Advances in Brief

Endothelin-1 Production and Decreased Endothelin B Receptor Expression in Advanced Prostate Cancer

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Abstract

The potent vasoconstrictor endothelin-1 (ET-1) is at its highest concentration in the normal human ejaculate and is associated with the progression of metastatic prostate cancer. ET-1 protein expression is detected in situ in 14 of 14 primary cancers and 14 of 16 metastatic sites of human prostatic carcinoma. Exogenous ET-1 induces prostate cancer proliferation directly and enhances the mitogenic effects of insulin-like growth factor I, insulin-like growth factor II, platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor in serum-free conditions in vitro. The ETA-selective receptor antagonist A-127722 inhibits ET-1-stimulated growth, but the ETB-selective receptor antagonist BQ-788 does not. ET-3, an ETB-selective agonist, also had no effect on prostate cancer growth. No specific ETB-binding sites could be demonstrated in any established human prostate cancer cell line tested, and ETB mRNA, detected by reverse transcription PCR, was reduced. The predominance of ETB binding on human benign prostatic epithelial tissue is not present in metastatic prostate cancer by autoradiography. In human prostate cancer progression to metastases, ET-1 and ETB expression are retained, whereas ETA receptor expression is reduced.

Introduction

The lethal phenotype of androgen-independent human prostate cancer will claim an estimated 40,000 lives in the United States in 1995 (1). In an attempt to understand the cytokines involved in the lethal prostate cancer phenotype better, we identified ET-1 as an important factor in the pathophysiology of prostate cancer tumor biology previously (2). For example, plasma-immunoreactive ET concentrations are elevated abnormally in 58% of men with metastatic tumor burdens; every human prostate cancer cell line tested produces ET-1 at the levels of mRNA and protein, and exogenous ET-1 is a prostate cancer mitogen in vitro and enhances new bone formation in vivo. The ET family consists of three isoforms and four homologous cardiotoxic peptides (sarafotoxins) isolated from the venom of Atractaspis engaddensis; all have 21 amino acids and four conserved cysteine residues. ET-1 is the best characterized member of the ET family, and has potent synergy with many of the same peptide growth factors implicated in advanced prostate cancer progression (reviewed in Refs. 4–6).

This study tested the hypotheses that ET-1 expression in the androgen-refractory and lethal prostate cancer phenotype acts to enhance the effects of other autocrine peptide growth factors in prostate cancer proliferation through specific ET receptors, which can be targeted for therapy. Therefore, we tested whether ET-1 is produced in many primary and metastatic human prostate cancer tumors, evaluated the receptor pathways mediating the mitogenic and synergistic effects of ET-1 on prostate cancer proliferation in vitro, and quantified specific 125I-labeled ET-1 binding sites in benign, malignant, and metastatic human prostate tissues.

Materials and Methods

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (6-μm thick) were incubated with normal prostate tissue specimens (10 primary lesions), 16 prostate cancer metastases (7 bone, 2 liver, 2 adrenal, 2 lymph node, 1 lung, 1 splenic, and 1 subdural), and 4 primary prostate tumors from the autopsies of five men who died of prostate cancer. These sections were stained with a monoclonal (mouse) anti-ET-1 antibody (IgG1; clone TR.ET.48.5; Affinity Bioreagents, Neshanic Station, NJ) using an immunoperoxidase method (Vectastain avidin-biotin complex kit; Vector Laboratories, Inc., Burlingame, CA). To assure specificity, the primary antibody was: (a) preincubated with 4 nmol ET-1 (Sigma Chemical Co., St. Louis, MO) for immunoadsorption (negative control in all cases), (b) omitted, or (c) substituted with nonspecific mouse IgG. Immunostaining intensity was graded by two blinded observers.

Mitogenic Assays. Cell cultures and mitogenic assays were performed as described previously (2) with the following minor modifications. Five × 10^5–2 × 10^6 human prostate cancer cells were allowed to adhere in 96-well plates, washed, and exposed to RPMI 1640 media alone (negative control), RPMI 1640 supplemented with exogenous ET-1 or ET-3 (Sigma) or the ETA-selective [A-127722 (K1: ETA, 69 μM; ETB, 114 μM); Ref. 7] and ETB-selective [BQ-788 (50% inhibitory concentration: ETA, 1000 μM; ETB, 1 nM); Ref. 8] antagonists (10 pm to 100 nM). To determine whether ET-1 (10 nM) could enhance the effects of various growth factors on prostate cancer proliferation, the following human growth factors (10 ng) were screened in serum-free conditions in five human prostate cancer cell lines: EGF, basic fibroblast growth factor, IGF-I, IGF-II, and PDGF (R&D Systems, Inc., Minneapolis, MN). All conditions were run in series of four to six wells with a minimum of three experiments per cell line.

Cell Death Analysis. To determine whether ET-1 could inhibit apoptosis, quantification of DNA fragmentation indicative of apoptosis was performed in the prostate cancer cell lines following exposure to ET-1. Briefly, prostate cancer cell lines were exposed to ET-1 (1–100 nM) in RPMI 1640 or to RPMI 1640 alone for 2, 4, or 6 days. All cells were harvested (adherent and floating); half of the sample was subjected to centrifugation, precipitation, and quantification of high- and low-molecular-weight DNA as described previously (9, 10), and the rest was subjected to flow cytometric analysis (FACStar Plus; Becton Dickinson, Mountain View, CA) of apoptotic and nonapoptotic nuclei using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Dexamethasone (1 μM)-treated human peripheral blood lymphocytes, prostate cancer cell lines treated with phenylbutyrate (a known inducer of human prostate cancer apoptosis; Ref. 10), and terminal deoxynucleotidyltransferase enzyme exclusion acted as the positive and negative controls, respectively.

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3 The abbreviations used are: ET, endothelin; ET-1, endothelin-1; ETA, ET A receptor; ETB, ET B receptor; EGF, epidermal growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; Kd, dissociation constant; Bmax, maximal binding capacity; RT, reverse transcription; NO, nitric oxide.
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**125I-labeled ET-1-binding Studies.** Binding sites for ET-1 were evaluated using saturation and competitive binding techniques described previously for prostatic tissues (11) and cultured cells in situ (12, 13). Briefly, saturation studies were performed in triplicate at various concentrations (0.0625–2.0 nM) of 125I-labeled ET-1 (2000 Ci/mmoll; Amersham, Arlington Heights, IL). Nonspecific binding was determined in parallel assays (three times) in the presence of a final concentration of 1 μM nonradioactive ET-1. The apparent Kₐ and Bmax under these in situ conditions were calculated by Scatchard analysis of binding data. Competition binding studies were performed in the presence of 0.1 nM (final concentration) 125I-labeled ET-1, varying concentrations of unlabeled ET-1, ETA-selective (BQ-123 and A-127722), or ETB selective (sarafotoxin S6C and BQ-788) ligands (5 × 10⁻¹³–5 × 10⁻⁵) in HBSS containing 0.1% BSA. In both studies, conditions were added to confluent, washed monolayers of prostate cancer cells cultured in 24-well plates (Falcon, Oxnard, CA) or to 1 × 10⁶ cells in 1.5-ml Eppendorf tubes for poorly adherent prostate cancer cell lines. After 60 min of incubation at 25°C, cells were extensively washed and solubilized, and cell-bound radioactivity was determined on a gamma counter (Wallac Wizard 1470, Turku, Finland) with 78% efficiency.

**RT-PCR.** Total RNA was isolated from prostate cancer cell lines, an immortalized human neonatal prostatic epithelial line (267-B-1), a benign prostatic hyperplasia epithelial line (BPH-1), and human placenta by the guanidium thiocyanate-phenol-chloroform extraction method. The RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer/Cetus, Norwalk, CT) according to the manufacturers’ instructions. Briefly, 1 μg total RNA was reversed transcribed using the random hexamer primers. The cDNA was then amplified for 25 or 35 cycles using an intron-spanning, specific ETA or ETB set of primers. Each cycle consisted of 95°C for 1 min (denature), 60°C for 1 min (anneal), and 72°C for 2 min (extend). The primer sets (ETA, 5'-GATCACAATGAC1TGCTAC1GATC-3' and 5'-CTCAAGCTGCCATCTCTTCTGC-3'; ETB, 5'-CTAAAGGAGACAGGACGGCAGGATC-3') were based on published sequences of human ETA and ETB (14, 15). The RT-PCR samples were evaluated by agarose gel electrophoresis and Southern blot analysis, and the PCR products were cloned and sequenced to confirm product specificity.

**Autoradiographic Localization of 125I-labeled ET-1-binding Sites.** Autoradiography was performed as described previously (16). Briefly, five human prostate tissues containing benign and malignant epitheliums were obtained from radical prostatectomy specimens, and multiple prostate cancer liver metastases obtained at autopsy were frozen immediately and stored at −80°C. Six consecutive sections (20 μm) were incubated in one of the following solutions: total ET-1 binding [0.1 nM 125I-labeled ET-1 (label)], nonspecific binding (label + 1 μM ET-1), total ETB binding (label + 1 μM BQ-123 or A-127722), and total ETA binding (label + 0.1 μM sarafotoxin S6C or 1 μM BQ-788). Sections were exposed to Hyperfilm-1H with 20-μm-thick autoradiographic 125I Micro-Scales standards (Amersham). The autoradiographs were analyzed quantitatively by converting the median pixel density (Photoshop 3.0; Adobe Systems, Inc., Mountain View, CA) of sequential sections at the same histological location to radioactivity (nCi/mg), using a curve generated from the median pixel density of the calibrated standards. Specific radioactive densities were determined by subtracting nonspecific from total radioactive densities.

**Results and Discussion**

**Immunohistochemistry.** This study found that specific ET-1 immunoreactivity was present in human prostate cancer in vivo, independent of the clinical stage or hormonal milieu, as demonstrated by immunohistochemical staining of multiple prostate cancer lesions (Fig. 1). Specific cytoplasmic ET-1 immunostaining was present in every primary prostate cancer specimen (14 of 14) studied and in 14 of 16 lesions obtained at autopsies from 5 men who died of widely metastatic prostate cancer. This conclusion is supported by negative immunoreactivity following immunoperoxidase, omission of the primary antibody, or substitution of nonspecific mouse IgG. Endothelial

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**Fig. 1.** Representative examples of ET-1 expression in human prostate cancer. A, primary prostate cancer, with specific ET-1 staining of the neoplastic cells. B, loss of ET-1 staining in a section flanking A, following immunoperoxidase staining of the anti-ET-1 monoclonal antibody with ET-1. C–F, tissue obtained at autopsy from men dying of androgen-refractory prostate cancer. C, intense, specific ET-1 immunostaining of the neoplastic cells in primary prostate cancer; D, prostate cancer metastatic to lymph node; E, bone, with a typical osteoblastic response; and F, lung, A–F, × 40. Similar ET-1 staining was observed in every prostate cancer studied in 15 different men.
cells were a consistent positive internal control. These findings are consistent with the elevated levels of plasma-immunoreactive ET found in the majority of men with advanced metastatic disease and support our hypothesis that prostate cancer cell secretion may cause this increase (2). Interestingly, despite a decreased optimum antibody dilution (primary prostate cancer, 1:500; autopsy lesions, 1:1000) ET-1 immunostaining was generally more intense in the autopsy specimens; considering antibody dilution and staining intensity as rough indicators of peptide concentrations, levels of ET-1 are evidently higher in advanced prostate cancer specimens than in primary lesions.

ET-1-induced Prostate Cancer Cell Proliferation. In 4-day, serum-free growth assays, exogenous ET-1 induced proliferation in every established human prostate cancer cell line tested; the ratio of cell number to control (no ET-1 added) was significant (range, 10–50%) in each concentration (10 pM–100 nM) of ET-1 tested (data not shown). These data are in agreement with our previous observations conducted in the presence of insulin in the basal media (2), which can act as both a mitogenic factor and an inducer of ET-1 secretion (17). The ET-1-induced prostate cancer cell proliferation was blocked by the addition of the selective ET<sub>A</sub> antagonist A-127722 (Fig. 2, A and B) but not by the selective ET<sub>B</sub> antagonist BQ-788. These data support the hypothesis that the effects of ET-1 are mediated through ET<sub>A</sub> only. The predominant ET receptor on benign prostatic epithelium has been identified as ET<sub>B</sub> (16), yet an ET<sub>B</sub>-specific ligand, ET-3, which has a more than 3-log greater affinity for ET<sub>B</sub>, had no mitogenic effect on prostate cancer growth (data not shown).

ET-1 Does Not Decrease Prostate Cancer Cell Apoptosis. Low-molecular-weight DNA fragmentation is a marker of the endonuclease activity accompanying apoptosis. ET-1, in the dose range tested (1–100 nM), did not decrease the amount of DNA fragmentation when compared with cells grown in RPMI 1640 alone. This observation was confirmed by flow cytometric analysis of digoxigenin-labeled genomic DNA; ET-1-treated cells showed no decrease in apoptosis (data not shown). Taken together with the mitogenic data, ET-1 seems to cause an increase of prostate cancer cell number by inducing mitosis rather than decreasing apoptotic death; these modest growth effects in vitro may have significant consequences in the 30–50-year natural history of prostate cancer.

ET-1 Potentiates Multiple Growth Factors in Prostate Cancer Cell Proliferation. The proliferative effects of adding exogenous ET-1 (10 nM) to a polypeptide growth factor (10 ng) were tested in five prostate cancer cell lines. Synergistic prostate cancer cell growth was observed in 7 of 25 tested combinations, as demonstrated by a significant ($P < 0.05$) increase in cell number greater than the additive effects of ET-1 and the growth factor alone (Fig. 2C). Additive proliferative effects were observed in another 9 of 25 combinations, including EGF (LNCaP, PPC-1, and TSU), basic fibroblast growth factor (LNCaP), IGF-I (PC3 and PPC-1), IGF-II (PC3 and PPC-1), and PDGF (PC3). No synergistic growth effects were observed with EGF. The most significant increases were observed in the human prostate cancer cell line TSU and with the IGF-ET-1 combi-

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Fig. 2. A, ET-1-stimulated proliferation of PPC-1 cells grown in RPMI 1640 (vehicle; O) is inhibited by A-127722 (10 nM; O), a selective ET<sub>A</sub> antagonist. B, proliferation of PPC-1 cells grown in 1 nM ET-1 (O) is dose-dependently inhibited by A-127722 in the media. These data are representative of every prostate cancer cell line tested. Points, mean of quadruplicate determinations. C, synergistic growth effects of ET-1 (10 nM) added to a polypeptide growth factor (10 ng) (solid bar) compared with growth factor alone (open bar) and ET-1 alone (hatched bar) in the prostate cancer cell line TSU. The combinations (solid bar) represent a significant increase ($P < 0.05$, t test) over the additive effects of ET-1 and growth factor alone. Bar, mean of three experiments done in quadruplicate (±SE) as a percentage of basal growth (RPMI 1640 alone). Synergy was also observed between ET-1 and IGF-I, IGF-II, and PDGF in the human prostate cancer cell line DU145 (data not shown).

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ET Receptors: Binding Sites and mRNA in Prostate Cancer Cell Lines. The ET receptor saturation studies performed in situ demonstrated that the binding of $^{125}$I-labeled ET-1 was saturable and of high affinity in four of five human prostate cancer cell lines. Under the experimental conditions described, very little specific binding could be demonstrated in the prostate cancer cell line LNCaP; therefore, no definitive conclusions could be made about its $K_d$ or $B_{max}$. In the other cell lines, the approximate $K_d$s and $B_{max}$s ranged from 0.08 (TSU) to 0.62 (PPC-1) nM and from 500 (PC3) to 23,700 (PPC-1) binding sites/cell, respectively, and are consistent with those reported for prostate and a number of other tissues (11–13; reviewed in Ref. 18). Competition binding experiments using ET$_A$-selective (BQ-123 and A-127722) and ET$_B$-selective (sarafotoxin S6C and BQ-788) receptor ligands demonstrated specific ET$_A$ binding only (Fig. 3A). No ET$_B$ binding was demonstrated in any of the human prostate cancer cell lines tested.

RT-PCR of total mRNA from human prostate cancer cell lines, immortalized normal human neonatal prostatic epithelial and benign prostatic hyperplasia epithelial lines, and human placenta (positive controls for ET$_A$ and ET$_B$; Ref. 19) revealed a PCR product for both ET$_A$ and ET$_B$ after amplification for 35 cycles (Fig. 3B). The expected 427- and 547-bp PCR products were observed for the ET$_A$ and ET$_B$ primer sets, respectively, and the identity of these products was confirmed by Southern hybridization and sequence analysis. After amplification for 25 cycles, only an ET$_B$ product was detected in the prostate cancer cell lines (data not shown). To assure the quality of the cDNA for PCR, human glyceraldehyde-3-phosphate dehydrogenase was coamplified in each case (data not shown). Although low levels of ET$_A$ mRNA can be detected in the human prostate cancer cell lines, functional ET$_A$ binding sites could not. It has been suggested that ligand-induced ET$_A$ mRNA down-regulation may be due to increased message degradation (3); similar regulation in prostate cancer cells may account for low ET$_A$ mRNA and undetectable protein.

Autoradiographic Localization of ET$_A$ and ET$_B$ Binding in Benign and Malignant Prostate Tissues. The reported predominance of ET$_B$-binding sites on the benign prostate epithelium and ET$_A$-binding sites in prostatic stroma was confirmed by autoradiographic analysis of $^{125}$I-labeled ET-1 binding to prostate sections taken from five radical prostatectomy specimens (Fig. 4 and Table 1). The ratios of the densities of ET$_A$:ET$_B$-binding sites in the epithelium and stroma were 0.25 and 2.56, respectively, which are remarkably close to the ratios reported previously (0.20 and 2.54, respectively; Ref. 16). The infiltrating growth pattern of prostate cancer into an ET receptor-rich stromal background exceeded the autoradiographic resolution necessary to determine ET-binding sites on only malignant cells in the primary prostate cancer tissue sections examined. Despite this limitation, areas of cancer were quantified by pixel density and show decreased ET$_B$ binding relative to other tissues. Certain prostate cancer metastases, such as the liver metastases obtained at autopsy, are characterized by homogenous sheets of malignant cells, and background $^{125}$I-labeled ET-1 binding is not an issue. Clearly, ET$_B$ binding was decreased greatly in these lesions. The liver tissue itself had roughly equal ET$_A$ and ET$_B$ binding (2.15 nCi/mg), as reported previously (20).

The specific functional consequences of the reduced ET$_B$ expression in the human prostate cancer cell lines are unknown but provocative. The decreased expression or complete loss of ET$_B$ receptor-specific responses could impact the secretion, function, and clearance of ET-1 in prostate cancer. For example, circulating ET-1 is cleared by ET$_B$ receptors: $^{125}$I-ET-1 binding in vivo is inhibited by BQ-788 (an ET$_B$ antagonist), but not BQ-123 (an ET$_A$ antagonist; Ref. 8). Furthermore, an i.v. infusion of this ET$_B$ antagonist produced a significant increase in plasma ET-1 concentrations, suggesting that...
ETB blockade interrupts ET-1 clearance (8). A similar “hormonal buffer system” has been proposed for silent atrial natriuretic receptors (21). Finally, ET-1 secretion from cultured human keratinocytes, which express ETB exclusively, is inhibited by exogenous ET-1 (22). These observations may explain, in part, the increased tissue and plasma concentrations of ET-1 seen in advanced prostate cancer.

The loss of another ETB-specific response may impact prostate cancer progression. The vasodilatory effect of the ET isopeptides has been linked to ETB-mediated NO productioncounteracting otherwise unopposed ETA-induced vasoconstriction (23, 24). NO has been shown to counter-regulate ET-1 broadly, by blunting both receptor binding and subsequent Ca$^{2+}$ elevation and by inhibiting ET-1 production by the endothelium (25, 26). Although its exact role in tumor biology is still unclear, NO induces cytotoxicity and apoptosis in vitro and has been shown to both inhibit metastases and, conversely, to promote tumor growth in vivo (27–30). If the NO present in prostate cancer has an ET-1-secreting phenotype at metastatic sites as well as the primary tumor. As a mitogen and potentiator of other growth factors for prostate cancer proliferation, ET-1, acting through ETB, provides a distinct therapeutic target.

**References**


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