Gene Therapy for Prostate Cancer by Targeting Poly(ADP-Ribose) Polymerase

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ABSTRACT

Poly(ADP-ribose) polymerase (PARP) has strong affinity for DNA strand breaks and cycles on and off the DNA ends to allow DNA repair. A DNA-binding domain of PARP (PARP-DBD) acts as a dominant-negative mutant by binding to DNA strand breaks irreversibly and sensitizing mammalian cells to DNA-damaging agents. Therefore, expression of PARP-DBD in prostate carcinoma cells offers a strategy to achieve sensitization to genotoxic treatments. Toward this end, we developed recombinant plasmids expressing the PARP-DBD under the control of the 5′-flanking sequences of the human prostate-specific antigen (PSA) gene. Tissue specificity of PARP-DBD expression in human tumor cells was confirmed using the PSA-producing (LNCaP) and PSA-negative (PC-3) prostate cancer cells, as well as cells of nonprostate origin, Ewing’s sarcoma (A4573 cells). LNCaP cells stably transfected with the PSA-regulated cDNA for PARP-DBD exhibit an androgen-dependent induction of PARP-DBD expression as determined by Western blotting, reverse transcription-PCR, and in situ immunofluorescence. Furthermore, we found that PARP-DBD sensitized LNCaP cells to DNA-damaging agents, such as ionizing radiation and etoposide. Androgen (R1881) -dependent stimulation of PARP-DBD expression resulted in a 2-fold growth inhibition in LNCaP cells as compared with controls, and an augmented apoptotic cell death in response to ionizing radiation or etoposide. Taken together, the plasmid vector developed in this study permits the expression of the human PARP-DBD in an androgen-inducible and PSA-dependent fashion, and sensitizes prostate adenocarcinoma cells to DNA-damaging treatments. These results provide proof-of-principle for a novel therapeutic strategy for the treatment of prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men, and it is the second most fatal cancer resulting in ∼40,000 deaths annually in the United States. Whereas >80% of the tumors initially respond to androgen ablation, metastatic prostatic cancer inevitably progresses to an androgen-independent state. Once this happens, the disease is difficult to control, because hormonally independent tumors become resistant to additional hormonal manipulations as well as chemotherapy or radiotherapy (1).

PARP is a zinc finger-containing protein, allowing the enzyme to bind either double- or single-strand DNA breaks. Numerous studies have shown that PARP is involved in a variety of biological functions, all of which are associated with breaking and rejoicing of DNA strands, and plays a pivotal role in DNA damage repair (reviewed in Refs. 2–4). Recent advances in apoptosis research have identified PARP as one of the intracellular “death substrates,” and have demonstrated that limited proteolysis of PARP by caspases is an early event or perhaps a prerequisite for the execution of programmed cell death in a variety of cells (5). The caspase-specific DEVD motif resides adjacent to the nuclear localization signal of the PARP protein. Cleavage of PARP at this site results in the separation of the two zinc finger DNA-binding motif in the NH2 terminus of PARP from the automodification and catalytic domains located in the COOH terminus of the enzyme. Consequently, this cleavage excludes the catalytic domain from being recruited to the sites of DNA fragmentation during apoptosis and presumably disables PARP from coordinating subsequent repair of genome maintenance events. The biological function of the DNA-binding domain of PARP has been investigated by using stable cell lines expressing PARP-DBD protein (6). Data obtained from these experiments indicate that PARP-DBD expression in mammalian cells: (a) leads to trans-dominant inhibition of PARP; (b) has no effect on normal cell proliferation; and (c) sensitizes the cells to genotoxic agents and ionizing radiation. Exposure of the PARP-DBD-expressing cells to these DNA-damaging agents results in a marked reduction of cell survival, increased frequency of sister chromatid exchanges, inhibition of cell proliferation, and apoptosis induction (7). Thus, the DBD of PARP offers a potential for targeted sensitization of tumor cells to genotoxic agents and radiotherapy.

In the past few years several new approaches for treating advanced neoplasms have been proposed, including that of gene therapy. Differential expression of the desired gene product in the target tissue is central to the concept of gene therapy. One such approach is to use tissue-specific promoters to drive therapeutic genes. From this point of view, the promoter of the gene encoding the PSA represents a promising tool for prostate cancer-specific gene expression (8). Although low levels of PSA are detectable in the serum of men with normal prostates, PSA expression is increased in most patients with prostate cancer, regardless of tumor stage and hormone responsiveness. The promoter of the PSA gene has been cloned, and its two functional domains have been identified: a proximal promoter and a distal promoter, which can also function as an enhancer (9). Using LNCaP tumor xenografts in the nude mouse model it was demonstrated that the PSA promoter retained its tissue-specific properties in vivo (10). Furthermore, the PSA promoter was able to mimic the prostate-specific and androgen-regulated expression of the PSA gene in transgenic mice (11). Thus, the PSA promoter contains the features that are fundamental for the development of expression vectors for prostate-specific gene therapy: tissue specificity and androgen responsiveness.

The present study reports a novel approach for combination therapy that uses the tissue-specific (prostate) and DNA damage-specific (targeting the PARP function) gene therapy for prostate cancer. We describe the development of recombinant plasmids for expression of the DNA-binding domain of PARP under control of prostate tissue-specific promoter in PSA producing LNCaP prostate carcinoma cells. Our results show that enforced PARP-DBD expression significantly augments sensitivity of these cells to DNA-damaging treatments, presenting a novel strategy for gene therapy directed to prostate cancer.
MATERIALS AND METHODS

Cell Lines and Tissue Culture. The androgen-responsive prostate carcinoma LNCaP and androgen-independent PC-3 cell lines were obtained from American Type Culture Collection and maintained by serial passage in DMEM supplemented with 10% fetal bovine serum. Cells subjected to androgen stimulation tests were maintained in medium with 10% charcoal-stripped fetal bovine serum for 7 days before the addition of synthetic androgen R1881 (Perkin-Elmer Life Science). Ewing’s sarcoma cell line A4573 (kindly provided by Dr. Timothy Kinsella, University of Wisconsin, Madison, WI) was maintained in Eagle’s MEM (Life Technologies, Inc.). All of the irradiations were performed in air, using a $^{137}$Cs source in a JL Shepard MARK I laboratory irradiator at a dose rate of 3.85 Gy/min.

Plasmid Constructs. The 1.3-kb fragment that contains the upstream enhancer element (9, 10) of the PSA regulatory region (nt −745 to −2080) was cloned from human placenta using a PCR-generated probe corresponding to nts 1–200 of PSA cDNA. The PSA gene minimal promoter (nt −619 to +12) was obtained by performing PCR amplification using human placenta genomic DNA as a template, and set of primers 5′-GGTTGGAGAACAGGAGGTG (upstream) and 5′-TCTCCGGTGGACTGTTGAA (downstream) designed according to the reported sequence data for the 5′ region of the human PSA gene (9). The 0.7-kb fragment of the human PARP cDNA encoding for DNA binding domain (aa 1–234) was PCR amplified using plasmid pCD12 containing cDNA for human PARP as a template and primers designed according to the reported sequence data (12). The human cDNA coding for the DNA-binding domain of PARP (5′-EcoRI/HindIII) was inserted into pcDNA 3.1 (+) expression vector at the EcoRI/HindIII restriction sites downstream of the human CMV promoter/enhancer. Subsequently, PARP-DBD was tagged at its COOH terminus with a sequence encoding four Flag-epitope tags (13). The resulting recombinant plasmid, pCMV-DBD/F, permits constitutive expression of human PARP-DBD under control of the CMV promoter. To express the human PARP-DBD under control of the PSA gene regulatory elements, the CMV promoter sequences were replaced with a 1336-bp XhoI-EcoRV fragment of PSA enhancer fused with an EcoRI fragment containing 662-bp sequence of PSA promoter. The resulting plasmid, pPSA(e/p)-DBD/F is designed to express human PARP-DBD in androgen-inducible and PSA-dependent fashion.

Transient DNA Transfections. DNA transfections were carried out using an activated-dendrimer (Superfect; Qiagen) as described (13). Cells (2 × 10$^5$) were transfected with 5 μg of pCMV-DBD/F or pPSA(e/p)-DBD/F plasmids using a ratio of DNA to Superfect reagent of 1:10. Assays for PARP-DBD expression were performed 48 h after transfection.

Stably Transfected LNCaP Cell Lines. Cells were transfected with pPSA(e/p)-DBD/F, pCMV-DBD/F, or with control, neomycin-resistant expression vector pCMV-DDB/F, respectively, using Superfect reagent (Qiagen). The G418-resistant colonies from each replicated experiment were pooled to form polyclonal cell populations and were routinely maintained in medium containing 300 μg/ml G418.

PARP-DBD Immunodetection. Logarithmically growing cells were washed twice with cold PBS and lysed at 4°C for 30 min in buffer: 0.5% Triton X-100, 0.5% NP40, 2 mM Na$_2$OAc, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. For immunoprecipitation, cell lysates were normalized for protein content and incubated with anti-Flag M2 monoclonal antibody agarose affinity gel (Sigma), followed by Western blotting using polyclonal anti-PARP antibody (R&D System; 1:1000) directed against the aa 71–329 of PARP protein. The secondary antibody was donkey-antigoat IgG conjugated to horseradish peroxidase (Santa Cruz, 1:2000). Signals were detected using the ECL system (Amerham). For in situ PARP-DBD immunodetection, LNCaP cells (PSA-DBD) were grown on poly-L-lysine-coated glass slides (Fisher Scientific). After induction of PARP-DBD expression by synthetic androgen R1881 (10 nM) for 24 h, cells were subjected to fixation with 3.7% paraformaldehyde and incubated for 30 min with anti-Flag M2 monoclonal antibody (Sigma; dilution 1:200). Washes were followed by 30-min incubation with Cy5 conjugated secondary antibody (Jackson ImmunoResearch; dilution 1:200) in PBS, contained 10% donkey serum and 0.1% 300 Bloomgelatin. Transmitted and Cy5 fluorescence images were acquired using an IX 70 confocal microscope (Olympus, Melville, NY).

DNA Binding Assays. PARP and PARP-DBD affinity for DNA was assayed using double-stranded oligonucleotides coated onto magnetic beads. Briefly, 100 μg/mL streptavidin-coated Dynabeads (Dynal, ISS) were incubated with 120 pmols of 5′-biotinylated double-stranded plk (5′-GT-GAAAAGGTGAAAG) oligonucleotides (14) in accordance with the manufacturer’s instruction. Crude cell extracts in IP buffer were prepared from cells transiently transfected with pCMV-DBD/F or from parental LNCaP cells, and were normalized for protein content. Purified PARP protein (Alexis; specific activity 30 units/μg) or cell lysates were combined with plk-affinity beads and incubated for 30 min with gentle agitation at room temperature. The protein-bound beads were separated using a magnetic separator (Dynal), and bound proteins were eluted with 30 μl of 1 M NaCl and subsequently analyzed by Western blotting using goat polyclonal anti-PARP antibody (R&D Systems). Recombinant human PARP and streptavidin-coated beads containing no DNA were used as positive and negative controls, respectively.

RT-PCR Analyses. RNA was isolated from cells using TRIzol Reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. The primers for human DBD-Flag fusion protein were: sense, 5′-CTCCCTTACGTCTGCGT-3′; antisense, 5′-CTTTTATCACGTGATCGT-3′. RT-PCR was performed using 2 μg of total cellular RNA and the ThermoScript RT-PCR System (Life Technologies, Inc.)

RESULTS AND DISCUSSION

Human PSA Promoter/Enhancer Drives Expression of the PARP-DBD in LNCaP Cells. This study is focused on the unique properties of the DNA-binding domain of PARP as a potent molecular sensitizer to DNA-damaging treatments. Data from several investigations demonstrated that genetically engineered PARP-DBD is critically involved in DNA damage repair by acting as a trans-dominant inhibitor of PARP activity and that its overexpression in mammalian cells sensitizes them to DNA-damaging treatments (7). In this study we isolated and cloned the fragment of human PARP cDNA encompassing the region (aa 1–234) that encodes two zinc fingers of the enzyme as well as the KKKSKK nuclear localization signal (PARP-DBD). Subsequently, we developed plasmid vectors to express human PARP-DBD as a Flag-fusion protein in human prostate adenocarcinoma cells (LNCaP cell line) both constitutively and in androgen-dependent fashion (Fig. 1A). The recombinant plasmid, pCMV-DBD/F, permits constitutive expression of the PARP-DBD under control of the human CMV promoter. To achieve tissue-specific expression of the PARP-DBD in the androgen-sensitive LNCaP cells, we have constructed an expression vector, pPSA(e/p)-DBD/F, comprised of the coding region for the DNA-binding domain of PARP linked to 5′-flanked sequences (1.3 kb upstream enhancer and 0.6 kb minimal promoter) of the human PSA gene. The expression of the PARP-DBD Flag-fusion protein in LNCaP cells was confirmed in transient transfection assays (Fig. 1B). Immunoblot analysis of cell lysates revealed that exogenous PARP-DBD Flag fusion protein has a molecular mass of 31 kDa consistent with the length of corresponding cDNA, and is recognized by anti-Flag and anti-PARP antibodies. Functional activity of expressed PARP-DBD Flag-fusion protein was assayed in DNA binding reactions using double-stranded 5′-biotinylated oligonucleotides coupled to streptavidin-coated magnetic beads. These beads were used to recover DNA-binding proteins from LNCaP
cells transiently transfected with pCMV-DBD/F plasmid. We found (Fig. 1C) that both endogenous PARP and PARP-DBD fusion protein are captured effectively by DNA fragments, thus indicating that PARP-DBD retain its DNA-binding activity when expressed in LNCaP prostate carcinoma cells.

Androgen Responsiveness of the PARP-DBD Expression in LNCaP Cells. The 5'-regulatory sequences of the human PSA gene have been cloned and characterized (9). Deletion analysis of this region identified a minimal (core) promoter region (nt −320 to +12), strong upstream enhancer (nt −5824 to −3738), and the presence of down-regulating elements within the central region (nt −4136 to −541; Refs. 9, 10). Previous studies have identified the 5'-enhancer element linked to minimal core promoter of the human PSA gene as an effective combination of regulatory elements capable of driving the expression of reporter genes in PSA-producing prostate cancer cells both in vitro and in vivo (11, 15). Consequently, we developed and tested the construct, pPSA(e/p)-DBD/F, for its ability to drive the expression of PARP-DBD in a tissue-specific fashion, and its androgen responsiveness in prostate carcinoma cells.

The tissue specificity of PARP-DBD expression under control of the PSA promoter/enhancer was evaluated in transient transfection assays using the PSA-producing (LNCaP) and PSA-negative (PC-3) prostate cancer cells, as well as cells of nonprostate origin such as Ewing’s sarcoma (A4573 cell line). We found that PSA enhancer/promoter-driven expression of the human PARP-DBD was immunodetectable only in PSA-producing LNCaP prostate carcinoma cells but not in PSA-independent cell lines (Fig. 2). Although more PSA-producing cell lines need to be tested to elaborate a PSA dependence of the PARP-DBD expression, our data are consistent with findings reported previously that PSA promoter retains its tissue specificity both in vivo and in vitro (11, 15).

The 5' flanking region of the human PSA gene contains several androgen-responsive elements and is responsible for the androgen-dependent expression of PSA in benign and malignant prostate cells. To evaluate whether the PSA promoter/enhancer constructs support androgen responsiveness of PARP-DBD expression, LNCaP carcinoma cells were stably transfected with PARP-DBD expression vectors, and established polyclonal LNCaP sublines (PSA-DBD and CMV-DBD) were subsequently subjected to analysis of PARP-DBD expression levels. Cells were grown in medium containing charcoal-stripped serum for 7 days followed by incubation for 24 h in the absence or presence of the synthetic androgen, R1881 (0–10 nM). Western blot analysis and RT-PCR were performed to evaluate androgen-regulated expression of the human PARP-DBD in LNCaP cells (Fig. 3). Parental LNCaP cells and the LNCaP cell subline (CMV-DBD) were used as negative and positive controls, respectively, for PARP-DBD expression levels in these experiments. We found that expression of PSA-DBD cells to androgen (R1881) resulted in dose-dependent stimulation of PARP-DBD expression at levels of mRNA (Fig. 3B) and protein (Fig. 3A). No notable changes in the PARP-DBD expression levels have been observed in control cell lines exposed to R1881 at doses up to 10 nM (data not shown). Androgen-dependent regulation of PARP-DBD expression in PSA-DBD prostate carcinoma cells was additionally confirmed by in situ immunodetection of the PARP-DBD-Flag fusion protein using fluorescence microscopy (Fig. 3C). These data indicate that the pPSA(e/p)-DBD/F recombinant vector allows expression of functionally active DBD of PARP in vitro and that the androgen-dependent expression is specific to PSA-producing prostate carcinoma cells.

PARP-DBD Expression Sensitizes LNCaP Cells to DNA Damage. The PARP-DBD fragment acts as a trans-dominant inhibitor of PARP activity by competing with endogenous wild-type PARP for DNA strand breaks (6). Furthermore, using atomic force microscopy we have demonstrated recently that PARP-DBD binds to broken DNA strands irreversibly (16), making them inaccessible to DNA repair enzymes. These data suggest that forced expression of the PARP-DBD can impair the function of endogenous PARP in cellular responses to DNA damage leading to accumulation of sustained lesions in the genome, thereby overcoming cellular resistance to radio- and chemotherapeutic intervention. In support of this suggestion, the sensitization of the DBD-expressing mammalian cells to ionizing radiation and DNA-damaging agents has been demonstrated recently (7).
then to the presence of androgen in the growth medium. In fact, other studies have shown that androgens are potent stimulators of LNCaP cells growth in vitro (17).

We next examined whether the PARP-DBD-mediated sensitization of LNCaP cells to DNA damage is attributable to an increased rate of apoptosis. Quantitative measurements of cell death were carried out using annexin V-propidium iodide staining and mitochondrial depolarization assays. Previous studies (18) show that LNCaP cells are highly resistant to ionizing radiation and fail to activate classical apoptotic pathways in response to DNA-damaging treatments. In agreement with these findings, we found that parental LNCaP cells as well as the uninduced PSA-DBD cell subline exhibit only marginal levels of cell death after exposure to ionizing radiation or etoposide (Fig. 4). When PARP-DBD expression was induced by R1881, irradiated or etoposide-treated LNCaP (PSA-DBD) cells showed significantly (>2-fold) increased staining for annexin V (Fig. 4B) and depolarization of mitochondrial membrane (Fig. 4C) within 24 h of treatment. These data indicate that perturbation of PARP function via enforced expression of its dominant-negative mutant (PARP-DBD) results in enhanced sensitivity of prostate cancer cells to DNA-damaging treatments. Considering the fact that androgens block apoptosis in LNCaP cells triggered by diverse agents, including ionizing

To investigate whether PARP-DBD would increase the susceptibility of human prostate carcinoma to DNA-damaging treatments, the expression of PARP-DBD in LNCaP (PSA-DBD) cells was induced by R1881, and cells were subsequently exposed to ionizing radiation or etoposide (VP-16). We found that androgen (R1881)-dependent stimulation of PARP-DBD expression significantly enhanced (at least 2-fold) growth inhibition of PSA-DBD cells in response to DNA damage, compared with control cells (Fig. 4A). This inhibition can be attributed to PARP-DBD expression in LNCaP cells (Fig. 3) rather
radiation (19), our observations suggest that overexpression of the PARP-DBD augments apoptotic pathways in these cells in an androgen-independent fashion. Additional investigations are required to elucidate the mechanisms for PARP-DBD-mediated sensitization of LNCaP cells to DNA damage, as well as the enhanced apoptotic responses in DBD-expressing prostate cancer cells. The studies addressing these questions are currently underway.

Although many prostate cancer cells are deficient in DNA mismatch repair, they are resistant to ionizing radiation and DNA-damaging drugs. Therefore, targeting molecular components that are critically involved in maintenance of genome stability is a promising approach directed at overcoming intrinsic tumor cell resistance to DNA-damaging treatments. From this point of view, the strategy described here represents a novel starting point for the design of PARP-based molecular therapies targeting prostate cancer in vivo. First, this approach uses tissue-specific (prostate carcinoma) and treatment-specific (DNA damage) gene therapy for prostate cancer. Next, to avoid the potential side effects due to expression of PSA in tissues other than prostate, tumor cells are targeted using an agent that is not functionally active in the absence of massive DNA damage and, therefore, would not be toxic to cells outside of the irradiated volume or pose a genetic risk to the patient. Furthermore, PARP-DBD-mediated cell death is independent of cell proliferation states because both nondividing cells and rapidly proliferating cancer cells cannot survive the massive accumulation of long-lived damage in the genome (20). Thus, targeting tumor cells with the PARP-DBD can be beneficial especially for the control of prostate cancer, because prostate cancers usually grow very slowly. These properties of the PARP-DBD are in marked contrast to conventional chemotherapeutic drugs, which primarily target proliferating cells. In summary, the plasmid vector developed in this study permits the expression of the human PARP-DBD in an androgen-inducible and PSA-dependent fashion, and sensitizes prostatic adenocarcinoma cells to DNA-damaging treatments. These results provide a proof-of-principle for a novel therapeutic strategy to control prostate cancer.

ACKNOWLEDGMENTS

We thank David Andolino for technical assistance and Elaine North for help with manuscript preparation.

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