Neoplasia • Vol. 7, No. 7, July 2005, pp. 631–637

www.neoplasia.com

Endothelin-1 Inhibits Apoptosis in Prostate Cancer[1]

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Abstract

Endothelin-1 (ET-1), produced by the prostate epithelia, likely plays an important role in the progression of prostate cancer. ET-1 can bind two receptor subtypes; generally, binding of the endothelin receptor A (ET_A) induces a survival pathway, whereas binding of the endothelin receptor B (ET_B) mediates clearance of circulating ET-1 as well as promotes apoptosis. In prostate carcinoma, hypermethylation of the ET_B promoter results in repression of ET_B expression, thereby eliminating the negative growth response that ET-1 binding elicits through this receptor. Therefore, activation of ET_A exclusively provides a pathway for survival advantage. Our current studies examine the mechanisms by which activation of the ET_A may allow growth/survival. ET-1 treatment of prostate tumor cells significantly decreased paclitaxel-induced apoptosis through activation of the ET_A subtype. The anti-apoptotic effects of ET-1 are mediated, at least in part, through the Bcl-2 family. Although no significant changes in Bcl-2 expression occurred with ET-1 treatment, the pro-apoptotic family members Bad, Bax, and Bak all decreased significantly. Further analysis of the survival pathway demonstrated that phosphorylation of Akt occurs with ET-1 treatment in a time- and dose-dependent manner through phosphatidyinositol 3-kinase activation. These data support the combination of ET_A antagonists and apoptosis-inducing therapies for prostate cancer treatment.

Neoplasia **7,** 631–637

Keywords: Endothelin-1, apoptosis, prostate cancer, endothelin receptors, Akt.

Introduction

Prostate cancer is characterized by low rates of cell proliferation coupled with diminished rates of cell death [1]. This pattern has made prostate cancer among the most resistant of malignancies to cytotoxic chemotherapeutic agents. Furthermore, the cornerstone of the management of advanced prostate cancer, androgen deprivation therapy, relies on the effective induction of apoptotic pathways. The emergence of androgen refractory prostate cancer, which leads to the lethal form of the disease, indicates that these cells likely have developed survival mechanisms to escape death.

The potent vasoconstrictor endothelin-1 (ET-1) has been implicated in prostate cancer disease progression [2–4]. ET-1 expression occurs in almost every human prostate cancer tissue studied [4,5]. Moreover, patients with metastatic prostate cancer have elevated levels of plasma ET-1 compared with patients with organ-confined cancer as well as healthy individuals [4]. ET-1 binds to two receptor subtypes. Activation of the endothelin receptor A (ET_A) can lead to induction of a survival pathway, whereas activation of the endothelin receptor B (ET_B) can result in clearance of circulating ET-1 as well as in stimulation of apoptosis. However, the response to the binding of either receptor remains cell type–dependent. In prostate cancer, the expression of the endothelin receptors, ET_A and ET_B, is altered compared to the pattern seen in normal prostatic tissues [6,7]. The ET_B, predominant on benign prostatic epithelial cells, has a much lower expression on prostate cancer cells, owing, at least in part, to frequent hypermethylation of the ET_B gene, *EDNRB* [8]. Increased ET-1 expression, coupled with the increased ET_A expression that occurs with higher prostate tumor stage and grade, may produce a survival advantage for the prostate cancer cells. Indeed, in a phase II clinical trial of the ET_A antagonist, atrasentan, there was a significant delay in time to disease progression compared to placebo in men with hormone refractory disease [9,10].

In studies of endothelial and stromal cell populations, ET-1 acting through the ET_A inhibited apoptosis induced by a cytotoxic agent [11]. Given that endothelin receptor expression in prostate cancer favors the ET_A and the compelling results from the atrasentan clinical trials, it is our hypothesis that ET-1 can act as a survival factor for prostate cancer. Therefore, we studied ET-promoted survival in prostate cancer, and demonstrated that ET-1, acting through ET_A and the phosphatidyinositol 3-kinase (PI3-kinase)/Akt pathway, inhibited paclitaxel-induced apoptosis.

Materials and Methods

Cell Lines

Prostate cell lines DU145, PC3, LNCaP (American Type Culture Collection, Manassas, VA) PPC-1 [12], and TSU [13] were grown in RPMI 1640, and LAPC4 (gift from Dr. Robert

Abbreviations: ABT-627, atrasentan; ET, endothelin; ET_A, endothelin receptor A; ET_B, endothelin receptor B; *EDNRB*, endothelin receptor B gene; PI3-kinase, phosphatidyinositol 3-kinase

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[1]Support for the data analysis was provided by the Pharsight Academic License Program (for the WinNonlin software). This work was supported, in part, by the Mellam Family Foundation.
Received 16 December 2004; Revised 8 February 2005; Accepted 9 February 2005.

DOI 10.1593/neo.04787

Reiter, UCLA, Los Angeles, CA) cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS and penicillin/streptomycin.

Apoptosis Assay

Prostate cell lines were pretreated with 1.0 μM ABT-627 or A127722 (ET_A antagonists; Abbott Laboratories, Abbott Park, IL), 1.0 μM RES-701 (ET_B antagonist; American Peptide, Sunneyvale, CA), or A-192621 (ET_B antagonist; Abbott Laboratories), 200 nM wortmannin (Sigma Chemical Co., St. Louis, MO), 10 μM LY294002 (Sigma Chemical Co.), or 20 μM PD98059 (Calbiochem, La Jolla, CA) for 1 hour prior to ET-1 treatment in serum-free medium. ET-1 (100 nM) was added followed by 100 nM paclitaxel (Bristol-Myers Squibb, Princeton, NJ) or an antibody to fas (10 ng/ml; Signal Transduction Laboratories, Lexington, KY) and the cells were incubated for 4 to 24 hours. The cells were scraped from the plates and pelleted by centrifugation (200*g*). The cell pellet was then lysed using apoptosis lysis reagent and centrifuged at 10,000*g* for 10 minutes. A spectrophotometric ELISA-based assay was used to quantify histone-associated DNA fragments present in the cell lysates according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN).

Immunoblot Analysis

Prostate cells were plated in 100-mm dishes and treated with ET-1 (0.1 nM–1.0 mM) for 5 minutes to 24 hours in the presence or absence of: 100 nM ABT-627 or A127722; PI3-kinase inhibitors, 200 nM wortmannin and 10 μM LY294002; MEK inhibitor, 20 μM PD98059; and p70 S6 kinase inhibitor, 5 nM rapamycin. The cells were lysed in 20 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol, 1% Triton X-100, and 135 mM NaCl with fresh protease inhibitors. The proteins (40 μg) were separated by 10% or 12% SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked and incubated in primary antibody [phospho-Akt, Akt, phospho-p44/42 MAP kinase, BAD, 1:1000 dilution (NEB, Beverly, MA); Bcl-2, Bcl_{XL} phospho-Raf, 1:500 dilution (Transduction Laboratories); Bax, Bak, caspase3, caspase 9, 1:200 dilution (Oncogene, Boston, MA)] in TBST (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C. After washing, the blots were incubated in secondary antibody (goat anti-mouse or goat anti-rabbit HRP, 1:20,000; Roche Diagnostics) for 1 hour and washed in TBST. Immunoreactive proteins were visualized by ECL (Amersham, Piscataway, NJ) and Kodak (Rochester, NY) XAR film. Densitometry was performed using a Molecular Imager FX phosphorimaging system with Quantity One 4.1 quantitation software (Bio-Rad, Hercules, CA). Band densities were normalized to corresponding β-actin bands. EC_{50} for ET-1–induced Akt phosphorylation was determined from the nonlinear regression analysis of the densitometry data using WinNonlin Pro Academic v4.0.1 (Mountain View, CA).

Annexin V Staining and Flow Cytometry

PPC-1 cells were grown in 100-mm tissue culture dishes (Becton Dickinson, San Jose, CA). When cells were subconfluent, growth media were removed, cells were washed with RPMI, and serum-free media were added. Six groups were formed according to administered treatment: 1) serum-free media only; 2) ET-1 (10^{-7} M); 3) paclitaxel (10^{-7} M); 4) ET-1 for 1 hour followed by paclitaxel; 5) ABT-627 (10^{-6} M), followed by ET-1 for 1 hour, and then followed by paclitaxel; 6) A192621 (10^{-6} M), followed by ET-1 for 1 hour, and then followed by paclitaxel. Each group was treated for a total of 4 hours. In addition to paclitaxel, these studies were carried out using a fas antibody (10 ng/ml for 4 hours) to induce cell death with and without ET-1 treatment. Cells were collected in 24 hours and evaluated for apoptosis by Annexin V staining.

For flow cytometry using the Annexin V assay, cells were collected and double-stained with fluorescein isothiocyanate–conjugated Annexin V (PharMingen, San Diego, CA) and propidium iodide (PI) (Sigma Chemical Co.). Cells were counted and 10^5 cells for each condition (in 100 μl of Annexin V binding buffer) were placed in 5-ml round-bottom tubes (Becton Dickinson). Each condition was done in duplicate. Annexin V was added according to the manufacturer's recommendations. PI was used at a final concentration of 5 μg/ml. Annexin V–positive cells were considered apoptotic and their percentage of the total number of cells was calculated. Cells taking up vital dye PI were considered dead. Samples of 10,000 cells were analyzed by FACScan flow cytometer with LYSYS II software package (Becton Dickinson).

Results

ET-1 Inhibits Prostate Cancer Apoptosis In Vitro

Treatment of the prostate cancer cell PPC-1 with paclitaxel induces apoptosis as demonstrated by an ELISA-based apoptosis assay as well as by flow cytometric analysis of Annexin V staining (Figures 1 and 2). In the presence of ET-1, however, there is a significant reduction in the amount of cell death. The addition of the selective ET_A antagonist, A-127722, or its racemic compound, ABT-627, reversed the ability of ET-1 to inhibit apoptosis. The selective ET_B antagonist, A192621, did not significantly affect the ability of ET-1 to inhibit apoptosis (Figure 2). The cells treated with A192621 + ET-1 + paclitaxel showed a slightly higher rate of apoptosis (31.63%) compared with ET-1 + paclitaxel (23.44%); however, this increase was not statistically significant ($P = .558$) and may be the result of partial nonspecific blockade of ET_A by using 10 μM ET_B antagonist in these studies. A similar pattern was seen in the other prostate cancer cell lines tested, all of which have no detectable ET_B expression due to *EDNRB* methylation. The anti-apoptotic actions of ET-1 were also blocked by LY294002, an inhibitor of the PI-3 kinase pathway, but not by PD98059, an inhibitor of the MEK1 pathway (Figure 1). To confirm these results, prostate cancer cell lines were exposed to agonistic antibodies to fas, a potent inducer of apoptosis [14,15]. The addition of ET-1 significantly inhibited fas-induced apoptosis, whereas ET_A antagonists blocked this effect (data not shown).