Differentiation (2003) 71:425–433 © International Society of Differentiation 2003

ORIGINAL ARTICLE

Esmond J. Sanders · Eve Parker

Retroviral overexpression of bcl-2 in the embryonic chick lens influences denucleation in differentiating lens fiber cells

Received June 16, 2003; accepted in revised form July 25, 2003

Abstract During the course of their differentiation, embryonic lens fibers undergo loss of their cytoplasmic organelles and nuclei. The denucleation process bears similarities to the nuclear breakdown that occurs during apoptosis. This has given rise to the hypothesis that this denucleation is analogous to apoptosis, but without the plasma membrane changes characteristic of apoptotic cell death. Previous work has shown that several members of the apoptotic cascade are active during denucleation. Here, we have overexpressed the anti-apoptotic molecule bcl-2 in developing lenses of the 8-day-old chick embryo *in ovo*, using the replication-competent retrovirus RCAS. We find that lenses over-expressing bcl-2 show varying degrees of distortion in comparison with untreated and negative insert controls, including a more spherical shape and disorganized fiber cells. All overexpressing lenses showed significantly higher numbers of smaller nuclei in the lens core, where denucleation begins. There was no change in cell size or pattern of proliferation. These *in vivo* results were confirmed *in vitro* using lens epithelial cell cultures, which differentiate into lentoids. The lentoids in treated cultures showed the same effect on nuclear number and size. We further found that in lenses overexpressing bcl-2 there was a reduction in the activation of caspase-9 and the cleavage of the caspase substrate DFF45, and, in the lens core, a failure of the nuclear chromatin to condense. These results provide strong support for the view that embryonic lens fiber cell denucleation is analogous to the nuclear degradation that occurs during apoptosis, and that similar control pathways are involved in both these phenomena.

Esmond J. Sanders (✉) · Eve Parker
Department of Physiology
University of Alberta
Edmonton, Alberta, Canada T6G 1Y6
Tel: (780) 492-5486
Fax: (780) 492-8915
e-mail: esmond.sanders@ualberta.ca

Key words lens · nuclear degeneration · bcl-2 · apoptosis

Introduction

The optical clarity of the ocular lens depends on the occurrence of several characteristic events in the differentiation of primary and secondary lens fiber cells. This clarity is achieved as a result of an elongation of the fiber cells perpendicular to the plane of light; the laying down of molecules of the crystallin family; and the loss of fiber cell organelles, including the nuclei (Piatigorsky, 1981; Wride, 1996, 2000; McAvoy et al., 1999). Control of these developmental events appears to involve the complex interplay of the actions of several differentiation factors, including those of the FGF (Le and Musil, 2001) and TGFβ (de Iongh et al., 2001) families, as well as TNFα (Wride and Sanders, 1998).

In the chick embryo, the denucleation phase of fiber cell differentiation commences between embryonic days (ED) 6 and 8 in the central region of the lens (Modak and Perdue, 1970; Sanwal et al., 1986), and expands centrifugally in a rapid process that is accompanied by a concomitant loss of the mitochondria (Bassnett and Beebe, 1992) and other organelles. In the secondary lens fibers, the demise of the nuclei occurs without their extrusion from the fiber cell cytoplasm, but with the typical morphological signs of nuclear breakdown, such as condensation and margination of the chromatin. These events are preceded by inter-nucleosomal nucleic acid degradation, similar to that which occurs during apoptotic cell death (Counis et al., 1998). This regulated mode of DNA fragmentation allows the degenerating fiber cell nuclei to be labeled by the same nick end-labeling techniques that are used to identify apoptotic nuclei (Chaudun et al., 1994; Wride and Sanders, 1998).

These similarities to apoptotic processes have led to the proposal that lens fiber cell denucleation is an

U.S. Copyright Clearance Center Code Statement: 0301–4681/2003/7107–425 $ 15.00/0

apoptotic event, which is not accompanied by the cytoskeletal and plasma membrane changes that lead to the characteristic shrinkage, blebbing, and fragmentation in truly apoptotic cells (Lang, 1997; Dahm et al., 1998; Dahm, 1999; Wride, 2000; Bassnett, 2002). This speculation is supported by experimental evidence, which shows that expected elements of the apoptotic cascade (Reed, 2000; Zimmermann et al., 2001) are expressed and activated in a developmentally regulated fashion during lens fiber cell denucleation *in situ* and *in vitro*. The first such study was that of Fromm and Overbeek (1997), who showed that bcl-2-overexpressing mutant mice have altered lens fiber cell differentiation and reduced lens fiber cell death in response to the induction of apoptosis by inhibition of p53. The bcl-2 mutants were also noted to show an inhibition of fiber cell denucleation, suggesting that denucleation in normal mice may depend on the activity of bcl-2-inhibitable genes. This view was supported by examination of the expression of several bcl-2 family molecules during embryonic chick lens differentiation, which was found to be temporo-spatially regulated during development (Wride et al., 1999).

The bcl-2 family of pro- and anti-apoptotic molecules controls the permeability of the mitochondrial outer membrane, regulating the activity of the voltage-dependent anion channel and the release of cytochrome c into the cytosol, where it collaborates with other molecules to activate downstream members of the apoptotic cascade (Harris and Thompson, 2000; Martinou and Green, 2001; Reed and Green, 2002).

A role for caspases and the cleavage of the caspase substrates poly(ADP-ribose) polymerase (PARP) and DNA fragmentation factor 45 (DFF45) in the denucleation process has also been demonstrated *in situ* and also *in vitro*, where cultured lens epithelial cells form small lens-like bodies called lentoids (Ishizaki et al., 1998; Wride et al., 1999). Furthermore, the sub-cellular dynamics of caspase-9 activation in denucleating fiber cells suggests that it associates with cytochrome c and Apaf-1 in the cytoplasm (Sanders and Parker, 2002), as it does in conventional apoptotic cells (Zhou et al., 1999). The failure of the apoptotic membrane events to follow denucleation in the fiber cells may be attributable to specialized features of the caspase-9 activation cascade in these cells, and the programmed removal of the nuclei and mitochondria (Sanders and Parker, 2002).

Despite this evidence suggesting that fiber cell denucleation is an apoptotic event, there are valid objections to this contention based on the clear differences between the two phenomena, and on the possibility that denucleation is triggered by the metabolic conditions in the center of the lens (Bassnett and Mataic, 1997; Bassnett, 2002).

In the present study, we have attempted to address these issues directly by overexpressing bcl-2 specifically in the developing lens of the chick embryo *in vivo* and *in vitro*, using the replication-competent retrovirus RCASBP(B) carrying the human bcl-2 transcript insert, and analyzing the resulting nucleation state of the fiber cells at ED 8, after the commencement of denucleation. We find that bcl-2 overexpression in lenses results in alterations in the denucleation of the lens fiber cells, characterized by decreased nuclear loss, decreased nuclear size, and disorganization of the denucleating fiber cells. Further, bcl-2 overexpression reduces the activation of caspase-9, and of DFF45 in the lens fiber cells, thus supporting the contention that denucleation is an apoptotic-like event.

Methods

Chick embryos

Fertilized White Leghorn hens' eggs were incubated at 37°C for the requisite number of days and the resulting embryos were staged according to Hamburger and Hamilton (1951). The embryos were removed and washed in Tyrode's saline. For Western blotting and cell culture, the lenses were removed to Tyrode's saline on ice and dissected if necessary using electrolytically sharpened tungsten needles without the use of digesting enzymes.

Preparation of the retrovirus

Retroviral vectors were produced as described previously (Logan and Francis-West, 1999; Keyes and Sanders, 2002). Briefly, pRCASBP(B) −/− and pRCASBP(B)/bcl-2 (supplied by Dr. S.H. Hughes, Frederick Cancer Research and Development Center, Maryland) were transfected into primary cultures of line 0 chick embryo fibroblast (CEFs) that had been produced from specific pathogen-free eggs (Hyvac, Adel, Iowa). The supernatant was collected and concentrated by ultracentrifugation at 25,000 rpm at 4°C for 2.5 hr. The supernatant was carefully removed and the remaining pellet was resuspended in 100 µl Optimem (GIBCO BRL Burlington, Ontario, Canada), which was then frozen in aliquots at −70°C. Controls included staining transfected CEFs for both the human bcl-2 transcript and the p19 viral coat protein, as well as immunoblotting for bcl-2. Viral titer was obtained by serial dilution and infection of CEFs for 48 hr. Cells were immunostained for the viral coat protein and the number of infectious virions was calculated as 5.0×10^7 per ml.

In ovo embryo culture and retroviral infection

Eggs were windowed after 2 days of incubation and microinjected *in ovo* with ~100 pl retroviral vector preparation containing the positive or negative bcl-2 insert into the right optic cup using a picoinjector (Medical Systems Corp.). The left optic cup was not treated and served as a control. Sub-blastodermal injection of India ink was used as a contrast agent. Following injection, the eggs were sealed with scotch tape and were reincubated for 6 days. After this time, the embryos were dissected and the heads were removed. A minimum of 12 injected and surviving embryos were analyzed for each treatment. The heads were fixed in Carnoy's fixative overnight at 4°C and then embedded in paraffin wax. The specimens were sectioned at 8 µm and stained with 0.5 µl/ml 4′,6-diamidino-2-phenylindole (DAPI, Eugene, Oregon, USA) for 4 min at room temperature for visualization of nuclei, and mounted with Vectashield mounting medium. The slides were examined using a Zeiss LSM510 confocal microscope (Toronto, Ontario, Canada)

equipped with an ultraviolet laser. The numbers of nuclei present in the core of the lens in sections cut through the equator of the lens was quantified on the confocal images. Nuclei were counted in the field of view defined by the confocal images shown in Figures 2B, 2D, and 2F. In addition, some sections were stained with hematoxylin to visualize the nuclei.

In vitro lens cell culture and retroviral infection

Dissociated lens cells were cultured as described previously (Menko et al., 1984; Wride and Sanders, 1998; Sanders and Parker, 2002). Briefly, lenses dissected from embryos at day 8 of incubation were trypsinized at 37°C in 0.1% trypsin in calcium- and magnesium-free Tyrode's solution for 20 min, or until all the lens capsules were ruptured. The cells were dissociated by repeated pipetting, pelleted to remove the supernatant, resuspended in medium 199 (GIBCO Life Technologies, Burlington, Ontario, Canada) containing 10% fetal calf serum (FCS; GIBCO) and gentamycin, and then plated onto coverslips (1.5 mm thickness, for optimum quality confocal microscopy) coated with Matrigel (1.25 mg/ml; Collaborative Biomedical Research, Mississauga, Ontario, Canada). Before plating the cells, the Matrigel was allowed to air dry on the coverslips for ~30 min before washing with medium 199 containing 10% FCS. The culture medium was changed each day, and the cultures were maintained at 37°C for 4 or 5 days.

Cultured cells were infected with virus after the first overnight incubation. The initial 100 µl medium was removed and replaced with fresh 100 µl complete medium containing 5 µl concentrated virus with 8 µg/ml polybrene. No other virus was added during the subsequent medium changes, but fresh polybrene was included in each medium change. After 4 or 5 days, cultures were fixed as above and stained with DAPI for confocal microscopy.

Lens epithelial cells, cultured under the conditions described here, freely form aggregates, or lentoids, that exhibit characteristics associated with lens fiber differentiation. The numbers of nuclei in lentoids in 4 day lens epithelial cultures was measured by observing DAPI-stained cultures using a 20 × objective and counting the nuclei in lentoids ~40 µm in diameter. This was carried out on a minimum of three different cultures for each treatment, and in a minimum of ten different lentoids per culture. Comparisons between different conditions were made using Tukey's multiple comparison test. The conditions were: untreated cells cultured in normal medium; untreated cells cultured in medium plus polybrene only; cells treated with RCAS −/−; and cells treated with RCAS/bcl-2.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting

PAGE and Western blotting were carried out as described previously (Wride and Sanders, 1998; Wride et al., 1999). Briefly, lens tissue was homogenized in protease inhibitor buffer containing 15 µg/ml aprotinin, 1 µg/ml leupeptin, 5 µg/ml pepstatin, and 1.74 mg/ml phenylmethyl-sulfonyl fluoride (PMSF). Protein concentrations were determined using the Bio-Rad Bradford-based protein assay method, and 20 µg of protein was added to each well of an 8% or 12% polyacrylamide gel. Proteins were transferred from the gels onto a supported nitrocellulose membrane at 100 V for 2 hr.

For immunoblotting, membrane blocking was carried out using 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20. Primary antibodies were incubated for 18 hr at 4°C followed by four washes for 10 min each, after which biotinylated secondary antibodies were used for 1.5 hr at room temperature. Bcl-2 was detected using a mouse monoclonal anti-human bcl-2 antibody (#100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:100 dilution). Viral coat protein was detected using undiluted supernatant containing mouse monoclonal antibody to avian myeloblastosis virus AMV-3C2 (Developmental Studies Hybridoma Bank). This antibody detects a 19 kDa protein carried by the RCAS viral coat (Potts et al., 1987). Cleavage of caspase-9 was demonstrated using a rabbit anti-human polyclonal antibody (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada; 1:500 dilution), which recognizes the 10 kDa cleavage fragment. DNA fragmentation factor (DFF45) was detected using a goat anti-human polyclonal antibody (C19; Santa Cruz Biotechnology, Inc.; 1:100 dilution), which recognizes the C-terminal end of the 45 kDa subunit of DFF45.

Proteins were peroxidase labeled using the Vectastain ABC reagent (Vector Laboratories, Inc. Burlington, Ontario, Canada) for 1.5 hr at room temperature, and visualized by adding luminol reagent (Santa Cruz Biotechnology, Inc.) for 1 min, and then exposing the blot to Hyperfilm ECL (Amersham International Plc, Baie d'Urfe, Quebec, Canada). Immunoblotting was repeated from one to three times for each experiment to ensure reliability. Representative examples are illustrated.

Assessment of fiber cell size and cell proliferation

In order to assess lens fiber size, sections were labeled with rhodamine-phalloidin (Molecular Probes, Eugene, OR), which stained the peripheral actin bundles in the cells (Lo et al., 1997). Sections were incubated with 20 units/ml rhodamine-phalloidin, containing 10 µl/ml Tween 20 to facilitate penetration, for 1 hr at room temperature.

An assessment of the patterns and extent of cell proliferation in control and treated lenses was carried out using the monoclonal antibody PC10 (Sigma Chemical Co., Oakville, Ontario, Canada), which is specific for proliferating cell nuclear antigen (PCNA) expressed only in proliferating cells (Waseem and Lane, 1990). Sections were incubated in monoclonal antibody at a dilution of 1:1000 in 4% goat serum for 1 hr at room temperature. After washing, the sections were incubated in biotinylated goat anti-mouse IgG at a dilution of 1:50 for 1 hr, and color was developed using the diaminobenzidine reaction.

Results

Bcl-2 overexpression in the embryonic lens

In order to assess the degree of bcl-2 overexpression achieved, whole lenses treated *in ovo* with RCAS-positive and -negative vectors were prepared for Western blotting and tested for immunoreactivity against the AMV coat protein at 19 kDa, for the level of infection, and against human bcl-2 protein at 25 kDa, for the level of bcl-2 expression (Fig. 1).

Transfection of lenses with the RCAS/bcl-2 vector (Fig. 1, lane 1) resulted in high levels of immuno-reactivity against both the AMV coat protein and human bcl-2 protein. Transfection with the control negative insert vector, RCAS −/−, resulted in a blot that was positive for AMV protein, but not human bcl-2 (lane 2). Untransfected control tissue was negative for both proteins (lane 3), while samples from the chick embryo fibroblasts (CEF) used to grow the virions (lane 4) were positive for both proteins and showed that they were at the same molecular size as the bands seen in lanes 1 and 2.

Nuclear effects of bcl-2 overexpression in whole lens

Lenses exposed to the negative insert virus (Figs. 2A, 2B) showed the oval shape characteristic of normal ED

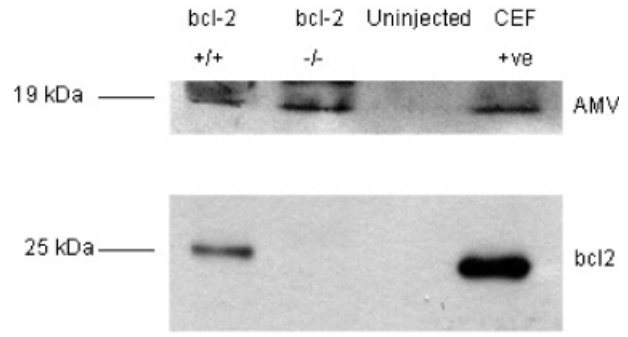


Fig. 1 Western immunoblot using lysates from virus-treated and -untreated lenses (lanes 1–3) and bcl-2-transfected chick embryo fibroblasts (CEF; lane 4). The samples were blotted with antibody against avian myeloblastosis virus coat protein (AMV; 19 kDa), and the membranes were stripped and then re-blotted with antibody against human bcl-2 (25 kDa). The two blots shown are therefore from the same sample. Lane 1: transfection with the bcl-2 insert results in expression of bcl-2 and viral coat protein; lane 2: transfection with the negative insert results in expression of the viral coat protein only; lane 3: non-transfected cells are negative for both proteins; lane 4: transfected chick embryo fibroblasts are positive for both proteins.

8 lenses. These samples showed the beginning of nuclear loss in the central core of the lens, where nuclei were more sparse, and a relatively ordered distribution of lens fiber cells, as judged by the distribution of their nuclei. The degenerating nuclei in the lens core showed condensation and margination of the chromatin (Fig. 2B, black and white inset), characteristic of apoptosis (see also Wride and Sanders, 1998).

Exposure to the RCAS/bcl-2 vector resulted in varying degrees of lens distortion (Figs. 2C–2F). All bcl-2-overexpressing lenses showed a more rounded appearance than the controls, suggesting a higher pressure within the lens capsule. Also, all treated lenses showed greater numbers of nuclei in the core region and varying degrees of disorganization of the fiber cell nuclei, in comparison with lenses exposed to the negative control and with uninjected lenses. For lenses treated with the negative insert virus, the mean number of nuclei in the core sections was 128.1 ± 9.9; for lenses treated with the bcl-2 insert, the number of nuclei was 288.3 ± 28.8 (significantly different, $P < 0.001$; $n = 10$). Although smaller, these nuclei did not show the chromatin condensation seen in the controls (Fig. 2D, black and white inset).

In more extreme cases, (Figs. 2E, 2F), a diffuse staining with DAPI was observed, suggesting the leakage of nucleic acids from the nuclei.

Nuclear effects of bcl-2 overexpression in cultured lentoids

Lens epithelial cells, cultured under the conditions described here, freely form aggregates, lentoids, which exhibit characteristics associated with lens fiber differentiation. After 4 or 5 days in culture, these lentoids appear in the cultures in a variety of sizes; in this study, lentoids of $\sim 40\,\mu m$ were selected for examination. Lentoids of this size are typically normally composed of 10–15 cells containing large rounded nuclei, each $\sim 3\,\mu m$ in diameter (Fig. 3A). In cultures transfected with bcl-2 (Fig. 3B), the lentoids had a different appearance, with approximately double the number of nuclei, which were approximately half the diameter of those in the untreated and negative insert controls. The smaller size of the nuclei reflected the same result obtained in whole lenses *in ovo*. The number of nuclei in the treated lentoids was significantly higher ($P < 0.001$) than that observed in any of the control conditions (Fig. 3C). Observation by phase contrast microscopy provided no evidence that the fiber cells in these lentoids were multinucleated.

Bcl-2 overexpression blocks the cleavage of caspase-9 and DFF45

Lysates of lenses treated with the RCAS/bcl-2 vector were immunoblotted against an antibody that recognizes the 10 kDa cleavage product of caspase-9, and compared with similar blots using lysates from lenses treated with RCAS $-/-$ and with untreated lenses (Fig. 4A). Overexpression of bcl-2 considerably reduced, but did not eliminate, the expression of the 10 kDa caspase-9 fragment (lane 2).

Lysates of lenses treated with the RCAS/bcl-2 vector were also immunoblotted against an antibody that recognizes the 28 kDa cleavage product of DFF45, and compared with similar blots using lysates from lenses treated with RCAS $-/-$ and with untreated lenses (Fig. 4B). Results indicated that the cleavage of DFF45 was reduced, but not eliminated, in the lenses that were overexpressing bcl-2.

Assessment of fiber cell size and cell proliferation

In order to determine whether bcl-2 overexpression influenced fiber cell size, sections were labeled with rhodamine-phalloidin (Figs. 5A, 5B), which stains the peripheral actin bundles and outlines the cells. The lens fiber cell diameter in both control (Fig. 5A) and bcl-2-overexpressing (Fig. 5B) samples was the same, at ~ 3–$5\,\mu m$.

Similarly, the pattern and extent of cell proliferation was unchanged by overexpression of bcl-2 (Figs. 5C, 5D). In both cases, lenses showed the presence of proliferating cells in the epithelium and in the transitional annular pad region, but not among the differentiating lens fiber cells.

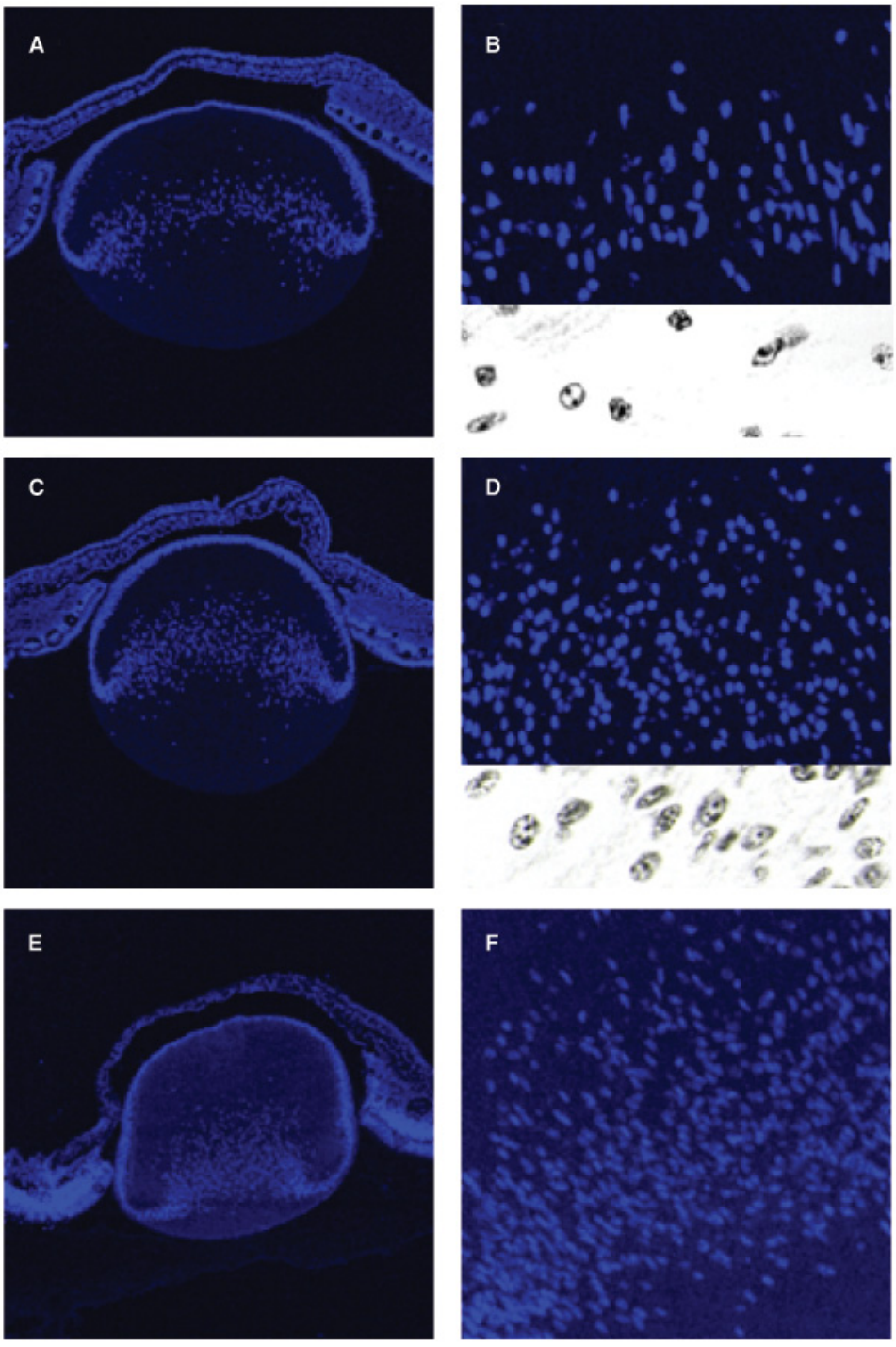


Fig. 2 Lenses treated with negative and positive bcl-2-expressing viruses, sectioned through their equator, and stained with DAPI. (**A** and **B**) Lenses treated with the negative insert virus. The black and white inset in (B) shows degenerating nuclei with condensed chromatin. (**C–F**) Lenses treated with the bcl-2-overexpressing virus. The black and white inset in (**D**) shows nuclei from the lens core with dispersed chromatin. Magnification: $\times 21$ (**A, C,** and **E**); $\times 42$ (**B, D,** and **F**); $\times 100$ (insets).

Discussion

The temporo-spatial modulation of apoptotic cell death during embryonic development is influenced by multiple signaling pathways (Sanders and Wride, 1995), but the tissue-specific expression of bcl-2, in particular, appears to have a major role in this control (Novack and Korsmeyer, 1994). There are a number of studies

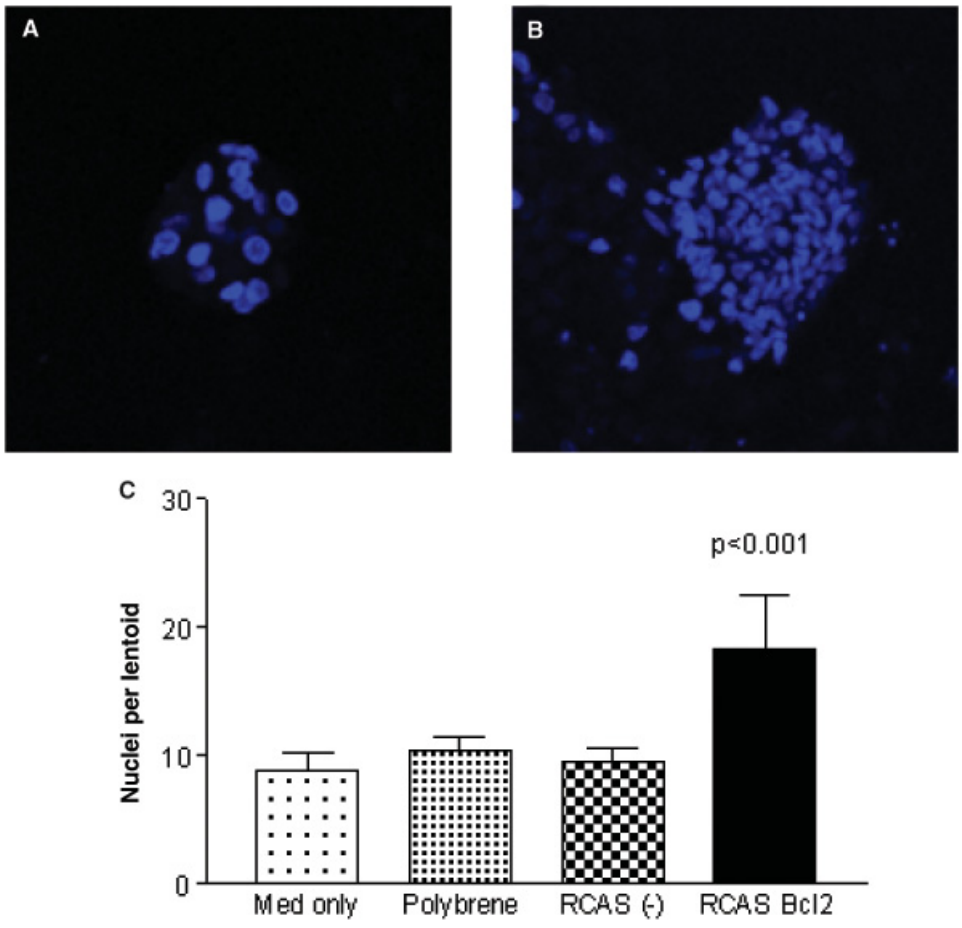
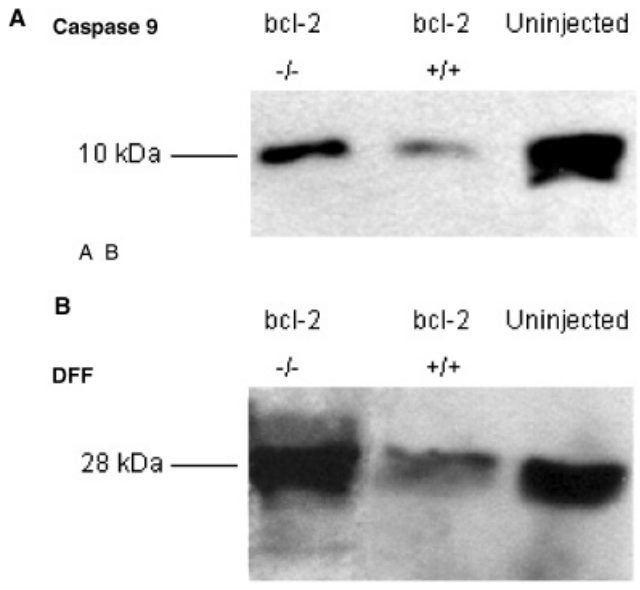


Fig. 3 Confocal images of typical lentoids from cultures treated with RCAS/bcl-2 negative insert (**A**) and RCAS/bcl-2 (**B**). The bcl-2-expressing lentoid shows many more, smaller, nuclei than the negative control. Magnification: ×90. (**C**) Histogram showing the frequency of nuclei per lentoid in the presence of untreated culture medium only; culture medium containing polybrene; RCAS virus with the negative insert; and RCAS virus with the bcl-2 insert. The bcl-2-overexpressing cultures are significantly different from all the other conditions ($P<0.001$).



showing that overexpression of bcl-2 in embryonic tissue can protect cells against death during development (Farlie et al., 1995; Sato et al., 2002).

In the lens, Fromm and Overbeek (1997) reported that transgenic overexpression of bcl-2 in mouse embryos inhibited p53-dependent fiber cell apoptosis, and inhibited the normal process of fiber cell denucleation. They concluded from their experiments that the normal process of lens fiber cell denucleation likely

Fig. 4 (**A**) Western immunoblot with an antibody against caspase-9, using lysate from lenses treated with the RCAS/bcl-2 negative insert (lane 1); with the bcl-2 positive insert (lane 2); and from uninjected lenses (lane 3). Expression of the 10 kDa cleavage fragment of activated caspase-9 is considerably reduced by overexpression of bcl-2 (lane 2). (**B**) Western immunoblot with an antibody against DNA fragmentation factor (DFF45), using lysate from lenses treated with the RCAS/bcl-2 negative insert (lane 1); with the bcl-2 positive insert (lane 2); and from uninjected lenses (lane 3). Expression of the 28 kDa cleavage product of DFF45 is reduced by overexpression of bcl-2 (lane 2).

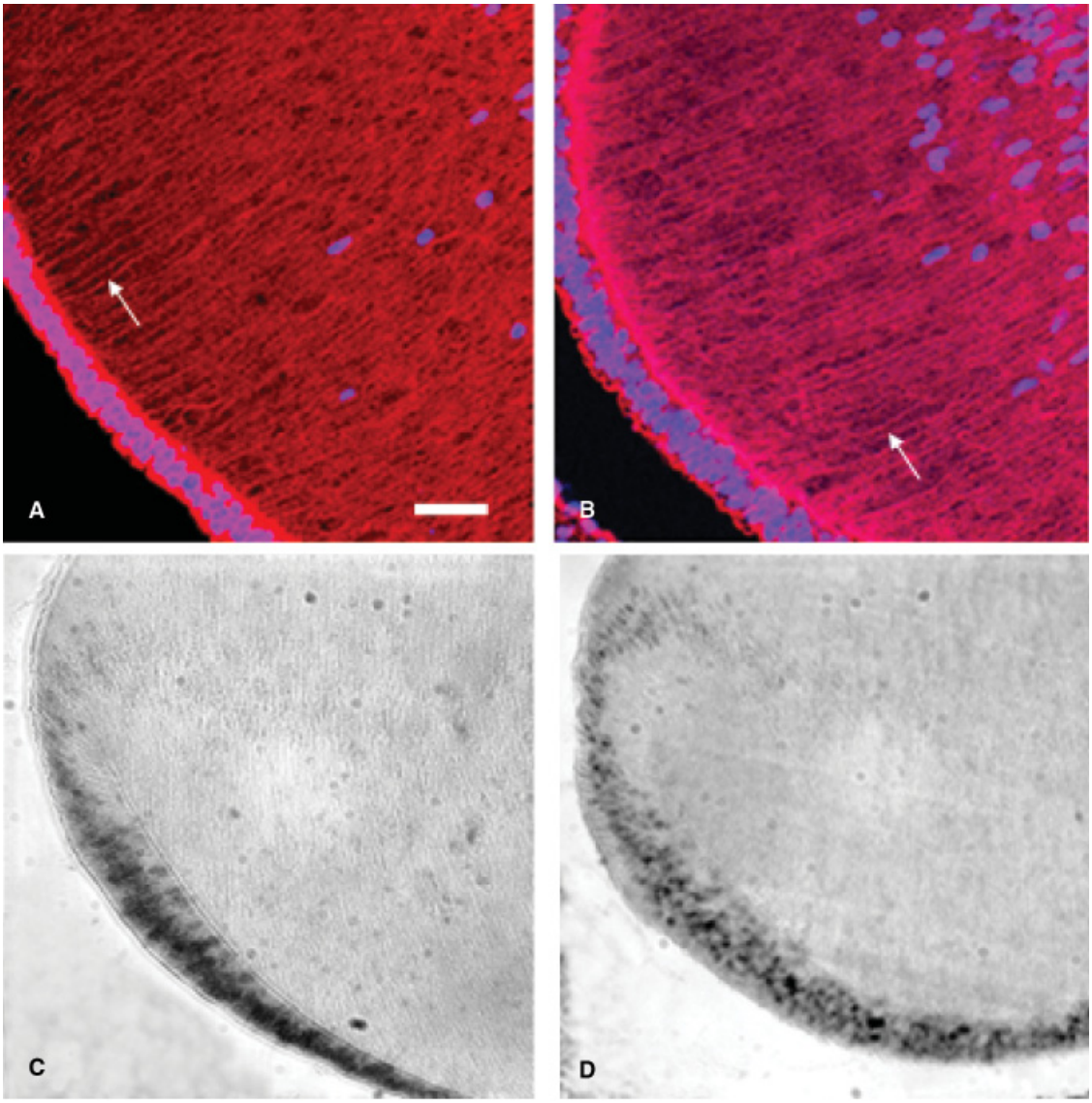


Fig. 5 (**A** and **B**) Lens sections stained with rhodamine-phalloidin to outline the fiber cells. Fiber cell width (arrows) in the untreated lenses (**A**) was ~3 μm, and not different from the width of cells in treated lenses (**B**). The scale bar represents 30 μm. (**C** and **D**) Lens sections stained for proliferating cell nuclear antigen (PCNA). Densely labeled nuclei in the untreated lenses (**C**) occurred in the epithelium and transitional annular pad regions, but not in the region of the maturing fibers. The pattern was the same in the treated lenses (**D**). Magnification: ×65 (**C** and **D**).

involves members of the apoptotic cascade, the activity of which is inhibitable by bcl-2. We have extended this work by directly overexpressing bcl-2 in differentiating chick embryo lenses *in ovo*, and concur with many of their conclusions. We find that overexpression of this molecule *in ovo*, in the chick embryo, disrupts the denucleation process and results in a phenotype with several distinct characteristics. After a total of 8 days of incubation, when denucleation is normally well under way in the lens core, the overexpressing lenses show a significantly larger number of nuclei in the core region in comparison with negative insert and uninjected controls. Furthermore, the bcl-2-overexpressing lenses consistently showed varying degrees of disorganization of the fiber cell orientation, and a more spherical shape than the controls, effects which were clearly not related to changes in fiber cell size or proliferation. It is possible that fiber cell disorganization results in greater pressure within the lens capsule and thus a more spherical lens. The spherical lenses observed after transfection may arise from increased fluid pressure in the lenses or from a weaker capsule. These possibilities require further investigation. Fromm and Overbeek (1997), using the TUNEL method for apoptotic nuclei, also described a diffuse staining suggestive of cell lysis in response to bcl-2 overexpression. We have shown the same effect with DAPI nuclear staining.

The *in ovo* results were substantiated using the *in vitro* culturing of lens epithelial cells. In this culture system,

the dissociated epithelial cells differentiate into lens-like structures, lentoids, the development of which, in many respects, parallels the development of intact lenses. Thus, the lens fiber cells in the lentoids show several of the characteristics associated with denucleation in the intact lens, including changes in mitochondrial membrane permeability, caspase activation, and cytochrome c translocation (Wride et al., 1999; Sanders and Parker, 2002). Furthermore, the developmentally regulated expression of bcl-2 in both intact lenses and in lentoids has implicated this molecule in the differentiation of the fiber cells (Wride et al., 1999). In the current experiments, bcl-2 overexpression in lens epithelial cell cultures resulted in a significant increase in the number of nuclei in the lentoids, as well as in a reduction in their size.

The reduction in the size of the nuclei in transfected lentoids, in comparison with untreated and negative insert controls, reflected a similar observation *in ovo* in intact lenses, and is enigmatic. It may indicate that the nuclei have started to follow an apoptotic-like pathway, which would include extreme shrinkage (Counis et al., 1998), but have been unable to complete the process, presumably due to the influence of the overexpression of bcl-2. Although the final disappearance of the nuclei in the normal lens is a relatively rapid process (2–4 hr; Bassnett and Beebe, 1992), a progressive reduction in nuclear size is seen in the untreated lens (Wride and Sanders, 1998). The mechanisms leading to shrinkage of the nucleus in apoptotic cells are unclear, but presumably they are related to the downstream effects of caspase activation. In the present work, we can show only incomplete inhibition of caspase-9 cleavage in response to bcl-2 overexpression. The residual activation of this caspase may therefore be related to the partial shrinkage of the nuclei.

Previous studies have implicated several caspases in the fiber cell denucleation process (Wride et al., 1999), including the regulatory caspase-9 (Sanders and Parker, 2002) and a downstream caspase-3-like member of the caspase family (Ishizaki et al., 1998). That overexpression of bcl-2 can block caspase activation and consequent cleavage of downstream caspase substrates has also been demonstrated previously (Fulda et al., 2002). Here, we have shown that bcl-2 overexpression reduces the cleavage, and therefore activation, of caspase-9, and also reduces the cleavage of the caspase substrate DFF45. These results, together with our previous findings (Wride et al., 1999), confirm that DFF45 and its cleavage by caspase-9 constitute a major pathway in the denucleation process. Whether or not the incomplete cleavage of DFF45 is related to the failure of the chromatin to condense in the nuclei of the transfected cells (Liu et al., 1998; Widlak, 2000) remains to be determined. As we have previously commented (Sanders and Parker, 2002), we have been unable to confirm a role for caspase-3 in the denucleation process. We further suspect (unpublished observations) that there may be a significant caspase-independent component to the cytoplasmic and nuclear changes during lens fiber cell differentiation.

Taken together, these results, both *in vivo* and *in vitro*, support the contention of Fromm and Overbeek (1997) and Wride et al. (1999) that bcl-2 is implicated in the denucleation process, and, by the same token, also support the view that denucleation is an apoptotic event (Dahm, 1999; Wride, 2000).

Acknowledgments We thank Drs. Mike Wride and Bill Keyes for valuable discussion, and the Canadian Institutes of Health Research for an operating grant in support of this work.

References

Bassnett, S. (2002) Lens organelle degradation. Exp Eye Res 74:1–6.

Bassnett, S. and Beebe, D.C. (1992) Coincident loss of mitochondria and nuclei during lens fiber cell differentiation. Dev Dyn 194:85–93.

Bassnett, S. and Mataic, D. (1997) Chromatin degradation in differentiating fiber cells of the eye lens. J Cell Biol 137:37–49.

Chaudun, E., Arruti, C., Courtois, Y., Ferrag, F., Jeanny, J.C., Patel, B.N., Skidmore, C., Torriglia, A. and Counis, M.F. (1994) DNA strand breakage during physiological apoptosis of the embryonic chick lens: free 3′OH end single strand breaks do not accumulate even in the presence of a cation-independent deoxyribonuclease. J Cell Physiol 158:354–364.

Counis, M.F., Chaudun, E., Arruti, C., Oliver, L., Sanwal, M., Courtois, Y. and Torriglia, A. (1998) Analysis of nuclear degradation during lens cell differentiation. Cell Death Differ 5:251–261.

Dahm, R. (1999) Lens fibre cell differentiation—a link with apoptosis? Ophthalmic Res 31:163–183.

Dahm, R., Gribbon, C., Quinlan, R.A. and Prescott, A.R. (1998) Changes in the nucleolar and coiled body compartments precede lamina and chromatin reorganization during fibre cell denucleation in the bovine lens. Eur J Cell Biol 75:237–276.

de Iongh, R.U., Lovicu, F.J., Overbeek, P.A., Schneider, M.D., Joya, J., Hardeman, E.D. and McAvoy, J.W. (2001) Requirement for TGFβ receptor signaling during terminal lens fiber differentiation. Development 128(supplement): 3995–4010.

Farlie, P.G., Dringen, R., Rees, S.M., Kannourakis, G. and Bernard, O. (1995) bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc Natl Acad Sci USA 92:4397–4401.

Fromm, L. and Overbeek, P.A. (1997) Inhibition of cell death by lens-specific overexpression of bcl-2 in transgenic mice. Dev Genet 20:276–287.

Fulda, S., Meyer, E. and Debatin, K.-M. (2002) Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene 21:2283–2294.

Hamburger, V. and Hamilton, J.L. (1951) A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Harris, M.H. and Thompson, C.B. (2000) The role of the bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ 7:1182–1191.

Ishizaki, Y., Jacobson, M.D. and Raff, M.C. (1998) Role for caspases in lens fiber differentiation. J Cell Biol 140:153–158.

Keyes, W.M. and Sanders, E.J. (2002) Regulation of apoptosis in the endocardial cushions of the developing chick heart. Am J Physiol Cell Physiol 282:C1348–C1360.

Lang, R.A. (1997) Apoptosis in mammalian eye development: lens morphogenesis, vascular regression and immune privilege. Cell Death Differ 4:12–20.

Le, A.C. and Musil, L.S. (2001) FGF signaling in chick lens development. Dev Biol 233:394–411.

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T. and Wang, X. (1998) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc Natl Acad Sci USA 95:8461–8466.

Lo, W.K., Shaw, A.P. and Wen, X.J. (1997) Actin filament bundles in cortical fiber cells of the rat. Exp Eye Res 65:691–701.

Logan, C. and Francis-West, P. (1999) Gene transfer in avian embryos using replication-competent retroviruses. Methods Mol Biol 97:539–551.

Martinou, J.C. and Green, D.R. (2001) Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2:63–67.

McAvoy, J.W., Chamberlain, C.G., de Iongh, R.U., Hales, A.M. and Lovicu, F.J. (1999) Lens development. Eye 13:425–437.

Menko, A.S., Klukas, K.A. and Johnson, R.S. (1984) Chicken embryo lens cultures mimic differentiation in the lens. Dev Biol 103:129–141.

Modak, S.P. and Perdue, S.W. (1970) Terminal lens cell differentiation. Exp Cell Res 59:43–56.

Novack, D.V. and Korsmeyer, S.J. (1994) Bcl-2 protein expression during murine development. Am J Pathol 145:61–73.

Piatigorsky, J. (1981) Lens differentiation in vertebrates: a review of cellular and molecular features. Differentiation 19:134–153.

Potts, W.M., Olsen, M., Boettiger, D. and Vogt, V. (1987) Epitope mapping of monoclonal antibodies to gag protein p19 of avian sarcoma and leukemia viruses. J Gen Virol 68:3177–3182.

Reed, J.C. (2000) Mechanisms of apoptosis. Am J Pathol 157: 1415–1430.

Reed, J.C. and Green, D.R. (2002) Remodeling for demolition: changes in mitochondrial ultrastructure during apoptosis. Mol Cell 9:1–3.

Sanders, E.J. and Parker, E. (2002) The role of mitochondria, cytochrome c and caspase-9 in embryonic lens fibre cell denucleation. J Anat 201:121–135.

Sanders, E.J. and Wride, M.A. (1995) Programmed cell death in development. Int Rev Cytol 163:105–173.

Sanwal, M., Muel, A.S., Chaudun, E., Courtois, Y. and Counis, M.F. (1986) Chromatin condensation and terminal differentiation process in embryonic chicken lens *in vivo* and *in vitro*. Exp Cell Res 167:429–439.

Sato, N., Sakuma, C., Kato, H., Milligan, C.E., Oppenheim, R.W. and Yaginuma, H. (2002) Bcl-2 rescues motoneurons from early cell death in the cervical spinal cord of the chicken embryo. J Neurobiol 53:381–390.

Waseem, N.H. and Lane, D.P. (1990) Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. J Cell Sci 96:121–129.

Widlak, P. (2000) The DFF40/CAD endonuclease and its role in apoptosis. Acta Biochim Pol 47:1037–1044.

Wride, M.A. (1996) Cellular and molecular features of lens differentiation: a review of recent advances. Differentiation 61:77–93.

Wride, M.A. (2000) Apoptosis as seen through a lens. Apoptosis 5:203–209.

Wride, M.A. and Sanders, E.J. (1998) Nuclear degeneration in the developing lens and its regulation by TNFα. Exp Eye Res 66:371–383.

Wride, M.A., Parker, E. and Sanders, E.J. (1999) Members of the Bcl-2 and caspase families regulate nuclear degeneration during chick lens fibre differentiation. Dev Biol 213:142–156.

Zhou, H., Li, Y., Liu, X. and Wang, X. (1999) An Apaf-1-cytochrome c multimeric complex is a functional apoptosome that activates procaspase 9. J Biol Chem 274:11549–11556.

Zimmermann, K.C., Bonzon, C. and Green, D.R. (2001) The machinery of programmed cell death. Pharmacol Ther 92:57–70.