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Review

Analysis of nuclear degradation during lens cell differentiation

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Abstract

Lens cells demonstrate a terminal differentiation process with loss of their organelles including nuclei. Chromatin disappearance is characterised by the same changes as most apoptotic cells, i.e. condensation of chromatin and cleavage into high molecular weight fragments and oligonucleosomes. The endo-deoxyribonucleases (bicationic (Ca^{2+}, Mg^{2+}), mono-cationic (Ca^{2+} or Mg^{2+}) and acidic non-cationic dependent nucleases) are present in lens fibre cells. Our results suggest that the acidic non-cationic nuclease (DNase II) plays a major role in chromatin cleavage. This nuclease, known to be lysosomal, is found in lens fibre nuclei and only an antibody directed against DNase II inhibits the acidic DNA cleavage of lens fibre nuclei. In addition, there must be another DNase implicated in the process which is not DNase I but appears to be a Ca^{2+}, Mg^{2+} dependent molecule. Regulation of these DNase activities may be accomplished by the effect of post-translational modifications, acidic pH, mitochondrial release molecules, growth factors or oncogenes. Finally, fibre cells lose organelles without cytoplasmic elimination. The survival of these differentiated cells might be due to the action of survival factors such as FGF 1.

Keywords: lens cells; differentiation; nuclear apoptosis; genome cleavage; DNases

Abbreviations: AIF, apoptosis inducing factor; AP, annular pad; DAPI, 4′,6-diamidino-2-phenylindole; DNase, deoxyribonuclease; DSB, double strand breaks; E15, embryonic day 15; EDTA, ethylene diamino tetra acetic; Ep, epithelia; FGF, fibroblast growth factor; HMG, High mobility group proteins; HMW, high molecular weight; HPV, human papiloma virus; ICE, interleukin-1β converting enzyme; IF, inner fibre cells; NT, nick translation; OF; outer fibre cells; PMSF, phenyl methyl sulfonyl fluoride; Rb, retinoblastoma; RPN, ribonuclear proteins; SDS, sodium dodecyl sulphate; SSB, single strand breaks; SV, simian virus; TNF, tumour necrosis factor; TUNEL, TdT-mediated dUTP-biotin nick end labelling; UV, ultraviolet

Introduction

The eye lens is a useful model for the examination of many fundamental processes occurring during embryonic development (reviewed by Cvekl and Piatigorsky, 1996; Wride, 1996; Zelenka *et al*, 1996; Lang, 1997). Cellular differentiation of the lens is accompanied by nuclear degeneration similar to that appearing during apoptosis.

This avascular organ (Figure 1), surrounded by a capsule, is composed of a layer of epithelial cells and internally, a mass of elongated cells, the fibres. In the equatorial region of the lens, the epithelial cells elongate into fibres, DNA synthesis is arrested and high concentrations of crystalline proteins accumulate (Piatigorsky, 1981). The loss of organelles is extensive in these terminally differentiating lens fibres. It affects the mitochondria (Bassnett and Beebe, 1992), the endoplasmic reticulum (Bassnett, 1995) and some components of the cytoskeleton such as intermediate filaments and microtubules (Kuwabara and Imaizumi 1974; Bradley *et al*, 1979; Vrensen *et al*, 1991; Sandilands *et al*, 1995). One of the most striking features is nuclear degeneration, first described by Modak (Modak *et al*, 1969; Modak and Perdue, 1970) that mimics nuclear oligonucleosomal degradation (Appleby and Modak, 1977), often described in apoptosis (Wyllie, 1980; Wyllie *et al*, 1980). Despite these changes, the anucleate fibre cells remain within the lens throughout the life span of the individual.

Unlike apoptotic cells which die randomly, the fibre cells differentiate, following a highly ordered pattern of temporal progression. This allows the study of nuclear fate and DNase activation (Counis *et al*, 1989a). Microdissection of an embryonic or post-hatch chicken lens separates the nucleated, undifferentiated epithelial cells attached to the capsule from the underlying postmitotic, differentiated fibre cells. Thus, cells from the same lineage, yet differing in metabolism and differentiation state can be compared.

Modification of lens nuclei during terminal differentiation

Lens fibre differentiation from epithelia to fibre cells exhibits a remarkable change in nuclear shape and morphology (Figure 2A – D). Epithelial nuclei (Figure 2A) and outer fibre nuclei are slightly round, then, as they mature, the nuclei elongate

(Figure 2B and C) and shrink to a globular and highly condensed shape (Figure 2D) (Sanwal *et al*, 1986). In 1970, Modak and Perdue demonstrated by Feulgen and UV microspectrophotometric analysis the loss of DNA from the central and pycnotic fibre cell population. The nuclei from these cells contain amounts of DNA equivalent to the 1C value, while that of epithelial and annular pad cell populations range between 2–4c. We have confirmed this result (Yamamoto *et al*, 1990) using microfluorometry with Hoechst 33258 fluorochrome, in the presence of NaCl to increase accessibility of DNA to dye. We have shown a decrease in the amount of DNA in lens fibre nuclei during embryonic and postnatal development not observed in epithelial cell nuclei. In the nuclei of mouse central lens fibres, Vrensen *et al* (1991) describe an accumulation of osmiophilic or dense bodies in the nucleoplasm. These appear to be extruded into the cytoplasm and then the extracellular space. This material could represent fragments of chromatin or RNP particles as Sanwal *et al* (1986) observed earlier.

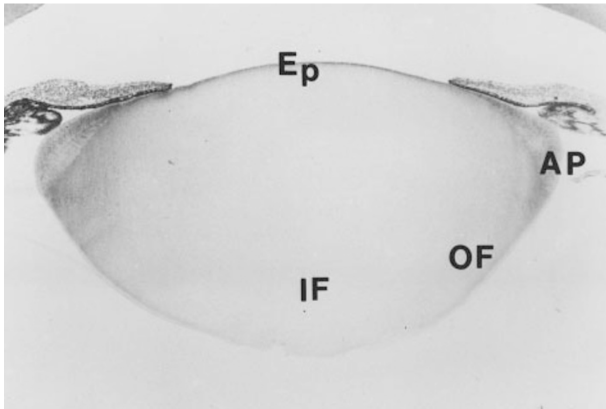

Figure 1 Embryonic chick lens central section at E18 stained with 1% toluidine blue (×25) showing Ep: epithelia. AP: annular pad, OF: outer fiber cells with elongated nuclei. IF: inner fiber cells with round nuclei

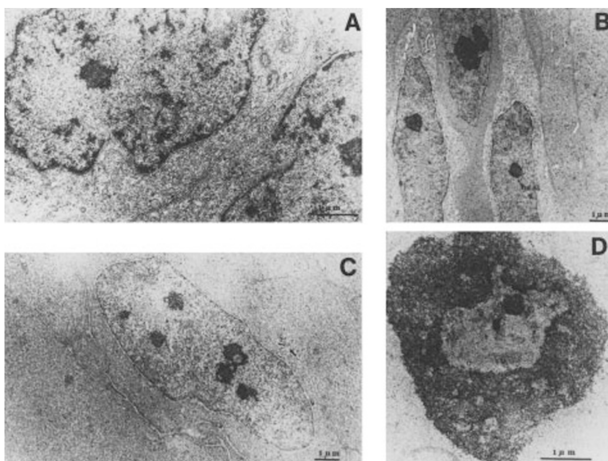

Figure 2 (**A**, **B**, **C** and **D**) transmission electron microscopy of nuclei from embryonic chick lens cells. Equatorial epithelial nuclei at E11 (**A**). Outer fibre nuclei at E11 (**B**) and E18 (**C**). Central fibre nuclei at E18 (**D**) (Sanwal *et al*, 1986)

Cleavage of the chromatin may be modulated by the structure of DNA itself, affected by the nuclear protein environment. Among the different proteins implicated in chromatin stability, histone H1 plays a fundamental role at the linker of the nucleosome and in compacting polynucleosome chains into higher-order structures. HMG-14 nuclear protein has different DNA binding domains and shows affinity for DNA within the nucleosomal core (Trieschmann *et al*, 1995; Lovell-Badge, 1995). Thus with specific antibodies raised against histone H1 and HMG-14, we studied the loss of both nuclear proteins during lens cell development and differentiation with the idea that the linker protein would be lost first and this, would increase DNase efficacy. Curiously, HMB-14 is lost quite early during embryonic development (Table 1; Figures 3 and 4). HMG-14 is observed in all lens nuclei at 11 day of embryonic development, E11 (Figure 3), yet is completely absent in central round nuclei at E18 and at older stages (Figure 4). Histone H1 is conserved, and can still be observed at 11 day post-hatching but is completely absent 7 days later (Table 1). Histone H1 belongs, in fact, to a family of proteins that can be resolved into different subfractions. The histone variant H1-2 (Table 1) is not observed in embryonic fibres though it is seen in epithelial cells at the same age (Roche *et al*, 1992). These results may indicate that DNA is depleted of protective proteins during lens fibre differentiation, a feature which may favour the actions of endonucleases.

Table 1 Nuclear proteins of lens fibre chromatin

	HMG 14 (1)		Histone H1 (1)			
Stages	OE	CR	OE	CR	Histone (2)	
in days	nuclei		nuclei		H1-1	H1-2
E 6	++	no CR	nd	nd	nd	nd
E 11	++	++	++	+	+	–
E 14	nd	nd	nd	nd	+	–
E 18	++	–	++	+	+	–
PH 4	++	–	nd	nd	nd	nd
PH 11	++	–	++	+	nd	nd
PH 18	++	–	++	–	nd	nd
PH 32	++	–	nd	nd	nd	nd

OE: outer elongated nuclei; CR: central round nuclei; E: embryonic stage; PH: post hatch stage. (1) Results obtained by immunofluorescence with polyclonal antibodies directed against HMG 14 and Histone H 1. (2) PAGE analysis revealed by Coomassie, autoradiography and Western blot (Roche *et al*., 1992). nd: not determined

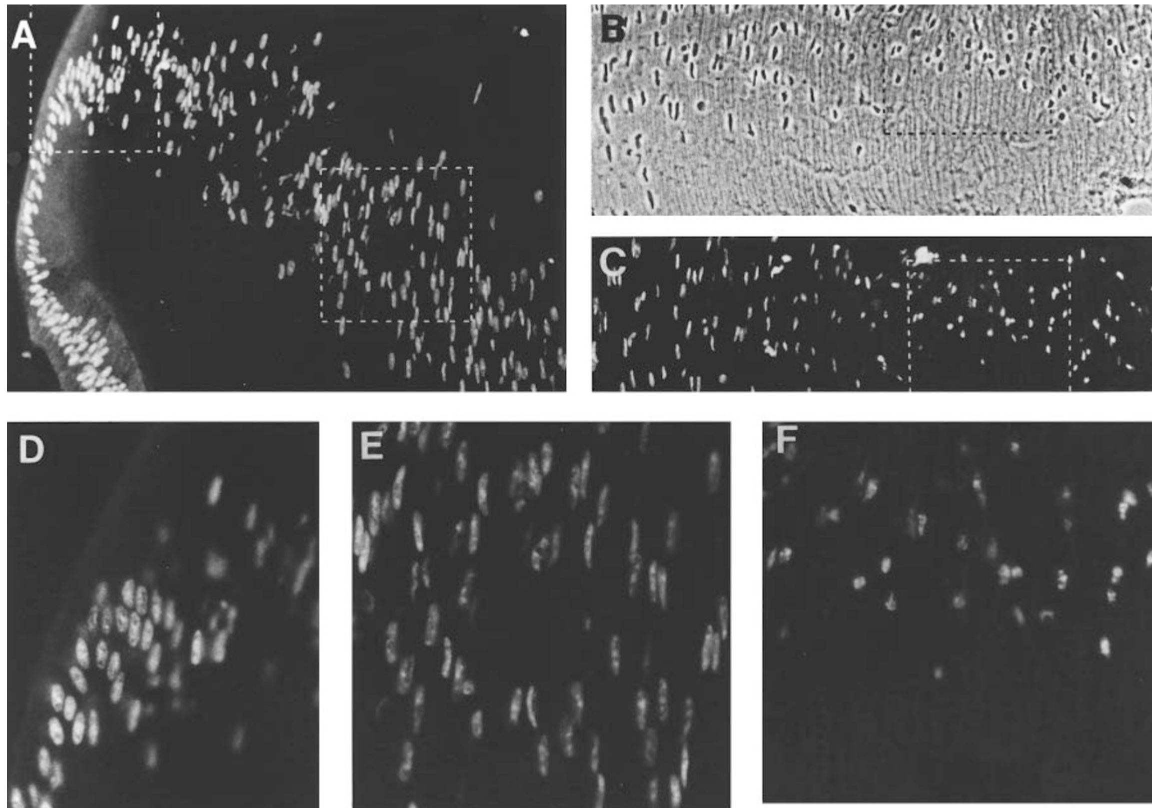

Figure 3 Indirect immunofluorescence of E11 lens sections incubated with a polyclonal antibody against HMG14. (**A**) annular pad and outer fibres (×16). (**B**) and (**C**) central fibres with elongated and round nuclei (×16). **B** corresponding phase contrast to **C**. (**D**, **E**, **F**) higher magnification (×40) of insets from **A** (**D** and **E**) and **B**, **C** (**F**): (**D**) annular pad, (**E**) outer fibres and (**F**) central fibres

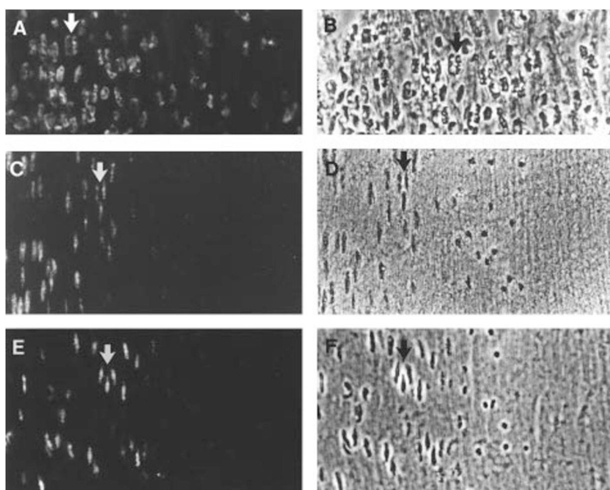

Figure 4 E6 (**A**, **B**), E18 (**C**, **D**) and 4 day post hatch lens (**E**, **F**) sections incubated with a polyclonal antibody against HMG14 showing central, elongated and round fibre nuclei (×40). Indirect immunofluorescence (**A**, **C**, **E**) and corresponding phase contrast (**B**, **D**, **F**). Arrows show the same group of cells in each pair of pictures

Nevertheless, the data are not sufficiently definitive to state whether the protein depletion precedes or parallels DNA degradation.

DNA cleavage

Non-random DNA degradation occurs in terminally differentiating lens cells; fragment size has been determined by sucrose gradient analysis or agarose gel electrophoresis. DNA ladders derive from large fragments of DNA, coming themselves from high molecular weight DNA. In a healthy lens epithelial cell, the DNA has a high molecular weight of >165 S and sediments to the bottom of an alkaline sucrose gradient as a homogenous population (Counis *et al*, 1977). As differentiation progresses, it appears in the middle region of the same gradient, a polydisperse DNA population with an average molecular weight of 64 S. This last population is also observed when lens cells are X-irradiated, and corresponds to the high molecular weight DNA populations observed during apoptosis resolved by pulse field gel analysis (Cain *et al*, 1994). The same specific sites of DNA cleavage were detected as in apoptotic cells.

From an historical point of view, DNA degradation in fibre cells was first studied *in vitro* using isolated nuclei. In 1977, Appleby and Modak showed that the cells located in the central regions of the lens contained DNA with an oligonucleosome banding pattern when analyzed in neutral agarose gels. This is detected at E15 and later stages. We have recorded a similar pattern (Muel *et al*, 1986) in isolated fibre cell nuclei, present here at E11 and E18 (Figure 5). DNA cleavage of these nuclei, incubated for

different times in 10 mM Tris HCl, pH 7.4 containing 0.34 M sucrose, 15 mM 2-mercaptoethanol and 0.05 mM PMSF, is also visualized in neutral agarose gels. An oligonucleosomal ladder is only recorded with lens fibre chromatin; this DNA cleavage is first visible at E11 and at subsequent stages.

As early as the late 1970s, it was apparent to us that the pattern of DNA cleavage was progressive, with a multi-step mechanism, from monodisperse, high molecular weight DNA to a polydisperse DNA population (Counis *et al*, 1977). Only a fraction of the total DNA was cleaved into oligonucleosomal fragments that characterise the DNA ladder. The accumulation of DNA strand breaks could be the result of either an increase in DNase activity or of an impairment in DNA repair. It is probable that both processes play an active part in the genome loss. To this end, we have shown a gradual decrease of the DNA synthesising enzymes. At E10, the DNA polymerase α activity is lower in chick lens fibres than in epithelial cells. In 14.5 month old chick fibres, there is a total disappearance of DNA polymerases (α and β) and DNA ligases (Counis *et al*, 1981). This loss of polymerases may be more likely related to decreased cell cycle traverse. The typical DNA cleavage frequently observed in many examples of programmed cell death has led to the hypothesis that there is also present an active endodeoxyribonuclease capable of producing double stranded breaks (DSB) in the DNA of differentiating lens fibre nuclei.

Endodeoxyribonuclease activity in lens fibre cells

There are several arguments that suggest the existence of an endonuclease activity in lens fibre cells. Oligonucleosomes are only observed in fibre nuclei (Figure 5A and B) incubated in neutral buffer with no exogenously added cations. In the same conditions, no nucleosomal ladder is observed in epithelial cells. This results in DSB suggesting the presence of a nuclease (Appleby and Modak, 1977; Muel *et al*, 1986). However, it has also been demonstrated that oligonucleosomal ladder formation is strictly dependent on the presence of cations. Increasing amounts of EDTA or EGTA prevent nucleosomal formation. This nuclease activity cleaves completely, after a lag of 4 h, several genes such as α crystallin, β tubulin and vimentin (detectable in nuclei, at E11, by Southern blot (Muel *et al*, 1989)). A nuclear or cytoplasmic extract from lens fibres contains a nuclease, the activity of which is capable of cleaving a supercoiled PM2 DNA (Counis *et al*, 1986). This endonuclease activity increases in the nuclear fraction from E15 to E18, leading to a decrease of the cytoplasm/nuclear ratio from 100 to 16.

However, in epithelial cells with an intact nucleus, there is also an endonuclease activity capable of cleaving a supercoiled PM2 DNA. This activity is present in the cytoplasm at E15 and E18 but is always higher in fibres (Counis *et al*, 1986). In epithelial nuclei incubated as above, no oligonucleosome formation can be seen, just a smear of high molecular weight DNA appears after incubation (Muel *et al*, 1986). Evidence of nucleosome distribution, however, has been seen in epithelial nuclei. If epithelial lens cells are irradiated with X-rays, and the nuclei prepared as above, it is then possible to observe faint oligonucleosome formation after a delay of 20 h (Trevithick *et al*, 1987).

In conclusion, differentiating fibre cells contain endodeoxyribonuclease(s) capable of cleaving supercoiled DNA substrate and fibre chromatin into nucleosomal ladders. Epithelial cell nuclei also contain endonuclease activity which can be activated. However, we do not yet know if both compartments share the same nuclease molecules.

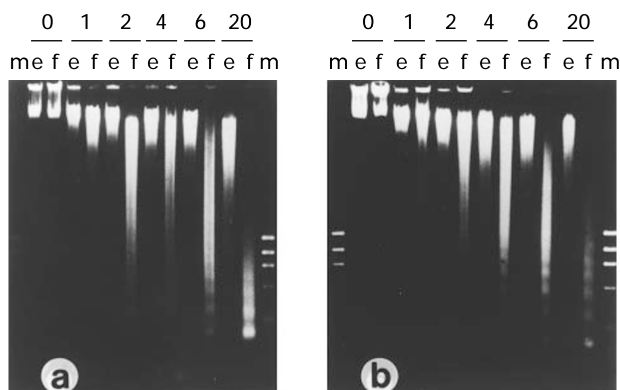

Figure 5 Electrophoretic analysis on 1% neutral agarose gel of oligonucleosomes produced by autodigestion of lens fibre nuclei (f) at E11 (**a**) and E18 (**b**). Compare the f lanes with e lanes corresponding to epithelial cell nuclei (Muel *et al*, 1986). Nuclei were incubated during 0, 1, 2, 4, 6 and 20 h in 10 mM Tris-HCl (pH 7.4), 0.34 M sucrose, 0.05 mM PMSF and 15 mM 2-mercaptoethanol. Markers (m): f × 174 RF-DNA *Hae*III digests (1352, 1078, 872, 603, 311, 271, 234, 194 bp)

Classification of lens DNases

DNase activities may be classified into three main groups. The first group is the class of DNase I-like molecules, with an absolute Ca^{2+}, Mg^{2+}-dependence. The second group includes the acidic or DNase II-like nucleases, with no cation dependence. The third group contains DNases with dependence on only one cation, such as Mg^{2+}, Mn^{2+} or Ca^{2+}.

The Ca^{2+}, Mg^{2+}-dependent DNases

The first group, of which the best known enzyme is DNase I, comprises all Ca^{2+}, Mg^{2+}-dependent enzymes. DNase I has been reviewed by Moore (1981). First purified from bovine pancreas, this 30 kDa polypeptide has a Ca^{2+}, Mg^{2+}-dependence with an optimal activity at neutral pH. It has a natural and specific inhibitor, G-actin, and is also inhibited by zinc and aurintricarboxylic acid (ATA). DNase I cleaves DNA by producing single (SSB) and double (DSB) strand breaks with free 3′ OH ends (Mannherz, 1992). It has been cloned and sequenced from different species: rat (Polzar and Mannherz, 1991), bovine Worrall and Connolly, 1990) and human (Shak *et al*, 1990). It is the best characterised eukaryotic nuclease.

Hewish and Burgoyne (1973) first demonstrated that chromatin from liver nuclei could be autodigested, in the presence of divalent cations (Ca^{2+}, Mg^{2+}), into regular sub-

structures which appear to be multiples of the smallest size unit. Since then, Wyllie (1980) and others, including ourselves have studied Ca^{2+}, Mg^{2+}-dependent DNase, suggesting that it plays a role in oligonucleosomal ladder formation. Wyllie and coworkers isolated this activity from thymocytes as a protein of 110–130 kDa, which they reported to be closely related to one subunit of topoisomerase II (Arends *et al*, 1990; Arends and Wyllie, 1991). Lack (1981) identified in nuclease activity gels or zymograms, a 30 kDa protein, whose activity was Ca^{2+}, Mg^{2+}-dependent, in tissues such as parotid and submaxillary salivary glands and small intestine, lymph node, kidney, heart, prostate gland and seminal vesicles. Ucker *et al* (1992) using the same technique, showed a doublet at 40 kDa, with nuclease dependent Ca^{2+} and Mg^{2+} activity, in NIH3T3 fibroblast nuclei and in (SV40)-transformed fibroblast nuclei. Gaido and Cidlowski (1991) have isolated an 18 kDa nuclease from rat thymus which can also be classified as belonging to the DNase I family. Recently, Pandey *et al* (1997) purified a novel 97 kDa endonuclease.

In lens fibre cell extracts, neutral Ca^{2+}, Mg^{2+}-dependent nuclease activity was measured (Figure 6A) by a specific assay capable of discriminating the Ca^{2+}, Mg^{2+}-nucleases from acidic, non-cationic DNases (Torriglia *et al*, 1995). This bi-cationic activity represented in fact several DNase activities. After isoelectrofocusing electrophoresis followed by an activity transfer to an agarose gel containing DNA, two polypeptides with an acidic charge (pI 5.2 and 5.3) displayed this activity. By comparison, commercial DNase I has a pI of 5.3 (Counis *et al*, 1989b). Using a gel activity technique (i.e. Laemmli gels containing DNA), two Ca^{2+}, Mg^{2+}-dependent activities corresponding to 30 and 60 kDa polypeptides were observed (Figure 6B) in a Tris-SDS lens fibre extract (Arruti *et al*, 1995). These cationic activities, noted in both chicken embryonic lens fibre cells and completely anucleate fibre cells (hen of 1.5 years old), are dependent strictly on Ca^{2+} and Mg^{2+} and inhibited by a specific inhibitor of DNase I, G-actin (Arruti *et al*, 1995). It has been hypothesised that these cationic DNase activities accumulating in lens fibre cells at an older stage could

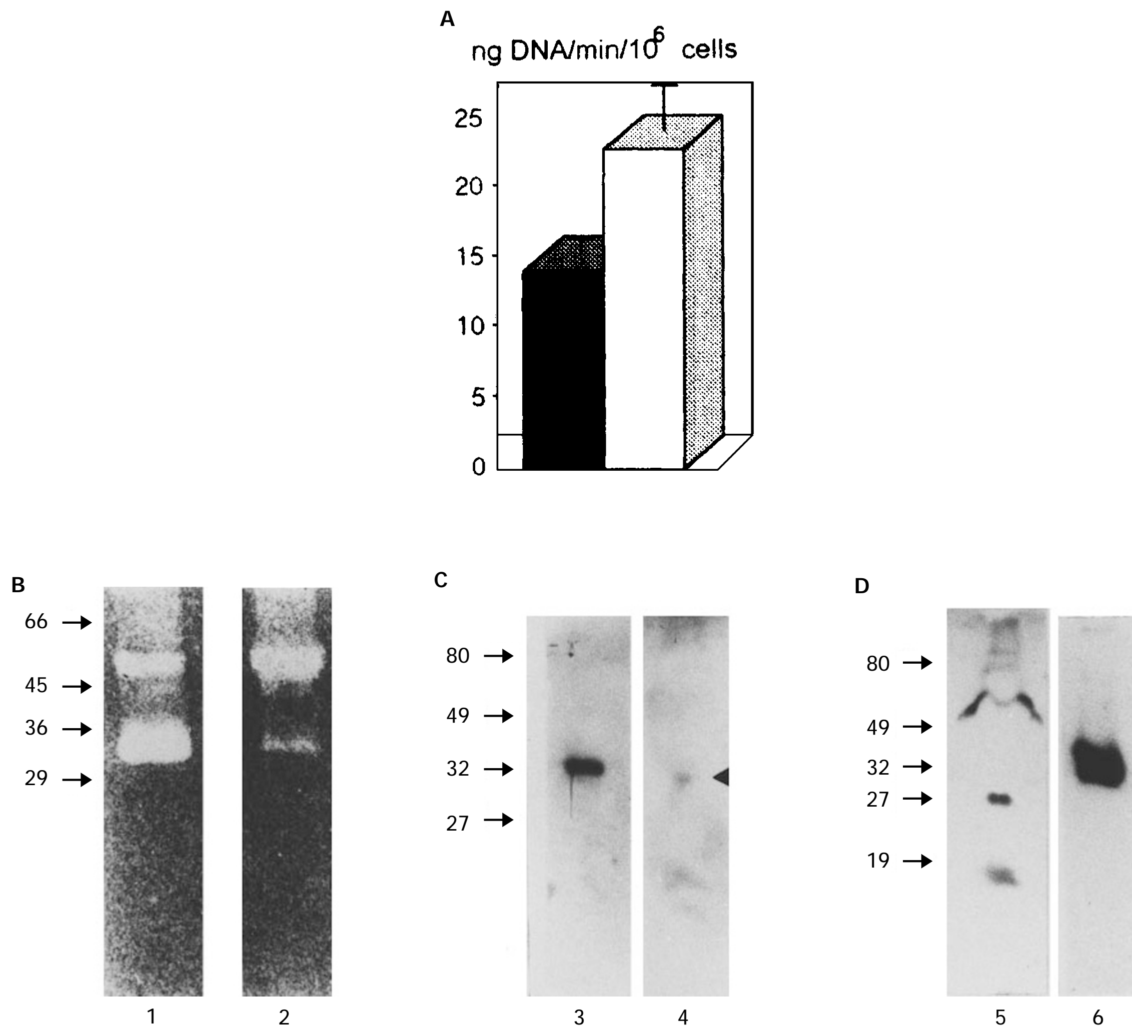

Figure 6 DNase content in chick lens fibre cells. (**A**): DNase activity assay, depicting the presence of neutral Ca^{2+}, Mg^{2+} (dark bar) and acidic-noncationic (white bar) DNase activities at E18 (Torriglia *et al*, 1995). (**B**): Non radioactive activity gel showing cationic (Ca^{2+}, Mg^{2+}) DNase activities in outer (1) and central (2) 2 month old lens fibre extracts (Arruti *et al*, 1995). E18 lens fibre DNase I (**C**) and DNase II (**D**) detected by Western blot with specific anti-DNases. Control DNase I (3). DNase I (4) and DNase II (5) detected in a E18 fibre extract. Control DNase II (6) (Torriglia *et al*, 1995)

have another function as nuclei have long disappeared. They may be linked to α crystallin, via G-actin, and play a role in ultrastructural organisation of the lens fibre itself (Gopalakrishnan and Takemoto, 1992).

These DNase I-like enzymes were further immuno characterised using an anti-DNase I raised against bovine pancreatic DNase I. This antibody inhibits DNase I activity *in vitro* (Torriglia *et al*, 1995). A single band of 32 kDa was detected by Western blot (Figure 6C, lane 4). When considering the immunochemistry data, DNase I (Figure 7, panel 2) was found in the nuclei of E18 fibre cells (corresponding nuclear staining DAPI, Figure 7, panel 1).

In epithelial cells, we have observed a Ca^{2+}, Mg^{2+}-dependent nuclease activity which was reduced compared to that in fibres, when expressed per cell number. Three main polypeptides were labelled with an antibody directed against DNase I, in Western blot (60, 32 and 18 kDa) (Torriglia *et al*, 1995). These DNase I-like molecules were found mainly in the nuclei (Figure 7, panel 4; corresponding nuclear staining DAPI, panel 3) of epithelial cells in lens sections, when observed by immunohistochemistry (Torriglia *et al*, 1995).

Different techniques suggest that several Ca^{2+}, Mg^{2+}-dependent nucleases exist in lens fibre cells, characterised either by their pI or by their molecular weight as in apoptotic cells (30 kDa DNase I, NUC 18 and the new 97 kDa). If we consider that the active DNase I is the 30 kDa polypeptide, other Ca^{2+}, Mg^{2+} nuclease activities exist, probably not related to DNase I. It is noteworthy that all these polypeptides require millimolar concentrations of Ca^{2+} and Mg^{2+} when assayed *in vitro*, but the intracellular Ca^{2+} level is in the nanomolar range. This is well below the concentration required to activate the enzymes *in vitro* (Barry and Eastman, 1992).

Non-cationic DNases

The second group of DNases corresponds to DNase II-like activity. DNase II has been described by Bernardi (1971) and Liao *et al* (1989). Initially purified from porcine spleen, it has a molecular weight of 40–46 kDa, and is composed of two subunits of 35 and 10 kDa. DNase II cleaves DNA in sodium acetate buffer (150 mM) and in the presence of EDTA (10 mM), with an optimal acidic pH (4.6–5), produces DSB and SSB with 3′ phosphate ends. Having been neither sequenced nor cloned; it is not as well known as DNase I and few authors have studied this molecule. Yasuda *et al* (1992) purified a 32 kDa protein with DNase II activity. This activity can be detected in many human tissues including liver, kidney, spleen, lung, heart, pancreas, thymus and salivary fluid suggesting an ubiquitous nuclease.

In lens fibre cells, the assay discriminating the acidic (pH 5.5), non-cationic nuclease activity from cationic and neutral DNase has shown an acidic nuclease activity (Figure 6A) in fibre cells (Torriglia *et al*, 1995). We have not been able to use the denaturating gel activity technique to detect any DNase II activity in tissues or any purified commercial preparations even if the reducing agent is omitted. This differs from the findings of Mezzina (1989) in lymphocytes and Pandey *et al* (1997) in rat hepatoma cells. We have prepared a polyclonal antibody against DNase II (27 kDa from Worthington) having a high titre. It does not cross-react with DNase I and is able to inhibit DNase II activity *in vitro* (Torriglia *et al*, 1995). In fibre cells, DNase II

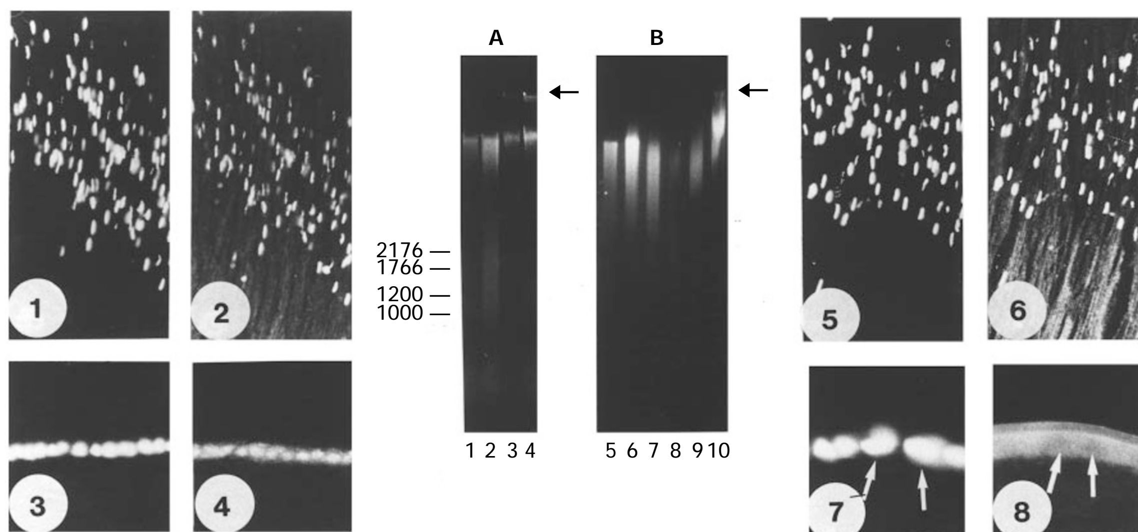

Figure 7 Localisation and function of cation dependent (Ca^{2+}, Mg^{2+}) and acidic, non cationic DNases (Torriglia *et al*, 1995). Nuclear localisation of DNase I in lens outer fibre nuclei (panel 2) or in epithelial nuclei (panel 4). Nuclear Dapi stain of the same section (panels 1 and 3). Nuclear localisation of DNase II in lens outer fibre nuclei (panel 6) and cytoplasmic localisation in epithelial cells (panel 8). Nuclear Dapi stain of the same section (panels 5 and 7). Effect of the anti-DNase I and anti-DNase II antibodies on DNA cleavage: 10^6 lens epithelial (**A**) or fibre (**B**) nuclei were incubated 6 h in neutral cationic or in acidic, non-cationic medium in absence or in presence of an anti-DNase I or DNase II antibody. Arrow indicate the sample well. (**A**) Acidic non-cationic DNase nuclease activity in E18 epithelial cells (lane 3) is not inhibited by anti-DNase II (lane 4) nor is cationic nuclease activity in same tissue (lane 1) by anti-DNase I (lane 2). (**B**) Acidic non cationic DNase activity in E18 fibre nuclei (lane 8) is inhibited by anti-DNase II antibody (lanes 9–10) while cationic nuclease activity in E18 fibre nuclei (lane 5) is not inhibited by anti-DNase I antibody (lanes 6–7)

antibody recognises three major bands (60, 23 and 18 kDa) which are detected, at least for two of them (60 and 23 kDa) only in these differentiating cells as compared to epithelial cells (Figure 6D, lane 5). Immunolocalization (Figure 7, panel 6; corresponding nuclear staining DAPI, panel 5) shows the presence of DNase II, highly concentrated, in all populations of fibre nuclei programmed to degenerate, although the DNase II is known to be a lysosomal enzyme (Liao *et al*. 1989). Other authors have also found this enzyme in nuclei (Slor and Lev, 1971).

In non-differentiating lens epithelial cells, there is also some acidic and non-cationic nuclease activity, to a lesser extent than in differentiating fibre cells. Western blotting shows two immunoreactive bands of 100 and 18 kDa which are located in cytoplasm (Figure 7, panel 8; corresponding nuclear staining DAPI, panel 7) when recorded by immunofluorescence (Torriglia *et al*, 1995). The DNase II immunoreactivity is also restricted to the basal membrane.

These new results are of interest in studies on DNase II. The appearance of several DNase II bands recognised by the specific DNase II antibody suggests that DNase II may have HMW precursors, or may be linked with regulatory proteins.

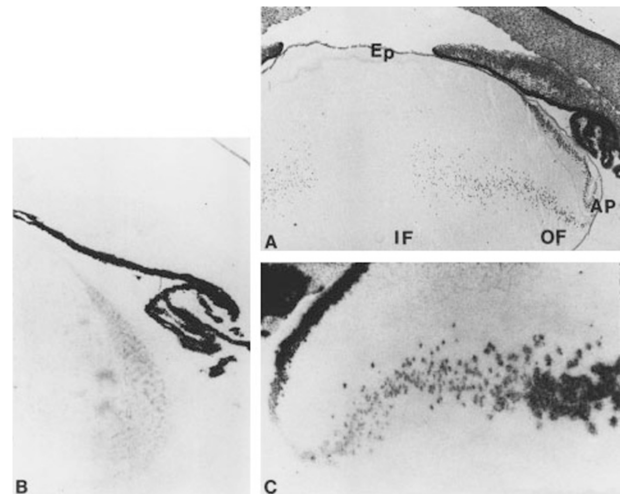

Figure 8 Histological staining and nick translation (NT) on sections of embryonic chick lens at E18. (**A**) ($\times 50$) Embryonic chick lens section stained with hematoxylin and eosin at E18 showing the different cell types: epithelia (Ep), annular pad (AP), outer fibre cells (OF) and inner fibre cells (IF). (**B** and **C**) ($\times 95$). Autoradiographs of sections fixed with methanol/acetic acid before NT in the absence (**B**) or in the presence (**C**) of DNase I. In (**B**), the cells show no single-strand breaks with 3′ OH termini (Chaudun *et al*, 1994)

DNases with a single cation requirement

The most important among these enzymes are probably the Mg^{2+}-dependent enzymes, with a key role in DNA repair (Wallace, 1988). Basnak'yan *et al* (1989) have also described a Mn^{2+}-dependent DNase (30 kDa) fractionated from rat liver chromatin, and Nikonova *et al* (1982) a Ca^{2+}-dependent DNase in rat thymocytes. Recently, a calcium-dependent 15 kDa endonuclease has been described in rat renal proximal tubules subjected to hypoxia/reoxygenation injury (Ueda *et al*, 1995). We have also observed in lens fibre cell nuclei, DNase activity dependent on one cation, either Ca^{2+} or Mg^{2+} (Muel *et al*, 1986). Lens fibre nuclei incubated 4 h in neutral Tris HCl medium, containing EDTA, EGTA and either Ca^{2+} or Mg^{2+}, show a smear of DNA degradation by electrophoresis, indicating nuclear activation of one cation dependent DNases.

Participation of these different nuclease activities in DNA cleavage

The mechanism of DNA degradation and chromatin condensation is not well understood and the existence of several endonucleases has been postulated: DNase I (Peitsch *et al*, 1994). DNase II (Barry and Eastman 1992, 1993; Eastman, 1994), NUC 18 identified as cyclophilin (Gaido and Cidlowski, 1991; Montague *et al*, 1994) and the 97 kDa endonuclease (Pandey *et al*, 1997).

In differentiating lens fibre cells where nuclei are physiologically programmed to disappear, we have observed all the types of DNases described in the literature. In an attempt to identify which endonucleases may be responsible for the various levels of endogenous DNA fragmentation, several studies were performed.

Using *in situ* nick translation reaction, we showed in chick lens fibre cells (E 18, Figure 8A) (Chaudun *et al*, 1994) that there was no accumulation of SSB with free 3′ OH ends on DNA (Figure 8B), as would be expected if a DNase of type I was responsible for DNA degradation (Figure 8C). In addition, Morgenbesser *et al* (1994) and Robinson *et al* (1995), recorded no DNA labelling by the TUNEL method, indicating that an addition to SSB, no DSB with free 3′ OH were obtained suggesting a modest participation of DNase I-like nuclease. These results have led to the hypothesis that another DNase may be responsible for DNA cleavage.

When nuclei of epithelial cells are isolated (Figure 7A) in acidic medium (lanes 3–4), or in the presence of Ca^{2+}, Mg^{2+} (lanes 1–2), a mild DNA degradation is seen. This cleavage is inhibited neither by anti-DNase II (lane 4) nor by anti DNase I (lane 2). In contrast, when fibre nuclei are incubated in an acidic medium (Figure 7B, lanes 8–10), the medium devised for DNase II activity, there is strong DNA degradation (lane 8) that can be only blocked by the antibody directed against DNase II (Figure 7B, lanes 9–10).

Thus, these results strongly implicate DNase II in this process. We believe that DNases of type II could be responsible for the early events of DNA cleavage during this physiological process, since no nucleosomal ladder is seen. A Mg^{2+}-dependent endonuclease has been observed to cleave the high molecular weight DNA in other tissues e.g. liver and thymus (Cain *et al*, 1994; Cohen *et al*, 1994; Walker and Sikorska, 1994). This is not in contradiction with our results, since DNase II has been described to be active in the presence of Mg^{2+} (Bernardi and Sadron, 1964).

This type II nuclease from lens fibres is probably related to the deoxyribonuclease II described by Barry and Eastman (1993) in chinese hamster ovary cells. However, we have no reason to think that it may be a DNase

producing nucleosomes such as the DNase I reported by Peitsch *et al* (1994) or the 97 kDa nuclease from Pandey *et al* (1997). The DNase I is present in lens fibre nuclei but during *in vitro* nuclei incubation, this nuclease appears to have weak activity, since the Ca^{2+}, Mg^{2+}-dependent activity recorded (Figure 7B, lane 5) is not inhibited by a specific inhibitory antibody directed against DNase I (lanes 6–7; Torriglia *et al*, 1995).

In addition, we have shown the presence of different nuclease polypeptides active in Ca^{2+}, Mg^{2+} medium. These results raise the possibility that there must exist another Ca^{2+}, Mg^{2+}-dependent DNase activity different from DNase I which may be implicated in the process of nucleosomal formation.

These results suggest that at least two different enzymes may be responsible for complete DNA degradation. This is in good agreement with the two activities described by Walker and Sikorska (1994) in the process of apoptosis, indicating a general pathway of concerted activation of DNases. We can then consider that among the two Ca^{2+}, Mg^{2+}-dependent fibre nuclease activities we have noted, one may play a role in nucleosomal formation. This DNase could be from the DNase I class, as identified by its Ca^{2+}, Mg^{2+}-dependence, but may not be related to DNase I. In addition, the Ca^{2+}, Mg^{2+}-dependent NUC 18 described by Cidlowski and coworkers (Gaido and Cidlowski, 1991; Montague *et al*, 1994) may be involved as well as a hypotonic-extracted 97 kDa nuclease recorded by Pandey *et al* (1994, 1997). This apparent diversity of DNase molecules suggests different signalling pathways as Segal-Bendirdjan and Jacquemin-Sablon (1995) proposed.

Modulators of nuclear degeneration while fibre cell cytoplasm remain all along the life-span

We must remember that lens fibre cells lose their organelles, while the cells are still present in the organ, throughout their life-span. Thus some controls must exist in these differentiating cells that do not appear in cells committed to die.

Several factors can delay DNA cleavage. For example, Zn^{2+} is capable of blocking all DNases, including DNase II (Torriglia *et al*, 1997). Culture of E11 chick lenses in a medium lacking tryptophan delays DNA degradation (Counis *et al*, 1984), perhaps by inhibiting DNase synthesis or precursors of the DNA breakage cascade. Alternatively, acidic pH can accelerate the phenomenon of genomic cleavage (Barry and Eastman, 1992, 1993; Gottlieb *et al*, 1995). As fibre cells are acidophilic (Zwaan and Williams, 1968), this state could contribute to DNase II activation. It is important to note that even if DNase II has an optimal pH of activity in the acidic range, it is still able to degrade DNA at a neutral pH (Figure 9). Mitochondria may also induce DNA disappearance. In lens fibre cells, mitochondria are lost coincidentally with nuclei (Bassnett and Beebe, 1992) just before DNA degradation (Bassnett and Mataic, 1997) and could contribute, by liberating factors such as apoptotic inducing factor (AIF) or cytochrome C capable of inducing DNA breakdown (Kroemer *et al*, 1997).

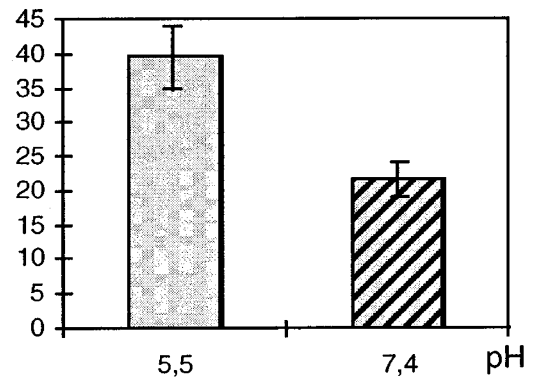

Figure 9 DNase II activity in acidic and neutral pH. 100 ng of DNase II (Worthington) were incubated with 1 μg of ^{3}H-labelled DNA for 30 min at 37°C, as described (Torriglia *et al*, 1995), in the presence of 10 mM Tris, 10 mM EDTA, pH 5.5 (grey column) or in the presence of 10 mM Tris, 1 mM EDTA, pH 7.4 (hatched column)

In the lens, there are various growth factors and/or oncogenes that can modulate the whole process of differentiation. Some of them may maintain cell cytoplasm survival while others may act to delete organelles. We have found fibroblast growth factors (FGF 1 and FGF 2) in embryonic chick retina at E11 and E18 (Mascarelli *et al*, 1987) and hypothesised, as have others, (Lovicu *et al*, 1995) that exogenous FGFs may induce lens differentiation. McAvoy and Chamberlain (1989) have shown that exogenous FGF induces lens epithelial cells, in explant culture, to proliferate, migrate and differentiate into fibre cells in a progressive concentration-dependent manner. We have observed during lens development an accumulation of FGF 1 in embryonic chick fibre cells, particularly in outer fibre cells (expressed as ng/10^8 cells; Table 2). The distribution of FGF 1 as well as the corresponding mRNA (Philippe *et al*, 1996) can thus be correlated with the pattern of differentiation in the lens, the greatest amount being concentrated in the nuclei of the outer fibres, a pattern not seen with FGF 2 (Cirillo *et al*, 1990). Lens fibre cells could be dependent on FGFs for their survival and differentiation (Chow *et al*, 1995) via either their receptor (Robinson *et al*, 1995; De Iongh *et al*, 1996) or an autocrine loop, since FGF 1 has no signal peptide (Renaud *et al*, 1996).

Lens cell differentiation as a model for apoptotic events

DNA degradation during fibre cell differentiation bears a number of similarities to apoptotic cells. Recently, Zelenka *et al* (1996) and Lang (1997) have suggested that lens cell terminal differentiation may be a special adaptation of the apoptotic process. Wride (1996) in another context, highlights the possible role of TNFα in loss of the lens cell nucleus and the role of apoptotic molecules e.g. ICE, bax and bcl2, suggesting a strong link between both processes. On the other hand Bassnett and Mataic (1997), using various

Table 2 Amount of FGF1 in lens cells during differentiation

	Epithelial FGF 1 ng/ml protein	Epithelial FGF 1 ng/ml/10^8 cells	Fibre FGF 1 ng/mg protein	Fibre FGF 1 ng/ml/10^8 cells
E 11 total tissue	2.4±0.4	5.1±0.6	0.44±0.03	11.9±0.9
E 18 total tissue	202.6±62.4	31.1±5	65.6±5.7	201.0±27.2
outer fiber			101.9	
inner fiber			24.6	
PH 1 d total tissue	208.0±55.1		58.5±0.7	
outer fiber			71.6	
inner fiber			29.4	
PH 3 m total tissue	89.6±28.7		27.2±10.9	
outer F			24.1	
inner F			5.5	

E: embryonic stage. PH: post hatched stage. d: day, m: month

morphological and chronological criteria believe that the process of lens cell differentiation is distinct from apoptosis. One of their major arguments is that in lens fibre cells, the organelles disappear 2–3 days before DNA fragmentation while in apoptotic cells, including lens epithelial cells (Li *et al*, 1995), the organelles are still present after DNA has been degraded. In addition, they point out that there is no membrane blebbing or formation of apoptotic bodies. Nevertheless, evidence of: the accumulation of G1 cyclins without initiating DNA synthesis; the expression of $p34^{cdc2}$ in fibre cells (Zelenka, 1996); the induction of apoptosis in lenses from $p53^{-/-}$ mice in which the Rb function is inactivating by the HPV E7 oncoprotein at the time that nuclear degeneration begins (Pan and Griep, 1995), and the degradation pattern of DNA in fibre cells, support the idea of a biochemical link between apoptosis and loss of the fibre cell nucleus. This idea is also supported by recent work. Transgenic mice were generated that overexpress bcl-2 in a lens specific fashion. Overexpression of bcl-2 was sufficient to interfere with normal lens cell differentiation. A cell disorganisation is seen, as well as inhibition of loss of the lens cell nucleus (Fromm and Overbeek, 1997).

Recent evidence has demonstrated that mitochondria are required for apoptosis, probably through release of cytochrome C (Kroemer *et al*, 1997; Yang *et al*, 1997; Kluck *et al*, 1997). The resulting activation of caspases degrades many proteins in both the cytoplasm and the nucleus. If caspases are activated in the lens, it would appear that terminally differentiated cells would have no chance of long term survival. Hence, by deleting the mitochondria before the nuclei instead of afterwards, the cells are able to survive. This might be the difference between differentiation and apoptosis in the lens. This would also suggest that in the lens a different pathway is required to activate nucleases because the cells now lack mitochondria.

Conclusion

In conclusion, the lens is an interesting model for studying patterns of nuclear degeneration and DNA cleavage. Its fibre cells contain or receive the information which leads to their nuclear disappearance, while the cytoplasm is conserved throughout the life-span. It is, thus, a differentiating system which is interesting to compare with a true apoptotic one. In 1977, we had shown that DNA was broken into multi-step fractions, i.e. high molecular and oligonucleosomal DNA fragments. The chromatin was condensed in the last steps before nuclear disappearance, mimicking apoptotic nuclei. Lens fibre nuclei, as all nuclei, contain several kinds of DNases and the understanding of their presence and function is highly complex. The activity of these nucleases must be partially blocked, implicating subtle regulation, probably via proteases, but no studies have been done on these molecules.

Among several Ca^{2+}, Mg^{2+}-dependent DNases including DNase I, mono-cationic (Ca^{2+} or Mg^{2+}) nucleases and non-cationic DNase (DNase II), we have been able to strongly implicate DNase II in this process. DNase II regulation is poorly understood. It is supposedly a lysosomal enzyme, while in our model, it has been found located in the nuclei of these differentiating cells. It is active in fibre nuclei when incubated in acidic buffer, a medium devised only for non-cationic DNase. Under these conditions, only the anti-DNase II antibody can inhibit the acidic DNA degradation. If DNase II seems very important, we cannot exclude a complementary role for DNase I, located in all fibre nuclei. However, it is probably a minor one as the DNA degradation observed in a Ca^{2+}, Mg^{2+} buffer, specific for cationic DNases cannot be inhibited by anti-DNase I. Thus, we presume that another DNase of the Ca^{2+}, Mg^{2+} class may be an additional factor in this complex process. This regulation may also involve multiple additional factors including post-translational modifications of the DNases, pH, mitochondrial release factors, oncogenes and/or trophic factors including FGF 1.

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