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Cell proliferation, DNA repair, and p53 function are not required for programmed death of prostatic glandular cells induced by androgen ablation

(apoptosis/S-phase progression/DNA damage)

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ABSTRACT **Androgen ablation induces programmed death of androgen-dependent prostatic glandular cells, resulting in fragmentation of their genomic DNA and the cells themselves into apoptotic bodies. Twenty percent of prostatic glandular cells undergo programmed death per day between day 2 and 5 after castration. During this same period, <1% of prostatic glandular cells enter the S phase of the cell cycle, documenting that >95% of these die in G_0. During the programmed death of these G_0 glandular cells, a futile DNA repair process is induced secondary to the DNA fragmentation. This futile DNA repair is not required, however, since inhibition of this process by >90% with an appropriately timed hydroxyurea dosing regimen had no effect upon the extent of the programmed death of these cells after castration. Likewise, p53 gene expression is not required since the same degree of cell death occurred in prostates and seminal vesicles after castration of wild-type and p53-deficient mice.**

Metastatic prostatic cancer is often highly responsive to androgen ablation therapy (1). After an initial response, unfortunately, relapse occurs due to the growth of androgen-independent prostatic cancer cells. This relapse occurs even if complete androgen blockage is used, and thus androgen ablation is rarely curative (2). The realization that the relapse is due to the continuing growth of androgen-independent prostatic cancer cells for which there is no effective therapy (3) has led to a search for new methods of controlling these cells. A lead for such an approach is based upon the understanding that the rapid involution of the normal prostate induced by androgen ablation is due to the activation of the process of cellular suicide, termed programmed cell death or apoptosis (4–6). Programmed death induced in the prostate is cell type specific. Only glandular epithelial cells and not basal epithelial cells or stromal cells are androgen dependent and thus undergo programmed cell death following castration (7). These glandular cells constitute ≈85% of the total cells in the ventral prostate of an intact adult male rat, and ≈80% of these glandular cells die within 1 week after castration (7). This is due to the activation of the programmed cell death pathway normally repressed by androgen in the prostate (7–16). This programmed death pathway involves an energy-dependent cascade in which the cells undergo an epigenetic reprogramming, which leads to double-stranded fragmentation of their DNA followed by cellular fragmentation into apoptotic bodies (4, 6, 9, 12). These apoptotic bodies are then phagocytosized by either intraepithelial macrophages or other glandular cells (4, 11, 17).

Androgen ablation induces a similar active reprogramming process within androgen-dependent prostatic cancer cells leading to their self-induced DNA fragmentation and death (18). Due to this induction, androgen-dependent prostatic cancer cells are effectively eliminated by androgen ablation. Androgen ablation does not result in activation of the programmed death of the androgen-independent prostatic cancer cells present within a patient (19). While androgen-independent prostatic cancer cells do not activate programmed cell death following androgen ablation, death may be induced in these cancer cells by a variety of chemotherapeutic agents if these cells are proliferating (20). Unfortunately, the rate of cell proliferation of human prostatic cancer cells *in vivo* is remarkably low (i.e., ≥1–2% per day) (21, 22). This low rate of tumor cell proliferation *in vivo* is one of the major reasons why androgen-independent human prostatic cancer cells are not effectively managed by presently available chemotherapeutic agents (3).

These observations raise a series of critical issues. *First*, can programmed cell death occur without requiring the entrance of cells into the proliferative cell cycle (i.e., can cells undergo programmed death in G_0)? It is critical to resolve this issue to validate the potential for the programmed cell death pathway as a therapeutic target for nonproliferating prostatic cancer cells. DNA damage induced by a variety of chemical and radiation treatments is known to activate DNA repair (23). Since double-stranded fragmentation of genomic DNA is induced during programmed cell death (6, 16, 18), this raises a *second* issue of whether DNA repair is activated and whether such a futile process is required for cell killing. Resolving this issue is critical to predict whether inhibitors of DNA repair are a logical choice for combination with other physical DNA-damaging agents (e.g., x-radiation) to potentiate programmed death of nonproliferating prostatic cells. Previous studies have demonstrated that enhanced p53 expression is induced by DNA damage (24), and a series of additional studies has suggested a critical role for p53 expression in programmed cell death (25–27). Since DNA damage process is activated during programmed cell death, this raises a *third* issue of whether p53 protein expression is required for this process. Resolving this issue is critical since androgen-independent prostatic cancer cells can lose the ability to express p53 protein (28). To resolve these three issues, the programmed death of androgen-dependent normal prostatic glandular cells was used as a model system.

MATERIALS AND METHODS

Animals. Inbred Copenhagen adult male rats were obtained from Harlan–Sprague–Dawley. p53-deficient (i.e., p53

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Abbreviations: HU, hydroxyurea; t.i.d., three times a day.

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knockout) mice carried germ-line disruptions of both of their p53 genes, preventing any production of p53 protein by homologous recombination as described (29). Mice used were both p53 wild-type (i.e., C57BL/6) and p53-deficient mice. Mice were 5 weeks of age at initiation of the experiments. Castration and androgen replacement to induce proliferative regrowth of the regressed prostate were performed as described (8).

Quantitation of Prostatic Cell Death Induced by Androgen Ablation. To quantitate the percentage of the glandular cells undergoing programmed death in the ventral prostate at various times after castration, formalin-fixed, paraffin-embedded histological sections were terminal transferase end-labeled as described by Gavrieli et al. (30). The percentage of prostatic glandular cells dying per day by apoptosis was calculated by dividing the percentage of terminal transferase-labeled glandular cells detectable at any particular time point by the half-life of terminal transferase-labeled cells in the prostate (i.e., 0.167 days). The half-life for terminal transferase-labeled glandular cells was determined by castrating rats and then 36 hr later giving these rats daily s.c. injections of 2 mg of testosterone. At 3-hr intervals after testosterone replacement, the percentage of terminal transferase-labeled glandular cells was determined. This percentage continued to increase for the first 12 hr after testosterone replacement before decreasing between 12 and 24 hr after androgen replacement. Based upon the plot of the logarithm of the percentage of terminal transferase-labeled glandular cells vs. time of androgen replacement, the half-life for terminal transferase end-labeled glandular cells was determined to be 0.167 days.

Determination of the Percentage of Prostatic Cells Entering S Phase per Day. Intact rats or animals castrated for various times were injected i.p. with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (20 Ci/mmol; Amersham) per gram of body weight. One hour after i.p. injection, ventral prostates were harvested, an aliquot was analyzed for the dpm of thymidine incorporated into prostatic DNA as described (8), and an aliquot was processed for radioautography as described (7). The percentage of glandular cells entering the S phase per day was calculated by dividing the percentage of glandular cells labeled by the length of the S phase in prostatic glandular cells expressed in days [i.e., 0.38 days (31)].

Quantitation of DNA Repair During Prostatic Regression. Bromodeoxyuridine (BrdUrd) was injected i.p. at a dose of 50 mg/kg into rats 6 hr before being killed. Rats were killed at 24-hr intervals after castration; their ventral prostates were removed, fixed in buffered formalin, and processed for anti-BrdUrd immunocytochemical staining using a mouse monoclonal antibody against BrdUrd (Accurate Chemicals), according to manufacturer's protocol. The percentage of prostatic glandular cells positively stained for BrdUrd incorporation into their nucleus was quantitated by the random sampling technique (7).

Determination of the DNA Content of Prostatic Cells Incorporating BrdUrd After Castration. Groups (three to five) of rats were castrated; 66 hr later, the animals were injected i.p. with 50 mg of BrdUrd per kg. Six hours later, ventral prostates were harvested, and single-cell suspensions were prepared by mincing the tissue and then incubating at 37°C in Hanks' balanced salt solution containing Dispase (50 caseinolytic units/ml; Collaborative Biomedical Products, Bedford, MA) and 0.04% DNase I (Sigma). Cells (10^7) were fixed in 70% ethanol and processed for fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-BrdUrd antibody staining according to the manufacturer's (Becton Dickinson) recommendations for flow cytometry analysis. Based upon anti-BrdUrd FITC fluorescence and appropriate single-cell size, the 7% of the cells with the highest level of anti-BrdUrd FITC fluorescence were sorted by fluorescence-activated cell sorting, since 7% of glandular cells incorporated BrdUrd into their DNA on day 3 postcastration (see Table 2). Sorted cells were stained with propidium iodide (5 μg/ml) and analyzed for their DNA content.

Statistical Analysis. Values are expressed as the mean ± SE. Statistical analyses of significance were made by a one-way analysis of variance with the Newman–Keuls test for multiple comparisons.

RESULTS

Kinetics of Programmed Cell Death and Entrance into S Phase Induced by Androgen Ablation. By using the terminal transferase method of Gavrieli et al. (30) to identify prostatic glandular cells undergoing programmed death in tissue sections and adjusting for the half-life of these dying cells, the percentage of glandular cells dying per day via programmed death in the prostate of intact and castrated rats was determined (Table 1). In intact rats, 1.2% of the glandular cells die per day via programmed death. Within the first day after castration, this percentage increases, and between days 2 and 5 after castration, 17–21% of the glandular cells die per day via programmed death. By using [^{3}H]thymidine pulse labeling, it was determined that 1.3% of prostatic glandular cells enter the S phase per day in intact control rats (Table 1). Within 1 day after castration, there is an 80% decrease ($P < 0.05$) in the percentage of glandular cells entering S phase. By 4 days after castration, there is a >90% reduction in this value. Analysis of the data in Table 1 demonstrates that >98% of prostatic glandular cells die after castration without entering the S phase (i.e., cells die out of cycle in G_0).

Percentage of Ventral Prostatic Cells Undergoing DNA Repair After Castration. To detect DNA repair in prostatic glandular cells, a high dose (i.e., 50 mg/kg)/long exposure (i.e., 6 hr) BrdUrd labeling method was used. This is a total BrdUrd dose per rat that is 3360 times higher than the precursor dose used in the [^{3}H]thymidine pulse labeling studies reported in Table 1. Such a high dose allows incor-

Table 1. Temporal changes in the prostatic daily rate of programmed cell death and cell entrance into S phase after androgen ablation

Time after castration, days	Percent of prostatic glandular cells dying per day via programmed death	Percent of glandular cells entering the S phase per day
0 (intact control)	1.2 ± 0.1	1.33 ± 0.18
1	3.0 ± 0.2*	0.27 ± 0.06*
2	21.4 ± 1.9*	0.32 ± 0.07*
3	20.5 ± 2.7*	0.13 ± 0.08*
4	19.8 ± 3.1*	<0.13*
5	17.0 ± 2.6*	<0.13*
7	4.8 ± 1.8*	<0.13*

There were four rats per group.
*$P < 0.05$ compared to intact controls.

poration of the precursor for several hours after injection (32). This long period of incorporation (i.e., 6 hr) coupled with the use of the highly sensitive immunocytochemical detection of BrdUrd maximizes detection of both scheduled S-phase DNA synthesis and unscheduled DNA repair (23). By using this high dose/long exposure protocol, there is a 3- to 4-fold increase in BrdUrd labeling by as early as day 2 after castration, which peaks on day 4 after castration (Table 2). The distinguishing feature between scheduled S-phase DNA synthesis and unscheduled DNA repair is that during S-phase DNA synthesis there is a net accumulation of nuclear DNA content. In contrast, during G_0 DNA repair, no net accumulation occurs and the cells have a diploid content of DNA (23).

To determine whether BrdUrd incorporation was part of a futile G_0 DNA repair process or due to S-phase DNA synthesis, a flow cytometric method was used to analyze the DNA content of prostatic cells from rats 3 days after castration that have incorporated BrdUrd. These studies demonstrated that 82.5% ± 6.9% of BrdUrd-positive prostatic cells had a diploid (G_0) compliment of DNA. The original single-cell suspension used for these analyses included prostatic stromal cells, basal epithelial cells, and intraepithelial macrophages in addition to glandular cells. These cell types are not androgen dependent, and they continue to enter the S phase after castration (12, 33). In addition, Evans and Chandler (17) demonstrated that between 2 and 3 days post-castration there is a major increase in the proliferation of the intraepithelial macrophages coupled to a major decrease in the percent of prostatic glandular cells entering S phase. The continuing proliferation of these androgen-independent prostatic cells after castration is detectable in this analysis. Thus the detection of ≈20% of the BrdUrd positively labeled cells having a non-G_0 complement of DNA is not unexpected. These results demonstrate that the vast majority (i.e., >80%) of BrdUrd incorporation into prostatic glandular cells after castration is due to G_0 DNA repair and not to entrance into S phase.

Neither Progression Through S Phase nor G_0 DNA Repair Is Required for Programmed Death Induced by Androgen Ablation. The previous data raise the issue of whether a futile G_0 DNA repair process induced by androgen ablation is associated with, but not causally required for, prostatic cell death. To resolve this issue, rats were injected i.p. with 500 mg of hydroxyurea (HU) per kg every 8 hr for 5 days. The ability of this three times a day (t.i.d.) HU dosing regimen to prevent progression through S phase was validated by treating rats previously castrated for 1 week with 3 days of exogenous androgen replacement to recruit glandular cells into S phase (8) simultaneously with and without the t.i.d. HU regimen. These studies demonstrated that the HU regimen prevented ($P < 0.05$) the [^{3}H]thymidine incorporation into prostatic DNA by >90% (i.e., [^{3}H]thymidine incorporation into DNA in castrated rats, 110 ± 18 dpm per 100 μg of DNA per hr; in castrated rats given androgen for 3 days, 2165 ± 150 dpm per 100 μg of DNA per hr; in castrated rats given both androgen and HU for 3 days, 198 ± 25 dpm per 100 μg of DNA per hr).

The ability of this t.i.d. dose of HU to prevent unscheduled G_0 DNA repair was documented by castrating rats and injecting the animals every 8 hr with either HU or the saline vehicle and then injecting each rat with 50 mg of BrdUrd per kg 66 hr after castration. Six hours after the BrdUrd injection (i.e., 72 hr after castration), the percentage of glandular cells labeled was determined. t.i.d. HU dosing completely prevented the increase in BrdUrd labeling induced by castration (i.e., 0.5% ± 0.1% BrdUrd-labeled cells in both intact and castrated animals given HU vs. 7.0% ± 1.1% in HU-untreated castrated rats). When intact male rats were treated with this t.i.d. HU regimen for 1 week, there was no indication of an increase in programmed cell death in the prostate based upon the lack of an increase in morphologically detectable apoptotic bodies or terminal transferase end-labeled cells or loss of DNA content.

These combined results document that the t.i.d. HU treatment inhibits by >90% both S-phase DNA synthesis and G_0 DNA repair without itself inducing programmed cell death in the prostate. Rats were castrated and injected i.p. every 8 hr with either 500 mg of HU per kg or saline vehicle. Five days after castration, the ventral prostate DNA content was reduced 51% ± 2% in the animals not treated with HU vs. 47% ± 3% in castrated rats receiving the t.i.d. HU treatment. Histological analysis also demonstrated an identical atrophic morphology for the prostates from both groups, and an identical percentage of glandular cell was detected as apoptotic bodies in both groups of prostates. These data demonstrate that the programmed death of prostatic glandular cells induced by androgen ablation does not require either progression through S phase or G_0 DNA repair.

p53 Expression Is not Required for Androgen Ablation-Induced Cell Death. p53-deficient mutant mice were established by using homologous recombination to produce null mutations in both of the p53 alleles, thus preventing any production of p53 protein in these mice (29). Wild-type (p53 expressing) mice and p53-deficient mice were castrated, and after 10 days the animals were killed, their seminal vesicles and prostates were removed and weighed, and aliquots were used for DNA determination and histological analysis. Due to the small starting weight and DNA content of the prostatic lobes (i.e., anterior, ventral, and dorsolateral lobes) and the fact that these lobes are androgen dependent, all of the prostatic lobes from one animal were combined for the weight and DNA determinations. These analyses demonstrated that there is an identical decrease in the wet weight and DNA content in both the seminal vesicles and prostate from

Table 2. Temporal changes in the percentage of prostatic glandular cells incorporating BrdUrd after androgen ablation

Time after castration, days	Percent of prostatic glandular cells labeled during 6 hr of BrdUrd exposure
0 (intact control)	0.5 ± 0.1
1	0.6 ± 0.2
2	2.1 ± 0.2*
3	7.0 ± 1.1*
4	10.4 ± 1.8*
5	2.0 ± 0.4*
7	1.0 ± 0.2*

There were four rats per group.
*$P < 0.05$ compared to intact controls.

Table 3. Response of the seminal vesicles and prostate to androgen ablation in wild-type vs. homozygous p53 null mutant mice

	Intact		Ten days after castration	
Tissue	Wet weight, mg	DNA, μg	Wet weight, mg	DNA, μg
Seminal vesicle				
Wild type	37 ± 3	190 ± 5	6 ± 1*	92 ± 5*
p53 homozygous mutant	36 ± 5	175 ± 7	6 ± 1*	88 ± 10*
Prostate				
Wild type	30 ± 4	147 ± 7	6 ± 1*	78 ± 8*
p53 homozygous mutant	24 ± 3	149 ± 5	9 ± 2*	89 ± 10*

There were five mice per group.
*$P < 0.05$ when compared to the corresponding values for the intact wild-type mice.

wild-type and p53-deficient mice (Table 3). Histological analysis likewise demonstrated an identical percentage of terminal transferase end-labeled prostatic glandular cells in the two groups of animals. Thus, androgen ablation-induced programmed death of androgen-dependent cells does not require any involvement of p53 protein expression.

DISCUSSION

Androgen ablation-induced programmed death of prostatic cells involves the double-stranded fragmentation of their genomic DNA (6, 9, 12). As documented by the present and previous studies (17, 34), this DNA fragmentation and programmed death occurs in prostatic glandular cells out of cycle (i.e., cells in G_0) without their entrance into S phase. There is precedence for DNA fragmentation and programmed death of G_0 cells without a requirement for entry into the S phase. The programmed death of thymocytes induced by exposure to the appropriate doses of either glucocorticoid or DNA topoisomerase I or II inhibitors occurs while these cells are in G_0 with no progression even into early G_1 of the proliferative cell cycle (35). In addition, it has been demonstrated that when androgen-dependent PC-82 human prostatic cancer cells are grown as a xenograft in nude mice and the host is castrated, the percent of both BrdUrd and Ki-67 positively labeled tumor cell nuclei decreased by >90% within a 1-week period after castration (36). Since these DNA synthesis markers are decreasing during the period when these PC-82 cancer cells are undergoing DNA fragmentation and programmed cell death (18), these results demonstrate that androgen-dependent prostatic cancer cells, like normal prostatic glandular cells, do not require progression into S phase to undergo programmed death.

While not requiring entrance into the S phase, the DNA fragmentation induced by androgen ablation during the programmed death of the prostatic glandular cells does activate a process of DNA repair in which nucleotide precursors are incorporated into the fragmenting DNA. This is futile G_0 DNA repair, however, since eventually the DNA is degraded into nucleosomal size pieces. Activation of such a futile DNA repair process is consistent with the observation of Colombel *et al.* (37) that there is an enhanced immunochemical staining in prostatic glandular cell nuclei for both BrdUrd and proliferation-specific nuclear antigen (PCNA) after castration. PCNA is required for both S-phase DNA synthesis and DNA repair (38, 39), and it has been previously documented that PCNA expression can increase without a corresponding increase in S-phase DNA synthesis or Ki-67 expression (40).

The activation of such futile DNA repair is not unexpected since a variety of agents that produce DNA damage induce DNA repair (23). This futile G_0 DNA repair is not, however, required for programmed death of nonproliferating prostatic glandular cells since blocking this DNA repair does not affect the death of these cells induced by castration. Likewise, expression of the p53 protein is not required for the androgen ablation-induced proliferation-independent programmed cell death since this process occurs to the same extent in wild-type and p53-deficient animals, which express no p53 protein. Similarly, we have observed that TSU-pr1 human prostatic cancer cells can be induced to undergo proliferation-independent programmed cell death (Y.F., P. Lundmo, and J.T.I., unpublished data) even though in these cells one of the p53 alleles is physically deleted and the other allele is mutated to a form incapable of producing p53 protein (28). An additional demonstration that p53 expression is not required for generalized programmed cell death has been provided by the observation that glucocorticoid- or calcium ionophore-induced programmed death occurs normally in thymocytes from p53-deficient mice (29, 41).

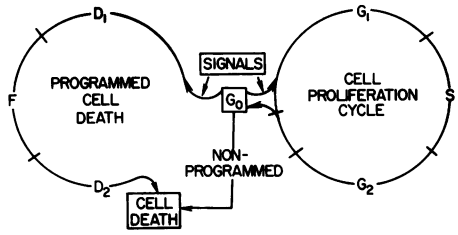

FIG. 1. Revised cell cycle denoting the options of a G_0 prostatic glandular cell. D_1 denotes the period during which new gene and protein expression required for induction of the DNA fragmentation period (denoted F) occurs as part of the programmed cell death pathway. D_2 denotes the period during which the cell itself fragments into apoptotic bodies as part of its programmed death.

With the realization of the importance of programmed cell death, the idea that prostatic cell number is determined by the proliferative cell cycle alone has been modified. Based upon this modification, a redefined "cell cycle" has been proposed (42). The overall cell cycle controlling cell number is composed of a multicompartment system in which the prostatic glandular cells have at least three options (Fig. 1). The glandular cell can be (*i*) secretorially active and not undergoing either proliferation or death (G_0 cell), (*ii*) undergoing cell proliferation (G_0–G_1–S–G_2–mitosis), or (*iii*) undergoing cell death by either the programmed (G_0–D_1–F–D_2–cell death) or nonprogrammed (i.e., necrotic) pathway. In conclusion, these observations have identified the programmed cell death pathway as target for therapeutic drug development for the management of androgen-independent prostatic cancer, since it can be induced without p53 expression in nonproliferating prostatic cells.

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