Expression of Clostridium Perfringens Enterotoxin Receptors Claudin-3 and Claudin-4 in Prostate Cancer Epithelium

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ABSTRACT

The mRNA for Rvp.1 (rat ventral prostate) increases in abundance before gland involution after androgen deprivation. Rvp.1 is homologous to CPE-R, the high-affinity intestinal epithelial receptor for Clostridium perfringens enterotoxin (CPE), and is sufficient to mediate CPE binding and trigger subsequent toxin-mediated cytolsis. Rvp.1 and CPE-R (claudin-4) are members of a larger family of transmembrane tissue-specific claudin proteins that are essential components of intercellular tight junction structures regulating paracellular ion flux. However, claudin-3 and claudin-4 are the only family members capable of mediating CPE binding and cytolsis. The present study was designed to study the expression of claudin-3 and claudin-4 in human prostate tissue as potential targets for CPE toxin-mediated therapy for prostate cancer. On human multiple-tissue Northern blot analysis, mRNAs for both claudin-3 and claudin-4 were expressed at high levels in prostate tissue. In normal prostate tissue, expression of claudin-3 was localized exclusively within acinar epithelial cells by in situ mRNA hybridization. Compared with expression within prostate epithelial cells in surrounding normal glandular tissue, expression of claudin-3 mRNA remained high in the epithelium of prostate adenocarcinoma (10 of 10) and prostatic intraepithelial neoplasia (five of five). Prostate adenocarcinoma cells metastatic to bone were obtained from a patient with disease progression during antiandrogen therapy. These metastatic cells were prostate-specific antigen-positive by immunohistochemical staining and also expressed functional CPE receptors as measured by sensitivity to CPE-induced cell lysis. The persistent high level of claudin-3 expression in prostate adenocarcinoma and functional cytotoxicity of CPE in metastatic androgen-independent prostate adenocarcinoma suggests a new potential therapeutic strategy for prostate cancer.

INTRODUCTION

Mortality attributable to prostate cancer is the second leading cause of cancer-related death among United States males (1). The failure rate of local therapy for early stage disease combined with the limited duration of effective palliative management with hormonal manipulation for advanced metastatic disease beg the development of newer therapeutic agents and strategies (2). Recent advances in the use of combination chemotherapy for advanced hormone-refractory prostate cancer are encouraging but remain inadequate (3).

The cDNA for Rvp.1 was initially cloned based on an increase in mRNA abundance within the Rvp immediately after castration and preceding prostate gland involution (4). The characterization of Rvp.1 as a specific receptor for the potent microbial cytotoxpin protein, CPE (5, 6), suggested that CPE may have potential utility as a therapeutic agent in advanced, hormone-refractory prostate cancer.

CPE triggers a multistep mechanism leading to efficient lysis of susceptible mammalian target cells within 10–20 min (7). This process requires a specific target cell membrane protein for initial CPE binding, a function mediated by CPE-R and Rvp.1. Mammalian cells that do not express either Rvp.1 or CPE-R fail to bind CPE and are resistant to CPE cytotoxicity; however, such resistant cells become susceptible to CPE cytotoxicity after transfection with cDNA for either CPE-R or Rvp.1 (5, 6).

Subsequent to target cell receptor binding by CPE, a Mt ~90,000 small membrane complex is formed in the target cell plasma membrane, which contains intact CPE. Additional target cell proteins then associate with the small complex to form a large, M >160,000 membrane complex that likely allows partial insertion of CPE into the target cell membrane with resultant initiation of massive small molecule permeability changes, osmotic cell ballooning, and lysis (8, 9).

Functional domain mapping of the full-length 319 amino acid CPE protein (10–12) demonstrates that CPE290–319 COOH-terminus fragment is sufficient for high affinity binding to target cell receptor and small complex formation, although this fragment is incapable of initiating large complex formation and cytolsis. Indeed, CPE290–319 COOH-terminus fragment inhibits cytolsis of susceptible target cells by full-length CPE. CPE residues 45–116 are essential for large complex formation and cytotoxicity, whereas deletion of the NH2 terminus generates a CPE45–319 fragment with enhanced large membrane complex formation and cytotoxic activity.

Rvp.1 and CPE-R are members of a large multigene family of claudin proteins (currently 18 members) implicated in tight junction structure and function (13–15); however, only claudins 3 and 4 are capable of binding CPE to mediate toxin-dependent cytolsis. Other claudin family members include endothelial cell-specific claudin-5 (TMVCF), mutations which are responsible for velo-cardio-facial syndrome (16), and claudin-16 (paracellin-1), mutations which are responsible for hereditary renal hypomagnesemia (17).

Recently, SAGE analysis demonstrated that claudin-3 and claudin-4 were among the six highest differentially up-regulated mRNAs in primary ovarian carcinoma cells compared with normal ovarian cells (18). Immunohistochemical analysis confirmed that claudin-3 and -4 protein expression was essentially undetectable in normal ovarian stroma and ovarian surface epithelial cells, whereas both claudin-3 and -4 were highly expressed on the membranes of ovarian carcinoma cells (18). The present study was designed to determine whether the human orthologue of Rvp.1 (hRVP/claudin-3) is expressed in malignant epithelial cells of human prostate cancer to serve as a potential target for a CPE-mediated therapeutic strategy.

MATERIALS AND METHODS

Cloning and Purification of NH2 Terminus His-tagged CPE. Clostridium perfringens obtained from American Type Culture Collection strain 2917 (Manassas, VA) was grown from a single colony and used to prepare bacterial DNA with the InstaGene kit, according to manufacturer’s directions (Bio-Rad). The bacterial DNA fragment encoding full-length CPE gene was PCR amplified (primer 1, 5′-AGG TGT TAA TCA TAT GAT GCT 3′; primer 2, 5′-AGG ATC CTT AAA ATT TTT GAA 3′; primer 3, 5′-AGG ATC CTT AAA ATT TTT GAA 3′). The PCR products were digested with the restriction enzymes Ndel/BamHI and cloned into a Ndel/BamHI-digested pet16 (Novagen) expression vector to generate an in-frame NH2-terminus His-tagged CPE expression plasmid, pet16-(His)5-CPE. His-tagged CPE toxin was prepared from pet16-(His)5-CPE transformed Escherichia coli BL-21

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3 The abbreviations used are: Rvp, rat ventral prostate; CPE, Clostridium perfringens enterotoxin; CPE-R, clostridium perfringens enterotoxin receptor; PSA, prostate-specific antigen; PN, prostate intrapithelial neoplasia.
Primary Culture of Metastatic Prostate Adenocarcinoma and in Vitro Quantitation of His-CPE-mediated Cytolysis. After written informed consent was obtained according to a Human Investigations Committee-approved protocol, prostate adenocarcinoma cells metastatic to bone were obtained by bone marrow core biopsy from the posterior iliac crest of a patient with pancytopenia attributable to the progression of metastatic prostate carcinoma while on antiandrogen therapy. A sample of the bone marrow biopsy submitted for standard pathological examination revealed that the marrow was replaced by >95% adenocarcinoma cells, which were positive for PSA and negative for synaptophysin by immunohistochemical stains.

Under sterile conditions, the remainder of the biopsy specimen was immediately minced and dispersed by collagenase IV (Sigma Chemical Co.; 10 mg/ml). The cells were cultured in keratinocyte-serum-free medium supplemented with bovine pituitary extract (10 mg/ml), epidermal growth factor (10 ng/ml), cholera toxin (10 ng/ml), 5% FCS, and 1% antibiotic solution (10,000 units/ml penicillin and streptomycin). After 3 days in primary culture, purified His-CPE was added to the medium to a final concentration of 3.4 μg/ml, and cytotoxicity was measured after 30 min at 37°C. Percentage of cytotoxicity was determined by comparison of adherent nonbalked cell counts within a demarcated 1-cm² grid under phase-contrast microscopy at time 0 and at 30 min post-CPE addition as described previously (5).

RESULTS

Tissue-specific Expression of hRVP/Claudin-3 and CPE-R/Claudin-4. Preliminary steps in determining the feasibility of CPE toxin therapy for prostate cancer included demonstrating a sufficient level of CPE receptor expression in malignant cells (see below) and also determining which normal tissues express CPE receptors that could restrict the therapeutic index. To examine the normal tissue-specific expression of hRVP/claudin-3 and CPE-R/claudin-4, probes corresponding to the unique 3′ untranslated regions of each human mRNA were used to probe human multiple tissue Northern blots (Fig. 1). The 1.6-kb hRVP/claudin-3 mRNA expression levels were highest in prostate. Moderate levels of hRVP/claudin-3 mRNA were also found in colon, small bowel, and pancreas. The 2.2-kb CPE-R/claudin 4 mRNA was expressed at highest level in colon, with intermediate levels in prostate, placenta, lung, and pancreas and low levels in small bowel, kidney, and uterus. Thus, gut, pancreas, and lung are tissues in which dose-limiting toxicity might be expected in prostate cancer patients treated systemically with CPE.

Cellular Expression of hRVP/Claudin-3 within Normal Human Prostate Tissue and Prostate Adenocarcinoma. Within the normal prostate gland, expression of hRVP/claudin-3 mRNA was restricted exclusively to glandular epithelial cells (Fig. 2, A–D). Prostate stromal tissues and endothelial cells showed essentially background levels of...
silver grain deposition that were no higher than acellular areas of the tissue section.

In prostate adenocarcinoma, hRVP/claudin-3 mRNA expression was also restricted to malignant epithelial cells. Human prostate biopsy specimens (26) were examined for hRVP/claudin-3 expression by in situ mRNA hybridization. Areas of normal epithelium were present in all specimens and contained 20–30 silver grains/cell (3+). Seven of the 26 specimens contained foci of prostate adenocarcinoma, 2 contained foci of PIN, and 3 contained both adenocarcinoma and PIN. The remaining 14 specimens contained only normal prostate tissue. In all cases where prostate adenocarcinoma or prostatic intraepithelial neoplasia was present, expression of hRVP/claudin-3 mRNA was equal to (6 of 10 adenocarcinoma and five of five PIN) or greater than (4 of 10 adenocarcinoma, 4+) expression levels in surrounding normal epithelial cells within the same tissue section (Fig. 2, E–H, representative).

Sensitivity of Androgen-independent Metastatic Prostate Cancer Cells to CPE-mediated Cytolysis. On the basis of previous in vitro studies, it was expected that any mammalian cell expressing either hRVP/claudin-3 or CPE-R/claudin-4 would be sensitive to CPE-mediated cytolysis. However, it was important to demonstrate this directly for human prostate adenocarcinoma cells, particularly in a clinically relevant stage of disease for which current therapy is ineffective and newer treatment modalities are critically needed.

For this purpose, we examined short-term in vitro primary cultured prostate adenocarcinoma cells obtained from the bone marrow of a patient during rapid disease progression while on antiandrogen therapy 2 years after initial diagnosis of poorly differentiated prostate adenocarcinoma, Gleason grade VII, involving seminal vesicles at suprapubic prostatectomy. Antiandrogen therapy with Lupron and Casodex was initiated 1 year after initial diagnosis for painful bone metastasis associated with a rising PSA. Androgen-independent disease progression occurred after 11 months of antiandrogen therapy and was associated with profound pancytopenia attributable to bone marrow replacement with >95% prostate adenocarcinoma cells on bone marrow biopsy. The prostate origin of these metastatic cells was directly confirmed by positive PSA and negative synaptophysin immunohistochemical staining.

The sensitivity of these primary cultured prostate adenocarcinoma cells to CPE-mediated cytolysis was tested along with appropriate positive control CPE-R-expressing Vero cells and negative control LNCaP and PC-3 human prostate cancer cell lines that do not express detectable levels of either hRVP/claudin-3 or CPE-R1/claudin-4. This experiment demonstrated that primary cultures of bone marrow androgen-independent metastatic prostate carcinoma cells were as sensitive as Vero cells to CPE-mediated cytolysis (Fig. 3). Lack of hRVP/claudin-3 and CPE-R1/claudin-4 expression in LNCaP and PC-3 cells was demonstrated by both Northern blot and by absence of CPE290–319 fragment binding (data not shown).

To demonstrate that the lack of claudin expression was responsible for the resistance of PC-3 and LNCaP to CPE-mediated cytolysis, each cell type was transfected with the cDNA encoding full-length claudin-3 fused in-frame to the green fluorescent protein (pcDNA3–claudin-3-GFP). Transfected cells displayed strong membrane-associated green fluorescence. PC-3 and LNCaP cells expressing claudin-3-green fluorescent protein were sensitive to CPE-mediated cytolysis (>98% cytotoxicity in the CPE cytolysis assay).

**DISCUSSION**

The demonstrated persistent in vivo expression of hRVP/claudin-3 in human prostate carcinoma epithelium and the in vitro cytotoxic effect of CPE on primary androgen-independent metastatic prostate carcinoma cells indicate CPE is likely to be cytotoxic in vivo to prostate carcinoma cells that express hRVP/claudin-3 and/or CPE-R/claudin-4. Absence of hRVP/claudin-3 or CPE-R/claudin-4 expression by PC-3 or LNCaP human prostate carcinoma cell lines may indicate that only a fraction of prostate cancers continue to express CPE receptors. However, it is also possible that loss of hRVP/claudin-3 and CPE-R/claudin-4 expression may have occurred during extended passage of PC-3 and LNCaP cells in culture, as demonstrated for other genes known to be expressed in both normal and
primary malignant prostate epithelium. Recent studies demonstrating >80-fold up-regulation of hRVP/claudin-3 and CPE-R/claudin-4 expression in ovarian cancer by SAGE analysis and immunohistochemistry in three of three primary ovarian cancer specimens are particularly relevant to this issue. These studies also demonstrated that none of the ovarian cancer cell lines examined (A2780, ES-2, OV1063, and POOL) expressed detectable levels of hRVP/claudin-3 or hCPE-R/claudin-4 mRNA or protein (18).

Normal intestinal, pancreatic, and lung tissues would be most likely to sustain dose-limiting toxicity on systemic exposure to CPE. The feasibility of developing a strategy for local or systemic use of CPE as an anticancer therapy will depend critically on toxicity studies in animals and humans. Such data are at present restricted to clinical toxicology and pathophysiology of epidemic diarrhea in humans and animals (19) and is beyond the scope of this study. In addition to standard Phase I-type determination of dose-limiting CPE toxicity, strategies to limit toxicity to nonprostate tissues include local delivery of the blocking CPE<sub>290...315</sub> peptide fragment to gut and lung via enteral and inhalation routes.

The results herein have shown that it is feasible to directly test for functional CPE-mediated cytotoxicity in primary cultures of tumor specimens from metastatic sites. Thus, it is now possible to prospectively determine the proportion of androgen-independent prostate carcinomas that remain sensitive to CPE-mediated cytolysis in vitro. In conjunction with independently published work documenting high-level up-regulation of claudin-3 and claudin-4 in primary ovarian carcinoma cells, the results also provide a rationale for in vitro testing of metastatic ovarian carcinoma cells for CPE-mediated sensitivity.

Fig. 3. CPE-mediated cytotoxicity of prostate adenocarcinoma cells from bone marrow metastasis site. In A, short-term cultured prostate adenocarcinoma cells (BMCaP) were compared with positive control Vero cells or negative control PC-3 or LNCaP cells for cytotoxicity after 30 min after the addition of CPE to the media. In B, CPE-mediated cytotoxicity of the same cell types was quantitated as described in “Materials and Methods.”

REFERENCES

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