Trk Receptor Inhibition Induces Apoptosis of Proliferating but not Quiescent Human Osteoblasts

Jacek Pinski, Ashani Weeraratna, Aarti R. Uzgare, Julia T. Arnold, Samuel R. Denmeade, and John T. Isaacs

Johns Hopkins Oncology Center, Baltimore, Maryland 21231-1000

Abstract

Prostate cancer frequently metastasizes to the skeleton, producing painful osteoblastic lesions, which are associated with significant morbidity and mortality. This bone tropism involves the bidirectional paracrine interactions between prostate cancer cells and osteoblasts. These interactions enhance prostate cancer cell survival and proliferation of osteoblasts. Therefore, agents that can induce apoptosis of prostate cancer cells and proliferating osteoblasts would be highly advantageous. Previously, we have documented that the unique survival pathway for prostate cancer cells involves a neurotrophin/Trk receptor autocrine pathway. The indocarbazole compounds, CEP-701 and CEP-751, are potent inhibitor of this Trk receptor survival signaling and thus selectively induces apoptosis of prostate cancer cells in various in vitro and in vivo models. In this study, we documented the effects of CEP-751 on the conditionally immortalized osteoblastic cell line, hFOB. In vitro, at the permissive temperature of 34°C, these cells express large T antigen, indicating their continuous proliferation, whereas at 39°C, T antigen is degraded and the cells stop proliferating without undergoing apoptosis. Trk receptors are expressed in hFOB cells, as determined both by reverse transcription-PCR and Western blots. These osteoblasts were shown to produce nerve growth factor into the medium than hFOB cells cultured at 39°C. Because the Trk/neurotrophin axis is present in both proliferating and quiescent (i.e., nonproliferating) osteoblasts, the effects of 48 h of exposure to various doses of CEP-751 on cell viability and apoptosis of hFOB cells were assessed by trypan blue exclusion assays and 4',6-diamidino-2-phenylindole nuclear staining. Cell viability and apoptosis of hFOB cells at 34°C were significantly and dose-dependently decreased compared with untreated proliferating cells. In contrast, even the highest concentration of CEP-751 (200 nM) did not affect cell viability and apoptosis of quiescent hFOB cells cultured at 39°C. This trk inhibition-induced cytotoxicity was confirmed using early-passage, proliferating normal (i.e., non-SV40-transformed) human osteoblasts, which also express Trk receptor protein. These combined results demonstrate that proliferating osteoblasts acquire a sensitivity to trk inhibition-induced apoptosis not shared with normally quiescent osteoblasts.

Introduction

Prostate carcinoma is the most common male malignancy in the United States and the second leading cause of cancer death (1). A significant event contributing to mortality and morbidity associated with prostate cancer is the development of skeletal metastases. Medical or surgical castration can only provide remission, and most patients with advanced prostate metastases to the bone will relapse (2). The growth of prostate cancer cells in bone lesions can be influenced by growth factors derived from the bone marrow, osteoblasts, or from the bone matrix products released by osteoelastic resorption (3). Previously, we documented that coculture of prostate cancer cells, LNCaP cells, with conditionally immortalized hFOB osteoblasts caused enhanced p53, p27, and p21 expression, leading to a decrease in the number of LNCaP cells entering the cell cycle (4). This osteoblast-induced enhanced G1-G2 checkpoint control affected the chemosensitivity of LNCaP cells (4). On the other hand, prostate cancer cells produce peptides with selective mitogenic activity for osteoblasts, such as urokinase-type plasminogen activator, transforming growth factor β, bone morphogenic proteins, and others, resulting in the development of very painful osteoblastic tumors in metastatic bone lesions (5–10). In this context, the role of neurotrophins and their trk receptors in osteoblasts has not been clearly determined. Our previous studies indicate that the neurotrophin/trk signal transduction axis might be involved in the regulation of a unique survival pathway in prostate cancer cells (11). Normal prostatic epithelial cells do not secrete significant amounts of neurotrophins but express trkA and p75 NTR receptors (11). Normal prostatic tissue from patients without prostate cancer contains substantial levels of NGF, which is produced in a paracrine manner by stromal cells (11). These stromal cells lack both trk and p75 NTR receptors (11). Although the NGF/trkA/p75 NTR axis is present in the normal prostate, normal prostatic epithelial cells do not depend on this axis for their survival. In contrast, malignant prostatic epithelial cells directly secrete a series of neurotrophins and express at least one of the trk receptor proteins (i.e., trk A, B, and/or C), while losing the expression of p75 NTR receptors (11). In addition, treatment with trk receptor tyrosine kinase inhibitors induces apoptosis of malignant prostate cells but not normal prostatic epithelial cells (11). In the past, mouse osteoblasts were shown to express trk receptors and to secrete significant levels of neurotrophins (12–14).

In this study, we documented the role of the neurotrophin/trk receptor axis in human hFOB osteoblasts. This cell line has been conditionally immortalized by the expression of a temperature-sensitive SV40-large T antigen, inducing their continuous proliferation at the permissive temperature of 34°C. In contrast, at 39°C, T antigen is degraded, and the cells stop proliferating and enter a quiescent state without undergoing apoptosis (15). Using this cell line, we demonstrated the efficacy of the trk receptor tyrosine kinase inhibitor, CEP-751, in apoptosis induction of proliferating but not quiescent hFOB osteoblasts. To rule out the effects of large T antigens, other than inducing continuous proliferation, these studies were repeated using early-passage proliferating cultures of normal (i.e., non-SV40-transformed) human osteoblasts.

Received 9/4/01; accepted 12/28/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by Grant 2P50-CA58236 from National Cancer Institute, NIH, and TAP Holdings, The Johns Hopkins University, in accordance with its conflict of interest policies, is managing the terms of this arrangement.

2 To whom requests for reprints should be addressed, at The Johns Hopkins University, Room 1 M44 Bunting Blaustein Building, 1650 Orleans Street, Baltimore, MD 21231. E-mail: isasscjo@jhmi.edu.

3 The abbreviations used are: trkA, high affinity tyrosine receptor kinase-A; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; p75NTR, 75-kilodalton-low affinity neurotrophin receptor; DAPI, 4',6-diamidino-2-phenylindole; BrdUrd, 5-bromo-2-deoxyuridine; NT-3, neurotrophin-3.
Materials and Methods

**Cell Culture.** The human prostate cancer epithelial cell lines CWR22Rv1 and DU-145 were maintained in culture in RPMI 1640 with 10% FCS as described previously (11). WI-38 human fetal lung fibroblasts were obtained from American Type Culture Collection and cultured in Eagle’s balanced salt solution supplemented with 10% FCS. Normal human osteoblasts (NHOst) were obtained from Clonetics (BioWhittaker, Inc., Walkersville, MD) and maintained in Osteoblast Growth Media Bullet kit obtained from Clonetics. The NHOst cells used were between passages 5 and 6. The conditionally immortalized human fetal osteoblastic cell line, hFOB, was also obtained from American Type Culture Collection and maintained in a 1:1 mixture of phenol-free DMEM/Ham’s F-12 medium, containing 10% (v/v) FBS supplemented with geneticin (300 μg/ml) at 34°C, the permissive temperature for the expression of the large T antigen. The medium was changed every 3 days. FOB cells used in all of the experiments were between passages 8 and 10. Cells from 70–80% confluent cultures were seeded into Costar 96-multwell plates (Costar, Cambridge, MA) at a density of 2.5 × 10^3 cells/well and cultured either at 34°C or 39°C. After 48 h (day 0), media were replaced with fresh media containing 25–200 nM CEP-751. CEP-751 was provided by Cephalon, Inc. (West Chester, PA). At the indicated time points, the total number of viable cells was determined by trypan blue exclusion assay. To do this, cells were trypsinized, and 100-μl samples were diluted 1:1 with 0.2% trypan blue dye. More than 200 cells were counted/sample, and the percentage of cells excluding dye was calculated.

To confirm that the loss of viability of hFOB cells was via apoptosis, cells were fixed in methanol containing 1 μg/ml DAPI (Sigma Chemical Co.) to label nuclear DNA, and the percentage of apoptotic cells was determined by evaluating nuclear morphology by epifluorescence microscopy.

For experiments with normal (i.e., non-SV40-transfected) human osteoblasts, 2.5 × 10^3 NHOst cell wells were seeded into 96-multwell plates in Osteoblast Growth Media and allowed to go untreated for 48 h at 37°C. After 48 h, the number of starting viable NHOst cells was determined on 12 wells containing 10^3 NHOst cell wells were seeded into 96-multiwell plates in Osteoblast Growth Media and allowed to go untreated for 48 h at 37°C. After 48 h, the number of starting viable cells was determined on 12 wells containing 10^3 NHOst cell wells was determined on 12 wells using the CellTiter 96 Cytotoxicity kit obtained from Promega Corp. (Madison, WI), according to the manufacturer’s protocol. At this time (i.e., time 0), remaining cells were replaced with media containing either vehicle (i.e., n = 12 wells) or 200 nM CEP-751 (i.e., n = 12 wells). After an additional 4-day treatment period, the number of viable NHOst cells was determined using the CellTiter 96 Cytotoxicity kit. The overall percentage of cytotoxicity induced by CEP-751 was calculated by dividing the numbers of viable NHOst cells after 4 days exposure to CEP-751 by the number of viable cells at time 0 and multiplying this by 100.

**Determination of Percentage of Cells in S Phase.** The incorporation of BrdUrd into genomic DNA, using cell proliferation kits obtained from Amersham (Buckinghamshire, United Kingdom), was used to identify cells in the S-phase of the cell cycle. This method uses immunocytochemical detection of BrdUrd incorporated into DNA using anti-BrdUrd antibody-coupled peroxidase staining. To obtain the percentage of cells in S-phase, 1000 cells were counted on each slide and the percentage was calculated.

**Expression of Trk Receptors in hFOB Osteoblasts.** FOB osteoblasts were harvested from in vitro culture. RNA was isolated from these samples using TRIzol reagent (Life Technologies, Inc., Bethesda, MD). cDNA was transcribed from 2 to 3 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Ambion). RT-PCR was performed using oligonucleotide primers, which were specific for human trkA as described previously (16).

For detection of Trk receptor protein, Western blots were performed on NHOst and hFOB cells (the latter grown at 34°C). To do this, cells were lysed in PBS containing 2% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1:20 dilution of complete TM containing EDTA (Roche Molecular Biochemicals, Indianapolis, IN), 5 μg/ml pepstatin, 80 mM glycerophosphate, 1 μM microcystin, 1 mM sodium orthovanadate, and 1 mM DTT. Forty μg of each cell lysate were separated by SDS-PAGE under reducing conditions. The proteins were blotted onto a polyvinylidene difluoride membrane, and probed with anti-pan trk antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Cat SC-7208) at a dilution of 1:100, as per the manufacturer’s instructions. Blots were further probed by horseradish peroxidase-linked antiumune secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) at a dilution of 1:5000, and proteins were visualized with the help of chemiluminescent reagents (ECL) from Amersham Pharmacia Biotech.

**Results**

**Neurotrophin/Trk Receptor Axis in hFOB Osteoblasts.** Previously, we have demonstrated that human prostate cancer cells express various neurotrophins (i.e., NGF, BDNF, and NT-3) and their respective cognate receptors (i.e., trk A for NGF; trk B for BDNF; trk C for NT-3; Ref. 11). To determine whether the proliferating hFOB cells (i.e., grown at 34°C) express trk A receptors, RT-PCR analysis was performed using the CWR22Rv1 and DU-145 human prostate cancer cells as positive controls and the WI-38 human lung fibroblasts as a negative control. These results demonstrate that hFOB osteoblasts express trk A receptor mRNA (235-bp band; Fig. 1A). To confirm that the trk mRNA is translated in the cells, Western blot analysis was performed. These results (Fig. 1B) demonstrated that proliferating hFOB cells express the trk receptor protein. Also, hFOB osteoblasts synthesize and secrete into the media NGF and BDNF but not NT-3 (Fig. 2). When maintained at the growth-permissive temperature of 34°C, hFOB cells undergo exponential growth with a 34-h doubling time. The continuously cycling nature of these cells at 34°C is reflected by a 29.2 ± 2.3% S-phase fraction as determined by BrdUrd incorporation. When these cells are exposed to the nonpermissive temperature of 39°C, they stop cycling continuously and entered a quiescent state. This was documented by the observation that the S-phase fraction dropped to 1.7 ± 1.1% by 48 h of exposure to 39°C. This growth cessation is associated with a >4-fold decrease in NGF and BDNF secretion by the hFOB cells at 39°C as compared with 34°C (Fig. 2). This raised the issue of whether this autocrine production of NGF and BDNF is involved in the mitogenic or survival response of these cells. To resolve this, growing hFOB cells were shifted to 39°C for 3 days to allow these cells to enter a low growth

---

**ELISA Analysis of Neurotrophin Expression in hFOB Osteoblasts.** Media conditioned for 48 h by hFOB osteoblasts cultured at 34°C or 39°C were assayed for neurotrophin levels. All starting media (i.e., not exposed to cells) were assayed and found to be consistently negative for any neurotrophin content. The neurotrophins were detected using the NGF, BDNF, or NT-3 E-Max Immunoassay kit from Promega, according to the manufacturer’s instructions and as described previously (11).

**Statistical Analysis.** The data obtained were analyzed by one-way ANOVA. Values represent the mean ± SE. P < 0.05 was considered significant. For determination of IC₅₀, linear regression was used.

---

**Fig. 1. A.** RT-PCR for the trkA receptor. Positive controls used include CWR22Rv1 and DU-145 human prostate cancer cells. Negative controls used are WI-38 human lung fibroblasts. **B.** The expected 235-bp band for trkA using the given primers. Left, molecular weights of the marker. Actin RT-PCR was also performed as a loading control. **B.** expression of trk proteins in osteoblasts. Trk protein was detected in normal human osteoblasts (NHOst) and those conditionally transformed by SV40 T antigen (hFOB grown at 34°C) by Western blotting using anti-Trk antibodies.
phase and then 10 nM NGF and 10 nM BDNF were added to the culture medium. Such addition of exogenous neurotrophins did not induce a stimulation in cell number, which remained constant over the next 4 days at 39°C.

**Effect of Trk Receptor Inhibition on Survival of Proliferating versus Quiescent Osteoblasts.** To resolve whether neurotrophin/trk signaling affects the survival of hFOB cells, the pan trk inhibitor, CEP-751, was used. This compound inhibits the tyrosine signaling activated by neurotrophins binding to all three of these trk receptors (i.e., trk A, B, and C; Refs. 16–18). Exposure to 100 nM CEP-751 decreased the viability of hFOB cells growing at 34°C (Fig. 3A). DAPI nuclear staining demonstrated that this decrease in viability was attributable to apoptosis induced by 100 nM CEP-751 (Fig. 3B). The cytotoxic response to trk inhibition by exponentially growing hFOB cells was confirmed using dose-response studies. These studies demonstrated that the IC50 (i.e., concentration required to induce apoptosis of 50% of proliferating hFOB cells) was 110 nM (Fig. 4A). In contrast, when hFOB cells were maintained in a quiescent state by culturing at 39°C, even the highest concentration of CEP-751 (i.e., 200 nM) did not increase the apoptosis of the cells (Fig. 4B).

In hFOB cells at the permissive temperature of 34°C, large T antigen induces continuous proliferation, but it also binds and thus neutralizes p53 function. This raised the issue of whether the sensitivity of hFOB cells to apoptosis induced by CEP-751 is attributable only to the fact that these cells are proliferating or that it also requires neutralized p53 function in these proliferating cells. To resolve this issue, early-passage proliferating cultures of normal human osteoblasts (NHOst) were used because they express the trkA protein (Fig. 1B) and because they are not transfected with SV40, and thus having wild-type p53 function, the NHOst cells were exposed to either vehicle or 200 nM CEP-751 for 4 days. In the vehicle-treated cultures over this 4-day period, the number of viable cells increased (P < 0.05) by 4-fold [i.e., going from 7,000 ± 545 cells (n = 12) to 28,425 ± 1,543 cells (n = 12)]. In contrast, over this same 4-day exposure to 200 nM CEP-751, the number of viable cells decreased (P < 0.05) by <50 ± 4.0% [i.e., going from 7,000 ± 545 (n = 12) to 3,495 ± 282 (n = 12)]. Because in these NHOst cells p53 function is not neutralized, these results demonstrate that the cytotoxicity of CEP-751 to proliferating human osteoblasts does not require simultaneous loss of p53 function.

**Discussion**

Metastatic prostatic cancer is a lethal disease primarily because it metastasizes to the bone, where it characteristically produces osteoblastic, not osteoclastic, lesions. These osteoblastic metastases involve
the abnormal proliferation of osteoblasts induced by their interaction with prostatic cancer cells within the bone lesions. Because of the importance of the neurotrophin/trk receptor survival pathway in prostate cancer and the potential role of neurotrophins as antiapoptotic factors in proliferating osteoblastic cells (14), interruption of the neurotrophin/trk receptor axis could represent a novel and rational approach for the treatment of bone metastases in prostate cancer patients. To test this possibility, the indocarbazole analogue CEP-751 was used because it is a potent and selective tyrosine kinase inhibitor of the neurotrophin-specific trk receptors that has demonstrated anti-cancer activity in various in vivo and in vitro models of prostate cancer (11, 16–19).

As a model system for such testing, the conditionally immortalized human osteoblastic cell line, hFOB, was used initially. This line was immortalized by transfection with a temperature-sensitive SV40 large T antigen (15). When cultured at the permissive temperature of 34°C, these cells express large T antigen and undergo continuous proliferation. In contrast, when cultured at the nonpermissive temperature of 39°C, large T antigen does not accumulate, and these cells stop proliferating, become quiescent, and express typical markers for differentiated osteoblasts such as type I collagen, osteocalcin, osteopontin, osteonectin, vitamin D receptor, parathyroid receptor, and alkaline phosphatase and produce extracellular matrix (15). In the present study, we have documented that hFOB osteoblasts express trk receptors and produce significant amounts of the neurotrophins NGF and BDNF. Our results are consistent with previous observations that a neurotrophin/trk receptor autocrine pathway is expressed in the mouse osteoblastic cell line MC3T3-E1 (14–12).

This hFOB cell line has several advantages over primary cultures of osteoblasts derived from normal human bone. The first advantage is that the hFOB cells do not become senescent, unlike normal human osteoblast (NHOst) cells, which can only be serially passage for ~10 population doublings. The second advantage is that the hFOB cells can be easily placed in or out of continuous proliferation simply by a shift in temperature. Using these hFOB cells, we have demonstrated that the trk inhibitor CEP-751 induces apoptosis of proliferating hFOB osteoblasts (i.e., at 34°C) without affecting the viability of nonproliferating hFOB osteoblasts (i.e., at 39°C). These results demonstrate that the neurotrophin/trk receptor autocrine pathway signaling is required for the survival of proliferating but not for quiescent hFOB osteoblasts. Although convenient, the use of the hFOB cells does have a limitation. At the permissive temperature of 34°C, the expressed large T antigen not only induces continuous cell proliferation, it also binds and thus neutralizes p53 protein function. This raises the issue of whether the acquired cytotoxicity response of proliferating hFOB cells to CEP-751 requires simultaneous loss of p53 function. If loss of p53 function is a critical requirement for such apoptosis of proliferating osteoblasts, this would severely limit the therapeutic efficacy of trk inhibitors against osteoblastic metastases. This is because osteoblasts in a bone metastasis of prostate cancer presumably retain their p53 wild-type function. To resolve the role of p53 function in trk inhibition-induced apoptosis, early-passage proliferating cultures of normal human osteoblasts, which express trkA receptors and but not transfected with SV40 large T antigen and thus retain p53 function, were treated with CEP-751. These studies demonstrated that trk inhibition induced the death of such p53-competent proliferating human osteoblasts. These combined results demonstrated that only proliferation and not loss of p53 function is required for osteoblasts to acquire a sensitivity for trk inhibition-induced apoptosis. These data support the rationale for the use of such trk inhibition to target the apoptotic death of both the prostatic cancer cells and the proliferating osteoblasts in bone metastases. This is particularly appealing because such apoptosis should be possible without inducing the unwanted apoptosis of quiescent osteoblasts in areas of normal bone not involved in metastatic deposits.

References
Trk Receptor Inhibition Induces Apoptosis of Proliferating but not Quiescent Human Osteoblasts

Jacek Pinski, Ashani Weeraratna, Aarti R. Uzgare, et al.