

Programmed Cell Death during Regression of PC-82 Human Prostate Cancer following Androgen Ablation¹

Natasha Kyprianou, Hugh F. English, and John T. Isaacs²

The Johns Hopkins Oncology Center, Baltimore, Maryland 21205 [N. K., J. T. I.], and the Divisions of Endocrinology and Urology, Hershey Medical Center, Hershey, Pennsylvania 17033 [H. F. E.]

ABSTRACT

To study the mechanism of regression of human prostatic cancer following androgen ablation, the androgen-responsive PC-82 human prostatic adenocarcinoma xenograft was used as a model system. Castration of male nude mice bearing PC-82 xenografts results in a 50% tumor regression by 2 wk following androgen ablation. This regression is due to a sequence of biochemical and morphological events that results in both the cessation of cell proliferation and activation of programmed death or apoptosis of the androgen-dependent prostatic cancer cells. Associated with this response are an enhanced expression of the transforming growth factor β_1 gene, a potent inhibitor of cell proliferation, and testosterone-repressed prostatic message 2 (designated TRPM-2), a programmed cell death-associated gene. Fragmentation of tumor DNA into nucleosomal oligomers and histological appearance of apoptotic bodies are characteristic early events that preceded the dramatic reduction in tumor volume following androgen ablation. These results suggest that androgen-dependent human prostatic cancer cells, like normal prostatic cells, retain the ability to inhibit proliferation and to activate programmed cell death in response to androgen ablation. Clarification of the biochemical pathway involved in the activation of this programmed cell death should identify new targets of therapy for even androgen-independent human prostatic cancer.

INTRODUCTION

Androgen has the dual ability to stimulate cell proliferation and inhibit cell death of normal rat prostatic glandular epithelial cells (1). Androgen ablation induces a series of discrete biochemical events that leads to a cessation of cell proliferation and the activation of programmed death of these androgen-dependent prostatic cells, ultimately resulting in the involution of the gland (2-9). Within 12 h after castration of adult male rats, the serum testosterone concentration decreases to below 2% of the value present in intact hosts (2, 10). This rapid decline in serum androgen results in the ventral prostatic dihydrotestosterone concentration decreasing within the first 24 h following castration to below a critical threshold value which results in cessation of proliferation and the death of the androgen-dependent ventral prostatic glandular epithelial cells (2, 10, 11).

The death of the prostatic glandular epithelial cells induced by androgen ablation occurs as an active energy-dependent process which involves a cascade of biochemical changes, collectively referred to as programmed cell death (2-5). The majority of the glandular epithelial cells which die via the programmed cell death pathway are not in the proliferative cell cycle (*i.e.*, G₀ cells) nor is proliferation required for these cells to undergo this suicide process (2). It is the activation of this programmed cell death pathway in G₀ glandular epithelial cells which results in the rapid involution of the rat ventral prostate following castration. Associated with this programmed cell

death is enhanced expression of a series of genes within the prostate. This series includes an increase in the expression of the TGF- β_1 gene (5), *c-myc* protooncogene (12), *c-fos* protooncogene (12), *M*, 70,000 heat shock gene (13), the TRPM-2 gene (14), and the glutathione *S*-transferase Xb₁ gene (13).

The exact function of any of these epigenetic changes, activated following androgen ablation, is not entirely clear. It is known, however, that, like other systems in which programmed cell death occurs (15-18), this type of cell death initially involves fragmentation of genomic DNA. This fragmentation involves enzymatic degradation of the genomic DNA into the nucleosomal oligomers (*i.e.*, multiples of a 180 nucleotide base pair subunit) lacking intranucleosomal breaks in the DNA. This fragmentation of prostatic DNA is a result of activation of a Ca²⁺-Mg²⁺-dependent endonuclease present within the nucleus induced by elevation of intracellular free Ca²⁺ occurring following androgen ablation (3, 19, 20). This Ca²⁺-Mg²⁺-dependent nuclease selectively hydrolyzes prostatic DNA at sites located between nucleosomal units, thus resulting in the stereotypic ladder of DNA fragments (2, 3). This DNA fragmentation is subsequently followed by irreversible morphological changes, histologically defined as apoptosis, which characteristically involve chromatic condensation, nuclear disintegration, cell surface blebbing, and eventually cellular fragmentation into a cluster of membrane-bound apoptotic bodies (21-24). In contrast to this programmed cell death process in which the death of the prostatic cells is the result of the activation of an energy-dependent biochemical pathway, there is another type of cell death process termed necrotic death (25). Necrotic cell death can be induced by a variety of nonphysiological agents [*i.e.*, HgCl₂, iodoacetate, cyanide freezing and thawing, detergents, etc.] which create an osmotic imbalance in the cell leading to the release of mitochondrial and lysosomal enzymes of the cell (25). This enzyme release stops catabolic activities while initiating autolysis of the cell, producing its death. In necrotic cell death, the cell osmotically lyses in an energy-independent fashion, and any DNA degradation, which occurs, does so after the cell is already dead via the released lysosomal hydrolyases. Comparisons of the temporal induction of DNA fragmentation, appearance of apoptotic bodies, and prostatic regression following castration have demonstrated that the fragmentation of genomic DNA is actively involved in the death of the androgen-dependent rat ventral prostate glandular cells and does not occur as a result of the cells already being dead (2, 4). Thus, the death of the glandular epithelial cells within the ventral prostate of the rat following androgen ablation occurs via programmed, not necrotic, cell death.

Androgen ablation is standard therapy for metastatic prostatic cancer, since nearly all men with metastatic prostatic cancer have an initial response to such treatment (26). This positive response often involves a partial regression of the cancer within individual patients. Tumor regression occurs only

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² To whom requests for reprints should be addressed.

³ The abbreviations used are: TGF- β_1 , transforming growth factor β_1 ; TRPM-2, testosterone-repressed prostatic message 2; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; TE buffer, 10 mM Tris-HCl (pH 8.0):1 mM EDTA.

when the rate of tumor cell death is greater than tumor cell proliferation. The question therefore arose as to whether androgen-dependent human prostatic cancer cells retain the same mechanisms involved in inhibiting proliferation and activating programmed cell death as observed in normal rat prostatic glandular cells in response to androgen ablation. To answer this question, the PC-82 human prostatic adenocarcinoma xenograft model was used. The PC-82 prostatic tumor is derived from a primary adenocarcinoma of the prostate (27) and mimics many of the important properties of clinical prostate cancer when maintained in athymic mice, including its ability to regress following androgen ablation (28).

The parameters used to determine if the human PC-82 cancer cells retain a similar mechanism of responsiveness to androgen ablation as normal rat prostatic cells included: (a) histological appearance of mitotic *versus* apoptotic bodies; (b) pattern of DNA fragmentation and its temporal relationship with regression of the PC-82 tumor volume following androgen ablation; and (c) level of expression of two genes (*i.e.*, TGF- β_1 and TRPM-2) which increase in the normal rat prostate following castration. The selection of these two genes is based upon the following data. Normal androgen-dependent rat prostate cells contain receptors for TGF- β_1 and also express TGF- β_1 mRNA, both of which parameters increase by more than 2-fold within the first 3 days following castration (5, 29). In addition, TGF- β has been demonstrated to inhibit cell proliferation of normal rat prostate cells and certain rat prostatic cancer cells in *in vitro* cell culture (30). With regard to TRPM-2, there is an excellent temporal correlation between the expression of the TRPM-2 gene and activation of programmed cell death in rat ventral prostate following androgen ablation (14). In addition, the TRPM-2 gene, originally cloned from the involuting rat prostate (31), has been demonstrated to be induced during programmed cell death in a large variety of other tissue systems (32).

MATERIALS AND METHODS

Animals. Five- to 6-wk-old male athymic Ncr-*nu* mice (National Cancer Institute) were anesthetized with Metofane (Pittman Moore, Washington Crossing, NJ) and then implanted s.c. with a 2- to 3-mm³ piece of the PC-82 tumor. The tumor volume in individual animals was determined at various times using methods described previously (33). When the tumors reached a volume of 500 mm³, 5 of the animals were killed, their PC-82 tumors were harvested, 40 tumor-bearing animals were castrated under anesthesia, and an additional group of 5 intact animals was allowed to go untreated to evaluate their tumor growth behavior. At 1, 3, 7, and 14 days following castration, 5 of these castrated mice were killed, and their PC-82 tumor was removed and used for assays described later. Five of the castrated mice were allowed to go untreated for 4 wk following castration with their tumor volume being determined during the involution period. Fifteen of the castrated mice were allowed to go 1 wk, then they were anesthetized, and a 1-cm-long testosterone-filled Silastic capsule, produced as described previously (11), was implanted s.c. in the flank of each animal. These 1-cm-long testosterone-filled capsules restored the serum testosterone level to 6 to 8 ng/ml (28). The tumor volumes were determined for each of these animals, and after either 1, 2, or 3 wk of testosterone replacement, 5 of these animals were killed at each interval, and the PC-82 tumors were harvested.

Quantitative Histological Analysis of PC-82 Tumor. Following death of the host mice, PC-82 tumors were removed rapidly, weighed, fixed with 4% paraformaldehyde and 5% glutaraldehyde, and then diluted 1:1 with 0.1 M cacodylate buffer (pH 7.3) at 4°C. After 10 min thin slices were obtained and placed in a small quantity of fresh fixative on a sheet of dental wax. These pieces were diced into cubes (≤ 1.5 mm³)

and placed in fresh fixative. After 2 h of primary aldehyde fixation, the tissue was washed in 0.1 M cacodylate buffer and postfixed for 1 h in 1% osmium tetroxide. The tissue was then dehydrated through a gradient of increasing ethanol concentrations and embedded in PolyBed 812 (Polysciences, Inc., Warrington, PA). Sections (1 μ m) were obtained with a glass knife on a Porter-Blum MT2-B ultramicrotome and dried onto clean glass microscope slides. The sections were stained with 0.5% toluidine blue in 0.5% aqueous sodium borate. The percentage of PC-82 prostatic cancer cells which were mitotic or apoptotic was determined using morphometric methods as described previously (4). The data are expressed as the group (*i.e.*, $n = 5$) mean \pm standard error. The significance of difference was determined by analysis of variance using the Newman-Keuls test to allow for multiple comparisons.

Northern Analysis of TGF- β and TRPM-2 mRNA Expression. Polyadenylate-containing RNA was isolated from xenografts removed from intact, castrated, and androgen-restored castrated hosts; size-fractionated (5 μ g/well) through 1.4% agarose gels; and transferred onto nylon membranes as previously described (5). DNA probes used for hybridization analysis in this study were kindly provided by the following individuals: human β -actin (34) from Dr. D. Cleveland (The Johns Hopkins University, Baltimore, MD); TRPM-2 cDNA (31) from Dr. M. Tenniswood (University of Ottawa, Ottawa, Ontario, Canada); and human TGF- β_1 cDNA (35) which includes the entire coding region for mature TGF- β_1 from Dr. R. Derynck (Genentech, Inc., San Francisco, CA). Plasmids were prepared using the alkaline lysis method as described by Maniatis (36). Insert-specific DNA was released by appropriate restriction enzyme digestion, and fragments were recovered by electrophoresis through low melting point agarose. Inserts were labeled to high specific activity (~ 5 to 6×10^8 dmp/ μ g of DNA) by oligo labeling using [α -³²P]dCTP and the multiprimer DNA labeling kit purchased from Amersham (Amersham Corp., Arlington Heights, IL) according to the method of Feinberg and Vogelstein (37). The labeled probes were separated from unincorporated radionucleotides using Sephadex G-50 (Pharmacia Chemicals, Montreal, Quebec, Canada) and spun column chromatography as described by Maniatis (36). Membranes were hybridized according to the protocol described by Church and Gilbert (38). Prehybridizations were performed in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, and 1% bovine serum albumin for 2 to 4 h at 65°C. After addition of the denatured probe, hybridizations were carried out at 65°C for 12 to 18 h with continuous shaking. The filters were washed first with 0.5 M sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA for 1 h at 65°C, then with 0.25 M sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA (30 min at 65°C), and finally with 0.1 M sodium phosphate, pH 7.2, 0.1% SDS, and 1 mM EDTA (30 min at 65°C). Autoradiography was performed by exposing the filters to Kodak XAR-5 film at -70°C with intensifying screens for 6 h to 4 days. Filters which were initially hybridized to either TGF- β_1 or TRPM-2 probes were washed free of radioactive probe in 50 M Tris-HCl, pH 8.0, 2 mM EDTA, and 0.1% SDS for 1 h at 65°C. After exposure to X-ray film to check for complete removal of radioactivity, filters were rehybridized as described above using the β -actin probe. Autoradiographs were analyzed with a scanning densitometer (Model 1650; Bio-Rad, Richmond, CA), and peak tracings were cut out and weighed. The results were normalized by expressing the units obtained for the TGF- β or TRPM-2 transcript relative to the units obtained for the β -actin transcript.

Analysis of DNA Fragmentation. PC-82 tumors were excised following various treatments of the host mice, and prostatic nuclei were purified as previously described (2). Nuclear pellets were resuspended in 10 mM EDTA, 150 mM NaCl, and 10 mM Tris-HCl buffer, pH 8.0 (10 ml of buffer/g of starting tissue), and lysed with 0.5% SDS in the presence of proteinase K (300 μ g/ml) (Sigma Chemical Co., St. Louis, MO) at 37°C for 18 h. Following sequential extraction with phenol and chloroform:isoamyl alcohol (24:1), DNA was precipitated in absolute ethanol in the presence of 10 mM MgCl₂ and 0.3 M sodium acetate, pH 5.2, at -20°C for 24 h. Samples resuspended in TE buffer were incubated with 100 μ g/ml of RNase A at 37°C for 1 h. Following extraction with chloroform:isoamyl alcohol (24:1), DNA was precipitated as described above, dissolved in TE buffer, electrophoretically analyzed on 1.6% agarose gels, and visualized by UV fluorescence after

staining the gels with ethidium bromide (0.5 $\mu\text{g/ml}$). As a control, the *in vitro* induction of necrotic cell death due to detergent disruption of PC-82 cancer cells was induced by homogenizing PC-82 tumor from intact hosts in 1% (v/v) Triton X-100 and then incubating the homogenate for 1 h at 37°C prior to DNA extraction.

RESULTS

Castration of intact animals bearing established PC-82 tumors results in a reduction of the tumor volume so that by 2 wk postcastration, the PC-82 tumor volume regresses by approximately 50% (Fig. 1). This regression is specifically due to androgen ablation, since testosterone replacement of 7-day-castrated hosts results in complete regrowth of the tumor (Fig. 1). Morphological analysis of the regressing tumor revealed a reduction in proliferative activity as indicated by the rapid decrease in percentage of PC-82 cells undergoing mitosis (Table 1).

Since TGF- β_1 is a potent inhibitor of proliferation of many epithelial cells (39, 40), including those of the normal rat prostate (3), the expression of TGF- β_1 transcripts was studied during castration-induced regression of the PC-82 tumor. Within the first day postcastration, there is a 5-fold increase in TGF- β_1 mRNA levels which increases to 8-fold by Day 7 prior to a decrease to constitutive expression levels by 14 days postcastration (Fig. 2). This rapid induction of TGF- β_1 thus precedes the dramatic decrease in the percentage of PC-82 cells undergoing mitosis observed by 3 days postcastration (Table 1).

Concomitant with the decrease in proliferative activity in the regressing PC-82 tumor, there is a parallel dramatic increase in cell death as indicated by an enhanced percentage of PC-82 cells undergoing apoptosis (Table 1). A 9-fold increase in the percentage of PC-82 cells undergoing apoptosis is obtained

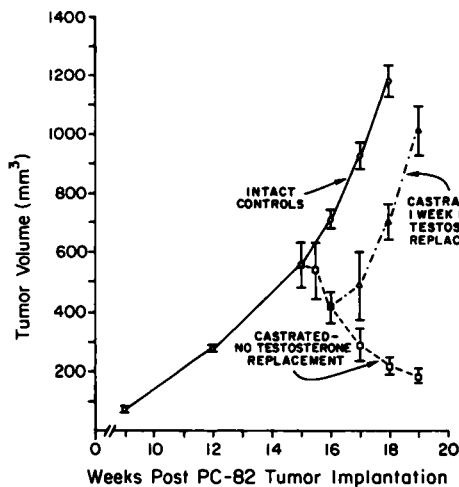


Fig. 1. Effect of castration and androgen administration on the growth of PC-82 tumor maintained in male nude mice. Points, mean; bars, SE.

Table 1 The percentage of PC-82 prostatic cancer cells which are mitotic versus apoptotic in intact and castrated tumor-bearing mice

Treatment	% of PC-82 cells undergoing	
	Mitosis	Apoptosis
Intact, none	0.17 \pm 0.06 ^a	0.14 \pm 0.02
1 day postcastration	0.24 \pm 0.07	1.17 \pm 0.31 ^b
3 days postcastration	0 ^b	3.65 \pm 0.87 ^c
7 days postcastration	0 ^b	3.30 \pm 0.33 ^c
14 days postcastration	0 ^b	0.45 \pm 0.05

^a Mean \pm SE.

^b $P < 0.05$ compared with values for the intact nontreated group.

^c $P < 0.01$.

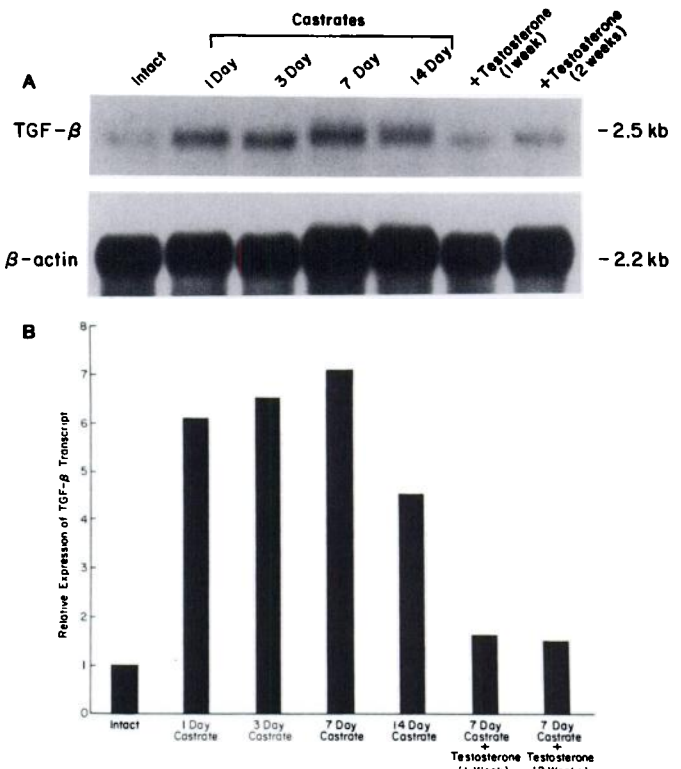


Fig. 2. Induction of TGF- β mRNA expression in human PC-82 tumors following androgen ablation. Polyadenylated mRNA (5 $\mu\text{g/well}$) isolated from the PC-82 tumors of intact, 1-day, 3-day, 7-day, and 14-day-castrated mice and 7-day-castrated mice given either 1 or 2 additional wk of exogenous testosterone replacement was size fractionated and blotted onto nylon membranes as described in "Materials and Methods." The blot was initially hybridized with a ^{32}P -labeled TGF- β_1 cDNA probe and subsequently with a ^{32}P -labeled β -actin cDNA probe (A). Molecular size in kilobases (kb) is indicated. Autoradiographs were quantitated, and results were normalized relative to the expression of β -actin transcript. Relative expression of TGF- β_1 is shown (B).

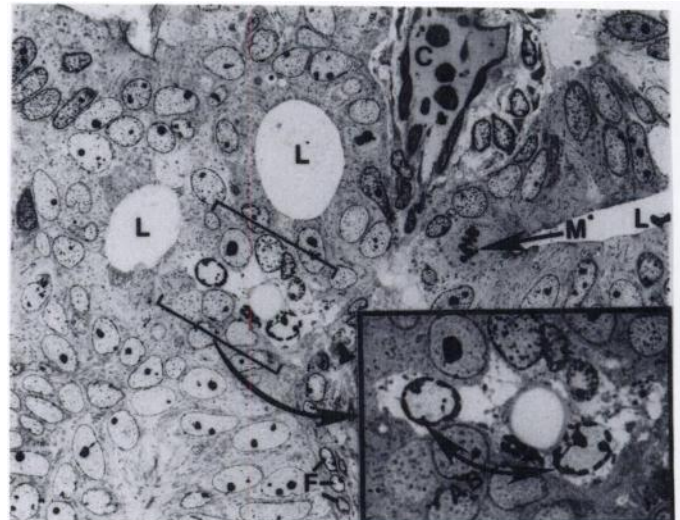


Fig. 3. Histology of PC-82 tumor xenograft from a 1-day-castrated host. The tumor consists of cuboidal cancer cells which are surrounded by occasional nontumorous cells identified as endothelial cells and fibroblast-like cells (F). Glandular lumen (L), blood capillaries (C), and a mitotic figure (M) are clearly identified. Two tumor cells with nuclei exhibiting early changes characteristic of apoptotic bodies are shown (inset: AB).

which declined by Day 14 postcastration (Table 1). Histological examination of PC-82 tumor xenografts 1 day after castration revealed that the tumor consists of cuboidal cancer cells surrounded by other noncancerous cells (Fig. 3). Individual tumor cells with nuclei displaying early apoptotic changes are easily

identified (Fig. 3). These changes include nuclear pyknosis and severe cytoplasmic vacuolization and are restricted to the cancer cells.

The pattern of DNA fragmentation in PC-82 human prostatic cancer cells was determined at various times following castration of tumor-bearing hosts. The characteristic ladder of DNA nucleosomal oligomers is evident by 1 day postcastration (Fig. 4, Lane 2). Progressive fragmentation of the higher molecular weight nucleosomal multimers into lower molecular weight oligomers continues between 3 and 14 days postcastration (Fig. 4, Lanes 3, 4, and 5, respectively).

The kinetics of DNA fragmentation induced by androgen ablation temporally correlated with the enhanced appearance of apoptotic bodies in PC-82 xenografts (Table 1). Both events preceded the dramatic reduction in tumor volume, a phenomenon characteristic of programmed cell death. Analysis of DNA isolated from tumors from intact mice reveals high molecular DNA with essentially no fragmentation (Fig. 4, Lane 1, and Fig. 5, Lane 1, labeled "Intact"). To examine the specificity of this nucleosomal pattern of DNA fragmentation, PC-82 tumors from intact hosts were exposed *in vitro* to detergent disruption (*i.e.*, Triton X-100) to induce necrotic cell death. DNA analysis following this *in vitro* treatment revealed random degradation of tumor DNA into a continuous spectrum of sizes (Fig. 5), as opposed to the nucleosomal ladder obtained *in vivo* following androgen ablation (Fig. 4).

Since an increased expression of the TRPM-2 gene is associated with programmed cell death in a large variety of systems (32), including regression of the normal rat prostate following castration, the expression of TRPM-2 transcripts was studied during castration-induced regression of the PC-82 tumor. Within the first day after castration, there is a 5-fold increase in TRPM-2 mRNA levels with a nearly 20-fold increase in expression occurring by 7 days postcastration (Fig. 6B). After

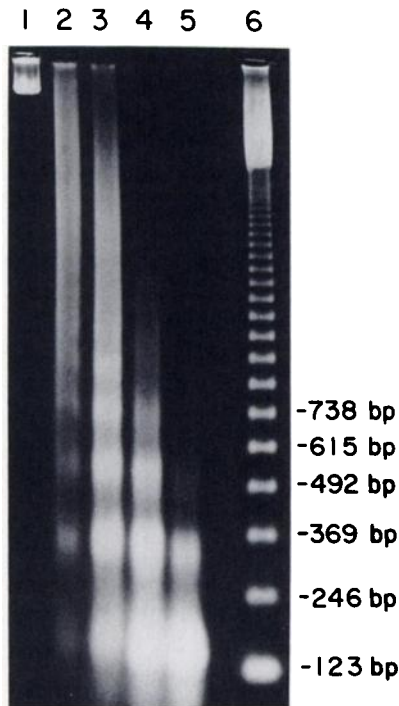


Fig. 4. Electrophoretic analysis of DNA isolated from PC-82 tumor xenografts from intact and castrated hosts. Lane 1, control PC-82 tumor DNA from intact hosts; Lanes 2, 3, 4, and 5 represent DNA isolated from tumors following 1, 3, 7, and 14 days postcastration. Lane 6, 123-base pair (bp) DNA ladder as a molecular weight marker.

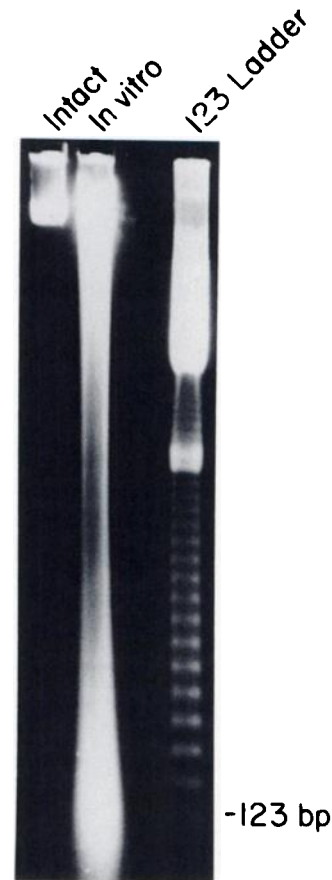


Fig. 5. Electrophoretic analysis of DNA isolated from PC-82 tumor tissue induced *in vitro* to undergo necrotic cell death. Lane labeled "Intact" is DNA obtained from PC-82 grown in an intact male mouse and directly analyzed. Lane labeled "In vitro" is from a PC-82 tumor grown in an intact host which was homogenized in 1% Triton X-100 with the homogenate then being incubated *in vitro* for 1 h at 37°C prior to DNA extraction.

2 wk of androgen ablation, there is a moderately small decrease in TRPM-2 mRNA levels (Fig. 6B). Androgen administration to 7-day-castrated mice suppresses TRPM-2 transcripts to a level comparable to its constitutive expression in intact hosts. The 2.3-kilobase molecular weight size of the TRPM-2 mRNA transcript from PC-82 human tumors (Fig. 6A) is indistinguishable from that of rat ventral prostate (31).

DISCUSSION

The present studies demonstrate that the mechanism of PC-82 tumor regression following androgen ablation involves an inhibition of cell proliferation coupled with an activation of programmed cell death. Elevation of TGF- β_1 following castration is probably involved as a negative regulatory signal to inhibit further cell proliferation once programmed cell death is induced. Enhanced TRPM-2 expression, fragmentation of DNA into nucleosomal oligomers, and the morphological manifestation of enhanced apoptosis following androgen ablation are characteristic early events that preceded PC-82 tumor regression. These results demonstrate that androgen-dependent human prostatic cancer cells retain the ability to activate a programmed cell death cascade similar to that induced in normal rat prostatic epithelial cells following androgen ablation (2–5, 14, 22). The evolutionary conservation of programmed cell death among different species (*i.e.*, rat and human), as well as the ability of both normal and malignant prostatic cells to undergo programmed cell death via a common suicide process,

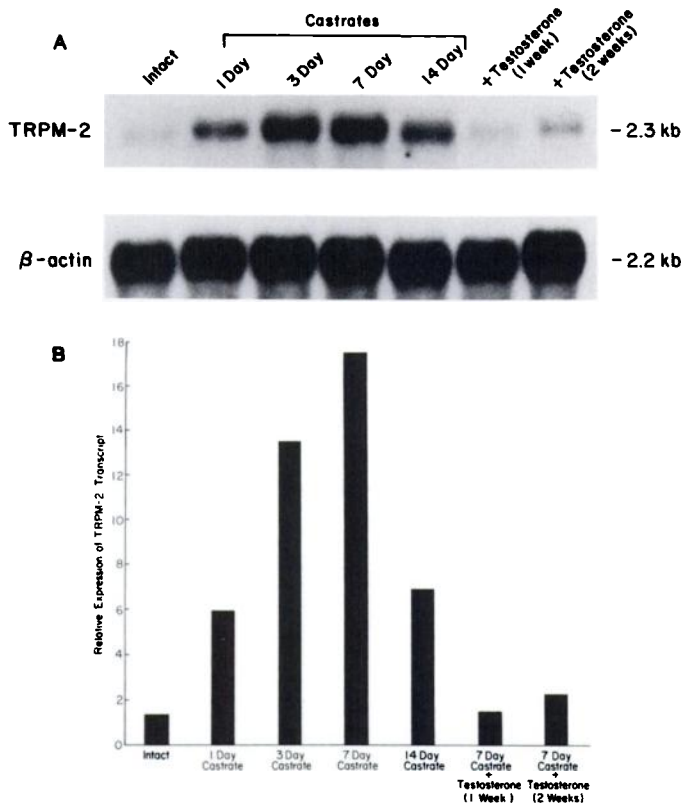


Fig. 6. Induction of TRPM-2 mRNA expression in human PC-82 tumors following androgen ablation. Northern hybridization analysis and autoradiography (A) and relative expression of TRPM-2 transcripts (B) were performed as described for Fig. 2. kb, kilobases.

suggests a well-defined genetic program for this mode of cell death.

Metastatic prostatic cancer is a fatal disease for which no therapy is available which effectively increases survival (41, 42). The major reason for the inability of androgen ablation monotherapy to increase survival in men with metastatic prostatic cancer is that the cancer within an individual patient is heterogeneously composed of clones of both androgen-dependent and -independent prostatic cancer cells even before therapy is initiated (43–46). Thus, androgen ablation alone does not affect the preexisting androgen-independent cancer cells already present. To affect all the heterogeneous prostatic cancer populations within an individual patient, effective chemotherapy specifically targeted against the preexisting androgen-independent cancer cells must be combined with androgen ablation to affect the androgen-dependent cancer cells (47, 48). Unfortunately, there are presently no highly effective chemotherapeutic agents which can eliminate androgen-independent prostatic cancer cells (42). Recent studies have demonstrated that even androgen-independent prostatic cancer cells retain the major portion of the biochemical cascade (*i.e.*, Ca^{2+} - Mg^{2+} -dependent endonuclease-induced fragmentation of DNA, TRPM-2 induction, etc.) that leads to the programmed cell death pathway, only there is a defect in these cells such that it is no longer activated by androgen removal (49). A new therapeutic approach for androgen-independent prostatic cancers therefore would be to develop some type of non-androgen ablation method to activate this programmed cell death cascade in androgen-independent prostate cancer cells distal to the point of the defect. Attempts at such an approach are presently under way.

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