Androgen Ablation Leads to an Upregulation and Intranuclear Accumulation of Deoxyribonuclease I in Rat Prostate Epithelial Cells Paralleling Their Apoptotic Elimination

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Abstract. After androgen ablation by castration, the epithelial cells of the rat ventral prostate are eliminated by apoptosis. The number of cells showing apoptotic chromatin degradation increases with time up to day 3 after castration as verified by in situ end labeling of fragmented DNA. Apoptotic chromatin degradation is catalyzed by a Ca\(^{2+}\), Mg\(^{2+}\)-dependent endonuclease. Recently, evidence has been presented that suggests deoxyribonuclease I (DNase I) is identical or very closely related to the apoptotic endonuclease (Peitsch, M.C., B. Polzar, H. Stephan, T. Crompton, H.R. MacDonald, H.G. Mannherz, and J. Tschopp. 1993. EMBO [Eur. Mol. Biol. Organ.] J. 12:371–377). Therefore, the expression of DNase I in the ventral prostate of the rat was analyzed before and after androgen ablation at the level of protein, enzymatic activity, and gene transcripts using immunohistochemical and biochemical techniques. DNase I immunoreactivity was detected only in a few single epithelial cells before androgen ablation. After castration, a time-dependent increase in DNase I immunoreactivity was observed within the epithelial cells. It first appeared after about 12 h in the apical region of a large number of epithelial cells. Up to day 3 after castration, the intracellular DNase I antigenicity continuously increased, and the cell nuclei gradually became DNase I positive. At day 5, almost all nuclei of the epithelium were stained by anti–DNase I. DNase I immunoreactivity was particularly concentrated in cells showing morphological signs of apoptosis, like nuclear fragmentation, and in many cases was found to persist in apoptotic bodies. DNase I gene transcripts were detected in control animals using dot and Northern blotting as well as RNase protection assay. After androgen ablation, the amount of DNase I gene transcripts in total extractable RNA was found unchanged or only slightly decreased up to day 5. Their exclusive localization within the epithelial cells was verified by in situ hybridization. Before castration, the DNase I gene transcripts were homogeneously distributed in all epithelial cells. At day 3, DNase I–specific mRNA was found to be highly concentrated in cells of apoptotic morphology. Using the zymogram technique, a single endonucleolytic activity of about 32 kD was detected in tissue homogenates before castration. After androgen ablation, the endonucleolytic activity increased about four-to sevenfold up to day 3. At day 5, however, it had dropped to its original level. At day 1, three new endonucleolytic variants of higher molecular mass were expressed. At day 3, the predominant endonucleolytic activity exhibited an apparent molecular mass of 32 kD. Enzymatic analysis of the endonucleases present in prostate homogenates before and after castration demonstrated properties identical to DNase I. They were inhibited by chelators of divalent cations, Zn\(^{2+}\) ions and monomeric actin. Immunodepletion was achieved by immobilized antibodies specific for rat parotid DNase I. A polyclonal antibody raised against denatured DNase I was shown by Western blotting to stain a 32-kD band after enrichment of the endonuclease from day 0 and 3 homogenates by preparative gel electrophoresis. The data thus indicate that androgen ablation leads to translational upregulation of an endonucleolytic activity with properties identical to DNase I in rat ventral prostate, followed by its intracellular retention and final nuclear translocation in those epithelial cells that are destined to apoptotic elimination.

Apoptosis or programmed cell death is a process by which cells in multicellular organisms are eliminated. This important physiological mechanism guarantees ordered tissue shaping during development and cellular homeostasis of adult organs. Cellular survival of many tissues depends on a constant supply of growth factors or hormones. The prostate is an androgen-dependent organ. Depletion of testosterone leads to rapid tissue involution due to apoptotic elimination of the glandular cells of the secretory epithelium (Kyprianou and Isaacs, 1988). It is estimated that ~80–90% of the epithelial cells

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of the prostate possess androgen receptors and depend on a constant supply of testosterone for their survival (Kyprianou and Isaacs, 1988; Furuya et al., 1994; Banerjee et al., 1995).

The rat ventral prostate has become a classical animal model in which apoptosis can experimentally be induced and analyzed after androgen ablation. Castration leads within a few days to apoptotic death of a large number of its androgen-sensitive epithelial cells. Thus, this system offers the possibility to study the biochemical events of apoptosis under physiological conditions. In view of the increasing incidence of malignancies of this organ, this model is also of paramount importance since the most effective treatment of carcinomas of the prostate is androgen ablation by castration or antiandrogen treatment. Under in vivo conditions, apoptotic cell death is always accompanied by internucleosomal DNA degradation that in many instances can be demonstrated by agarose gel electrophoresis of extracted DNA (DNA ladder formation). Thus, internucleosomal chromatin degradation has been shown to occur in the rat ventral prostate after castration (Kyprianou et al., 1988). The exact function of chromatin fragmentation is still unclear, but it has been proposed that it represents the irreversible step of apoptosis and facilitates removal of the apoptotic cell. In some instances it might also lead to the destruction of viral infectious DNA or chromatin containing deleterious alterations, guaranteeing genomic stability of the organism (Peitsch et al., 1994). Surprisingly, it has been reported that chromatin degradation does not occur in a number of in vitro systems (established tumor cell lines), although these cells exhibit apoptotic morphological alterations (Oberhammer et al., 1993; Otto et al., 1996). These cellular systems may, however, represent special cases in which the apoptotic pathway has become faulty either during the initial transformation or when establishing as a permanent cell line. Therefore, it remains of essential importance for our understanding of the biochemical and regulatory mechanisms of the execution of apoptosis to identify the endonuclease(s) involved in this step of the apoptotic pathway.

A number of candidate endonucleases have been proposed (Caron-Leslie et al., 1991; Barry and Eastman, 1993). Recently, Peitsch et al. (1992, 1993) presented evidence that in rat thymo- and lymphocytes, the apoptotic endonuclease is identical or very closely related to deoxyribonuclease I (DNase I). In the past, DNase I has generally been regarded as a secretory enzyme released from the pancreas and/or parotid gland (Roehr and Mannherz, 1978; Mannherz et al., 1995) into the alimentary tract to fulfill digestive functions. However, recent reverse transcriptase–PCR data have shown that DNase I is also expressed in a number of nonsecretory rat tissues and may even be a ubiquitous protein (Polzar et al., 1994). Particularly high expression of DNase I was observed in tissues with high cellular turnover containing terminally differentiating cells prone to elimination by apoptosis, like the enterocytes of the small intestine or the keratinocytes of squamous stratified epithelia (Polzar et al., 1994).

Our data demonstrate that in the rat ventral prostate, the expression of DNase I is upregulated at the translational level in the glandular cells destined for apoptosis after castration. The increased DNase I expression is accompanied by the accumulation of DNase I within these cells, followed by its transfer into the cell nucleus. Furthermore, data are presented indicating that the nuclear relocation of DNase I parallels the appearance of cells of apoptotic morphology and the induction of DNA fragmentation.

Materials and Methods

Materials

Bovine pancreatic DNase I was a commercial product from Worthington Biochemical Corp. (Freehold, NJ). Calf thymus DNA was obtained from Sigma (Munich, Germany). Terminal transferase was a commercial product of Boehringer Mannheim (Mannheim, Germany). Avian myeloma virus reverse transcriptase was obtained from United States Biochemicals (Cleveland, OH). Fluorescein (FITC)-labeled dUTP and dATP were purchased from Dupont/NEN (Bad Homburg, Germany) and Boehringer Mannheim, respectively. Protein A immobilized to Sepharose was obtained from Pharmacia, (Freiburg, Germany). All other reagents were of analytical grade.

Castration of Male Wistar Rats

Young adult male Wistar rats (250–300 g body weight) were purchased from Ivanovas (Kisslegg, Germany). The animals were kept in cages and fed Altromin rat chow and drinking water ad libidum. Castration of anesthetized animals was performed via a retroperitoneal approach as described previously (Bacher et al., 1993). After the time intervals indicated the animals were anesthetized and bled. The ventral prostates were excised as quickly as possible, washed with PBS, and stored at −80°C until use. For the preparation of tissue homogenates, the washed ventral prostates were homogenized in 50 mM potassium phosphate, pH 6.5, supplemented with 0.25 mM PMSF and 2,000 U aprotinin/ml. After centrifugation at 20,000 g for 15 min, the proteins of the supernatants were precipitated with ammonium sulfate (final concentration 85%) and centrifuged again at 20,000 g for 30 min. The precipitate was dissolved in 20 mM potassium phosphate, pH 6.5, and dialyzed overnight at 4°C against the same buffer. The obtained material was used directly for determination of endonucleolytic activity or further purified by preparative gel electrophoresis.

Preparative Procedures

Commercial bovine pancreatic DNase I (EC 3.1.21.1) was further purified as described by Mannherz et al. (1980). Rat parotid DNase I was isolated and purified as given by Kreuder et al. (1984). Bovine DNase I was immobilized on vinylsulphone agarose (Mini-Leak; KEM EN TEC, Copenhagen, Denmark). Rabbit skeletal muscle actin was prepared as given by Mannherz et al. (1980). Human gelsolin segment I was expressed by transformed bacteria and isolated as detailed in Way et al. (1989). The stoichiometric 1:1 complex of segment I and monomeric (G-)actin was obtained by mixing both proteins at equimolar ratio.

The specificity of the polyclonal antibodies against either bovine or rat DNase I has been described previously (Mannherz et al., 1982; Kreuder et al., 1984). They were further affinity purified as detailed by Polzar et al. (1988) and tested by Western blotting for monospecificity using rat parotid homogenate (not shown, but see Kreuder et al., 1984). For immunoblot absorption experiments, 50 μl of the affinity-purified polyclonal anti-rat DNase I antibody (2 μg/ml IgG) was incubated with 100 μl protein A–Sepharose for 2 h at room temperature and subsequently washed twice with 100 mM Tris-HCl, pH 8.0, following the protocol given in Harlow and Lane (1988). For immunoadsorption, 100 μg of the tissue homogenates were incubated with 50 μl of anti-DNase I bound to protein A–Sepharose. After 2 h of incubation at room temperature, the mixture was centrifuged for 10 min at 1,000 rpm. The supernatants were analyzed for endonucleolytic activity using the plasmid degradation assay (10 μl of supernatant containing 5 μg of protein) or incubating 80 μl of supernatant (40 μg protein) with 2 × 105 isolated substrate nuclei prepared from mycoplasma-free cells of the human mammary carcinoma MCF-7 line (Otto et al., 1996; Paddenberg et al., 1996). For controls, the samples were identically treated using unloaded protein A–Sepharose.

1 Abbreviations used in this paper: DNase I, deoxyribonuclease I; PAA, polyacrylamide.
Analytical Procedures

Protein concentration of purified Dnase I was determined either by absorbance measurement at 280 nm using an extinction coefficient of 1.23 cm−1 and mg−1 for the bovine enzyme or by the Bradford (1976) procedure for rat parotid Dnase I. Dnase I activity was tested either by the hyperchromicity test initially introduced by Kunitz (1950), by the radial diffusion test using 1% agarose gels soaked with 50 μg salmon sperm DNA/mL as detailed by Nadano et al. (1993), or the plasmid degradation assay (Pecht et al., 1992, 1993). DNA ladder formation was analyzed by agarose gel electrophoresis as given by Paddenberg et al. (1996).

Plasmid Degradation Assay

The presence of endonucleolytic activity in tissue homogenates was verified by measuring the time-dependent degradation of the circular plasmid pBluescript II KS(+) or pUC 18 (Pecht et al., 1992, 1993). The reaction mixture (30 μL total volume) contained 0.5 μg plasmid in 20 mM Tris-ace
tate, pH 7.4, 2 mM CaCl₂, and 2 mM MgCl₂, and was supplemented with either 20 mM EDTA, 20 mM EGTA, or 5 mM ZnCl₂. Samples of tissue homogenates of ventral prostates and parotid gland containing 5 and 0.5 μg of protein, respectively, were added to start the reaction. After 90 min, the reaction was stopped by adding sample buffer for agarose gel electrophoresis (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 0.05% formamide, 1 mM EDTA, and 20 mM MOPS). Fractions from the Prep Cell preparation (see below) were analyzed in identical buffer supplemented with 1% BSA to absorb the SDS present in the elution buffer and to facilitate renaturation of the endonuclease. The samples were incubated for 16 h at 37°C.

Dot and Northern Blotting and RNase Protection Assay

Total RNA from rat ventral prostates was prepared according to Chomczynski and Sacchi (1987). Dot and Northern blots were performed as described previously (Weber, 1993; Polzar et al., 1994) using as probe the cDNA of rat parotid Dnase I (Polzar and Mannherz, 1990). For the RNase protection assay, an antisense RNA was transcribed from a gel-purified AvaiII fragment of the Dnase cDNA cloned in pBluescript II KS(+) vector using T3-RNA polymerase (Stratagene, La Jolla, CA) and [32P]UTP. The unprotected labeled RNA fragment was 651 bp, and the protected fragment was 587-80 bp. RNA protection analysis with total RNA of the rat parotid and prostate glands was performed using the ribonuclease protection analysis kit II from Ambion (Austin, TX) following the instructions given in the manual. Protected fragments were analyzed on a 5% polyacrylamide/8 M urea gel and detected on x-ray film (X-AR; Kodak, Inc., Rochester, NY) after overnight exposure at −80°C using an intensifying screen.

Electrophoretic Procedures

Electrophoresis on polyacrylamide (PAA) gels in the presence of sodium dodecylsulfate (SDS-PAGE) was performed as given by Laemmli (1970). Electrophoresis on polyacrylamide (PAA) gels in the presence of sodium dodecylsulfate (SDS-PAGE) was performed as given by Laemmli (1970). Electrophoretic Procedures

Electrophoresis on polyacrylamide (PAA) gels in the presence of sodium dodecylsulfate (SDS-PAGE) was performed as given by Laemmli (1970). Silver nitrate staining of PAA gels was carried out following the protocol of Jagow, 1991; Schägger et al., 1994) was combined with an agarose overlay for in situ hybridization. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique. Tissue homogenates were evaluated using the standard zymogram technique.

In Situ Hybridization

To generate Dnase I-specific antisense and sense probes, the 334-bp HindIII fragment of rat parotid Dnase I specific cDNA (corresponding to positions 650–984 of the nucleotide sequence of rat parotid Dnase I according to Polzar and Mannherz (1990; these sequence data are available from GenBank/EMBL/DDBJ under accession number X56060) was subcloned into pBluescript II KS(+) vector (Stratagene). To obtain the antisense probe, the linearized was used with XbaI followed by transcription using T3-RNA polymerase; to generate the sense probe, the plasmid was linearized with HindIII and transcribed using T7-RNA polymerase (Zanotti et al., 1995). The RNA transcripts were labeled with fluorescein-UTP (RNA Detection Kit; Amersham Buchler GmbH, Braunschweig, Germany).

For in situ hybridization, small slices of ventral prostates were immediately fixed in 4% buffered paraformaldehyde after sacrificing the animal. After fixation, the tissues were paraaffin embedded. Sections of 5 μm were cut, mounted on silanated slides, deparaffinized in xylene, and rehydrated according to standard procedures. After successive treatment with 0.02 M HCl for 10 min and 0.1% Triton X-100 in PBS for 90 s and washing in PBS twice for 3 min, the specimens were digested with 100 μg/ml proteinase K for 4 min, treated with 2 μg/ml glycine for 5 min, followed by 20% acetic acid for 15 s. Hybridization was done in hybridization buffer containing 50% formamide with 500 ng probe/ml at 55°C overnight. Specimens were washed with 1× SSC, 0.1% SDS (twice for 5 min at room temperature) and 0.5× SSC, 0.1% SDS (twice for 10 min at 55°C). Then the slides were incubated overnight with antibioticatagged on alkaline phosphatase (RNA Detection Kit; Amersham Buchler GmbH). Visualization of bound alkaline phosphatase was achieved by using 5-bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium chloride as substrates following exactly the protocol given by the supplier. All solutions and glassware used for in situ hybridization were autoclaved or baked at 200°C to inactive RNase activities.

Histochemical Procedures

Fresh tissues were fixed with 4% formaldehyde and embedded in paraffin, and 5-μm-thick sections were spread on poly-L-lysine-coated slides. For immunohistochemistry, endogenous peroxidase was inactivated by 3% H₂O₂/methanol for 30 min at −20°C. After rehydration, the tissue sections were immersed in PBS for 5 min and then blocked with 10% goat serum for 30 min. Thereafter, they were incubated overnight (about 16 h) with an affinity-purified polyclonal primary antibody against rat parotid Dnase I (Kreuder et al., 1984; Pecht et al., 1993; Polzar et al., 1994) in a humid atmosphere at room temperature. The staining with streptavidin peroxi
dase/DAB was done according to the instructions of the manufacturer (DAKO, Hamburg, Germany). Control experiments were performed after affinity absorption of the antibody preparations onto immobilized bovine Dnase I. In all instances this treatment led to a complete suppression of the specific immunoreaction.

In situ labeling (ISEL) of free 3′-OH ends of fragmented chromatin in paraffin-embedded sections was performed as described previously (Gavrieli et al., 1992; Wijsman et al., 1993) with the modifications given by

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Polzar et al. (1994) using FITC-labeled dATP or dUTP at 40 μM as substrate for the terminal deoxynucleotidyl transferase.

Photographs were taken (HP5 film; Ilford, Mobberley Cheshire, UK or Ektachrome 400HC film; Kodak, Inc., Rochester, NY) using a microscope (model Axioskop; Carl Zeiss, Jena, Germany) equipped with epifluorescence optics.

**Results**

*Time-dependent Weight Decrease of the Ventral Prostate after Castration*

In agreement with previous data (Kyprianou et al., 1988), we found that the wet weight of the rat ventral prostate continuously decreased to about one fifth of its control value within the first 7 d after castration. Histological analysis showed a considerable reduction in the height of the secretory epithelium and the size of the secretory follicles at days 5 and 7 (see Fig. 2, e and f). In this study, we only used the ventral prostate, which was previously shown to be most affected by androgen withdrawal (Kyprianou et al., 1988; Furuya et al., 1994; Banerjee et al., 1995).

*In Situ End-labeling of Apoptotic Cells*

Cells containing fragmented DNA can be identified by in situ end-labeling of the free 3’-OH ends generated during the internucleosomal DNA-degradation using labeled dUTP and terminal deoxynucleotidyl transferase (Gavrieli et al., 1992; Wijsman et al., 1993; Polzar et al., 1994). Applying this technique on sections of rat prostate at different time intervals after castration, we found that the number of labeled cells increased up to day 3 (Fig. 1, b–e) in agreement with previous quantitative data showing that most of the

![Figure 1. In situ end-labeling of rat ventral prostate before and after castration. (a) Positive control of rat prostate at day 0. The section was pretreated with purified bovine pancreatic DNase I to fragment the chromatin of all nuclei. (b–e) In situ end-labeling after castration: (b) 12 h; (c) 24 h; (d) cluster of cells; and (e) single cell at day 3. Bars, 100 μm.](image-url)
cells are eliminated between day 3 and 5 (Kyprianou et al., 1988; Aumüller et al., 1995; Banerjee et al., 1995; Tenniswood et al., 1995). The strongly stained nuclei were of varying size (Fig. 1 d) or fragmented (Fig. 1 c). The fate of the apoptotic cells seemed to differ: In many instances, we observed positively end-labeled cells located underneath epithelial cells of normal appearance (Fig. 1 d). In some cases, the apoptotic cells were found to be extruded into the lumenal space (see also Fig. 3 b).

Immunohistochemistry Using Anti–DNase I

We have recently presented evidence that the apoptotic Ca\textsuperscript{2+},Mg\textsuperscript{2+}-dependent endonuclease is identical or very similar to DNase I (Peitsch et al., 1992, 1993). Therefore, the expression and distribution of DNase I in rat ventral prostate was analyzed before and after castration using polyclonal, affinity-purified antibodies (Kreuder et al., 1984; Polzar et al., 1994). In control organs, most of the epithelial cells were DNase I negative. In some instances, we detected a weak immunostaining located in the apical region of the epithelial cells (not shown). Occasionally, single cells with an apparently apoptotic morphology exhibited strong nuclear DNase I immunoreactivity (Fig. 2 a). 6 h after castration, a number of cells exhibited increased cytoplasmic DNase I immunoreactivity, although the number of presumed apoptotic cells with positive nuclear staining was not increased (Fig. 2 b). 12 h after castration, a clear increase in DNase I immunoreactivity already became detectable in the apical region of many epithelial cells (Fig. 2 c). It was often found to be clustered in a given region of a particular follicle (not shown). The staining intensity of this cytoplasmic location increased during the following 2 d. At day 3, the DNase I antigenicity was clearly increased. It was only present in the columnar epithelial cells (Fig. 2 d). It appeared as granular apical staining, i.e., in regions known to harbor the endoplasmic reticulum and the Golgi apparatus. In addition, an increasing number of nuclei were DNSase I positive (Fig. 2 d). At days 5 and 7, most of the nuclei of the epithelial cells were DNase I positive (Fig. 2, e and f). The diameter of the secretory follicles and the height of their epithelium were found to be significantly reduced (Fig. 2, e and f), and the alveoli frequently exhibited epithelial infoldings (Fig. 2 f). At day 7, a large number of the remaining epithelial cells still exhibited nuclear DNase I immunoreactivity, although a considerable number of cells were DNase I negative (Fig. 2 f). At day 3, we detected the highest number of cells showing apoptotic morphology (Fig. 3, a–d). Cells containing fragmented nuclei or apoptotic bodies were frequently detected in epithelial infoldings and shown to be DNase I positive (Fig. 3, a, c, and d). A few apoptotic cells with apparently less nuclear remnants were DNase I negative (Fig. 3 d). Most of the apoptotic cells were localized at the basal site of the epithelium, although in some instances we detected apoptotic cells that were apparently shed into the lumenal space (Fig. 3 b).

Localization of DNase I Gene Transcripts by In Situ Hybridization

In situ hybridization using DNase I–specific antisense and sense RNA was used to localize its gene transcripts and to analyze its time-dependent variation after castration. Sections of the ventral prostate before and after castration were stained using sense and antisense probes. The sense probe did not show any labeling at all when used on sections of control prostates and at any other time point after androgen ablation (Fig. 4 a). In control prostates, a specific staining (blue stain) of exclusively epithelial cells was obtained using the antisense probe (Fig. 4, b and c). Within the epithelial cells, the staining was most prominent at supranuclear and apical localization that was frequently found to be interrupted by a narrow unstained region (Fig. 4, c and d). From ultrastructural studies, it is known that the rough endoplasmic reticulum is interrupted and localized in supranuclear and apical positions (Aumüller et al., 1980). Using the antisense probe, the homogeneous staining of the epithelial cells remained until day 2 (Fig. 4 d). At day 3, a considerable number of apoptotic cells were detected whose fragmented nuclei were in many instances strongly labeled (Fig. 4, e and f, black stain). The intensity of the specific stain decreased at day 5 (Fig. 4 g) and was further reduced at day 7 (not shown).

Analysis of DNase I–specific Gene Transcripts

The results obtained by in situ hybridization indicated the presence of DNase I gene transcripts in rat ventral prostates even before castration. Total RNA was isolated from ventral prostates before and at different time points after castration. Using the cDNA of rat parotid DNase I as probe on dot blots of total RNA of control and day 1, 3, and 5 prostates, the presence of DNase I gene transcripts was verified (Fig. 5 a). Similar results were obtained by Northern blotting (Fig. 5 c). One single mRNA band was obtained for days 0, 1, 3, and 5 that was identical in length to the one present in rat parotid RNA (1, 1 kb). Both assays indicated a slight decrease in the concentration of the DNase I–specific gene transcripts after androgen withdrawal. Furthermore, RNase protection analysis was used to attempt a more reliable, relative quantification of the DNase I gene transcript using total prostatic RNA (see Materials and Methods). A DNase I–specific signal was obtained for rat prostate that was identical in size to the one for rat parotid gland, although with an ~20-fold lower intensity (Fig. 5 d). Again, a clear decrease in the concentration of DNase I gene transcript was seen during the 5 d after castration.

Analysis of DNase I Specific Activity

Using the zymogram technique (Lacks, 1981) with the modifications described in Materials and Methods, endonucleolytic activities were detected in prostatic tissue homogenates before and after castration (Fig. 6 a). This procedure also allowed us to determine the molecular mass of the endonuclease(s) and to estimate changes of its activity after castration. Control prostates contained a single endonucleolytic entity of 32 kD and low activity (Fig. 6 a). After castration at day 1, three endonucleolytic entities of higher molecular mass were detected. However, at day 3 the pattern seemed to revert to the original one with a high activity of ~32 kD and minor activities of higher molecular mass, whereas at day 5 only very little activity of 32 kD was detected. Densitometry of the gel revealed that
the total activity had increased about sevenfold at day 3 (Fig. 6b). The molecular mass of the endonucleolytic activity in control animals and at day 5 was ~32 kD, almost identical to that of purified rat parotid DNase I or the activity present in rat parotid homogenate. This pattern of endonuclease isoforms was observed repeatedly using different animals (n = 6), and we assume that the shifts of apparent molecular mass at day 1 are due to increased synthesis of proforms that are processed during the following days. This analysis indicates the presence of at least four different isoforms: three at day 1 in addition to the 32-kD isoform. At day 3, the 32-kD variant predominates, although the ones with higher molecular mass were still visible. Even at day 5, isoforms of molecular mass greater than 32 kD were still detectable on the original gel. The endonucleolytic activity of all entities was inhibited by addition of either 20 mM EDTA, 20 mM EGTA, or 5 mM ZnCl₂ into the reaction buffer (not shown), indicating identical enzymatic properties.

The zymogram technique necessitates heating and denaturation steps to prepare the samples for SDS-PAGE, which could have led to an at least partial irreversible inactivation of the endonuclease(s). Therefore, we developed a modified zymogram procedure using native blue gel electrophoresis (Schägger and von Jagow, 1991; Schägger et al., 1994) to verify the observed changes in relative activity after castration. As described in Materials and Methods, samples of homogenates of prostates before and after castration were first subjected to nondenaturing gel electrophoresis. Thereafter, the gel was placed on a 1% agarose gel containing calf thymus DNA and ethidium bromide. After diffusion of the separated proteins, the agarose was incubated in 40 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, and 5 mM MgCl₂, and the location of DNA degrading activities was identified by UV light (as detailed in Materials and Methods). A single, broad band of endonucleolytic activity was detected in all homogenates that exhibited a migration behavior identical to the endonucleolytic activity present in rat parotid gland (Fig. 6c) or purified rat parotid DNase I (not shown). It was also evident that after castration, the activity present in prostate homogenates exhibited the identical time course, i.e., increasing up to day 3 followed by a considerable decrease at day 5. The densitometric analysis of these gels indicated a similar time-dependent quantiative increase of the endonucleolytic activity (Fig. 6d). Using this technique, we also analyzed the pH dependence of the endonucleolytic activities present in homogenates from days 0, 1, 3, and 5. In the range of pH 6 to 9, we observed only one nucleolytic activity comigrating with DNase I that was inhibited by Zn²⁺ ions, EDTA, or EGTA (not shown). At pH 5.5, an additional band appeared that comigrated with commercial DNase II and was not inhibited by Zn²⁺ ions, EDTA, or EGTA (not shown).

The difference in endonucleolytic activity between control and day 3 prostates was also verified using the plasmid degradation assay (Peitsch et al., 1992, 1993). A sequential dilution analysis of the tissue homogenates revealed that a 1:2 and 1:8 dilution diminishes the endonucleolytic activity present in day 0 and 3 prostates, respectively, to a similar extent (almost no degradation), indicating an at least fourfold higher activity in day 3 prostate in reasonable agreement with the densitometric analyses (Fig. 6e).

### Specific Tests for the Presence of DNase I in Rat Prostate Homogenates

A number of specific tests for DNase I were performed comparing prostatic homogenates (5 μg) of day 0 and 3. Using the plasmid degradation assay, the ionic requirements of the prostatic endonuclease were further investigated (Fig. 7a). It can be seen that the endonucleolytic activity present in the homogenate of control and day 3 prostates was active in the presence of 2 mM CaCl₂ and 2 mM MgCl₂ but completely inhibited after addition of 5 mM ZnCl₂, 20 mM EDTA, or 20 mM EGTA. This ion dependence parallels the known behavior of the Ca²⁺, Mg²⁺–dependent apoptotic endonuclease or DNase I.

DNase I is the only endonuclease whose enzymatic activity is specifically inhibited by monomeric (G-) actin (Lazarides and Lindberg, 1974; Mannherz et al., 1975; Hitchcock, 1980). Since actin polymerizes at high ionic strength and loses its ability to inhibit rat DNase I (Kreuder et al., 1984), G-actin was complexed with gelsolin segment 1, which stabilizes actin in its monomeric form even in the presence of high salt without interfering with DNase I binding (Kabsch et al., 1990; McLaughlin et al., 1993). Thus, stabilized monomeric actin was found to inhibit the endonuclease activity present in control and day 3 prostatic homogenates as determined by the plasmid degradation assay (Fig. 7b). It was found that higher amounts of actin:segment 1 complex were necessary to attain complete inhibition of the endonucleolytic activity present in day 3 homogenate (Fig. 7b).

Furthermore, the polyclonal, affinity-purified antibody was immobilized to protein A–Sepharose and used for immunoabsorption. The endonuclease present in day 0 and 3 homogenates was almost completely depleted after preincubation with immobilized anti–DNase I, as verified by analyzing the Sepharose supernatants by using the plasmid assay (Fig. 7c). We also tested the ability of the prostatic homogenates to catalyze the internucleosomal chromatin degradation of endonuclease-free substrate nuclei (prepared from mycoplasma-free MCF-7 cells). It can be seen that day 3 homogenate induces a typical DNA ladder (Fig. 7d, lanes 7 and 8). After immunoabsorption using the protein A–immobilized DNase I antibody, DNA ladder formation was completely suppressed (Fig. 7d, lane 9), indicating that the endonucleolytic activity immunodepleted
from day 3 homogenate is the enzymatic entity able to catalyze the internucleosomal chromatin degradation.

In contrast, day 0 homogenate (Fig. 7d, lanes 4–6) was not able to induce DNA ladder formation under identical incubation conditions. It is presently unclear whether this inability is due to its lower concentration or to the presence of modifications of the DNase I of day 0 homogenate or to specific inhibitors. Day 0 homogenate catalyzes chromatin degradation to high–molecular mass fragments that are just able to migrate into the agarose gel (Oberhammer et al., 1993). This activity is not precipitated by the anti–DNase I antibody (Fig. 7d, lanes 6 and 9). It cannot be excluded that after immunoprecipitation, the endonucleolytic activity of possibly residual DNase I was too low for producing a DNA ladder, although the result may also suggest that two different enzymatic entities are responsible for the production of the high–molecular mass fragments and the internucleosomal DNA degradation. Future experiments will concentrate on these questions.

Identification of DNase I by Western Blotting

Using the affinity-purified polyclonal antibody, attempts were made to identify DNase I in prostate homogenates by Western blotting. Most probably because of the low abundance of this enzyme, no positive reaction was obtained when using whole tissue homogenates. Therefore, the endonucleolytic activity was partially purified and enriched from tissue homogenates of control and day 3 prostates by preparative SDS-PAGE applying 10 mg of protein.

Figure 3. Immunohistochemical staining of rat ventral prostate with anti–DNase I. Top row of color plate: (a–d) 3 d after castration. (a) An epithelial infolding containing three apoptotic cells positively stained with anti–DNase I, marked by arrows. (b) A single apoptotic cell that is apparently extruded into the lumen. (c and d) Apoptotic cells with fragmented nuclei that are positively stained by anti–DNase I (arrows). In d, a presumed late apoptotic cell containing only minute nuclear remnants, which are DNase I negative, is marked by an arrowhead. a–d were counterstained with hematoxylin. Bar, 10 μm.

Figure 4. In situ hybridization of rat ventral prostates using DNase I–specific sense and antisense probes. Sections were treated as given in Materials and Methods. Using the sense probe: (a) No staining. (b–g) Antisense probe. (b and c) Before; (d) 2 d after; (e and f) 3 d after; and (g) 5 d after castration. Note cytoplasmic positive reaction at supranuclear and apical position (blue) in (b–d). At day 3 (e and f), a large number of apoptotic cells are detectable that exhibit strong staining of their fragmented nuclei, (arrows). At day 5, most nuclei are negative; only weak cytoplasmic, supranuclear reaction is obtained. Bars, 10 μm.
The fractions collected were analyzed for endonuclease activity by the plasmid degradation assay (Fig. 8, a and b). Analysis of the activity containing fractions by SDS-PAGE indicated a molecular mass range between 32 and 35 kD (Fig. 9, a and c) in agreement with the zymogram analysis. These fractions most probably represent a mixture of different prostatic proteins of this molecular mass range. Fractions containing the highest endonucleolytic activity of control and day 3 eluate were subjected to SDS-PAGE followed by standard Western blotting. A band of ~32 kD apparent molecular mass was stained by the polyclonal, affinity-purified antibody (Fig. 9, b and d). A much stronger immunoreaction was observed for the day 3 fraction. Densitometry indicated a fivefold increase in intensity at day 3 using the constant amount of purified rat parotid DNase I applied as internal standard (not shown). In agreement with the histochemical data, this result indicates that the amount of DNase I gene product was increased in day 3 homogenate. No staining was observed of fractions void of endonucleolytic activity (Fig. 9, b and d lanes 0' and 3'). In this experiment we used purified rat parotid DNase I as control, which for unknown reasons always gave a higher apparent molecular mass (~33 kD) than when present in whole tissue extracts (see also Fig. 6).

**Discussion**

The induction of apoptotic elimination of prostate epithelial cells after testosterone withdrawal represents a well-established in vivo model system for the analysis of the biochemical and molecular mechanisms of programmed cell death (Kyprianou et al., 1988; Furuya et al., 1994). It is estimated that ~80% of the epithelial cells of the rat ventral prostate are eliminated after castration. Recent data (Kyprianou et al., 1988) demonstrated that internucleosomal chromatin degradation (DNA ladder formation) becomes first detectable at day 1, and DNA degradation reaches its maximum at days 3 and 4 after castration. These authors attributed the DNA fragmentation to the parallel activation of a Ca^{2+}-Mg^{2+}-dependent endonucleolytic activity. The exact nature of this endonucleolytic activity has remained elusive so far. Here we present evidence at the level of specific mRNA and protein that DNase I is expressed in the rat ventral prostate. The zymo-
The enzymatic analysis (zymograms and plasmid assays) and Western blots indicated that the endonucleolytic activity is only attained in the presence of 8 µg of actin:segment 1. (c) Immunodepletion of the endonucleolytic activity in day 0 and 3 homogenates by immobilized anti–DNase I (see also Materials and Methods). Lane 1, plasmid on its own; lane 2, day 0 using 5 µg of homogenate after preincubation with unloaded protein A–Sepharose; lane 3, day 0 homogenate after preincubation with protein A–Sepharose loaded with antibodies against rat DNase I; lanes 4 and 5, identical experiment using 5 µg of day 3 homogenate (for details see Materials and Methods). (d) Immunodepletion of DNA ladder catalyzing activity in day 0 and 3 homogenates by immobilized anti–DNase I. Lane 1, molecular weight marker; lane 2, 2 × 10^5 substrate nuclei were incubated in the presence of 20 mM EDTA and (lane 3) 5 mM CaCl_2 and MgCl_2 for 24 h at 37°C. Note the absence of DNA: it was impossible to pipette the highly viscous DNA clot into the gel slot indicating the absence of endogenous endonucleases. Lanes 4-6, 40 µg of day 0 homogenate was incubated with 2 × 10^5 substrate nuclei for 24 h at room temperature: (lane 4) homogenate on its own, (lane 5) after preincubation with protein A–Sepharose alone, and (lane 6) after preincubation with protein A–Sepharose complexed with anti–DNase I. Note that high molecular mass DNA fragments are generated in all samples. Lanes 7-9, identical experiment using day 3 homogenate. Note that after preincubation with immobilized anti–DNase I, ladder formation is suppressed (lane 9), although high molecular weight DNA fragments are generated. Lane 1 gives phage λ treated with HindIII as molecular mass marker (from top to bottom: 23,130; 9,460; 6,557; 4,322 [top four closely spaced bands]: 2,200; and 2,027 bp).
Activity is considerably upregulated after castration at day 3. Our data obtained from immunohistochemistry demonstrate a significant increase of DNase I antigenicity after castration, which was exclusively localized in the prostatic epithelial cells. DNase I antigenicity was only rarely detected in control animals. In contrast, the data obtained by in situ hybridization demonstrate that even under control conditions, prostatic epithelial cells express DNase I gene transcripts, whose concentration was shown to slightly decrease after castration by dot and Northern blots as well as RNase protection. In situ hybridization indicates that DNase I–specific mRNA is localized within the cytoplasm of all prostatic epithelial cells before androgen ablation. After castration, however, the DNase I gene transcripts were found to be particularly concentrated in cells undergoing apoptosis, although this might have been because of the concomitant reduction in cell volume. The overall decrease in epithelial cell mass might also explain the reduction in total DNase I–specific mRNA as observed by the RNase protection assay. Indeed, it has been reported that the level of total RNA drops faster after castration than the cell number (Furuya et al., 1994). Since, however, our data also indicate no gross alteration in the gene transcript concentration of the house-keeping protein β-actin, we assume that DNase I expression is translationally upregulated after androgen withdrawal. Regulation at the translational level appears to be plausible, since: (a) in control animal, there is very little DNase I expression in spite of the presence of its gene transcripts in the epithelial cells; and (b) the increase in DNase I expression occurs well before the elevation of the rate in apoptotic cell elimination. Indeed, the DNase I gene product is considerably increased at day 3 as determined by immunohistochemistry and the biochemical procedures, when a decrease in the concentration of DNase I gene transcripts is obtained. Recently, it was demonstrated that a member of enzyme system necessary for mRNA maturation (the 70-kD U1 subunit of the snRNP-particle) is proteolytically degraded during the onset of apoptosis (Casiola-Rosen et al., 1994). This will lead to a decreased rate or even arrest of mRNA maturation and would necessitate the regulation of the expression of proteins needed for the execution of apoptosis at the level of translation. Additional support for a translational regulation of DNase I expression comes from recent data obtained on the human mammary adenocarcinoma cell line MCF-7 that demonstrate the presence of DNase I gene transcripts in these cells, but a lack of expression of the mature protein even after conditions supposed to induce apoptosis-like treatment with the antioestrogen tamoxifen (Otto et al., 1996).

In the past, DNase I has only been regarded to be a secretory enzyme, although a number of data indicate that it is ubiquitously expressed (Malicka-Blaszkiewicz and Roth, 1983; Polzar et al., 1994). Its cDNA cloned from rat parotid contains a signal sequence (Polzar and Mannherz, 1990). Indeed, under control conditions, DNase I antigenicity was sometimes observed in prostatic secretion, and enzymatic analysis demonstrated the presence of low activity in the secretory product of control prostates (data not shown). The mechanism of its intracellular accumulation is presently not fully understood. It is possible that the alterations and collapse of the cytoskeleton during apoptosis arrests the normal exocytotic pathway and leads to the retention of the increasingly expressed DNase I. It would then be intracellularly accumulated and finally be relocated into the cell nucleus. This intracellular routing...
has already been observed after transient transfection of the cloned DNase I cDNA and its overexpression in COS cells (Polzar et al., 1993). Furthermore, perinuclear localization or even storage of DNase I was demonstrated for a number of cell systems that constitutively express this endonuclease (Peitsch et al., 1993, 1994; Mannherz et al., 1995). Alternatively, induction of apoptosis may lead to the expression of variants of DNase I that are specifically transferred from the endoplasmic reticulum to the perinuclear space and finally into the cell nucleus. The zymograms demonstrated the appearance of three endonucleolytic bands of increased apparent molecular mass at day 1. Since we observed only a single form of DNase I transcript by Northern blots, this may indicate the formation of unprocessed preforms during the time period of increased translation. In addition, the existence of a number of DNase I variants possessing different apparent molecular masses because of differences in glycosylation is well documented (Kreuder et al., 1984; Nadano et al., 1993; Yasuda et al., 1994). At day 3, the dominant band is 32 kD and of high activity. This would suggest a switch to the variant that is also normally found in other tissues (Polzar et al., 1994). We tentatively assume, however, that this switch in isoform pattern is due to the maturation of newly expressed enzyme, but further experiments using in vitro translation systems will be necessary to decide this question.

The in situ end-labeling technique demonstrated that even at day 3, only a small fraction of these cells possess fragmented DNA. This indicates that like in the small intestine or stratified epithelia, the prostatic epithelial cells express DNase I in advance of their apoptotic elimination (Polzar et al., 1994; Zanotti et al., 1995). The migration of enterocytes along the villar surface or keratinocytes within epithelia resolves their fate spatially and temporally. In these tissues, a similar intracellular relocation of the increasingly expressed DNase I was observed, namely an initial cytoplasmic, then perinuclear, and finally nuclear accumulation that could be correlated to the migration of these terminally differentiating cells towards the location of their final apoptotic elimination.

In summary, the data obtained by immunohistochemistry and in situ hybridization demonstrate a clear correlation of the intracellular accumulation of DNase I to the increase in the rate of apoptotic cell elimination and DNA.
fragmentation of prostatic epithelial cells after castration. Our data obtained by immunohistochemistry demonstrate the presence of DNase I in cells with apoptotic morphology. We furthermore demonstrate that DNase I also fulfills one of the main requirements for being the apoptotic endonuclease, namely its intranuclear localization. The fact that it is expressed at an elevated level and retained intracellularly only after induction of apoptosis may be part of safeguard mechanisms that prevent its untimely contact with nuclear chromatin.

Our data may also be of clinical relevance since carcinomas of the prostate occur with increasing frequency. Their successful treatment is of paramount importance. They originate in most cases from epithelial cells and initially maintain their testosterone responsiveness, i.e., testosterone ablation can lead to tumor regression most probably by apoptotic elimination. Androgen ablation is the most effective form of therapy, at least during the initial stage of prostatic cancer therapy. Therefore, it will be interesting to analyze the expression of DNase I in these tumors in response to treatment with antitestosterone or other chemotherapeutic reagents and to evaluate the prognostic value of DNase I expression.

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