A Role for Caspases in Lens Fiber Differentiation

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Abstract. There is increasing evidence that programmed cell death (PCD) depends on a novel family of intracellular cysteine proteases, called caspases, that includes the Ced-3 protease in the nematode *Caenorhabditis elegans* and the interleukin-1β–converting enzyme (ICE)-like proteases in mammals. Some developing cells, including lens epithelial cells, erythroblasts, and keratinocytes, lose their nucleus and other organelles when they terminally differentiate, but it is not known whether the enzymatic machinery of PCD is in-

volved in any of these normal differentiation events. We show here that at least one CPP32 (caspase-3)-like member of the caspase family becomes activated when rodent lens epithelial cells terminally differentiate into anucleate lens fibers in vivo, and that a peptide inhibitor of these proteases blocks the denucleation process in an in vitro model of lens fiber differentiation. These findings suggest that at least part of the machinery of PCD is involved in lens fiber differentiation.

P ROGRAMMED cell death (PCD)[1] eliminates unwanted cells in most animal tissues at some stage of their development (for reviews see Glucksmann, 1951; Ellis et al., 1991; Jacobson et al., 1997). It is thought to involve an intracellular proteolytic cascade, mediated by members of the Ced-3/interleukin-1β–converting enzyme (ICE) family of cysteine proteases, now called caspases, that cleave one another and various key intracellular target proteins to kill the cell neatly and quickly (Yuan et al., 1993; Martin and Green, 1995; Alnemri et al., 1996; Chinnaiyan et al., 1996). Some developing cells, such as lens epithelial cells, erythroblasts, and keratinocytes, lose their nucleus and other organelles when they terminally differentiate, and it seems possible that they use some elements of the caspase-based death program in doing so. Here we provide evidence that this is the case for lens cells.

Primary lens fibers develop from lens epithelial cells that form the posterior half of the early lens vesicle. Secondary lens fibers develop later from epithelial cells at the equatorial region of the lens, adding to the original primary fibers

that remain as a core at the center of the lens (Goss, 1978; McAvoy, 1980). In the formation of both primary and secondary fibers, the cell nucleus and other organelles are lost as each lens fiber forms, but the intracellular mechanisms involved are unknown. There are conflicting reports about the nature of the nuclear loss, especially in the formation of secondary fibers. Modak and colleagues reported that the nuclear changes during terminal differentiation of chick secondary lens fiber cells resemble those seen in PCD: the nucleus becomes pyknotic and the DNA is degraded into low molecular weight fragments of discrete sizes (Modak and Perdue, 1970; Appleby and Modak, 1977). Kuwabara and Imaizumi, however, reported that the nucleus does not become pyknotic during secondary lens fiber cell differentiation, but instead just fades away (Kuwabara and Imaizumi, 1974). Moreover, several papers (Fromm et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Robinson et al., 1995; Chow et al., 1995a) report that nuclei of differentiating secondary fiber cells in the mouse lens are not labeled by the in situ end-labeling TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP-biotin nick-end labeling) technique, which should label nuclei containing fragmented DNA (Gavrieli et al., 1992), although a recent study demonstrated DNA fragmentation in differentiating chick lens fiber cells by both the TUNEL technique and in situ electrophoresis (Bassnett and Mataic, 1997).

In the present report we present three lines of evidence that lens fiber differentiation involves components of the machinery of PCD: (*a*) the nucleus in differentiating sec-

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1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; P, postnatal day; PCD, programmed cell death; PARP, poly(ADP-ribose) polymerase; STS, staurosporine; TUNEL, terminal deoxynucleotidyl transferase [Tdt]-mediated dUTP-biotin nick-end labeling.

ondary lens fiber cells can be labeled by the TUNEL technique, suggesting that DNA fragmentation occurs during the differentiation process; (*b*) poly-(ADP-ribose) polymerase (PARP), a known substrate for caspase-3 and some caspase-3–like caspases (Lazebnik et al., 1994; Alnemri et al., 1995*a,b*; Nicholson et al., Tewari et al, 1995; Duan et al., 1996; Schlegel et al., 1996), is cleaved during lens development in a similar way to the cleavage that occurs in PCD, suggesting that caspase-3 or a related caspase is activated during the process; and (*c*) a peptide caspase inhibitor inhibits denucleation and PARP cleavage in differentiating lens cells in culture, suggesting that caspase activation is required for these processes.

Materials and Methods

Cell and Explant Cultures

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Explant cultures of lens epithelium were prepared from postnatal day 11 (P11) Sprague-Dawley rats (Ishizaki et al., 1993), and cultured in serum-free medium containing basic fibroblast growth factor (bFGF, 250 ng/ml) plus insulin (5 μg/ml), in the presence or absence of the cell-permeable peptide caspase inhibitor Z-Val-Ala-Asp (O-Me)-CH2F (zVAD-fmk), or the chemically similar cathepsin B inhibitor Z-Phe-Ala (O-Me)-CH2F (zFA-fmk, Rasnick, 1985; both from Enzyme Systems Products, Dublin, CA), and used at 50 μM. In some experiments, lens explant cultures in protein-free medium were treated with 2 μM staurosporine (STS) in the absence or presence of 50 μM zVAD-fmk for 24 h and then fixed, and cell viability was assessed by nuclear morphology after staining with propidium iodide as described below. Dissociated lens epithelial cell cultures were prepared as described previously (Ishizaki et al., 1993). Briefly, lens epithelial cells were harvested by trypsinization of explant cultures. The suspended cells were filtered through nylon gauze, washed, suspended in a 1:1 mixture of DME and Ham's F-12 medium (DME/F-12) containing 0.5% BSA, and 20 cells were plated in 10 μl of medium in each well of a culture dish (model Terasaki; Nunc, Naperville, IL). After 30 min, one quarter of the wells received bFGF (250 ng/ml) plus insulin (5 μg/ml), one quarter received 10% FCS, one quarter received zVAD-fmk (50 μM), and one quarter were left untreated. After 2 d, cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983), as described below. Rat-1 cells, an immortal rat fibroblast cell line, were maintained as previously described (Ishizaki et al., 1995).

Propidium Iodide or Bisbenzimide Staining

Lens explants, unfixed (for bisbenzimide staining) or fixed in 70% ethanol for 10 min at −20°C (for propidium iodide staining), were stained with either bisbenzimide (Hoechst 33342; Frankfurt, Germany; 40 μg/ml in DME/F-12), or propidium iodide (4 μg/ml in PBS containing 100 μg/ml DNase-free RNase A for 30 min at 37°C, and then examined in a fluorescence microscope (model BH2-FLPC; Olympus, Tokyo, Japan).

MTT Assay

MTT was dissolved in DME/F-12 at 5 mg/ml and sterilized by passage through a 0.22-μm filter (Millipore Corp., Waters Chromatography, Milford, MA). This stock solution was added (1:10 parts of medium) to each microwell of the Terasaki plate, and then the plate was incubated at 37°C for 1 h. Viable cells cleave the tetrazolium ring of the MTT into a visible dark blue formazan reaction product, whereas dead cells remain uncolored. The total numbers of live and dead cells were counted in an inverted microscope (model OMT2; Olympus). No proliferation of lens epithelial cells was observed 2 d after plating, even in the presence of FCS or bFGF plus insulin.

TUNEL Assay

A P11 rat lens was fixed in 4% paraformaldehyde, equilibrated in 30% sucrose, frozen in Tissue-Tek O.C.T. compound (Miles, Inc., West Haven, CT),

and cut into 10-μm tangential sections with a cryostat (model Jung Frigocut 2800E; Leica, Inc., St. Gallen, Switzerland). The sections were collected onto gelatin-coated glass microscope slides, air dried, fixed again in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then treated with proteinase K (20 μg/ml) for 30 min at 37°C. After extensive washing, the sections were preincubated for 10 min at room temperature in the reaction buffer for TdT (Boehringer GmbH, Mannheim, Germany). The reaction was started by removing the preincubation buffer and adding the reaction mixture containing 500 U/ml TdT, 2.5 mM CoCl₂, and 40 μM biotinylated dUTP. Control sections were incubated in the reaction mixture without TdT. After 2 h at 37°C, the reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate. The sections were incubated in 2% BSA in PBS for 10 min and then in streptavidin–fluorescein (diluted 1:100; Amersham Intl., Little Chalfont, UK) for 60 min in the dark. After extensive washing in PBS, the sections were mounted in Gel Mount (Biomeda Corp., Foster City, CA) and examined in a fluorescence microscope (model BH2-FLPC; Olympus). In some experiments, lens epithelial explants cultured for 10 d were processed in the same way as described above, but in these cases the sections were examined in a confocal laser scanning microscope (model MRC-1024; Bio-Rad Laboratories, Hercules, CA). Four fields were randomly selected in the sections of explants from each experimental group (control, bFGF plus insulin-treated, and bFGF plus insulin plus zVAD-fmk-treated), and the numbers of TUNEL-negative and -positive nuclei were counted (100–150 total nuclei were assessed in each field).

Western Blot Analysis

Lenses from developing BALB/c mice were sonicated in SDS sample buffer, run on 7.5% SDS-PAGE gels, transferred to nitrocellulose, incubated in the monoclonal anti-PARP antibody C-2-10 (Lamarre et al., 1988; donated by W. Earnshaw [University of Edinburgh, Edinburgh, Scotland] and G. Poirier [Laval University, Ste-Foy, Québec]), and visualized by enhanced chemiluminescence (Amersham Intl.). Lenses from adult (6-wk) mice were dissected (using a dissecting microscope [model Stemi 2000; Carl Zeiss, Jena, Germany]) into two parts—anterior epithelium and outer lens fibers—and each was processed as described above. As controls, Rat-1 cells were incubated in DME/F-12 containing 10% FCS in the absence or presence of STS (4 μM) for 8 h; the cells were then scraped off the culture dishes, sonicated in SDS sample buffer, and processed as above. Lens epithelial explants were sonicated in SDS sample buffer, run either on 7.5% SDS-PAGE gels (for PARP cleavage analysis) or on 15% SDS-PAGE gels (for crystallin analysis), transferred to nitrocellulose, incubated in the monoclonal anti-PARP antibody, or in either a rabbit anti–α-, or anti–β-crystallin antiserum (donated by J. McAvoy, University of Sydney, Sydney, Austrialia), and visualized by enhanced chemiluminescence. The protein concentration of each extract in SDS sample buffer was assayed after precipitation in trichloroacetic acid using a protein assay kit (Bio-Rad Laboratories; Bradford, 1976)

Results

Caspase Inhibition Blocks Epithelial Cell PCD in Culture

We showed previously that rat lens epithelial cells undergo PCD when they are either deprived of survival signals or treated with high concentrations of the protein kinase inhibitor STS (Ishizaki et al., 1993). As shown in Fig. 1 *A*, when dissociated lens epithelial cells were cultured for 2 d at low density in medium containing BSA as the only protein, >80% of the cells died with the characteristic morphological features of PCD; the peptide caspase inhibitor zVAD-fmk (Chow et al., 1995*b*; Zhu et al., 1995; Jacobson et al., 1996) inhibited the PCD, as did FCS or the combination of bFGF and high insulin. We showed previously that lens epithelial cells in explant cultures can live for months in protein-free medium but undergo PCD rapidly in response to STS (Ishizaki et al., 1993); as shown in Fig. 1 *B*, zVAD-fmk markedly inhibited the PCD induced by STS in such explant cultures. These findings suggest
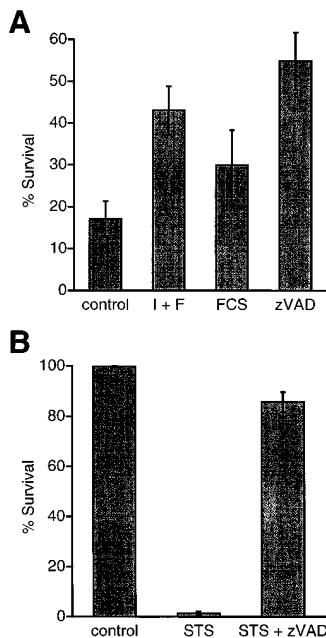
Figure 1. Suppression of PCD in cultured lens epithelial cells by zVAD-fmk. (*A*) Dissociated lens epithelial cells were prepared from P11 rats and cultured at low cell density (20 cells/10 μl) in medium containing BSA and no other protein (*control*), bFGF and insulin (*I+F*), 10% FCS (*FCS*), or 50 μM zVAD-fmk (*zVAD*). After 2 d, cell viability was assessed by the MTT assay. (*B*) Lens explants were treated with 2 μM STS in the absence or presence of 50 μM zVAD-fmk for 24 h. The cells were then fixed and cell viability was assessed by nuclear morphology after staining with propidium iodide. The results are shown as mean ± SEM.

Figure 2. TUNEL labeling of developing lens. (*A*) Drawing of the rat lens. Lens epithelial cells cover the anterior half of the lens; those just above the equator proliferate, migrate inward, and differentiate into elongated postmitotic lens fibers, which eventually lose their nucleus. (*B*) Fluorescence micrograph of a frozen section of a P11 rat lens in the region indicated in *A*, labeled by the TUNEL technique. Bar, 100 μm.
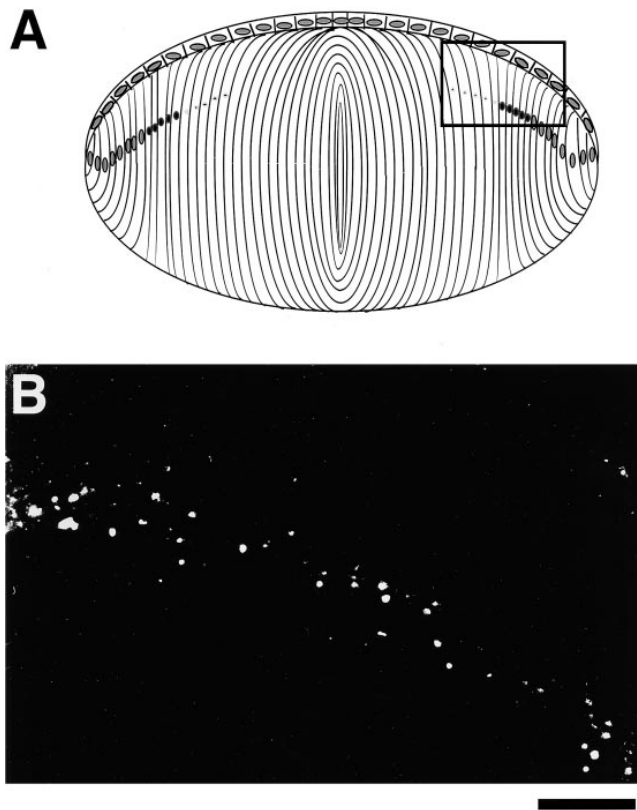
that caspases mediate PCD in lens epithelial cells, as they do in many other cell types (Weil et al., 1996).

Nuclei of Differentiating Lens Fibers Are TUNEL Positive

As discussed in the Introduction, there has been some controversy about the mechanism of nuclear loss during lens fiber differentiation. To help resolve this, we cut frozen sections of P11 rat lens and stained them by the TUNEL technique. As shown in Fig. 2, nuclei in differentiating secondary lens fiber cells were clearly labeled, whereas nuclei in the anterior epithelium were not, suggesting that DNA fragmentation occurs as the lens fiber cells terminally differentiate. No labeling was seen in control sections incubated in the reaction mixture without TdT (data not shown). After these studies were completed, Bassnett and Mataic (1997) reported that differentiating chick lens fiber cells could be labeled by the TUNEL technique.

PARP Is Cleaved during Lens Fiber Differentiation

To determine whether caspases become activated during lens fiber formation, we studied PARP, a known substrate of caspase-3 (Lazebnik et al., 1994; Nicholson et al., 1995; Tewari et al., 1995; Schlegel et al., 1996) and some caspase-3-like caspases (Fernandes-Alnemri et al., 1995*a,b*; Duan et al., 1996). PARP, a 116-kD protein, is cleaved to yield an 85-kD product during PCD (Kaufmann, 1989; Kaufmann et al., 1993; Gu et al., 1995; Nicholson et al., 1995; Tewari et al., 1995). As shown in Fig. 3, when extracts of developing mouse lenses were analyzed by Western blotting with a monoclonal anti-PARP antibody (Lamarre et al., 1988), PARP cleavage was just detectable at P0, and was readily detectable at P5 and P14, when many of the nuclei of secondary lens fiber cells are pyknotic. By P14, very little uncleaved PARP could be detected, although the 85-kD PARP cleavage product could readily be seen. We dissected the lens from a young adult (6-wk-old) mouse into

anterior epithelium and outer lens fibers (comprised of newly formed secondary fibers), and analyzed extracts of each by Western blotting with the anti-PARP antibody. The 85-kD PARP cleavage product was seen clearly in the outer lens fibers, but not in the anterior epithelium (Fig. 3). These findings suggest that caspase-3, and/or a closely related caspase, is activated during lens fiber development.

A Caspase Inhibitor Blocks Denucleation in Differentiating Lens Fibers In Vitro

To determine if the activation of caspases is required for lens fiber differentiation, we analyzed lens fiber development in explant cultures of anterior epithelium isolated from P8–P13 rat lens, as originally described by McAvoy and Richardson (1986). In this culture system, the epithelial cells differentiate into fibers under the influence of either retina-conditioned medium or bFGF. The cells proliferate and migrate to form a multilayered structure in which the upper layer of cells elongate and show many of the morphological changes that are characteristic of lens fibers, including nuclear pyknosis, loss of organelles, and the formation of ball and socket junctions; in addition, the cells initiate the synthesis of β- and γ-crystallins. Transparent
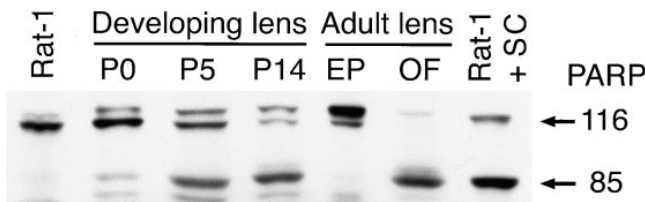
Figure 3. Western blot showing PARP cleavage during lens development. Rat-1 cells, either untreated or treated with 2 μM STS and 10 μg/ml cycloheximide (*SC*) for 12 h, were used as normal and PCD controls, respectively. The arrows indicate the position of uncleaved (116 kD) and cleaved (85 kD) PARP. Sample loading was adjusted to give similar amounts of total PARP in each lane. *EP* indicates epithelial cells; *OF* indicates outer fibers.

lens-like (lentoid) bodies begin to appear in the cultures after ~10 d (Peek et al., 1992). We observed the same sequence of events. Moreover, when examined after 10 d by the TUNEL technique, the proportion of TUNEL-positive nuclei was much greater in the explants treated with bFGF and insulin then in untreated explants (see below), and by 14 d lentoid bodies covered almost the complete surface of the treated explants (Fig. 4 *D*). By contrast, in untreated explants, the epithelial cells did not proliferate or change their shape, the explants remained as a monolayer, and lentoid bodies did not develop (Fig. 4 *C*). When we stained 14 d bFGF and insulin treated cultures with the fluorescent nuclear dye bisbenzimide, we found that all of the lentoid bodies, including the smallest ones, did not contain nuclei (Fig. 4 *B*), suggesting that they formed from differentiated fibers that had lost their nucleus.

When extracts of the explants were analyzed by Western blotting with an anti–β-crystallin antiserum, expression of β-crystallin was found to be much greater in the explants treated with bFGF and insulin (which contained lentoid bodies) than in untreated explants cultured for the same period (which did not contain lentoid bodies; Fig. 4 *F*), suggesting that the bFGF and insulin greatly enhanced lens fiber differentiation in these cultures, as previously described for bFGF (McAvoy et al., 1991; Peek et al., 1992). The small amount of β-crystallin in the untreated control explants may have come from cells at the periphery of the equatorial region of the explanted lens that had begun to differentiate at the time of dissection, or it may reflect a small amount of differentiation that occurred in the absence of bFGF and insulin. There was a smaller increase in α-crystallin in the treated cultures compared to the controls, which is not surprising as α-crystallin is expressed in undifferentiated lens epithelial cells, as well as in differentiated lens fibers (McAvoy, 1980; McAvoy et al., 1991).

The formation of lentoid bodies in the bFGF- and insulin-treated cultures was almost completely suppressed by the addition of the caspase inhibitor zVAD-fmk (Fig. 4 *E*), whereas the addition of the chemically similar cathepsin B inhibitor zFA-fmk (Rasnick, 1985) had no effect (data not shown). Moreover, zVAD-fmk greatly decreased the amount of nuclear pyknosis seen in the cultures treated with bFGF and insulin (data not shown), and, when explants that had been cultured for 10 d were labeled by the TUNEL technique, the percentage of TUNEL-positive nuclei was greatly reduced by zVAD-fmk treatment (mean ± SD: untreated

explants, 0.83 ± 0.31%; explants treated with bFGF plus insulin, 7.5 ± 2.67%; explants treated with bFGF plus insulin plus zVAD-fmk, 1.67 ± 0.69%). By contrast, neither the increased β-crystallin expression (Fig. 4 *F*) nor the increased cell proliferation (data not shown) seen in bFGF and insulin treated cultures was blocked by the addition of zVAD-fmk. When extracts of bFGF and insulin treated cultures (containing many lentoid bodies) were analyzed by Western blotting with anti-PARP antibody, PARP cleavage was readily detected, whereas PARP cleavage was not detected in cultures that were not stimulated with bFGF and insulin (Fig. 4 *G*). PARP cleavage in bFGF and insulin treated cultures was greatly reduced by the addition of zVAD-fmk (Fig. 4 *G*). These findings suggest that the activation of one or more caspases is required for denucleation to occur, at least in culture.

Discussion

We provide three lines of evidence that at least part of the machinery of PCD is involved in lens fiber differentiation. First, we show that the nucleus in developing secondary lens fiber cells can be labeled by the TUNEL technique, confirming the findings of Bassnett and Mataic (1997), who demonstrated DNA fragmentation in differentiating chick lens fibers by both the TUNEL technique and in situ electrophoresis. Together with the earlier findings of Modak and colleagues (Modak and Perdue, 1970; Appleby and Modak, 1977), these results indicate that DNA fragmentation, a characteristic feature of PCD, occurs as lens cells differentiate and lose their nucleus. It is uncertain why some previous studies failed to find TUNEL labeling of secondary lens fiber cell nuclei in the developing mouse lens (Fromm et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Chow et al., 1995b; Robinson et al., 1995). It is possible, as Bassnett and Mataic (1997) suggest, that the discrepancies in TUNEL labeling reflect differences in the way the labeling procedures were carried out. No labeling has been observed, for example, where the sections were not treated with proteinase K before incubation with TdT, while nonspecific labeling has been prominent when streptavidin–fluorescein was used without pretreatment with BSA.

The second line of evidence that components of the cell death program are involved in lens fiber differentiation is that PARP is cleaved in the developing lens. PARP cleavage is a widely used indicator of both PCD and the activation of caspase-3 and/or one or more of its close relatives such as caspase-7 (Alnemri et al., 1996). PARP is cleaved from a 116-kD form to an 85-kD fragment in many examples of PCD (Kaufmann, 1989; Kaufmann et al., 1993; Gu et al., 1995; Nicholson et al., 1995; Tewari et al., 1995), and it is cleaved in a similar way in the developing lens. This finding strongly suggests that caspase-3, and/or a closely related caspase, is activated during lens fiber differentiation, which in turn suggests that some parts of the machinery of PCD are used in normal lens development. It seems likely, for example, that caspase-6 becomes activated and cleaves one or more of the nuclear lamins (Fernandes-Alnemri et al., 1995a; Orth et al., 1996; Takahashi et al., 1996) to help remove the nuclear lamina when the nucleus degenerates in developing lens fibers.

The third line of evidence that lens fiber differentiation
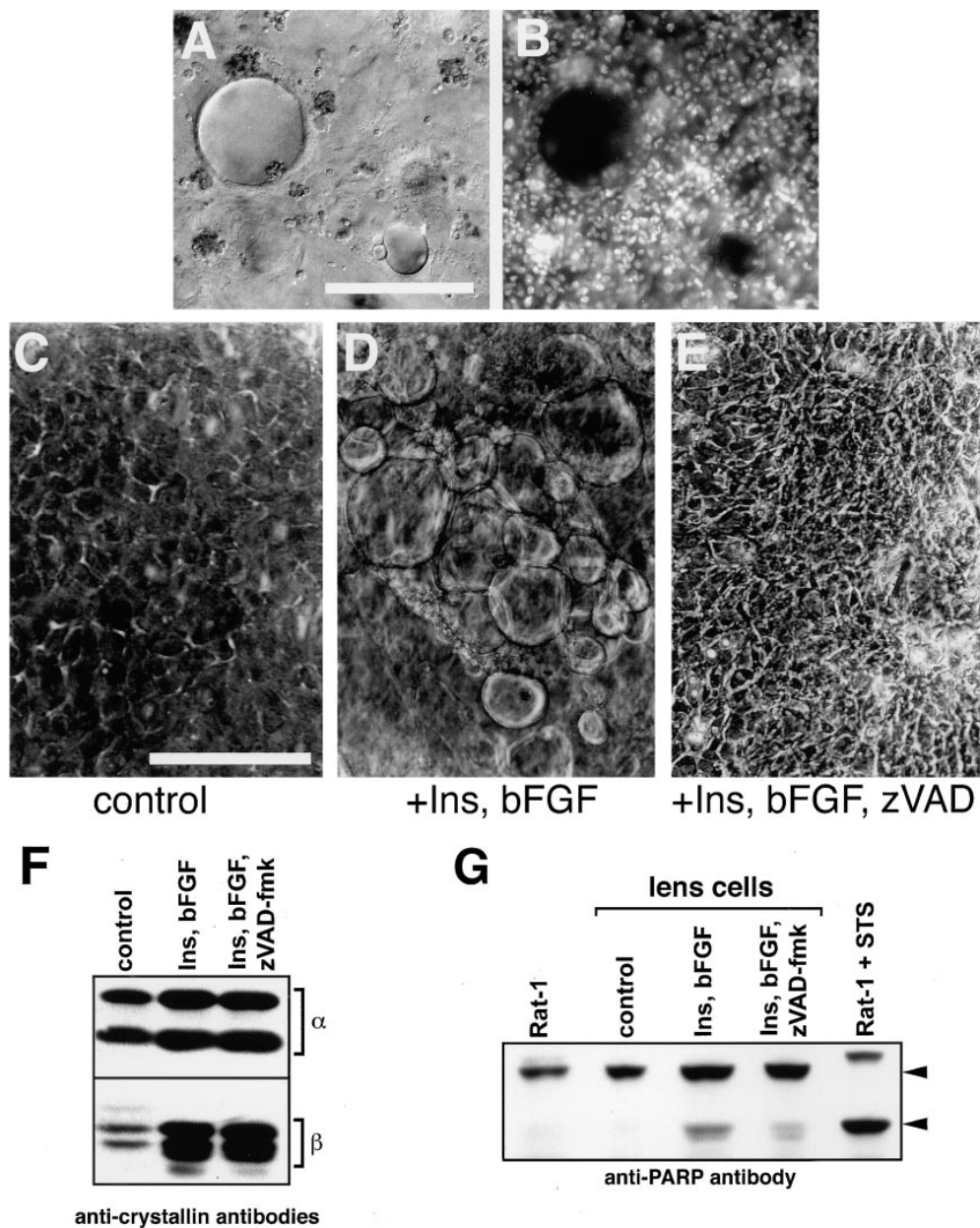
Figure 4. Lens fiber differentiation in culture. Explant cultures of lens epithelium were prepared from P11 rats and cultured in protein-free medium (*control*), medium containing bFGF (250 ng/ml) + insulin (5 µg/ml), or medium containing 50 µM zVAD-fmk + bFGF + insulin. (*A* and *B*) Lentoid bodies in 10-d cultures were stained with bisbenzimide (Hoechst 33342) and viewed by differential interference contrast (*A*) and fluorescence optics (*B*). Note the lentoid bodies do not contain nuclei. (*C–E*) Phase contrast micrographs of explants after 14 d in culture. (*F*) Western blot using rabbit anti–α- or anti–β-crystallin antiserum on extracts of explant cultures after 15 d in vitro. Sample loading was adjusted to give similar amounts of α-crystallin in each lane, which meant that 55.5 µg of total protein was loaded in the control lane, 14.6 µg in the insulin + bFGF lane, and 16.5 µg in the insulin + bFGF + zVAD lane. (*G*) Western blot using monoclonal anti-PARP antibody on extracts of explant cultures after 15 d in vitro. Arrows indicate uncleaved (116 kD) and cleaved PARP (85 kD). The same amount of total protein (∼10 µg) was loaded from each of the lens explant samples. Bars, (*A* and *B*) 200 µm; (*C–E*), 100 µm.

involves the machinery of PCD is that the caspase inhibitor zVAD-fmk, but not the chemically similar cathepsin B inhibitor zFA-fmk, inhibits both the formation of anucleate lentoid bodies and PARP cleavage in explant cultures of lens epithelial cells treated with bFGF and insulin. In this in vitro model of lens fiber differentiation, the production of β-crystallin increases, just as in vivo (Peek et al., 1992), and it is interesting that this increase is not suppressed by zVAD-fmk treatment. These findings suggest that, whereas PARP cleavage and the loss of the nucleus during lens fiber differentiation requires activated caspases, this is not the case for some other aspects of lens fiber differentiation, such as the increased expression of β-crystallin. Thus, lens fiber differentiation is not simply PCD, since the outcome is very different from that of classical PCD, where the cell usually shrinks, frequently fragments, and is rapidly phagocytosed and digested (Kerr et al., 1972; Wyllie et al., 1980); a lens fiber, by contrast, elon-

gates, fills up with crystallins, and persists for the lifetime of the animal.

Taken together, our results provide strong evidence that the differentiation of a lens epithelial cell into a lens fiber involves components of the basic machinery of PCD. It was recently reported that mice in which the caspase-3 gene was deleted by targeted gene disruption die perinatally with an excess of cells in the central nervous system, apparently as a result of decreased PCD in neuroepithelial cells (Kuida et al., 1996). Although PCD was reported to occur normally in other organs, it appears to us that there is an abnormally large number of nuclei in the core of the lens of the caspase-3–deficient mouse shown in Fig. 3 in Kuida et al. (1996), which could reflect a failure of fiber cell denucleation. If so, this finding would provide further evidence that lens fiber differentiation requires components of the basic machinery of PCD.

In addition to lens cell differentiation, there are at least

two other cases where mammalian cell differentiation is accompanied by the loss of the nucleus and other organelles—the development of erythrocytes and skin keratinocytes. It will be interesting to see if the differentiation in these cases is also caspase dependent. This seems likely in the case of keratinocytes, as overexpression of the PCD-suppressing gene *bcl-2* has been reported to inhibit human keratinocyte differentiation in culture (Nataraj et al., 1994).

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