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Enhanced expression of organic anion transporting polypeptides (OATPs) in androgen receptor-positive prostate cancer cells: Possible role of OATP1A2 in adaptive cell growth under androgen-depleted conditions

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ABSTRACT

The biological mechanisms underlying castration resistance of prostate cancer are not fully understood. In the present study, we examined the role of organic anion transporting polypeptides (OATPs) as importers of dehydroepiandrosterone sulfate (DHEAS) into cells to support growth under androgen-depleted conditions. Cell growth and mRNA expression of OATP genes were studied in human prostate cancer LNCaP and 22Rv1 cells under androgen-depleted conditions. The stimulatory effect of DHEAS on cell growth was investigated in LNCaP cells in which OATP1A2 had been silenced. Growth of both cell lines was stimulated by DHEAS and the effect was attenuated by STX64, an inhibitor of steroid sulfatase which can covert DHEAS to DHEA. OATP1A2 mRNA expression was increased most prominently among various genes tested in LNCaP cells grown in androgen-depleted medium. Similar results were obtained with 22Rv1 cells. Furthermore, the characteristics of [^{3}H]DHEAS uptake by LNCaP cells were consistent with those of OATP-mediated transport. Knockdown of OATP1A2 in LNCaP cells resulted in loss of the DHEAS sensitivity of cell growth. Our results suggest that enhanced OATP1A2 expression is associated with adaptive cell growth of prostate cancer cells under androgen-depleted conditions. Thus, OATP1A2 may be a pharmacological target for prostate cancer treatment.

1. Introduction

Gonadal androgens play a critical role in protein synthesis and cell survival in prostate tumors [1]. Therefore, androgen deprivation therapy (ADT) to remove gonadal testosterone or to antagonize androgen receptors is currently a mainstream treatment for prostate cancer. Once the disease progresses to castration-resistant prostate cancer (CRPC), it no longer responds to ADT. CRPC tumor progression is considered to involve enhanced androgen receptor (AR) function, mainly due to *AR* gene amplification/overexpression [2], stabilization of AR protein and increased sensitivity of AR to androgens [3], and constitutive, ligand-independent activation [4].

The organic anion transporting polypeptide (OATP/*SLCO*) family members generally mediate Na^+-independent transport of amphipathic organic anion compounds, including bile salts, steroid conjugates, thyroid hormones, and oligopeptides [5,6]. The physiological roles of these transporters are not yet fully understood, but there is compelling evidence that certain members of the OATP family promote cell proliferation and survival of human malignant tissues. We have previously shown that transport of estrone 3-sulfate via OATPs sustains the growth of hormone-dependent human breast cancer cells [7,8], because estrone 3-sulfate can be hydrolyzed by steroid sulfatase (STS) to estrone and eventually estradiol. We recently showed that OATP1B3 contributes to estrone 3-sulfate uptake in estrogen receptor-positive human breast cancer MCF-7 cells [9]. In addition

Abbreviations: AR, androgen receptor; STX64, 6-oxo-6,7,8,9,10,11-hexahydrocyclohepta[c]chromen-3-yl sulfamate; CRPC, castration-resistant prostate cancer; STS, steroid sulfatase; OATP, organic anion transporting polypeptide; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; ADT, androgen deprivation therapy; SRB, sulforhodamine B; PSA, prostate specific antigen; HPRT1, hypoxanthine guaninephosphoribosyltransferase; HSD, hydroxysteroid dehydrogenase; AP-1, activator protein-1; NTCP, Na^+-dependent taurocholate cotransporting polypeptide; E3S, estrone 3-sulfate; BSP, bromosulfophthalein; TCA, taurocholate; PRO, probenecid; SAL, salicylate; PAH, *p*-aminohippurate; TEA, tetra-ethylammonium; MPP^+, 1-methyl-4-phenylpyridinium.

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to estrone 3-sulfate, these OATPs translocate dehydroepiandrosterone sulfate (DHEAS) [10,11]. DHEAS is a thousand fold more abundant than testosterone in human serum [12], and is essentially unaffected by ADT. DHEAS is hydrolyzed to DHEA by STS [13], and DHEA can be converted to androstenedione in prostate cancer [14], resulting in activation of AR function [15]. More recently, clinical observations have suggested that several OATPs are upregulated in castration-resistant metastatic prostate tumor tissues derived from human patients [16]; however, it remains unclear whether OATPs play a role in prostate cancer cell survival under androgen-depleted conditions. We hypothesized that increased expression of OATPs does contribute to cell survival of androgen-dependent prostate cancer cells under androgen deprivation, by providing the cells with an increased supply of DHEAS as a precursor of active androgen.

In the present study, we investigated the expression of functional OATPs in, and the growth of, androgen-dependent prostate cancer cells. Among the OATP family members tested, OATP1A2 was found to be remarkably upregulated in AR-positive human prostate cancer cells cultured under androgen-depleted conditions. We propose that enhanced expression of OATP1A2 in these cells plays a role in progression of prostate cancer by increasing the availability of DHEAS as a precursor of active androgen, under conditions of androgen depletion.

2. Materials and methods

2.1. Materials

DHEAS, dihydrotestosterone (DHT), and [^{3}H]DHEAS sodium salt (3.50 TBq/mmol) were purchased from Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), and PerkinElmer Life Science (Boston, MA), respectively. Human prostate cancer LNCaP and 22Rv1 cell lines were purchased from American Type Culture Collection (Manassas, VA).

2.2. Cell culture and growth assays

LNCaP and 22Rv1 cells were cultured in RPMI1640 (Wako Pure Chemical Industries) with penicillin (100 units/mL, Nakalai Tesque, Kyoto, Japan), streptomycin (100 μg/mL, Wako Pure Chemical Industries) and 10% fetal bovine serum (FBS, Invitrogen). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. For the cell growth assay, cells were cultured in RPMI1640 with penicillin (100 units/mL), streptomycin (100 μg/mL) supplemented with FBS or charcoal-stripped FBS (CSS, Invitrogen) in the presence or absence of androgen and/or STX64 (also known as 667 coumate or 6-oxo-6,7,8,9,10,11-hexahydrocyclohepta[c]chromen-3-yl sulfamate, Sigma Aldrich, St. Louis, MO), which is a tricylic coumarin-based sulfamate that inhibits steroid sulfatase. The growth-stimulatory effect of androgens, including DHEAS, was evaluated by measuring cell growth after cells were plated at a density of 1.5–2.4 × 10^4 cells/cm^2 on a 96-well tissue culture plate. In general, once cells became attached to the plate, a test androgen or DMSO (at 0.1% of the final concentration) was added to the well, and cell growth was monitored for up to 9 days by means of sulforhodamine B (SRB, Sigma Aldrich) assay as previously described [17].

2.3. Gene expression profiling assay

LNCaP cells were cultured in RPMI1640 with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% CSS for 6 days. Then, total RNA was prepared using Isogen reagent (Nippon Gene, Tokyo, Japan). Microarray experiments were performed and data were analyzed according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). In brief, 300 ng of total RNA was subjected to linear amplification and Cy3 labeling using the Low RNA Input Linear Amplification Kit (Agilent Technologies), and then hybridized to a 44 K Whole Human Genome Microarray (Agilent Technologies). Data were extracted using Feature Extraction software (version 9.5.3) and analyzed using GeneSpring GX software (version 11.0).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from cells using Isogen reagent (Nippon Gene). mRNA expression of OATP1A2, OATP1B1, OATP1B3, OATP2B1, OAT2, OAT3, and OAT4 was measured by quantitative RT-PCR (qRT-PCR), using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies). The fold change in mRNA expression of transporter genes was normalized to hypoxanthine guaninephosphoribosyl-transferase 1 (HPRT1) using the 2-ΔΔ threshold cycles (Ct) method [18]. Primer sequences specific to transporter genes tested are listed in Table 1.

2.5. [^{3}H]DHEAS cellular uptake assay

Intracellular accumulation of DHEAS was evaluated by means of uptake assays as described previously [17]. Briefly, cells were plated at a density of 1.0 × 10^5 cells/cm^2 on a 24-well tissue-culture plate in culture medium supplemented with 10% FBS or CSS in the absence or presence of DHT (1 nM) two days before assay containing 0.1% DMSO of the final concentration. The assay was initiated by adding transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4,1.2 mM, $MgSO_4$, and 25 mM HEPES, adjusted to pH 7.4) containing 0.5 μCi/mL [^{3}H]DHEAS (10 nM) with or without inhibitors at 37 °C. Sodium dependence of uptake was evaluated by replacing Na^+ with Li^+, K^+ or N-methylglucamine (NMG^+). At the end of the assay, cells were washed with ice-cold transport buffer and solubilized in 1% (v/v) Triton X-100 (Wako Pure Chemical Industries). The radioactivity in the resultant cell lysate was measured using a liquid scintillation counter (Aloka, Tokyo, Japan). Intracellular [^{3}H]DHEAS accumulation was normalized by protein content determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and is shown as the ratio of accumulation to the initial concentration of DHEAS, i.e., cell-to-medium (C/M) ratio (μL/mg).

2.6. Western blot

To determine protein expression of OATP1A2, cells were collected and lysed in RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris at pH 8.0) by sonication. A 50 μg aliquot of total cell lysate was subjected to SDS polyacrylamide gel electrophoresis, and then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blots were probed with anti-human OATP1A2 rabbit polyclonal antibody (Assay Biotechnology Company, San Francisco, CA) at 1 to 500 dilution or with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) at 1 to 2000 dilution, followed by appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ).

2.7. Construction of OATP1A2-transfected LNCaP cells

LNCaP cells were transfected with pcDNA3.1/OATP1A2 or pcDNA3.1 alone (Invitrogen), and then selected with 800 μg/mL of G418 (Wako Pure Chemicals). Enhanced OATP1A2 expression