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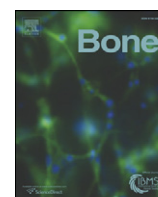

Original Full Length Article

Effect of transforming growth factor beta (TGF-β) receptor I kinase inhibitor on prostate cancer bone growth

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ABSTRACT

Transforming growth factor beta 1 (TGF-β1) has been implicated in the pathogenesis of prostate cancer (PCa) bone metastasis. In this study, we tested the antitumor efficacy of a selective TGF-β receptor I kinase inhibitor, LY2109761, in preclinical models. The effect of LY2109761 on the growth of MDA PCa 2b and PC-3 human PCa cells and primary mouse osteoblasts (PMOs) was assessed *in vitro* by measuring radiolabeled thymidine incorporation into DNA. *In vivo*, the right femurs of male SCID mice were injected with PCa cells. We monitored the tumor burden in control- and LY2109761-treated mice with MRI analysis and the PCa-induced bone response with X-ray and micro-CT analyses. Histologic changes in bone were studied by performing bone histomorphometric evaluations. PCa cells and PMOs expressed TGF-β receptor I. TGF-β1 induced pathway activation (as assessed by induced expression of p-Smad2) and inhibited cell growth in PC-3 cells and PMOs but not in MDA PCa 2b cells. LY2109761 had no effect on PCa cells but induced PMO proliferation *in vitro*. As expected, LY2109761 reversed the TGF-β1-induced pathway activation and growth inhibition in PC-3 cells and PMOs. *In vivo*, LY2109761 treatment for 6 weeks resulted in increased volume in normal bone and increased osteoblast and osteoclast parameters. In addition, LY2109761 treatment significantly inhibited the growth of MDA PCa 2b and PC-3 in the bone of SCID mice ($p<0.05$); moreover, it resulted in significantly less bone loss and change in osteoclast-associated parameters in the PC-3 tumor-bearing bones than in the untreated mice. In summary, we report for the first time that targeting TGF-β receptors with LY2109761 can control PCa bone growth while increasing the mass of normal bone. This increased bone mass in nontumorous bone may be a desirable side effect of LY2109761 treatment for men with osteopenia or osteoporosis secondary to androgen-ablation therapy, reinforcing the benefit of effectively controlling PCa growth in bone. Thus, targeting TGF-β receptor I is a valuable intervention in men with advanced PCa.

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Introduction

Prostate cancer (PCa), the second-leading cause of cancer-related death among men in the United States can be cured when it is confined to the gland, but when metastatic dissemination occurs, the prospect for cure decreases [1]. Androgen ablation is the most effective way to halt the growth of advanced PCa. However, responses are short lived, the disease then becomes castrate resistant, and only a modest survival advantage is achieved by administering chemotherapies. Bone is the primary site of castrate-resistant progression, and PCa is the only malignancy that consistently produces bone-forming metastases, although osteolysis is also an important component of the pathogenesis of the disease in bone [1]. The unique tropism of PCa cells for bone suggests that specific biologic interactions occur between those cells and the bone environment and that these interactions contribute to the lethal progression of the disease. To date, there is no effective treatment for bone metastases. One added burden for these patients is that androgen-ablation therapy is one of the causes of cancer treatment-induced bone loss, which increases the incidence of bone complications [2]. Thus, to reduce the suffering and prolong the lives of PCa patients, the development of effective therapies for the treatment and prevention of bone metastasis is urgently needed.

Abbreviations: PCa, prostate cancer; TGF-β1, transforming growth factor beta 1; TGF-β RI, TGF-β receptor type I; PMOs, primary mouse osteoblasts; rhTGF-β1, recombinant human TGF-β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BV, bone volume.

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Previous studies identified the plasma concentration of transforming growth factor beta 1 (TGF-β1) as a predictor of PCa progression and metastasis development [3–6]. TGF-β1 is a pleiotropic growth factor that regulates cellular proliferation, chemotaxis, differentiation, immune response, and angiogenesis [7,8]. Production of TGF-β by PCa-associated stroma has been shown to increase the growth and invasiveness of prostate epithelial cells [9]. Further, TGF-β was recently shown to favor osteoblastic bone metastases in experimental systems [10]. Bone is one of the most abundant reservoirs of TGF-β1, which can be released from the bone matrix during bone remodeling after PCa cells migrate to and grow there [11]. Thus, TGF-β is a candidate target for therapy of advanced PCa.

In humans, three isoforms of TGF-β have been described: TGF-β1, TGF-β2, and TGF-β3. Active TGF-β signals through a transmembrane receptor serine–threonine complex that comprises types I and II receptor kinases [12]. Binding of TGF-β1 to the type II receptor leads to the formation of a heterodimeric complex with the type I receptor, which is then phosphorylated. The receptor-associated Smads, Smad2 and Smad3, are subsequently recruited to the activated receptor I complex and are phosphorylated at the carboxyl terminus by the type I receptor. Phosphorylated Smad2/3 interacts with the co-Smad, Smad4, translocates to the nucleus, binds to specific DNA sequences, and recruits co-activators or co-repressors to regulate the transcription of TGF-β target genes [13]. Efforts in targeted drug discovery have thus led to the development of TGF-β receptor type I (TGF-β RI) kinase inhibitors [14].

In this study, we tested the antitumor efficacy of LY2109761, a new selective inhibitor of TGF-β1 RI kinases, on the growth of PCa cells in bone. We assessed its effects in two PCa cell lines that represent the osteoblastic and osteolytic components that are always present in bone metastases. Our findings support the development of therapies targeting TGF-β1 for advanced PCa.

Materials and methods

Cell lines and cultures

The human cell line MDA PCa 2b, a well-established osteoblastic PCa model developed in our laboratory [15], was propagated in BRFF-HPC1 medium (Athena Enzyme Systems, Baltimore, MD) with 20% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO). The other human cell line we used, PC-3, an osteolytic PCa model, was purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% FBS. Primary mouse osteoblasts (PMOs) were isolated from the calvaria of CD1 mouse pups as previously described [16]. All cells were incubated at 37 °C in 95% air and 5% CO_2.

TGF-β1 protein levels in conditioned medium

MDA PCa 2b and PC-3 cells and the PMOs were grown with complete growth medium in six-well plates. When the cells reached 85%–95% confluence, the medium was changed to serum free. Twenty-four-hour conditioned medium was collected, and the TGF-β1 concentration was measured by using a TGF-β1 ELISA kit (Enzo Life Sciences, Inc., Farmingdale, NY) and following the manufacturer's instructions. Measurements were performed in three biological replicates.

TGF-β RI kinase inhibitor

The TGF-β RI kinase inhibitor LY2109761 was synthesized and generously provided by Lilly Research Laboratories (Eli Lilly and Company, Indianapolis, IN). Its structure is shown in Fig. 1a. A stock solution of 5 mM LY2109761 was prepared in 100% DMSO and kept at −20 °C.

Mitogenic cell-proliferation assay

The human PCa cell lines MDA PCa 2b and PC-3 and the PMOs were seeded in six-well plates at densities of 4×10^5, 1×10^5, and 5×10^4 cells per well, respectively, so that they reached 60%–70% confluence after 72 h. At that time, fresh medium containing the indicated amounts of recombinant human TGF-β1 (rhTGF-β1; R&D Systems, Inc., Minneapolis, MN), LY2109761, or rhTGF-β1 + LY 2109761 was added. After 24 h of treatment, cell proliferation was assessed by incorporating [^{3}H]thymidine (NEN Life Science Products, Inc., Boston, MA) into the cells' DNA; the labeled thymidine was added for the final 3 h of culturing, and its degree of incorporation was measured as previously described [17].

Co-culturing of PMOs and human PCa cells

The PMOs were co-cultured with the PCa cells in a bicompartmental system in which two cell types share medium but are not in physical contact [16]. For controls, we used untreated PMOs and PCa cells, each growing alone in alpha-MEM with 2% FBS (Sigma-Aldrich). Culturing and co-culturing were performed with both the control cells and the cells treated as indicated. After 24 h of co-culturing, the numbers of PMOs and PCa cells were estimated by using the mitogenic assay described above.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA extracted from the cultured cells was treated with DNase I (Invitrogen), and RT was performed by using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was then amplified by PCR with gene-specific primers in standard reaction conditions, resulting in a 273-bp product. The primers for TGF-β RI were purchased from R&D Systems (cat. no. RDP-131). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The PCR products were resolved on 2% agarose gels.

Western blot analysis

Proteins extracted from MDA PCa 2b, PC-3, and PMO cell lysates were loaded into 4%–20% Tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes (Novex, San Diego, CA). TGF-β RI was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) after we incubated the membranes with anti–TGF-β RI antibody (R&D Systems) and then with the corresponding secondary antibodies. For detection of total and phosphorylated Smad2 (T-Smad2 and p-Smad2), cells were first grown to 70% confluence and then serum starved for 3 h. Next, we added rhTGF-β1 (2 ng/mL) with and without LY2109761 for an additional 24 h of incubation. T-Smad2 and p-Smad2 were detected by using mouse anti–T-Smad2 (BD Biosciences, Sparks, MD) and rabbit anti–p-Smad2 (Lilly Research Laboratories) primary antibodies, followed by the corresponding secondary antibodies.

In vivo PCa intrabone mouse models treated with LY2109761

Male SCID mice were obtained from Charles River Laboratories (Wilmington, MA) and housed in a certified specific pathogen-free facility. All animal experiments were conducted in accordance with accepted standards of humane animal care and were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

To generate the intrabone MDA PCa 2b PCa tumors, we injected 3 μL of medium containing 3×10^5 of the cells into the right femurs of 25 male SCID mice, as previously reported [16]. Four weeks after the cell injections, we determined tumor volumes in the femurs by

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