Acquisition of Chemoresistant Phenotype by Overexpression of the Antiapoptotic Gene Testosterone-repressed Prostate Message-2 in Prostate Cancer Xenograft Models

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ARTICLE

ABSTRACT

Testosterone-repressed prostate message-2 (TRPM-2) expression is highly up-regulated in normal and malignant prostate cells after androgen withdrawal. Although recent studies have suggested a protective role of TRPM-2 expression against apoptosis in several experimental models, the functional role of TRPM-2 in chemotherapy-induced apoptosis remains undefined. Here, we demonstrated that overexpression of TRPM-2 in human androgen-dependent LNCaP prostate cancer cells by stable transfection rendered them highly resistant to paclitaxel treatment than control LNCaP cells, with a 20-fold higher IC50 through the inhibition of apoptotic cell death. In mice bearing TRPM-2-overexpressing LNCaP tumors, tumor volume and serum prostate-specific antigen increased two to three times faster after castration and paclitaxel treatment compared with mice bearing control tumors. We then tested the efficacy of combined treatment with antisense TRPM-2 oligodeoxynucleotide (ODN) and paclitaxel in the mouse androgen-dependent Shionogi tumor model. Antisense TRPM-2 ODN treatment significantly enhanced paclitaxel chemosensitivility of Shionogi tumor cells in a dose-dependent manner, reducing the IC50 by 75%. Combined treatment of Shionogi cells with 500 nM antisense TRPM-2 ODN and 10 nM paclitaxel-induced apoptosis, either agent alone did not. Adjutant administration of antisense TRPM-2 ODN and polymorphic micellar paclitaxel after castration resulted in reduced TRPM-2 levels in vivo and a significant delay of emergence of androgen-independent recurrent Shionogi tumors compared with administration of either agent alone. Furthermore, combined treatment of mice bearing androgen-independent recurrent Shionogi tumors with antisense TRPM-2 ODN and micellar paclitaxel inhibited tumor growth compared with treatment with either agent alone. Collectively, these findings demonstrate that TRPM-2 overexpression helps confer a chemoresistant phenotype through inhibition of apoptosis, and that antisense TRPM-2 ODN may be useful in enhancing the effects of cytotoxic chemotherapy in hormone-refractory prostate cancer.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer deaths in men in Western industrialized countries. To date, no therapy exists that surpasses androgen withdrawal for men with advanced disease, with symptomatic and/or objective response in ~80% of patients. However, progression to androgen independence ultimately occurs in nearly all of these cases (1). Several hundred clinical studies using traditional cytotoxic chemotherapeutic agents document objective response rates of <10% and no improved survival rates (2). Accordingly, progression to androgen independence remains the main obstacle to improving the survival and quality of life in patients with advanced disease, emphasizing the need for novel therapeutic strategies that target the molecular mechanism of the androgen- and chemoresistant phenotype of prostate cancer.

TRPM-2, also known as clusterin, sulfated glycoprotein-2, or apolipoprotein J, was first isolated from ram rete testes fluid (3) and has been proposed to have various biological functions, including tissue remodeling, reproduction, lipid transport, and apoptotic cell death (4). TRPM-2 was initially regarded as a marker for cell death, because its expression is highly up-regulated in various normal and malignant tissues undergoing apoptosis (5–8). Recent studies, however, report conflicting findings on the association between enhanced TRPM-2 expression and apoptotic activity (9–11). Similarly, TRPM-2 expression is increased in regressing normal prostate after androgen ablation (5, 12), and its up-regulation has been shown to be associated with antiapoptotic activity and disease progression in prostate cancer (13–15). We have recently reported that TRPM-2 expression in prostate cancer cells has a protective role against castration-induced apoptosis (16). However, the functional significance of TRPM-2 expression in apoptosis induced by chemotherapeutic agents has not been investigated.

Controlled study of the complex molecular processes associated with progression to androgen independence in prostate cancer has proved difficult, because few animal models exist that reproducibly mimic the clinical course of the disease in men. The AD Shionogi mouse mammary carcinoma model is particularly useful for testing the efficacy of agents targeting castration-induced apoptosis and their effects on time to progression of androgen independence. AD Shionogi tumors in intact male mice undergo complete regression after castration but recur as rapidly growing AI tumors after 1 month in a highly reproducible manner (17). Of the available human prostate cancer cell lines, only the LNCaP tumors are AD when xenografted into male immunodeficient mice, PSA secreting, and immortalized in vitro. As in human prostate cancer, serum PSA levels in the LNCaP tumor model are initially regulated by androgen and directly proportional to tumor volume, with loss of androgen-regulated PSA gene expression after castration as a surrogate end point of progression to androgen independence (18).

In the present study, we evaluated the effects of TRPM-2 overexpression on time to progression of androgen independence after castration and paclitaxel treatment in the LNCaP tumor model. We then evaluated the effects of paclitaxel treatment on TRPM-2 gene expression in Shionogi tumor cells and the effects of antisense TRPM-2 ODN on paclitaxel chemosensitivity using the Shionogi tumor model.

MATERIALS AND METHODS

Paclitaxel. Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of paclitaxel (1 mg/ml) was prepared with DMSO and diluted with PBS to the required concentrations before each in vitro experiment.

Received 10/27/99; accepted 3/6/00.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Cancer Institute of Canada Grant 009002 and the American College of Surgeons George A. H. Clowes Career Development Award.

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3 The abbreviations used are: TRPM-2, testosterone-repressed prostate message-2; AI, androgen-independent; AD, androgen-dependent; PSA, prostate-specific antigen; ODN, oligodeoxynucleotide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase; CMV, cytomegalovirus; poly(A)+ mRNA, polyadenylated mRNA.
ment. Polymeric micellar paclitaxel used in the in vivo studies was generously supplied by Dr. Helen M. Burt (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada).

Antisense TRPM-2 ODN. Phosphorothioate ODN used in this study was obtained from Nucleic Acid-Protein Service Unit, University of British Columbia. The sequences of antisense TRPM-2 ODN corresponding to the mouse TRPM-2 translation initiation site were 5'-GCACAGCAGGAGAATCT-TCAT-3'. A 2-base TRPM-2 mismatch ODN (5'-GCACAGCAGGAGGATATTCAT-3') was used as control.

LNCaP Sublines. LNCaP cells were kindly provided by Dr. Leland Chung (University of Virginia, Charlottesville, VA) and maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS. Steroid hormone-depleted charcoal-stripped media were prepared as described previously (19). A pRC-CMV expression vector containing the 1.6-kb cDNA fragment encoding human TRPM-2 was kindly provided by Dr. Martin Tenniswood (W. Alton Jones Cell Science Center, Lake Placid, NY). The expression vector was transfected into LNCaP cells by the liposome-mediated gene transfer method as described previously (20). Briefly, 2 × 10^6 LNCaP cells were plated in 6-cm plates. The next day, 5 μg of purified TRPM-2-cloned pRC-CMV or pRC-CMV alone (as a control) were added to LNCaP cells after a preincubation for 30 min with 5 μg of LipofectAMINE reagent and 3 ml of serum-free Opti-MEM (Life Technologies). Drug selection, in 300 μg/ml Geneticin (Sigma), was begun 3 days after the transfection. Colonies were harvested 2 weeks after drug selection using cloning cylinders and expanded to cell lines.

Assessment of in Vivo LNCaP Tumor Growth and Determination of Serum PSA Levels. One million cells of each LNCaP subline were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Lincoln Park, NJ) in the flank region of 6- to 8-week-old male athymic nude mice (BALB/c strain; Charles River Laboratory, Montreal, Quebec, Canada). Each experimental group consisted of six mice. Mice were castrated via a scrotal approach when tumors reached 200–300 mm³ in volume, and from 10 to 14 days after the treatment, tumor volume was measured. Drug treatment began 7 days after the inoculation, and the results were compared with baseline data obtained from castrated mice. Each cell line was treated with 0.5 nM paclitaxel. After 48 h of incubation, DNA was extracted from culture cells, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and UV transillumination.

Proteins were extracted from each cell line after the same treatment as described in C and analyzed by Western blotting with an anti-PARP antibody. Uncleaved intact PARP, 116 kDa; cleaved PARP, 85 kDa.

Fig. 1. A, Western blot analysis of TRPM-2 protein in TRPM-2-transfected LNCaP cell lines. Protein was extracted from PC3 (positive control for the screening of TRPM-2 protein expression), LNCaP/P (parental cell line of LNCaP), LNCaP/C (vector-only transfected cell line), and four clones of TRPM-2 transfected LNCaP cells (LNCaP/T1 to LNCaP/T4), and TRPM-2 and β-tubulin levels were analyzed by Western blotting. Molecular mass: unprocessed form of TRPM-2, 60 kDa; mature form of TRPM-2, 40 kDa.

B, cytotoxic effect of paclitaxel treatment on LNCaP sublines in standard medium with 5% FCS. C, cytotoxic effect of paclitaxel treatment on LNCaP sublines in charcoal-stripped FCS. Each cell line in B and C was treated with various concentrations of paclitaxel for 72 h, and cell viability was then determined by in vitro mitogenic assay. Each data point represents the mean of three independent experiments ± SD. LNCaP/T1 and LNCaP/T2 showed significantly higher resistance to paclitaxel treatment than LNCaP/P and LNCaP/C (P < 0.01). D, DNA fragmentation assay of LNCaP sublines treated with paclitaxel. Each cell line was treated with 0.5 nM paclitaxel. After 48 h of incubation, DNA was extracted from culture cells, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and UV transillumination. E, Proteins were extracted from each cell line after the same treatment as described in C and analyzed by Western blotting with an anti-PARP antibody. Uncleaved intact PARP, 116 kDa; cleaved PARP, 85 kDa.
castration, 0.5 mg of polymeric micellar paclitaxel was administered once daily by i.v. injection. Tumor volume was measured once weekly and calculated by the formula length \( \times \) width \( \times \) depth \( \times 0.5236 \) (19). Blood samples were obtained with tail vein incisions of mice once weekly. Serum PSA levels were determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.2 \( \mu \)g/liter (Abbott IMX, Montreal, Quebec, Canada) according to the manufacturer’s protocol. Data points were reported as mean values \( \pm SD \).

**Shionogi Tumor Growth.** The Toronto subline of the transplantable SC-115 AD mouse mammary carcinoma was used in all experiments (21). Shionogi tumors were maintained in DMEM (Life Technologies) supplemented with 5% heat-inactivated FCS. For *in vivo* studies, \( 5 \times 10^6 \) cells of the Shionogi carcinoma were injected s.c. into adult male DD/S strain mice. When Shionogi tumors became 1–2 cm in diameter, usually 2–3 weeks after injection, castration was performed through an abdominal incision under methoxyflurane anesthesia. Details of the maintenance of mice, tumor stock, and operative procedures are described in a previous publication (22).

**Treatment of Cells with ODN.** *In vitro*-cultured cells were treated with various concentrations of ODN after a preincubation for 20 min with 4 \( \mu \)g/ml Lipofectin (Life Technologies) in serum free Opti-MEM. Media containing ODN and Lipofectin was replaced 4 h later with standard culture medium described above.

**Northern Blot Analysis.** Total RNA was isolated from *in vitro*-cultured cells and *in vivo* tumor tissues by the acid-guanidium thiocyanate-phenol-chloroform method. Poly(A)\(^+\) mRNA was then purified from total RNA using oligo(dT) cellulose (Pharmacia Biotech Inc., Uppsala, Sweden). Five micrograms of poly(A)\(^+\) mRNA from each sample were subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham, Arlington Heights, IL) overnight according to standard procedure (17). The RNA blots were hybridized with a mouse TRPM-2 cDNA probe labeled with \( [32\text{P}] \)dCTP by random primer labeling. After stripping, the membranes were rehybridized with a mouse G3PDH cDNA probe. These probes were generated by reverse transcription-PCR from total RNA of mouse brain using primers \( 5'\)-AATGAGCTCAGAAGCTG-TCCACT-3' (sense) and \( 5'\)-AAAGAGCGGTTGCTAGTGACCAGT-3' (antisense) for TRPM-2 and \( 5'\)-ATGTTGAAAGCGGTTGACGGAT-3' (sense) and \( 5'\)-AAAGTTGCTAGTGACCCT-3' (antisense) for G3PDH. Density of bands for TRPM-2 was normalized against that of G3PDH by densitometric analysis.

**Western Blot Analysis.** The expression of TRPM-2 and PARP protein in cultured cells and tumor tissues was determined by Western blot analysis as described previously (20). Briefly, samples containing equal amounts of protein (15 \( \mu \)g) were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were blocked in PBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 h with an anti-human TRPM-2 goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-human PARP mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-human PARP mouse monoclonal antibody (PharMingen, Mississauga, Ontario, Canada), or anti-rat \( \beta \)-tubulin mouse monoclonal antibody (Chemicon International Inc., Temecula, CA). The filters were then incubated for 30 min with horseradish peroxidase-conjugated anti-goat or mouse IgG antibody (Amersham), and specific proteins were detected using an enhanced chemiluminescence system (Amersham).

**In Vitro Cell Growth Assays.** The *in vitro* growth of LNCaP and Shionogi tumor cells was assessed by the *in vitro* mitogenic assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, respectively, as described previously (19, 20). Briefly, 3 \( \times 10^5 \) cells were seeded in each well of 96-well microtiter plates and allowed to adhere overnight. After treatment with various concentrations of paclitaxel and/or ODN, LNCaP cells were fixed with 1% glutaraldehyde (Sigma) and stained with 0.5% crystal violet (Sigma), and Shionogi tumor cells were treated with 20 \( \mu \)l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) in PBS, followed by incubation for 4 h at 37°C. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware) at 540 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percent of survival. Each assay was performed in triplicate.

**In Vitro Shionogi Tumor Growth.** To determine whether combined antisense TRPM-2 ODN and paclitaxel treatment delays time to AI recurrence after castration compared with either agent alone, male DDS mice bearing Shionogi tumors were castrated and randomly selected for treatment with antisense TRPM-2 ODN alone (group 1), mismatch control ODN alone (group 2), antisense TRPM-2 ODN plus paclitaxel (group 3), or mismatch control ODN plus paclitaxel (group 4). Each experimental group consisted of seven mice. Beginning the day of castration, 12.5 mg/kg antisense TRPM-2 or DNA Fragmentation Analysis. The nucleosomal DNA degradation was analyzed as described previously with a minor modification (20). Briefly, \( 1 \times 10^5 \) cultured cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After the indicated treatment with paclitaxel and/or ODN, cells were harvested and then lysed in a solution containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, and 0.5% SDS. After the centrifugation, the supernatants were incubated with 300 \( \mu \)g/ml proteinase K for 5 h at 65°C and extracted with phenol-chloroform. The aqueous layer was treated with 0.1 volume of 3 M sodium acetate, and the DNA was precipitated with 2.5 volumes of 95% ethanol. After treatment with 100 \( \mu \)g/ml RNase A for 1 h at 37°C, the sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

**Assessment of In Vivo Shionogi Tumor Growth.** To determine whether combined antisense TRPM-2 ODN and paclitaxel treatment delays time to AI recurrence after castration compared with either agent alone, male DDS mice bearing Shionogi tumors were castrated and randomly selected for treatment with antisense TRPM-2 ODN alone (group 1), mismatch control ODN alone (group 2), antisense TRPM-2 ODN plus paclitaxel (group 3), or mismatch control ODN plus paclitaxel (group 4). Each experimental group consisted of seven mice. Beginning the day of castration, 12.5 mg/kg antisense TRPM-2 or
mismatch control ODN was injected i.p. once daily into each mouse for 15 days. From 10 to 14 days after castration, 0.5 mg polymeric micellar paclitaxel was administered once daily by i.v. injection in groups 3 and 4. A second set of experiments was designed to evaluate the effects of combined treatment on established AI recurrent tumors. Castrate male DD/S mice bearing AI Shionogi tumors ~0.5 cm in diameter were randomly selected to receive three treatment regimens as described above. Tumor volume was measured twice weekly and calculated as described above. Data points were reported as average tumor volume ± SD.

**Statistical Analysis.** The in vitro cytotoxic effects of ODN and/or paclitaxel were analyzed using a repeated measure ANOVA model. AI recurrence-free survival curves were calculated by the method of Kaplan-Meier and evaluated with the Mantel-Cox log rank test. The remaining data were analyzed by Student’s t test. The level of statistical significance was set at \( P < 0.05 \), and all statistical calculations were done by use of Statview 4.5 software (Abacus Concepts, Inc., Berkeley, CA).

## RESULTS

### Increased Resistance to Paclitaxel by Overexpression of TRPM-2 in LNCaP Cells in Vitro

Western blot analysis was used to examine TRPM-2 protein expression levels in the LNCaP sublines. As shown in Fig. 1A, abundant levels of both unprocessed (60-kDa) and mature (40-kDa) forms of TRPM-2 protein were detected in TRPM-2-transfected clones (LNCaP/T1 to LNCaP/T4) at almost the same levels, whereas the parental LNCaP (LNCaP/P) and the control vector-transfected cell line (LNCaP/C) did not express detectable TRPM-2 protein levels. The four TRPM-2-transfected clones showed almost the same results in the subsequent experiments; therefore, we hereafter report only the data of LNCaP/P, LNCaP/C, LNCaP/T1, and LNCaP/T2.

To determine whether TRPM-2 overexpression confers a chemoresistant phenotype on LNCaP cells in vitro, the growth rates of LNCaP sublines after paclitaxel treatment were analyzed using normal and charcoal-stripped media. As shown in Fig. 1, B and C, LNCaP/T1 and LNCaP/T2 exhibited significantly higher resistance to paclitaxel compared with LNCaP/P and LNCaP/C both in normal and charcoal-stripped media (\( P < 0.01 \) for both). Overexpression of TRPM-2 in LNCaP cells increased the IC\text{50} of paclitaxel 5-fold (from 1.5 to 8 nM) in normal media and 20-fold (from 0.01 to 0.4 nM) in charcoal-stripped media (Fig. 1B).

The induction of apoptosis in LNCaP sublines in normal media treated with 1 nm paclitaxel for 72 h was assessed by DNA degradation assay and Western blot analysis of PARP protein, a substrate of the caspases activated during the process of apoptotic execution (23). The characteristic apoptotic DNA ladders were detected in LNCaP/P and LNCaP/C but not in LNCaP/T1 and LNCaP/T2 (Fig. 1D). Similarly, the \( M_t \) 116,000 intact form of PARP was observed in all of LNCaP sublines, whereas the \( M_t \) 85,000 PARP cleavage fragment was detected after paclitaxel treatment only in LNCaP/P and LNCaPC (Fig. 1E).

### Acquisition of Resistant Phenotype to Paclitaxel by Overexpression of TRPM-2 in the LNCaP Tumor Model in Vivo

To determine whether TRPM-2 overexpression confers resistance to paclitaxel treatment in vivo, \( 1 \times 10^6 \) cells of each cell line (LNCaP/P, LNCaP/C, LNCaP/T1, or LNCaP/T2) were inoculated s.c. in male nude mice. When tumors reached 200–300 mm\(^3\), mice were castrated. Beginning 10 days after castration, 0.5 mg of polymeric micellar paclitaxel was administered i.v. once daily for 5 days. LNCaP/P and LNCaP/C tumor growth decreased by 61 and 57%, respectively, by 4 weeks after castration and remained below precastrate volumes by 10 weeks after castration. In contrast, LNCaP/T1 and LNCaP/T2 tumor volume decreased by 11 and 28%, respectively, by 4 weeks after castration and thereafter increased 2.4- and 1.9-fold, respectively, by 10 weeks after castration (Fig. 2A). Serum PSA in mice bearing LNCaP/P and LNCaP/C tumors decreased by 77 and 75%, respectively, by 1 week after castration and remained below precastrate levels by 10 weeks after castration. In comparison, serum PSA in mice bearing LNCaP/T1 and LNCaP/T2 tumors decreased by 51 and 55%, respectively, before increasing 1.6- and 1.4-fold above precastrate levels, respectively, by 10 weeks after castration (Fig. 2B).

### Changes in TRPM-2 Expression in Shionogi Tumor Cells after Antisense TRPM-2 ODN and Paclitaxel Treatment

Northern blot analysis was used to determine the effects of paclitaxel treatment on TRPM-2 mRNA expression in Shionogi tumor cells. As shown in Fig. 3A, TRPM-2 mRNA induction increased in a dose-dependent manner by paclitaxel treatment at concentrations up to 10 nM. Time course experiments demonstrated that paclitaxel-induced TRPM-2 mRNA up-regulation peaked by 48 h after treatment and began decreasing by 72 h after treatment (Fig. 3B).

We then examined the effects of combined treatment with antisense TRPM-2 ODN and paclitaxel on TRPM-2 mRNA expression in...
Synergistic Effects of Antisense TRPM-2 ODN and Paclitaxel Treatment on Induction of Apoptosis in Shionogi Tumor Cells.

To examine whether treatment with antisense TRPM-2 ODN enhances the paclitaxel-induced cytotoxicity, Shionogi tumor cells were treated with various concentrations of antisense TRPM-2 ODN once daily for 2 days and then incubated with various concentrations of paclitaxel for 2 days. As shown in Fig. 4A, antisense TRPM-2 ODN treatment significantly enhanced paclitaxel chemosensitivity in a dose-dependent manner (P < 0.01), reducing the IC50 of paclitaxel from 100 to 25 nM, whereas mismatch control ODN had no effect. Dose-dependent synergy between antisense TRPM-2 ODN and paclitaxel was also observed by increasing the antisense ODN concentration when paclitaxel concentration was fixed at 10 nM (P < 0.01; Fig. 4B).

DNA fragmentation assay and Western analysis of PARP protein were used to evaluate effects of combined antisense TRPM-2 ODN (500 nM) and paclitaxel (10 nM) treatment on apoptosis induction. After the same treatment schedule described above, characteristic apoptotic DNA laddering was observed only after combined treatment with antisense TRPM-2 ODN and paclitaxel (Fig. 4C). Similarly, cleavage of PARP protein was detected only after combined antisense TRPM-2 ODN and paclitaxel treatment (Fig. 4D).

Delayed Hormone-refractory Recurrence of Shionogi Tumors in Vivo by Combined Antisense TRPM-2 ODN and Paclitaxel Treatment. Male mice bearing Shionogi tumors between 1 and 2 cm in diameter were randomly selected for treatment with either antisense TRPM-2 ODN alone, mismatch control ODN alone, antisense TRPM-2 ODN plus micellar paclitaxel, or mismatch control ODN plus micellar paclitaxel. Mean tumor volume was similar at the beginning of treatment in all four treatment groups. Beginning 10 days after castration, 0.5 mg of polymeric micellar paclitaxel was administered i.v. once daily for 5 days. During an observation period of 60 days after castration, AI tumors recurred in all mice after a median of 28, 39, and 42 days in the mismatch control ODN treatment group, antisense TRPM-2 ODN treatment group, and mismatch control ODN plus micellar paclitaxel treatment group, respectively (P < 0.01).
Mean tumor volume at day 60 after castration was 2789, 3481, 712, and 1276 mm$^3$ in the antisense TRPM-2 ODN, mismatch control ODN, antisense TRPM-2 ODN plus micellar paclitaxel, and mismatch control ODN plus micellar paclitaxel. Beginning the day of castration, 12.5 mg/kg antisense TRPM-2 ODN or mismatch control ODN was injected i.p. once daily for 15 days. Beginning 10 days after castration, 0.5 mg of micellar paclitaxel was injected i.v. once daily for 5 days. Tumor volume was measured twice weekly and calculated by the formula length $\times$ width $\times$ depth $\times$ 0.5236. Each point represents the mean tumor volume in each experimental group containing seven mice $\pm$ SD. Mean tumor volume in mice treated with antisense TRPM-2 ODN plus micellar paclitaxel 60 days after castration was significantly smaller than that in mice treated with antisense TRPM-2 ODN alone or mismatch control ODN plus micellar paclitaxel ($P < 0.001$). A, AI recurrence-free survival curves in mice treated as described in A. The time to progression of androgen independence in mice treated with antisense TRPM-2 ODN plus micellar paclitaxel was significantly delayed compared with mice treated with antisense TRPM-2 ODN alone or mismatch control ODN plus micellar paclitaxel ($P < 0.01$).

**Fig. 5. Effects of adjuvant administration of antisense TRPM-2 ODN and polymeric micellar paclitaxel after castration on Shionogi tumor growth.** A, mice treated with antisense TRPM-2 ODN alone, antisense TRPM-2 ODN plus micellar paclitaxel, and mismatch control ODN plus micellar paclitaxel. B, AI recurrence-free survival curves in mice treated as described in A. The time to progression of androgen independence in mice treated with antisense TRPM-2 ODN plus micellar paclitaxel was significantly delayed compared with mice treated with antisense TRPM-2 ODN alone or mismatch control ODN plus micellar paclitaxel ($P < 0.01$).

**Fig. 6. Effects of combined treatment with antisense TRPM-2 ODN and polymeric micellar paclitaxel on AI Shionogi tumor growth.** A, mice bearing AI recurrent Shionogi tumors were randomly selected for treatment with antisense TRPM-2 ODN alone, antisense TRPM-2 ODN plus micellar paclitaxel, and mismatch control ODN plus micellar paclitaxel. Treatments and measurement of tumor volume were performed using methods described above. Consistent with the in vitro results, antisense TRPM-2 ODN treatment resulted in a substantial reduction in TRPM-2 mRNA in AI Shionogi tumors (Fig. 6B). Furthermore, the $M_r$ 85,000 PARP cleavage fragment was de-
and observed that TRPM-2 transfectants were more highly resistant to paclitaxel chemosensitivity using TRPM-2-transfected LNCaP cells. The emergence of AI tumors beyond that achieved with either agent alone and enhanced regression of established AI tumors. Although early adjuvant antisense TRPM-2 ODN therapy after castration delayed progression of androgen independence, treatment with antisense TRPM-2 ODN alone had no effect on growth rates of established AI tumors. However, combined treatment with antisense TRPM-2 ODN plus paclitaxel decreased TRPM-2 mRNA expression and accelerated apoptosis induction in AI Shionogi tumors in vivo. These findings illustrate the efficacy of combined antisense TRPM-2 ODN and paclitaxel treatment for cooperatively delaying progression to androgen independence.

Integration and appropriate timing of combination therapies, based on changes in expression of functionally relevant genes after androgen ablation, may help delay progression to androgen independence. The results in the present study provide proof of principle for two potential strategies to delay emergence of the AI phenotype. The first strategy would initiate treatment earlier to enhance castration-induced apoptosis by targeting the antiapoptotic TRPM-2 gene up-regulation by androgen ablation with antisense TRPM-2 ODN. The second strategy would attempt to enhance sensitivity to conventional chemotherapy by reduction of TRPM-2-mediated chemoresistance with antisense TRPM-2 ODN. The preclinical data presented here provide support for clinical studies with combined antisense TRPM-2 ODN and paclitaxel therapy for advanced prostate cancer.

ACKNOWLEDGMENTS

We thank Mary Bowden and Howard Tearle for excellent technical assistance.

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Cancer Res 2000;60:2547-2554.

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