor growth (Fig. 4A). Figure 4B shows that tumors in non-Dz13 cohorts of animals were more aggressive, lysing bone cortex and establishing in the surrounding soft tissue (muscle around tibia), as well as invading more readily into the growth plate cartilage of the proximal tibiae.

In all, these data provide proof-of-principle that c-Jun down-regulation via Dz13 decreases osteosarcoma growth, in part due to the induction of apoptotic death in these cells.

This study has shown the efficacy due to c-Jun down-regulation afforded by a DNAzyme in osteosarcoma. By using this dissecting tool, we have shown the dependence on c-Jun of osteosarcoma cells for survival and tumor progression in vivo. 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 administered systemically; refs. 9, 10). Inhibition due to c-Jun knockdown was largely due to apoptosis, mediated via caspase-1, caspase-2, and caspase-8. Down-regulation of c-Jun may therefore be beneficial in perturbing the growth of osteosarcoma via stimulation of apoptosis in cancer cells.

FIGURE 1. c-Jun expression in osteosarcoma. A. Intense immunohistochemical staining of intranuclear c-Jun protein is present in primary human osteosarcoma tissue. B. c-Jun transcript is elevated in primary and secondary (lung) SaOS-2 tumors at 5 wk post–cell injection as determined by semiquantitative reverse transcription-PCR.

FIGURE 2. c-Jun down-regulation causes apoptosis in an osteosarcoma cell panel. A. Dz13-mediated dose-dependent increase in apoptosis at 24 h in osteosarcoma cells as determined by terminal nucleotidyl transferase–mediated nick end labeling assay (*, P < 0.01; **, P < 0.001; n = 4). B. Specific down-regulation of c-Jun at 96 h confirmed with Western blotting. C. Dz13-mediated induction of apoptosis in SaOS-2 cells at 12 h involves caspase-1, caspase-2, and caspase-8 but does not involve Fas/FasL (*, P < 0.001; n = 4).
Materials and Methods

Cell Culture and Cell-Based Assays

All cell lines were obtained from American Type Culture Collection and used within 10 passages. The apoptosis assay was done using the DeadEnd terminal nucleotidyl transferase–mediated nick end-labeling assay as per the manufacturer’s instructions (Promega). The apoptosis study with caspase inhibitors was done according to the manufacturer’s instructions (R&D Systems). Results were confirmed with the Annexin V-FITC Apoptosis detection kit I (BD Biosciences). The Fas/FasL study using monoclonal antibodies (Becton Dickinson) was done at an antibody concentration of 1 μg/mL.

Proliferation and bone mineral deposit formation assays were done as previously described (11). Briefly, for proliferation, 20,000 cells were seeded in 24-well plates at 37°C/5% CO2 in medium containing 10% FCS and cells trypsinised and enumerated on days 3 and 5 post-seeding using trypan blue exclusion and a hemocytometer chamber. Briefly, for the mineralized nodule formation assay, cells were seeded in 24-well plates at a density of 4,000 cells/well in complete growth medium supplemented with 1.4 mmol/L of CaCl$_2$, 10 nmol/L of dexamethasone, and 50 μg/mL of ascorbic acid. On day 3, 10 mmol/L of β-glycerophosphate was added, and on day 7, cells were fixed for 30 min with 4% p-formaldehyde before rinsing with distilled water. Nodules were stained with 5% AgNO$_3$ solution, and plates placed under UV light for 30 min. Wells were rinsed once with water, and nodules were enumerated under low-power magnification and photographed.

Transfection

Transfection with LipofectAMINE and fluorescence microscopy were done as before, as was transfection with Fugene-6 (12). Briefly, the indicated mass of oligonucleotides were complexed with the manufacturer-recommended mass of transfection reagent, and this complex was added to the cells. Cells were transfected for 24 h for the apoptosis assay, and 96 h for Western blotting.

Molecular Analyses

Immunohistochemistry and Western blots were done using Santa Cruz Biotechnology antibodies (13). Briefly, a primary rabbit anti-human antibody dilution of 1:100 (4°C, 24 h) and secondary goat anti-rabbit horseradish peroxidase antibody dilution of 1:400 (room temperature, 1 h) was used for immunohistochemistry of archival formalin-fixed/paraffin-embedded osteosarcoma specimens. Sections were blocked with goat

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serum. For the negative controls, the primary antibody step was omitted. Oligonucleotides were synthesized and prepared as before (7). Semiquantitative reverse transcription-PCR was done (11) using 5′ gagagcggaccttatggctacagta and 3′ gcccgttgctggactggattatcag primers for c-Jun mRNA amplification.

In vivo Studies

Ethics for the use of human tissue and mice were obtained from the St. Vincent’s Health, Human and Animal Ethics Committees, respectively. All animal procedures were done as previously described (11). Mice were checked daily, weighed, and tumors measured twice weekly using digital calipers. At commencement, throughout and at termination of the study, mice legs were X-rayed at 35 kV for 30 s using a cabinet system (Faxitron Corp.). Mice were euthanized using CO2 asphyxiation.

Statistical Analysis

All data were analyzed using the one-way Student’s t test with unequal variances.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Correction: Article on C-Jun and the Progression of Osteosarcoma: Proof in an Orthotopic Spontaneously Metastasizing Model

The article on C-Jun and the progression of osteosarcoma: Proof in an orthotopic spontaneously metastasizing model, in the June 2008 issue of Molecular Cancer Research contains significant similarities to an article published by the same authors in the June 2008 issue of Cancer Biology & Therapy (Downregulation of c-jun results in apoptosis-mediated anti-osteosarcoma activity in an orthotopic model (vol 7, pp 1033-1036, 2008).

Specifically, the Introductions and Abstracts of both papers are almost identical. The Results and Discussion sections and Figs. 1 and 3 are very similar, although Fig. 1 in Molecular Cancer Research uses different patient specimens than Cancer Biology & Therapy to show the levels of c-Jun. The Cancer Biology & Therapy paper characterizes and uses an in-house formulation of liposomes, whereas the paper in this journal uses a commercial transfection reagent. In Cancer Biology & Therapy the authors show medium grade OS, whereas Molecular Cancer Research shows OS (no particular grade indicated). Figure 3 in Molecular Cancer Research presents data from a different cell line, albeit one for OS, that behaves similarly to the untransformed cell line in SaOS-2. SaOS-2 cells are non-transformed, but the 143B cells discussed in the Cancer Biology & Therapy article are. These cells also behave differently in vivo. The authors also looked at a dose-dependent effect of Dz13 in Molecular Cancer Research, but only one dose in Cancer Biology & Therapy. In the Molecular Cancer Research paper, the authors exclusively examined the effects of Dz13 (the c-Jun downregulating oligonucleotide) in assays using human bone marrow cells and formation of bone mineral deposits by SaOS-2 cells.

Despite what they judge to be important differences, the authors believe that the overlap in the papers is substantial and could be confusing to readers. They regret that it occurred.

Molecular Cancer Research

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