Development of a Prostate-Specific Promoter for Gene Therapy against Androgen-Independent Prostate Cancer

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Androgen ablation has been the standard treatment for metastasized prostate cancer. In most cases, however, prostate cancer cells eventually lose androgen dependency and become refractory to the conventional endocrine therapy. Androgen-independent prostate cancer is characterized by a heterogeneous loss of androgen receptor (AR) expression among tumor cells. Prostate-specific promoters such as prostate-specific antigen and rat probasin (rPB) promoters have been examined in the development of gene therapy targeted to prostate cancer. However, those promoters require binding of the androgen–AR complex to the androgen-response element and are active only in the androgen-dependent prostate cancer cell lines and not in the androgen-independent cell lines. To target transgene expression in androgen-independent prostate cancer, we designed a prostate-specific promoter that is activated by the retinoids–retinoid receptor complex instead of the androgen–AR complex. The modified rPB promoters expressed transgenes in response to retinoid in both androgen-dependent and androgen-independent prostate cancer cells and not in other cancer cell lines or in human normal cells, in vitro and in vivo. Furthermore, the combination of retinoid treatment and adenovirus-mediated gene transfer of the modified rPB-driven HSV-tk gene resulted in a significant growth suppression of the androgen-independent prostate cancer cells in the presence of the prodrug ganciclovir. This study suggests that tailoring of the hormone-responsive elements may offer a new therapeutic opportunity against the hormone-refractory stage of prostate cancer.

Key Words: rat probasin promoter, androgen-independent, prostate cancer, gene therapy, adenovirus

INTRODUCTION

Prostate cancer is one of the most common malignancies in men, and the incidence is increasing especially in developed countries [1]. Although prostate cancer begins as an androgen-dependent tumor that undergoes clinical regression in response to androgen ablation therapy, the tumor frequently progresses to an androgen-independent state within 2 years, from which point there is no effective treatment [2]. The molecular basis for the development of androgen-independent prostate cancer is poorly understood, but the proposed mechanisms include amplification or overexpression of the androgen receptor [3,4], ligand-independent activation of the androgen receptor through “cross talk” with other signal transduction pathways [5], and alterations in androgen receptor ligand binding [6]. Although androgen-independent prostate cancer may continue to express androgen receptor (AR), a heterogeneous loss of AR expression is often observed among tumor cells, and a high proportion of AR protein-negative cancer cells has been correlated with a poor response to hormone ablation treatment and short survival [7,8]. Methylation of the AR promoter CpG island may be associated with loss of AR expression in prostate cancer cells [9].

Prostate-specific antigen (PSA) and rat probasin (rPB) promoters have been examined to target therapeutic genes to the prostate cancer cells [10,11]. However, the promoters may not be useful for the treatment of hormone-refractory prostate cancers, because the promoters require binding of the androgen–AR complex to the androgen-response element (ARE) and are active only in the androgen-dependent prostate cancer cell lines but not in the androgen-independent cell lines [12,13]. In terms of previous studies to overcome the limitations of prostate-specific promoters for androgen-independent prostate cancer, Martiniello-Wilks et al. reported that although the PSA promoter had low activity in androgen-independent
cells, the promoter could sufficiently activate a suicide gene in vivo [14]. They also demonstrated that the rPB promoter was more potent than the PSA promoter, and the rPB promoter combined with an enhancer from simian virus (SV) 40 showed high activity in androgen-independent cells in vitro and in vivo [15, 16]. In this study, we designed novel prostate-specific promoters that can direct transgene expressions to AR-negative androgen-independent prostate cancer cells. We chose the rPB promoter as a base because it was more potent than the PSA promoter in our previous study [12]. Two cis-acting ARE have been identified within the 5′-flanking region of the rPB gene [17], and the 5′-regulatory area of the androgen-response region is important for prostate-specific gene expression [18]. Hormone-responsive elements can be grouped into two categories: the TGACC motif confers regulation by estrogen, retinoic acid, and thyroid hormone and the TGTTCCT motif by glucocorticoids, progesterone, and androgen [19]. We have modified the rPB promoter by the substitution of the retinoic acid-response element (RARE) for ARE to utilize the retinoids-retinoic receptor complex in place of the androgen–AR complex, since retinoid receptors are ubiquitously expressed in human tissues. The growth inhibitory effect of retinoids per se has been documented in a wide variety of tumor cell types, including prostate cancer [20, 21], and the compounds are already being used clinically. Thus, the combination of all-trans-retinoic acid (ATRA) and prostate-specific gene therapy may be a reasonable and realistic choice for prostate cancer therapy.

Results
Expression of Retinoid Receptors in Prostate Cancer Cells
First, we examined expression of AR and the retinoid receptor family in prostate cancer cell lines. Although the androgen-dependent prostate cancer cell line (LNCaP) expressed AR, the androgen-independent prostate cancer cell lines (PC3, DU145) did not express AR (data not shown) as previously reported [25]. Retinoids transduce signals through the interaction of two families of retinoid receptors, retinoic acid receptors (RARs; RARα, β, γ) and retinoid X receptors (RXRs; RXRα, β, γ) [26]. The retinoid receptor binds as homodimer (RXR–RXR) or heterodimer (RAR–RXR) and binds with RARE to activate transcription. All three prostate cancer cell lines expressed RARβ and RXRα and LNCaP and PC3 cells expressed RARγ and RXRγ (Fig. 1). On the other hand, the expression of RARβ and RXRβ was not detected in prostate cancer cells, which was consistent with a previous report that RARβ and RXRβ mRNAs were selectively lost in prostate cancer [27].

Modified rPB Promoters Regulated by Retinoic Acid
To develop specific promoters for AR-negative androgen-independent prostate cancer, we modified the rPB promoter by substituting RARE for its ARE, and the promoter activities were compared with that of the rPB promoter in prostate cancer cells. Although the activity of the rPB promoter was significantly enhanced by the addition of synthetic androgen in LNCaP androgen-dependent cells [12], both rPB-DR(3) and rPB-DR(6) promoters lost any responsiveness to androgen in LNCaP cells (data not shown), suggesting that two AREs are required for a sufficient response of rPB promoter to androgen stimulation. The modified promoters were responsive to ATRA stimulation, and rPB-DR(6) showed a higher luciferase expression than rPB-DR(3) in a dose-dependent manner in all three prostate cancer cell lines (Fig. 2). Then, to investigate the prostate specificity of the modified rPB promoters, the plasmids were transfected into nonprostate cancer cells, HCT15, MCF-7, and MIAPaCa-2 cells. The luciferase activity was not significantly observed in nonprostate cancer cells in the presence of ATRA (Fig. 2), demonstrating that the modified rPB promoters retain their prostate specificity. To enhance the promoter activity, we inserted the human 4F2 heavy chain transcriptional enhancer sequence into the rPB-DR(6) plasmid [24]. The 4F2 enhancer could enhance luciferase expression (approximately threefold), but it also caused the loss of the prostate specificity (data not shown).

Prostate Specificity of ADV-rPB-DR(6)-AP in Vitro
To confirm further that the rPB-DR(6) promoter restricted expression of the alkaline phosphatase (AP) gene to prostate cancer cells, PC3 cells were infected with 100 or 300 m.o.i. of the ADV-rPB-DR(6)-AP. The cells were cultured in the presence of 3 μM ATRA for 2 days. The concentration of ATRA was attainable up to 6 μM in human serum by the maximum dose of oral medication [28]. Expression of the AP reporter gene in the presence of ATRA was readily observed, but no reporter gene activity was detected without ATRA in PC3 cells (Fig. 3A, top). To assess the AP activity of ADV-rPB-DR(6)-AP in the primary cultures of human normal nonprostate cells, ADV-rPB-DR(6)-AP or ADV-CAG-AP was used to infect smooth muscle cells, hepatocytes, and human umbilical vein endothelial cells (HUVECs). When normal cells were trans-
FIG. 2. Cell type- and ATRA-dependent activation of the modified rPB promoters. The prostate, colon, breast, and pancreatic cancer cell lines were transfected with the luciferase expression plasmids driven by the modified rPB promoters. The cells were grown in the presence of ATRA (0.1, 1, or 10 µM) for 2 days, and luciferase activity of the cell lysates was measured.
FIG. 3. Prostate specificity of the ADV-rPB-DR(6)-AP vector in vitro. (A) The PC3 cells and human normal primary culture cells were infected with ADV-rPB-DR(6)-AP or ADV-CAG-AP at m.o.i. 100 and 300 (PC3 cells) or m.o.i. 10 (normal cells) and were grown in the presence of ATRA for 2 days. (B) Effect of the rPB-DR(6)-driven suicide gene on the growth of PC3 cells. PC3 and MIAPaCa-2 cells were transduced with ADV-rPB-DR(6)-TK (m.o.i. 300) and cultured for 7 days. Twenty-four hours after the infection, 2 μM ATRA and 10 μM GCV were added. OD450-595 absorbance at 450 to 595 nm. *P < 0.001.
duced with the positive control ADV-CAG-AP vector at a m.o.i. of 10, the AP gene was expressed in more than 90% of the cells, which was compatible with the vector-infected PC3 cells at a m.o.i. of 300. The brown-stained cells were not detected in the ADV-rPB-DR(6)-AP-infected normal cells at the same m.o.i., even in the presence of ATRA (Fig. 3A, bottom).

To illustrate that, although the rPB-DR(6) promoter has lower activity than the CAG promoter, it is sufficiently active to achieve a therapeutic effect in androgen-independent prostate cancer, we infected PC3 and MIAPaCa-2 cells with ADV-rPB-DR(6)-TK, and ganciclovir (GCV) sensitivity was examined by cell proliferation assay. The addition of GCV significantly suppressed the growth of ADV-rPB-DR(6)-TK-infected PC3 cells only in the presence of ATRA, whereas ADV-rPB-DR(6)-TK infection did not show the growth suppression in MIAPaCa-2 cells (Fig. 3B).

Transgene Expression from ADV-rPB-DR(6)-AP Is Restricted to Prostate Cancer Cells in Vivo
To determine whether transgene expression under control of the rPB-DR(6) promoter was restricted to prostate cells in vivo, we transplanted the PC3 prostate cancer cells and the HCT15 colon cancer cells into the subcutaneous space of nude mice and injected the resulting tumors with the ADV-rPB-DR(6)-AP vector. The positive control ADV-CAG-AP resulted in transgene expression in approximately 90% of PC-3 and HCT15 tumor cells. Injection of the ADV-rPB-DR(6)-AP vector led to the expression of the AP transgene in 60–70% of the cells after administration of ATRA, but in its absence the AP gene expression was significantly suppressed (Fig. 4, top). The HCT15 subcutaneous tumor did not show any AP staining after the injection of ADV-rPB-DR(6)-AP with the administration of ATRA (Fig. 4, bottom). Major organs such as the brain, heart, lung, liver, spleen, kidney, pancreas, stomach, intestine, testis, and skeletal muscle of the ADV-rPB-DR(6)-injected mice did not show any AP activity (data not shown).

DISCUSSION
Various gene therapy strategies for prostate cancer have been examined. Eastham et al. reported that the direct intratumoral injection of an adenovirus vector encoding the herpes simplex virus thymidine kinase (HSV-tk) under the regulation of the Rous sarcoma virus long terminal repeat regressed nude mouse subcutaneous tumors of prostate cancer cells [29]. Hall et al. demonstrated the cooperative therapeutic effects of androgen ablation and HSV-tk gene transfer plus GCV treatment [30]. Blackburn et al. developed a heat-inducible fusion gene comprising cytotoxic daminase and HSV-tk genes and provided a unique application for hyperthermia in prostate cancer therapy [31]. However, targeting the prostate epithelium remains an important issue in the development of prostate cancer gene therapy. The prostate is unique in the sense that the organ is not necessary in adult life other than its role in male reproduction. The use of prostate-specific promoters may allow the substantial but safe escalation of the vector dosage [24] and may enable the effective delivery of toxin or apoptosis-inducing genes to the prostate cells and to the cancer cells, which may be disseminated or metastasized in the body.

Two prostate-specific promoters (PSA and rPB promoters) have been examined in the development of gene therapy targeted to prostate cancer. Human prostatic epithelial cells constitutively secrete PSA, which is a normal component of the seminal plasma. The PSA expression is controlled by androgen, and its promoter contains both an androgen-response element and an androgen-response region. The PSA promoter was used to express antisense RNA against the DNA polymerase α and topoisomerase II α genes in prostate cancer cell lines to inhibit cell growth [32]. Recently, Latham et al. reported that an adenoviral vector expressing the nitroreductase gene under the control of the PSA promoter sensitized the LNCaP cells to an alkylating cytotoxic agent, CB1954 [11], and Yu et al. demonstrated that the PSA-based promoter in the lentivirus vector drove the expression of diphtheria toxin A in prostate cells with satisfactory efficiency and specificity [33]. The rPB gene encodes an androgen- and zinc-regulated protein specific to the dorsolateral epithelium of the prostate [34]. In contrast to the PSA promoter, which has attracted the most attention, the evaluation of the rPB promoter has been limited in the context of gene therapy application. In this study, we chose the rPB promoter as a base for the promoter tailoring, because we previously showed that the rPB promoter activity was more potent than the PSA promoter activity in androgen-dependent prostate cancer cells [12]. Moreover, information on the 5'-regulatory region of the rPB gene is accumulating [18].

The experimental models of androgen-independent human prostate cancer growth often include the PC3 and DU145 cells, which fail to express AR or PSA [35]. However, LNCaP cells, which contain a mutant AR, may also reflect some of the androgen-independent cancer cells, because the amplification and ligand-dependent activation of AR and alterations of AR ligand binding were proposed as a mechanism of androgen independence [3–6]. Actually, LNCaP-type and PC3-type cells might coexist in hormone-refractory cancers. Thus, the development of a prostate-specific promoter, which is functional in both types of cells with retention of prostate specificity, should be useful for gene therapy in hormone-refractory prostate cancer. Furthermore, retinoids exert pleiotropic effects on the transformation of cells and tissues in culture, and the growth-inhibitory effects of retinoids per se
have been documented in a wide variety of tumor cell types, including prostate cancer [23,36]. Therefore, the combination of ATRA and prostate-specific suicide gene therapy might be a promising therapeutic strategy for advanced prostate cancer. This approach may also be applicable to the hormone-refractory stage of other cancer cells such as breast cancer by utilizing a different hormone-receptor signaling pathway. Promoter activity of tissue-specific promoters is generally lower than that of viral promoters such as CMV and SV40 promoters. In fact, the luciferase activity of rPB-DR(6) was approximately 10-fold lower than that of the SV40 promoter (data not shown). However, our promoter was potent enough to exert an unequivocal growth-suppressive effect when combined with the HSV-tk/GCV system (Fig. 3B), urging a series of the next-step preclinical studies on the modified rPB promoter in various therapeutic systems such as enzyme-directed prodrug therapy, proapoptotic gene transfer, and conditionally replicating virus vectors.

Recent events in human gene therapy trials have renewed the emphasis on safety as the primary criterion for the development of a novel gene therapy strategy [37]. The efficiency and specificity of the modified rPB promoter suggest that the promoter will play an important role in the future treatment of androgen-independent prostate cancer.

![FIG. 4. Expression of the AP transgene following intratumoral injection of ADV-rPB-DR(6)-AP. PC3 and HCT15 subcutaneous tumors were injected with 1 × 10⁹ pfu of ADV-rPB-DR(6)-AP. Starting 24 h before the injection, mice were treated with 1.5 mg/kg/day of ATRA at 12-h intervals for a total of seven times. Representative histological sections are shown (original magnification ×100).](image-url)
MATERIALS AND METHODS

Cell culture. Three human prostate cancer cell lines (LNCaP, PC3, DU145), a human colon cancer cell line (HCT15), and a human breast cancer cell line (MCF-7) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). All cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Primary cultures of human normal cells such as HUVEC, smooth muscle cells, and hepatocytes were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan) and cultured according to the suppliers’ instructions.

Western blot analysis. The cancer cells were lysed in RIPA buffer (10 mM Tris–HCl, pH 7.4, 1% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Forty micrograms of cell lysates were heated at 95°C for 5 min, size-fractionated by 8–16% SDS–polyacrylamide gel (TEFCO, Tokyo, Japan), and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed with rabbit polyclonal anti-RARα, anti-RARβ, anti-RARγ, anti-RXRα, anti-RXRβ, or anti-RXRγ antibodies (Santa Cruz, CA) using an enhanced chemiluminescence system (Amersham Bioscience, Buckinghamshire, UK).

Isolation of rPB promoter and construction of luciferase expression plasmids. We cloned the rPB (−426/+28) promoter region as previously described [12]. The promoter fragment was subcloned into a promoterless luciferase plasmid, pGL3 Basic vector (Promega, Madison, WI) and designated pPB-luci. The rPB promoter region contains two AREs (−236/−223 and −140/−117) and the 5′-flanking region of ARE, which appears essential for prostate-specific gene expression [15] (our data not shown). We substituted three copies of synthetic oligonucleotides representing RARE (direct repeat type: AGGTCAAGGTCA) for each ARE, so that the modified promoter can be activated in response to retinoids (Fig. 5). The rPB promoter regions containing one region (three copies) or two regions (total of six copies) of RAREs were designated rPB-DR(3) or (6), respectively (Fig. 6). One ARE (−236/−223) remained with rPB-DR(3). Transfections of luciferase expression plasmids. To compare the relative activity of the tailored promoters in prostate cancer cells, luciferase expression plasmids were transfected by use of polyethyleneimine as de-
scribed [22]. Forty-eight hours after DNA transfection, the culture medium was changed to RPMI 1640 supplemented with 5% charcoal-treated FBS, and cells were seeded at 5 × 10^3 (LNCaP: 1 × 10^4) well per six-well plates. Each well received 2 µg of luciferase expression plasmids. After a 2-h incubation, cells were grown in the absence or presence of ATRA for 48 h. The cells were then lysed with 100 µl of cell lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton-X) and incubated at room temperature for 20 min. Three microliters of cell lysate were mixed with 100 µl of Luciferase Assay Reagent (PicoGene; Toyo, Inc., Tokyo, Japan), and the light unit of the luciferase activity was measured using a luminometer (MINILumat LB 9506; EG&G Berthold, Vilvoorde, Belgium). The data were expressed as the relative luciferase activity, which is the chemiluminescence elicited from cells transfected with each luciferase expression plasmid in the presence of ATRA, divided by the chemiluminescence of the transfected cells in the absence of ATRA. The analyses (carried out in triplicate) were repeated a minimum of two times and the means ± standard deviations were plotted.

Construction of recombinant adenovirus vectors. The recombinant adenovirus encoding AP or HSV-tk genes under the control of ADV-PDR(6)-AP or ADV-PDR(6)-TK, respectively. The adenoviral vectors were prepared by Cre/loxP-mediated recombination of a sub360 adenoviral cosmid, which is an Ad5 derivative with a deletion of the E3 region, with an AP or HSV-tk adenoviral shuttle plasmid as described [23]. A recombinant adenovirus encoding AP driven by the CAG promoter, which consists of the cytomegalovirus immediate-early enhancer sequence and the chicken β-actin promoter, and β-globin hybrid promoter, was designated ADV-CAG-AP and used as a positive control. A cesium chloride-purified virus was desalted using sterile BioGel 100G desalting medium (EconoPac DG10; Bio-Rad, Richmond, CA) and diluted for storage in a 13% glycerol/phosphate-buffered saline solution.

Effect of ADV-PDR(6)-TK on proliferation of PC3 cells. PC3 cells were seeded at 2 × 10^5 per well in 96-well microtiter plates in the culture medium with 5% charcoal-treated FBS and infected with ADV-PDR(6)-TK at m.o.i. of 300. Twenty-four hours after adenovirus infection, 10 µM prodrug GCV and 2 µM ATRA were added. The cell numbers were assayed by a colorimetric cell viability assay using a water-soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS); Promega, Madison, WI) and incubated at room temperature for 20 min. Three microliters of the absorbance was determined by spectrophotometry using a plate reader.

Gene transfer of AP into subcutaneous tumor. Five-week-old male BALB/c nude mice were obtained from Charles River Japan (Kanagawa, Japan), and the PC3 cell suspension (5 × 10^6) was injected subcutaneously into the back of the mice. When a subcutaneous tumor nodule reached the size of 10 mm in diameter, 1 × 10^7 PFU of ADV-PDR(6)-AP was injected into the tumors. Starting 24 h before the vector injection, each mouse was treated with a subcutaneous injection of 1.5 mg/kg/day ATRA at 12-h time intervals and the means ± standard deviations were plotted.

REFERENCES


