Involvement of DNase II in Nuclear Degeneration during Lens Cell Differentiation*

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Alicia Torriglia, Elisabeth Chaudun, Françoise Chany-Fournier, Jean-Claude Jeanny, Yves Courtois, and Marie-France Counis

From XR 118 INSERM, Association Claude Bernard, 29 rue Wilhem, 75016 Paris, France

The characterization of DNase II and DNase I activity was undertaken to discriminate their different roles in physiological nuclear degradation during lens fiber cell differentiation. The activity of both nucleases determined in a new assay allows to discriminate DNase II from DNase I in the same extract. In fibers, both types of nuclease activities are found and appear higher than in epithelial cells. Specific polyclonal antibodies directed against these two nucleases reveal by Western blot analysis the presence of various DNase isoforms. DNase II like-nuclease, present in fibers, is represented by three major bands (60, 23, and 18 kDa), which are not detected, at least for two of them (60 and 23 kDa), in epithelial cells. DNase I like-nuclease pattern in fiber cells shows a single 32-kDa band, while several bands can be detected in epithelial cells. Immunocytochemistry studies show both nucleases present in lens cell sections. DNase II is, as usual, in cytoplasm of epithelial cells, but it appears strikingly concentrated in the nuclei of fibers. DNase I is always concentrated in nuclei of epithelial and fiber cells. DNA degradation observed in agarose gels shows that DNase II-activating medium cleaves the DNA from fiber cells more efficiently than DNase I-activating buffer. In addition, DNase II antibody is able to prevent this degradation. These results suggest a specific involvement of DNase II in nuclear degradation during lens cell differentiation.

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† To whom correspondence should be addressed. Tel.: 33-1-45-25-21-93; Fax: 33-1-46-50-01-95.
polyclonal antibody against DNase II. DNase II showed some molecular forms (60 and 23 kDa) detected only in fiber cells. DNase II was immunolocalized in the nuclei of these cells while it remained cytoplasmic in epithelial cells. Moreover, only DNase II antibody was able to inhibit the in vitro DNA degradation in fiber cells. These results allow us to propose the participation of DNase II in lens cell differentiation.

**EXPERIMENTAL PROCEDURES**

Materials—Fertilized eggs of White Leghorn chickens were obtained from Hass (Kaltenhouse, France). Calf thymus DNA was from Pharmacia Biotech Inc. Purified DNase I was from Sigma, and DNase II was from Worthington. Deoxy (5′-3′)adenosine 5′-triphosphate, ammonium salt (code TRK 34, 778 Gb/mmol), and enhanced chemiluminescence kit (ECL) were from Amersham International. Immobilon P was obtained from Millipore. Escherichia coli DNA polymerase I (endonuclease free), dCTP, dGTP, and dTTP were from Boehringer Mannheim. Polyclonal antibody against DNase II was from Rockland, and biotinylated goat anti-rabbit IgG was from Coop Biomedica. The scintillation kit (ECL) were from Amersham International. Immobilon P was obtained from Millipore. Hanks' buffer was from Hass (Kalthenhouse, France). Calf thymus DNA was from Phar- macy. Yung, and the mounting material, Tissue Tek OCT, was obtained from Miles.

DNA Labeling—0.5 mg of calf thymus DNA was dissolved in 10 ml of 50 mM Tris buffer, pH 7.4, containing 5 mM MgCl2, 50 μM bovine serum albumin, 50 μM 1-mercaptoethanol, 50 μM EDTA,ube, and dTTP, 2.5 μM [3H]dATP, and 100 μl of DNA polymerase I. The reaction was allowed to proceed for 30 min at 15°C and was then stopped with 500 μl of 150 mM NaCl. The labeled DNA was then precipitated with 2 volumes of 100% ethanol for 2 h at 0°C. The precipitated DNA was then washed four times with 70% ethanol and centrifuged each time at 2500 rpm (Heraeus) for 10 min. The labeled DNA was then allowed to solubilize at 4°C in 1 ml of H2O. The amount of DNA solubilized was evaluated by spectrophotometric reading at 260 nm.

Measurement of DNase Activities—DNase I activity was measured in 200 μl of 10 mM Tris buffer, pH 7.4, containing 10 mM CaCl2 and 10 mM MgCl2. The reaction, started with 1 μg of [3H]DNA, was allowed to proceed for 30 min at 37°C and was then stopped by incubation for 1 h at 0°C with 200 μl of 10% trichloroacetic acid containing 1% sodium pyrophosphate (PP). The non-digested DNA was then collected on a GF/C filter (Millipore) previously embedded in 1% PP. The filter was rinsed five times with 2 ml of 5% trichloroacetic acid containing 1% PP, twice with 2 ml of 100% ethanol, and air dried. The remaining radioactivity was counted in a β-scintillation counter using 10 ml of scintil- lation solution. DNase II activity was measured in 200 μl of 10 mM Tris-HCl buffer containing 10 mM EDTA, pH 5.75. The reaction was allowed to proceed, stopped, and the non-digested DNA was measured as described above.

Preparation of Lens Extracts for DNase Activity Measurements—250 lenses from 18-day-old chick embryos were dissected under a binocular microscope and the epithelia from the fiber cells. The tissues were then homogenized in 2 ml of 10 mM Tris, pH 7.4, containing 1 mM NaCl, using a Potter homogenizer. The homogenate was then centrifuged at 10,000 g (15 min), the supernatant was used as enzyme source. The reaction was then started with 1 ml of 0.05 M NaHCO3-Na2CO3, pH 9.6) for 1 h at 37°C. Endogenous nuclease digestion was detected by adding 2 μg of protein in 1 ml of 10 mM Tris buffer, pH 7.4, containing 0.05 mM phenylmethylsulfonyl fluoride and 0.34 μM sucrose. 10 mM CaCl2, and 10 mM MgCl2, were added to some tubes for control experiments. An aliquot of nuclease preparation was incubated in 10 mM Tris-HCl, 10 mM EDTA, pH 5.75. Antibodies (2 or 5 μl) against DNase I or II were added in the different samples incubated in neutral, cationic, or acidic buffer. The reaction was allowed to proceed for 6 h and was then stopped by the addition of 10 μl of 50 mM EDTA, 1 mg/ml proteinase K, 50 μg/liter, and 2 μl of 10% SDS. The lysate was incubated overnight at 37°C and then cooled on ice, and 2 μl of 2 M KCl were added to precipitate SDS, which was then removed by centrifugation (15).

Agarose Gel Analysis of DNA—1% neutral agarose horizontal slab gels were made up in 40 mM Tris-acetic acid, pH 7.8, 20 mM NaCl, and 2 mM EDTA. The samples were loaded and run at 80 V until bromphe- nol blue reached 8 cm. Gels were then incubated for 30 min at 37°C in PBS(19 mM Na2HPO4, 1 mM KH2PO4, 140 mM NaCl, 15 mM KCl, pH 7.5) containing 5% fat-free dried milk. Afterward, the blot was incubated for 1 h at room temperature with anti-DNase I antibody (80 μg/ml) or anti-DNase II antibody (1/250) diluted in PBS containing 0.5% fat-free dried milk. Controls included the substitution of equivalent dilution of rabbit preimmune serum for the antisera. Binding of the antibodies was visualized after two washings (10 min each) with PBS containing 0.5% fat-free dried milk. Controls included the substitution of equivalent dilution of rabbit preimmune serum for the antisera. Binding of the antibodies was visualized after two washings (10 min each) with PBS containing 0.5% fat-free dried milk.

Preparation of Antiserum—Antisera were obtained from porcine spleen in Freund's complete adjuvant was injected at four weekly intervals. Bleeds of 10–20 ml were performed 8 days after each bleeds and controls were included for each bleed. The antibody was localized on the sections using a biotinylated sheep anti-rabbit IgG (1/100 dilution) in PBS containing 5% fat-free dried milk. The sections were then incubated in PBS containing 1% SDS, washed in water for 30 min, visualized on a UV transilluminator (302 nm, UV products Inc.), and photographed using a polaroid positive-negative film, type S5.

Tissue Preparation and Immunohistochemistry—Chick lenses were fixed in 1% paraformaldehyde for 1 h, washed in PBS, mounted in Tissue Tek OCT, frozen in liquid nitrogen, and stored at −80°C until use. Serial sections, 10 μm thick, were prepared from the central lens with a Bright cryostat at −20°C and mounted on gelatin-coated slides. After removal of OCT with PBS, tissue sections were permeabilized with 0.3% Triton X-100 in PBS for 30 min. After washing with PBS, sections were saturated for 1 h at room temperature with PBS containing 5% milk and then incubated for 1 h at room temperature with the specific antisera (1/250 dilution). This incubation was followed by five washes for 5 min in PBS-1% milk. The antibody was localized using a biotinylated sheep anti-rabbit IgG (1/100 dilution) in PBS-1% milk (1 h, room temperature) and then incubated 1 h, room temperature, in Extravidin conjugated to rhodamine isothiocyanate diluted to 1/100 in PBS-1% milk. Sections were then washed for 5 min in PBS and coverslipted with 50% glycerol in PBS. During the last wash in PBS, nuclei were stained with the fluorescent nuclear stain 4′,6-diamino-2-phenylindole (DAPI). The sections were evaluated using a Leitz Aristoplan microscope equipped with an epi-illuminator HBO and filters for rhodamine and DAPI fluorescence. They were photographed using an Ilford HP5 film (400 ASA).

1 The antibodies used were: PBS, phosphate-buffered saline; DAPI, 4′,6-diamino-2-phenylindole.
RESULTS

DNase Assay—We set up a method allowing the measurement of both DNase I and DNase II. This new method uses commercial DNA purified from calf thymus, which has been labeled by a modified nick translation reaction, using $[^3H]dATP$ as the labeled nucleotide (see “Experimental Procedures”). In contrast to the classical technique, we did not use DNase I to amplify the nicks since this DNA contains enough single strand breaks with 3’-OH ends. In addition, labeled DNA was purified using only ethanol. The specific activity was about 20,000–60,000 cpm/μg.

To distinguish between DNase I and DNase II in the same sample, their activities were measured at different pH and cationic conditions. Fig. 1 shows the DNase I activity in cationic buffer at neutral pH (dark bars) and in acidic, non-cationic medium (white bars). Note that no enzymatic activity was observed in Tris-EDTA buffer, but a regular decrease of non-digested DNA was measured when DNase I was incubated in a neutral buffer containing Ca$^{2+}$ and Mg$^{2+}$. Fig. 1 shows the DNase II activity in both buffers used above for DNase I. In this case, the DNase II digested the DNA when incubated in an acidic buffer, but it was inactive when incubated in a neutral, Ca$^{2+}$- and Mg$^{2+}$-containing buffer. We used this technique to measure both kinds of endonucleases in lens cells from 18-day-old chick embryos. Fig. 3 shows the DNase activities in the epithelial and fiber extracts presented in ng of DNA digested per min either per mg of protein (A) or per 10⁶ cells (B). Note that in both epithelial and fiber cell the DNase II-like activity appeared greater than the DNase I-like activity. In contrast, the DNase I/DNase II ratio was the same for both populations of cells, DNase II representing approximately 60% of the total DNase activity.

DNase Molecular Weights—To further study these nucleases, we prepared a polyclonal antibody against DNase II. Fig. 4A shows the results obtained with the anti-DNase II serum, and Fig. 4B shows the results obtained with the preimmune serum. The antibody recognized specifically DNase II, a protein of 27 kDa (4A, lane 2), and no cross-reactivity with DNase I was observed (4A, lane 1). The preimmune serum recognized neither DNase II nor DNase I (4B, lanes 5 and 6). We used this antibody to identify DNase II-like proteins in epithelial and fiber extracts. As seen in Fig. 4A, the epithelial cells contained two bands of 18 and 100 kDa (4A, lane 3). In contrast, 3 major bands of 18, 23, and 60 kDa were labeled in fiber cells (4A, lane 4). Note that the 60-kDa protein was disrupted by the presence of a high amount of δ crystallin (open diamond) constitutive in lens fiber cells. All these bands seem to be specific since no signal was obtained with the preimmune serum used at the same dilution (4B, lanes 7 and 8).

Similar experiments were performed using a commercial antibody against DNase I (Fig. 5). The DNase I antibody recognized specifically DNase I, a protein of about 32 kDa (lane 1), and did not react with DNase II (lane 2). In epithelial cells, three major bands of 18, 32, and 60 kDa were labeled (lane 3). In fiber cells, only a single band of 32 kDa was seen (lane 4). Note that the high amount of proteins loaded on the gel increased the background.

Inhibition of DNase Activity—We have shown by two different methods that DNase I and DNase II were present in lens cells. To characterize which of these enzymes was responsible for DNA degradation in lens fibers, we determined if the antibodies were able to block the enzymatic activities. To do this, we tested DNase I and DNase II activities in the presence of increasing amounts of anti-DNase I and anti-DNase II antibodies (Fig. 6). Both enzymatic activities were inhibited by these
antibodies. The controls in Fig. 6 correspond to the degradation of 1 μg of labeled DNA by 1 unit of DNase I (A) and 4 units of DNase II (B). As in the Western blot analysis, no cross-inhibition was observed between both nucleases, and the preimmune serum had no effect on DNase II activity (data not shown).

To investigate the involvement of DNases I and II in DNA degradation, we prepared lens epithelial nuclei (Fig. 7A) and lens fiber nuclei (Fig. 7B). The incubation of fiber nuclei in a neutral buffer containing 10 mM Ca²⁺ and Mg²⁺ generated a smear (lane 5), which was not inhibited by DNase I antibody (lanes 6 and 7). The same results were obtained with epithelial nuclei (lanes 1 and 2). Lanes 8, 9, and 10 have been loaded with fiber nuclei incubated in Tris-EDTA, pH 5.75. Under these acidic conditions, in the control, the DNA cleavage was seen along the tract (lane 8), and cleavage inhibition was noted in the presence of increasing amounts of DNase II antibody (lanes 9 and 10). In lane 10, there was no DNA smear, and the DNA of high molecular weight appeared protected by the antibody against DNase II. In epithelial nuclei, the DNA degradation obtained under acidic conditions was not modified by the DNase II antibody (lanes 3 and 4). In addition, the DNase II antibody was not able to inhibit the smear generated when both epithelial and fiber nuclei were incubated in a neutral, cationic buffer (data not shown).

Immunolocalization of DNases in the Lens—To localize DNases I and II to different subcellular compartiments of the lens, lenses from 18-day-old chick embryos were sectioned transversally. A central section of an embryonic lens stained with 1% toluidine blue showed the different structures (Fig. 8). Fig. 9 shows the immunolocalization of DNase I and DNase II.
in outer fiber cells in a similar section. DNase I was mainly localized to nuclei (Fig. 9b) since the labeling obtained with anti-DNase I antibody was superimposable with the nuclear stain, DAPI (Fig. 9a). Similar nuclear distribution of the staining was observed when the anti-DNase II antibody was used (Fig. 9d) as compared to the control DAPI staining (Fig. 9c). In this case, some cytoplasmic labeling was observed. The same nuclear localization in central round fiber nuclei was observed with DNase I and DNase II (data not shown). Fig. 9f shows the results obtained with the preimmune serum. No labeling was observed either in the nucleus (see the corresponding DAPI staining, Fig. 9e) or in the cytoplasm.

A similar study is shown in Fig. 10 for the central epithelial cells. The nuclear staining DAPI (Fig. 10a) shows that the DNase I antibody (Fig. 10b) labeled the nuclei of epithelial cells. In contrast, the DNase II antibody labeled the cytoplasm of epithelial cells (Fig. 10d), and no significant labeling of the epithelial cell nuclei was observed (Fig. 10, c and d, white arrows). Note that a strong labeling of the lens capsule was also observed.

**Fig. 7.** Effect of the anti-DNase I and anti-DNase II antibodies on DNA cleavage. 10⁶ nuclei from lens epithelial cells (A) or fiber cells (B) were incubated in both neutral cationic and acidic medium in the absence or in the presence of an anti-DNase I or DNase II antibody. Arrow heads show the position of the sample wells. Incubation in neutral, cationic medium: control, lanes 1 and 5. DNase I antibody added, lane 6 (2 µl) and lanes 2 and 7 (5 µl). Incubation in acidic medium: control, lanes 3 and 8. DNase II antibody added, lane 9 (2 µl) and lanes 4 and 10 (5 µl).

**Fig. 8.** Section of an 18-day-old chick embryo lens stained with 1% toluidine blue. Ep, epithelial cells; AP, annular pad; OF, outer fibers; IF, inner fibers. The black bar represents 300 µm.

**Fig. 9.** Immunolocalization of DNase I and DNase II in outer fiber cells. Transversal sections of lens from 18-day-old chick embryos were incubated with polyclonal antibody against DNase I (b), DNase II (d), or with preimmune serum (f). a, c, and e show the same sections stained with the nuclear stain, DAPI. White bar represents 30 µm.

**DISCUSSION**

Methods to Characterize DNase I and DNase II—Chick embryonic lenses were chosen to study the involvement of DNase I and DNase II since DNA degradation occurs during lens cell differentiation. Epithelial cells are mitotically and transcriptionally active, and the DNA extracted from their nuclei has a high molecular weight (22). In contrast, fiber cells are postmitotic and have started a terminal differentiation program, which leads to DNA degradation (13, 15, 23). Most authors, including ourselves, have used the zymogram method to record DNase activities (24–27). Nevertheless, we are unable to detect any DNase II activity in tissues or in any purified commercial preparations with this denaturing method. Thus, we set up a new procedure to measure both DNase I and DNase II activities. The novelty of our experimental procedures is related to the DNA-substrate labeling by a DNA polymerase I. A good ratio of labeling is obtained even in the absence of DNase I. The use of radioactive DNA increases the sensitivity of the method compared to hyperchromicity techniques (28, 29). In addition, as the working conditions for DNase I and DNase II are differ-
ent (e.g. pH and cation requirement), we are able to measure both enzymes in the same sample without any purification.

To further study DNases, immunological methods remain a very powerful tool. An antibody against DNase I is commercially available. DNase II antibodies with low titre have been obtained by two laboratories (9, 30). Thus, we prepared a polyclonal antibody against DNase II having a high titre, which does not cross-react with DNase I and is able to inhibit DNase II activity in vitro.

Nuclease Activity in Lens Cells—Both DNase I and DNase II-like activities are found in fiber and epithelial cells. An increase of both nuclease activities per cell are recorded in fibers compared to epithelial cells. This is not the case if enzymatic activity is expressed as a function of the protein concentration. Terminal differentiation of the lens cells leads to an accumulation of specific proteins, the crystallins (31). These proteins represent 80–90% of the soluble proteins of the lens; in consequence, they dilute all the other cell proteins including DNases.

An increase of DNase activities in fiber cells is in agreement with the involvement of these enzymes in nuclear degeneration. It is interesting to note that there is no change in the DNase I/DNase II ratio between epithelial and fiber cells. This means that, in terms of activity, we have no elements to involve preferentially one or the other nuclease in the differentiation process of lens fibers.

Immunohistochemistry of DNase I and DNase II—We found several forms of DNase I-like molecules in epithelial cells, since three major bands of 60, 32, and 18 kDa are recognized. In fiber cells, we record only a single band of about 32 kDa, localized just underneath \( \delta \) crystallin. Since the DNase I activity is higher in fibers than in epithelial cells, this 32-kDa protein could be the active form of the enzyme already described (32, 33). The presence of higher and lower molecular weight peptides in epithelial cells may be due to the existence of precursor forms and degradation products. Non-active precursors have already been described in eukaryotic cells (34). An alternative hypothesis may be that the presence of crystallins or cytoskeletal proteins impairs the detection of the other forms. Actually, it is known that DNase I can interact with actin (35). To clarify this question, purification of the enzyme from lens cells would be a necessary step.

Several forms of DNase II-like molecules have also been found. Two forms of 100 and 18 kDa are present in epithelial cells, and three major bands of 60, 23, and 18 kDa are detected in fiber cells. Two of these (60 and 23 kDa) are found only in fiber cells and may be incriminated in lens cell differentiation process.

When considering the immunohistochemistry data, DNase I is found mainly in the nuclei of both epithelial and fiber cells, an already described subcellular localization for DNase I (36). DNase II, known as a lysosomal enzyme (37–39), is observed in the cytoplasm of epithelial cells and also in the lens capsule. Strikingly, DNase II is highly concentrated in all fiber cell nuclei. This enzyme has already been seen in cell nuclei (40, 41), but its presence there has been related to DNA replication (42). This nuclear localization is seen only in fiber cells cleaving their DNA and may reflect a specific function of DNase II in DNA degradation.

Involvement of DNase I and DNase II in DNA Degradation—To discriminate the function of these enzymes in lens DNA degradation, fiber and epithelial cell nuclei were incubated in the two different buffers corresponding to the optimal activation conditions of DNase I or DNase II. Only the nuclei incubated in an acidic medium, the medium devised for DNase II, show a strong degradation of fiber cell DNA. Furthermore, this degradation could be inhibited by the addition of DNase II antibody. This result strongly implicates DNase II in the process of DNA degradation. Nevertheless, it is worthwhile to note that the DNA cleavage observed in cationic and neutral buffer cannot be inhibited by either DNase I or DNase II antibodies. This may suggest the presence of other nuclease activity.

Which DNase Is Implicated in the Internucleosomal DNA Cleavage?—Nuclear degradation has been mainly studied in apoptosis or programmed cell death. In this field, three different groups implicate different DNases in the degradation of DNA. Tschopp and co-workers (36) support the idea that DNase I is the enzyme involved in this phenomena. Cidlowski
and co-workers (12, 43) claim the activity of NUC 18, whereas Barry and Eastman (9) are in favor of DNase II. In the lens, nuclear degeneration follows the same morphological and biochemical changes as the nuclei of most apoptotic cells (13, 15, 22). In this differentiating tissue, DNase I is present in the fiber cell nuclei, and its participation in DNA cleavage cannot be completely ruled out, though DNA degradation seen in neutral cationic medium cannot be easily related to DNase I. This last observation is in agreement with previous results that indicate that no single strand breaks with free 3'-OH ends accumulate in differentiating fiber cells (17) as could be expected if DNase I was implicated in this process. On the contrary, DNase II seems to be very important in DNA cleavage as demonstrated by its capacity to degrade fiber cell DNA and its nuclear localization, even if the DNA degradation pattern obtained was not the typical nucleosomal ladder. These data suggest that DNase II cleaves the DNA in coordination with other nucleases to produce the internucleosomal degradation. The extension of these findings to other apoptotic tissues is essential to assess if this is a general phenomenon. If this is the case, DNase II activation maybe a key event in apoptosis, which would provide new insights into therapeutic applications.

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