Hormone-regulated Apoptosis Results from Reentry of Differentiated Prostate Cells onto a Defective Cell Cycle

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ABSTRACT

Castration initiates extensive apoptosis of the secretory epithelial cells lining the ducts of the rat ventral prostate, resulting in the striking regression of this male sexual accessory tissue. We had previously described the paradox of finding similar cascades of gene activity (c-fos > c-myc > hsp-70) induced during the early period of ventral prostate regression and during the regrowth of the ventral prostate gland initiated by testosterone replenishment. This common pattern of protooncogene expression during periods of predominant cellular apoptosis or proliferation caused us to examine further the possibility that the two cellular events occur through identical early molecular pathways. In the present study we demonstrate that apoptotic prostate epithelial cells incorporate bromodeoxyuridine into nuclear high-molecular-weight DNA prior to nuclear DNA fragmentation. The DNA synthetic activity occurs in coordination with a massive induction of proliferative cell nuclear antigen, a proliferation marker, in the nuclei of androgen-deprived prostatic epithelial cells. Moreover, this activity is also associated with the increased expression of mRNA encoding p53, a suppressor gene well known as a cell cycle-blocking agent. Our data indicate that quiescent (G0) prostate epithelial cells undergo apoptosis due to two sequential events initiated by testosterone depletion. The first event is the active reentry of these cells into the cell cycle. The second event is the apoptotic destruction resulting from the inability of the differentiated cells to successfully complete this cycle.

INTRODUCTION

One of the more unusual aspects of hormone action is the ability to induce an apoptotic response in cells of hormone-sensitive tissues. This unique form of cell death has increasingly become the subject of attention due to the biological implications of a natural cell death process that is genetically regulated (1). Apoptosis, also referred to as a "programmed death," has been recognized most often during embryogenesis of higher organisms, wherein it provides a sculpting force, giving shape to developing masses of cells (2). In adult tissues, the abnormal onset of apoptosis is known to be a determinant in the development of benign disease conditions (3). Moreover, based on several recent studies of human lymphomas (4, 5), we are becoming more cognizant of apoptosis as an influence that must be considered in our theories concerning the development of human tumors and in efforts to generate more effective cancer therapies.

Our current understanding of this process, especially as it is evoked by hormone signals, is limited to certain morphological, biochemical, and molecular characterizations. We can identify apoptotic cell death by the presence of apoptotic bodies: cells with condensed and fragmented nuclei, shrunken to the extent that they pull away from neighboring cells and the basement membrane (6, 7). The nuclear DNA of apoptotic cells demonstrates a degradation paradigm in which intranucleosomal DNA is preferentially digested, resulting in a visual "ladder" of DNA fragments in multimers of 180-base pair units upon electrophoresis (8, 9). However, the most intriguing characteristic of this form of cell death is the apparent need for genetic involvement. In many cases, apoptosis can be abrogated by RNA synthesis and protein synthesis inhibitors (10, 11), demonstrating the requirement for some recently synthesized gene product(s) for its progression.

Based on the latter characteristic, hormone-regulated apoptosis can be described as a genetic suicide process, potentially driven by the expression of a lethal gene product. Therefore reports of specific and seemingly novel gene products, such as sulfated glycoprotein 2 (12) or tissue glutaminase (13), that are highly induced during apoptotic cell death have generated some degree of interest. To date, none of the identified apoptosis-associated genes can be proved to be lethal to the cell that synthesizes them. Moreover, there is no current evidence that a novel nuclease is involved in hormone-induced apoptotic DNA degradation. Rather, we document here a study of androgen-regulated apoptosis in the prostate gland, potentially demonstrating that apoptosis results from the defective progression of a normal cellular process.

This study was initiated because of an earlier study in which castration-induced regression of the rat ventral prostate gland was shown to be accompanied by a protooncogene cascade (transient induction of c-fos prior to the induction of c-myc transcripts) (14) that is most notable because it is usually associated with cellular proliferative responses in vitro (15) and in vivo (16, 17). At least one of these molecular signals (induced c-myc) has been localized to the glandular epithelial cells (18), the apoptotic target associated with androgen depletion of the ventral prostate gland. Moreover, castration-induced apoptosis of ventral prostate cells can be suppressed by calcium channel-blocking agents (19, 20) in the same manner that these drugs inhibit androgen-dependent tumor cell proliferation (21).

This seeming paradox wherein apoptotic prostate cells respond in a manner similar to that of proliferating prostate cells might be resolved if apoptosis and proliferation were shown to share an early molecular response pathway. Indeed, this hypothesis would serve to consolidate our current understanding of apoptosis induced by chemotherapeutic agents wherein the cells must be in cycle to access the apoptotic pathway (22, 23). To determine the extent with which the progress of androgen-regulated prostate cell apoptosis might coincide with normal cell cycle activities, we searched for the presence of other proliferative markers in the rat ventral prostate gland subsequent to castration. The markers that we have studied indicate that prostate epithelial cells proceed at least through S phase prior to their apoptotic demise. The coordinately increased activity of a well-known cell cycle suppressor, the p53 gene product, suggests one potential reason that this cell cycle is never completed.

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Cells onto a Defective Cell Cycle

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MATERIALS AND METHODS

Laboratory Animals and Tissues. Mature (325–350 g) male Sprague-Dawley rats (Camn., Inc., Wayne, NJ) were utilized as a source of normal and regressing ventral prostate tissue. All laboratory animals were maintained according to the Guidelines for Laboratory Animal Use and Care, with food and water available ad libitum. Castration was accomplished through a scrotal incision under sodium pentobarbital anesthesia (25 mg/kg) as previously described (14). Sham castrations were performed through a similar operation, although the testis were never ligated or excised. At indicated times after castration, rats were sacrificed by a lethal overdose of sodium pentobarbital (100 mg/kg) to obtain ventral prostate tissue and sections of the small bowel. This tissue was fixed overnight in phosphate-buffered formalin and then dehydrated in graded alcohols prior to paraffin embedding for immunological procedures or was flash-frozen in liquid N2 and stored at −85°C for subsequent DNA and RNA extraction.

Detection of Bromodeoxyuridine Incorporation in the Regressing Ventral Prostate Gland. BrdUrd (Sigma Chemical Company, St. Louis, MO) was given, where indicated, as a 50 mg/kg i.p. bolus solution in sterile pyrogen-free water. Duplicate rats were utilized for each time point studied. For in situ immunocytochemical identification of BrdUrd incorporation into nuclear DNA, 5-μm sections were cut from paraffin-embedded control (sham-operated) and regressing ventral prostates and from sections of the small bowel to allow evaluation of the immunostaining procedures. Tissue sections were immunostained with a mouse monoclonal antibody against BrdUrd (MedScan Industries, Hollywood, FL) as previously described (24) using biotinylated goat anti-mouse IgG and a peroxidase-labeled ABC reagent (Vector Laboratories, Burlingame, CA). Sections were lightly counterstained with hematoxylin for photomicrography.

Ventral prostate tissue (1 gland/time point) recovered for DNA extraction was flash-frozen in liquid N2 and then pulverized. DNA was extracted from 50 mg frozen tissue powder using the protocol and materials supplied with the A.S.A.P. genomic DNA extraction kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Aliquots of DNA containing 10 μg were electrophoresed in adjacent lanes of a 1.5% agarose gel. The gel was treated for 15 min with 10 mM HCl and for 15 min with a denaturing solution containing 0.2 N NaOH and 0.5 M NaCl and was incubated for final 30 min in neutralizing solution containing 0.5 M Tris-HCl, pH 8.0, 0.5 M NaCl. The DNA in the gel was then transferred to a Nytran nylon filter (Schleicher and Schuell, Inc., Keene, NH) by blotting overnight and was fixed to the filter in an 80°C vacuum oven for 1 h. The filter was incubated with blocking reagent in 25 mM Tris, pH 7.5, 0.15 M NaCl containing 0.5% Tween 20 and 10 mM EDTA prior to incubation with anti-BrdUrd monoclonal antibody (1/200) in 25 mM Tris, pH 7.5, 0.15 M NaCl for 1 h at 37°C. After washing, the filter was incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Promega, Inc., Madison, WI), and the staining reaction was processed using the NBT and NCT substrate reagent (Promega, Inc.).

Detection of PCNA Expression in the Regressing Rat Ventral Prostate Gland. Sections from paraffin-embedded regressing ventral prostate glands and from the small bowel were treated with methanol and 10% H2O2 to suppress endogenous peroxidase activity, blocked with 10% horse serum, and incubated overnight with monoclonal anti-PCNA antibody (DAKO, Inc., Carpenteria, CA). Immunostaining was completed using a secondary biotinylated horse anti-mouse antibody and the ABC peroxidase complex (Vector Laboratories) as previously described (25). Immunostained sections were lightly counterstained with hematoxylin for photomicrography.

Extraction and Hybridization of mRNA. Polyadenylated mRNA was extracted from a series of flash-frozen ventral prostate glands obtained from unoperated male rats and from rats at sequential intervals after castration (pooled 3 glands/time point) using a LiCl precipitation procedure previously described (14). Polyadenylated mRNA was enriched by oligo(dT)cellulose chromatography and was quantitated by spectrophotometry at 260 nm. Aliquots of mRNA containing 5 μg from each time point were denatured and electrophoresed in adjacent lanes of a 1.2% formaldehyde-agarose gel as previously described (14). The integrity of the RNA was ascertained by visualization of the ethidium bromide-stained ribosomal RNAs through UV transillumination. The RNA in the gel was blotted overnight to a Nytran nylon filter to produce a Northern blot and was subsequently hybridized with a 32P-labeled complementary DNA fragment for murine p53 (26) prepared with a random prime labeling kit (Boehringer Mannheim Biochemicals) and washed as described (14). The hybridized blot was directly analyzed on a Betagen, Betascope 360 scanning densitometer to obtain a quantitative estimate of p53 transcript band density, prior to exposure to Kodak XAR-5 X-ray film for autoradiography. The blot was subsequently cleaned and rehybridized to a probe for 18S rRNA, washed, and analyzed on the Betascope 360 densitometer to correct for potential RNA loading inequities.

RESULTS

Evidence for Induced DNA Synthesis in the Regressing Rat Ventral Prostate Gland. Proliferating cells can be identified in vivo by functional assays that measure the incorporation of nucleoside analogues into high-molecular-weight nuclear DNA. A well-described immunoassay, in which BrdUrd is utilized as a thymidine analogue (24, 27), provided a means of identifying comparable nuclear labeling in the crypts (not shown).
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Fig. 2. Apoptotic bodies in the regressing ventral prostate epithelium stain for BrdUrd incorporation. Three photomicrographs were taken of sections from a 60-h castrated rat that received a 6-h bolus of BrdUrd before recovery of the ventral prostate gland for BrdUrd immunostaining. Sections were lightly counterstained with hematoxylin. Arrows, obvious apoptotic nuclei within the epithelium. × 400.

and quantitating cells undergoing DNA synthesis in the control and regressing rat ventral prostate gland. In this assay, rats were treated for 6 h with a BrdUrd solution (50 mg/kg), and then the ventral prostate gland was collected, fixed, and imbedded in paraffin for sectioning. Sections cut from these tissue were immunostained for BrdUrd incorporation with a monoclonal anti-BrdUrd antibody. For each specimen, the number of stained epithelial cell nuclei were counted microscopically for 25 individual prostatic ducts from three different sections and are presented here as the average number of BrdUrd-positive epithelial nuclei per prostatic duct. The BrdUrd labeling index of a section of the small bowel from the same rat was utilized to ensure appropriate BrdUrd treatment, and this index was similar for control and castrated rats (approximately 24–26% of crypt cells were stained following a 6-h BrdUrd treatment). The normal adult rat ventral prostate gland is known to have a low proliferation rate, and this was confirmed by our assay, in which we were able to stain less than 1 epithelial nuclei/prostatic duct with a monoclonal anti-BrdUrd antibody following 6 h of BrdUrd treatment. In contrast, an equivalent treatment labeled 12
epithelial morphology, but we attribute this to morphological ± 3 (SD) epithelial nuclei/duct when given to the rat at 12 h following a 2-h pulse of BrdUrd that was chased into the apoptotic DNA frag staining (left) demonstrated apoptotic DNA fragmentation (Lanes B and C). The DNA in the gel was blotted onto a nylon filter, and the resulting Southern blot was

electrophoresed in an agarose gel. Ethidium bromide staining of this gel demonstrated the 180-base pair "ladder" DNA degradation pattern

demonstrated in the photomicrographs of Fig. 2. Southern blots made from these gels were probed with anti-BrdUrd monoclonal antibody, and sub

sequent immunostaining of the blot revealed intense reaction only with the DNAs extracted from the prostates of castrated rats (Fig. 3). The staining of 2-h BrdUrd-treated DNA was restricted to the HMW DNA band; however, there was significant staining of the lower-molecular-weight DNA fragments in the 6-h-treated regressing prostate. Our results show that BrdUrd is initially incorporated into HMW DNA of the regressing ventral prostate and is then chased into lower-molecular-weight fragments characteristic of apoptosis. Based on the results of our in situ staining for BrdUrd incorporation in the regressing prostate gland and this modified blot assay, we believe that it requires at least 6 h from the initiation of DNA synthesis to the onset of DNA and nuclear fragmentation associated with prostate cell apoptosis. PCNA is a nuclear protein the expression of which during the cell cycle is correlated with transition through the S phase (25). This antigen is not detected in noncycling cells; however, it is highly induced in proliferating cells, wherein it functions as a cofactor for the DNA polymerase (29). For this reason, PCNA is also referred to as cyclin (30). To determine further whether DNA synthesis in the regressing prostate mimics that associated with cellular proliferation, we used an anti-PCNA monoclonal antibody to immunostain regressing ventral prostate glands. As demonstrated by the photomicrographs shown in

Fig. 4, only rare epithelial nuclei were stained with this antibody in the control (unoperated) rat ventral prostate. However, nuclei of epithelial cells of the regressing prostate are highly reactive for PCNA staining as early as 18 h after castration. Staining intensity increases through the 60-h castrated specimen that we examined. Moreover, the fragmented nuclei of apoptotic bodies in the 60-h castrated ventral prostate also stain intensely for this marker of proliferation (Fig. 4).

Induced Expression of a Cell Cycle Suppressor, p53, during Androgen-regulated Apoptosis. As established by the data presented here and from a series of earlier studies, the apoptotic ventral prostate cell shares a number of characteristics with the proliferating prostatic cell, including the induction of a protooncogene cascade (14), nuclear BrdUrd incorporation into HMW DNA (prior to fragmentation), expression of nuclear PCNA antigen, and increased tyrosine phosphorylation (31). Because of these shared characteristics, we propose that hormonally induced apoptosis results when noncycling (differentiated) prostate epithelial cells are driven back onto the cell cycle, early after androgens withdrawal (Fig. 5). This hypothesis might also explain the apparent necessity of RNA and protein synthesis for prostate apoptosis (32) based on the requirement of synthetic activity for reentry onto the cell cycle. The absence of mitotic figures in the postcastration period implies that this cycle is never completed, i.e., it is defective.

Based on the recent report demonstrating that reintroduction of wild-type p53 protein into a population of rapidly cycling cells was sufficient to initiate apoptosis (33), we tried to determine whether p53 expression in the rat ventral prostate gland was altered by castration. A Northern blot prepared using a series of polyadenylated mRNAs extracted from rat ventral prostates at sequential daily intervals after castration was probed for the p53 transcript. The abundance of the 2.2-kilobase transcript increased 3-fold within 24 h and reached a peak of greater than 5-fold enhancement by 7 days after castration (Fig. 6). We have detected a similar 3–5-fold increase in p53 protein in 24-h castrated ventral prostate tissue with the use of a Western blot detection procedure (not shown here). We believe that this is the first in vivo evidence that the cell cycle

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Fig. 3. Immunochemical analysis of a Southern blot demonstrates incorporation of BrdUrd into HMW DNA of the regressing prostate prior to its fragmentation. DNAs extracted from the ventral prostate gland of a sham-operated rat (A) 6 h after i.p. injection of BrdUrd or from 60-h castrated rats, 2 (B) or 6 (C) h after injection of BrdUrd, were electrophoresed in an agarose gel. Ethidium bromide staining (left) demonstrated apoptotic DNA fragmentation (Lanes B and C). The DNA in the gel was blotted onto a nylon filter, and the resulting Southern blot was

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Fig. 4. Immunostaining of PCNA in control rat ventral prostate gland (A); 18 h after castration (×400) (B); 60 h after castration (×200) (C); 60 h after castration (×400) (D). Arrows, apoptotic nuclei.

FIGURE 4

(A) Immunostaining of PCNA in control rat ventral prostate gland. (B) 18 h after castration. (C) 60 h after castration. (D) 60 h after castration. Arrows indicate apoptotic nuclei.

Fig. 5. Diagrammatic representation of the concept of hormone-regulated prostate cell apoptosis as reentry into a defective cell cycle.

DISCUSSION

Apoptosis, a natural process of cell death and elimination, is as important for the development and maintenance of multicellular organisms as are cell proliferation and differentiation. The cellular mechanism responsible for this programmed cell death is often initiated by changes in the hormone or growth factor environment and is dependent on cell signaling systems just like other cell action processes. Therefore, we can expect that this mechanism is as complicated as any of the other cellular responses. The question that was addressed in this study is whether this cell death mechanism must be distinct from other cell action processes or whether apoptotic cell death might actually result if a normal cell action pathway is defective. Our efforts to this end were influenced by different studies of chemotherapeutically induced apoptosis demonstrating the relationship of this phenomenon (apoptosis) to the cell cycle (22, 23). Moreover, we were also motivated by an earlier observation in which the regressing rat ventral prostate gland was shown to display a particular pattern of genetic activity (14) that was most striking due to its generic association with cell proliferative responses, in vitro (15) and in vivo (16, 17).

Our model for these studies was the mature rat ventral prostate gland. This organ is remarkably sensitive to androgenic steroids, and up to 85% of the epithelial cells lining the ducts and glands will undergo apoptosis within the 2 weeks following castration (34, 35). This model has already proved useful in identifying drugs that can block apoptosis (19, 20) and in identifying specific gene products associated with apoptosis (12). Here we have demonstrated with a functional assay (BrdUrd incorporation analysis) and with an immunoassay for a molecular marker of proliferation (PCNA expression) that the number of cells in the prostatic epithelium undergoing DNA synthesis is dramatically increased after castration. BrdUrd-labeled DNA is detectable in "normal"-appearing nuclei after a 2-h
BrdUrd treatment but can be chased into nuclei showing overt condensation and fragmentation associated with apoptotic body formation by 6 to 8 h. This apparent DNA synthesis is not likely to be the result of a repair process as was demonstrated by the use of a modified Southern blotting procedure to characterize newly synthesized DNA. Our assay showed that BrdUrd was initially incorporated into HMW DNA and that this labeled DNA was subsequently fragmented in a manner characteristic of apoptosis. Therefore, prostatic epithelial cells that express gene products characteristic of the G1 phase of the cell cycle (c-fos and c-myc) after castration are also entering the S phase as evidenced by highly induced expression of PCNA/cyclin and BrdUrd incorporation. It is clear, however, that this cell cycle is never completed.

While we have not yet identified a precise defect associated with prostate cell cycle transit leading to apoptosis, it is appropriate to note that, unlike the productive cell cycle, prostate apoptosis occurs with markedly enhanced expression of the tumor suppressor gene product p53. This was demonstrated here by Northern blot analysis of the regressing ventral prostate gland. In addition we have confirmed this observation both by Western blotting analysis and by immunohistochemical observations of regressing prostate tissues. The recent demonstration that a genetic manipulation of p53-deleted tumor cells causing them to reexpress the wild-type p53 protein induced widespread apoptosis in these cells (33) is relevant to our observations in this hormonally induced system of apoptosis and implies a common mechanism wherein high expression of p53 during the cell cycle can divert this cycle into apoptosis. Presumably this diversion is related to the characteristic degradation of apoptotic cell DNA, a common attribute of apoptosis.

This hypothesis raises other important questions related to the observation that quiescent prostate epithelial cells would reinitiate the cell cycle following androgen withdrawal. It is of interest to note the experiments of Sonnenschein and his colleagues (36) that identified a narrow window of concentration within which testosterone can stimulate proliferation of androgen-sensitive cultured LnCaP cells. Perhaps the metabolic decline in circulating testosterone levels during the first 12 h after castration provides a concentration that is conducive to cell cycle initiation before falling to a level which fails to support further progress in this cycle. Alternatively, cell cycle reentry could be initiated by some definable alteration in the growth factor environment of the ventral prostate during the early period after castration. Although transforming growth factor β is generally associated with proliferative inhibition, significant enhancement of the expression of this polypeptide factor has already been detected in regressing rat ventral prostate tissue (37).

Aside from its importance for development, apoptosis is being considered increasingly as a contributing factor to certain human disease states and as a means of inhibiting (or curing) human cancers. Therefore, our understanding of the molecular mechanism leading to programmed death could provide new targets to inhibit its progression or to ensure its occurrence in abnormally proliferating cells. Rather than searching for "lethal" gene products as were proposed in earlier hypotheses, our studies indicate that these targets may well be gene products important for the initiation and completion of the cell cycle. We have already mentioned the potential involvement of p53 in cell cycle abortion. Additionally, a number of different investigations have indicated that protein kinase C is an important factor in apoptosis. Activation of this enzyme with phorbol esters can abrogate glucocorticoid-mediated apoptosis of lymphocytes (38), cold-induced apoptosis of cultured synovial cells (39), and apoptosis of serum-depleted endothelial cells (40). The complexity of the cell cycle ensures that we will be able to identify other gene products involved in apoptosis in the future.

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