Control of Lens Epithelial Cell Survival

Yasuki Ishizaki, James T. Voyvodic, Julia F. Burne, and Martin C. Raff

Medical Research Council Developmental Neurobiology Programme, Department of Biology, University College London, London WC1E 6BT, United Kingdom

Abstract. We have studied the survival requirements of developing lens epithelial cells to test the hypothesis that most cells are programmed to kill themselves unless they are continuously signaled by other cells not to do so. The lens cells survived for weeks in both explant cultures and high-density dissociated cell cultures in the absence of other cells or added serum or protein, suggesting that they do not require signals from other cell types to survive. When cultured at low density, however, they died by apoptosis, suggesting that they depend on other lens epithelial cells for their survival. Lens epithelial cells cultured at high density in agarose gels also survived for weeks, even though they were not in direct contact with one another, suggesting that they can promote one another's survival in the absence of cell–cell contact. Conditioned medium from high density cultures promoted the survival of cells cultured at low density, suggesting that lens epithelial cells support one another's survival by secreting survival factors. We show for the first time that normal cell death occurs within the anterior epithelium in the mature lens, but this death is strictly confined to the region of the anterior suture.

Compared to the great effort that has been devoted to studying the control of animal cell growth and proliferation, remarkably little work has been done on the control of cell survival. Yet, for some vertebrate cell types at least, it is clear that signals from other cells are required for cell survival, just as they are required for cell growth and proliferation (Sato, 1979; Ham, 1981; Basserga, 1985). Developing neurons, for example, require neurotrophic factors (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; Purves, 1988; Barde, 1989; Oppenheim, 1991), myeloid cells require colony stimulating factors (Metcalf, 1989; Williams et al., 1990; Koury and Bondurant, 1990), endocrine-dependent cells require specific hormones (Kerr and Searle, 1973; Krypaniou and Issacs, 1988; Wyllie et al., 1973, 1980), and developing oligodendrocytes require specific growth factors (Barres et al., 1992). If deprived of their survival factors, these cells die by programmed cell death, usually with the morphological features of apoptosis, in which the cells shrink and often fragment (Kerr et al., 1972; Wyllie et al., 1980; Searle et al., 1982). The same morphological characteristics are displayed by most other cells that die in normal developing or adult tissues, raising the possibility that some of these naturally occurring cell deaths result from a lack of survival factors. Consistent with this possibility, some of these normal cell deaths can be prevented or postponed by experimentally increasing the level of survival factor in the animal (Hamburger et al., 1981; Hofer and Barde, 1988; Oppenheim et al., 1988; Barres et al., 1992).

One of the best understood examples of cell survival control in vertebrates is provided by developing sympathetic neurons, which require NGF to survive. They are produced in larger numbers than are needed and then seem to compete with one another for the limiting amounts of NGF released by the target cells they innervate; only about half the neurons get enough NGF to survive, while the others die (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; Purves, 1988; Barde, 1989; Oppenheim, 1991; Korsching and Thoenen, 1983; Levi-Montalcini, 1987). In this way the number of sympathetic neurons innervating a population of target cells is automatically regulated to match the number of target cells. A similar competition for target-cell-derived neurotrophic factors may operate widely in the developing vertebrate nervous system to match the number of presynaptic and postsynaptic cells during both evolution (Purves, 1988) and development (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; Purves, 1988; Barde, 1989; Oppenheim, 1991).

We recently speculated that a similar mechanism may also operate for non-neuronal cells to help match the numbers of different cell types in various organs (Raff, 1992). In the most extreme form of this hypothesis, it is proposed that all cells in higher animals, except for blastomeres (Biggers et al., 1971), are programmed to kill themselves unless they are continuously signaled by other cells not to do so. In our first test of this hypothesis we found that newly-formed oligodendrocytes in the rat optic nerve behave similarly to developing neurons: ~50% of them die during normal development,

Address correspondence to Dr. Raff at Medical Research Council Developmental Neurobiology Programme, Department of Biology, Medawar Building, University College London, London WC1E 6BT, United Kingdom.

they cannot live on their own in culture in the absence of survival signals produced by other cells, and experimentally increasing the concentration of one such survival factor, PDGF, in developing rats greatly decreases their death in the optic nerve (Barres, 1992).

In the present study, we set out to challenge the most extreme form of the death-by-default hypothesis by studying the survival requirements of rat lens epithelial cells. The lens forms from the ectoderm at the site where the developing optic vesicle makes contact with it: the ectoderm here thickens, invaginates and finally pinches off to form the lens vesicle (Goss, 1978; McAvoy, 1980). The epithelial cells at the back of the vesicle differentiate into elongated lens fibers, which become filled with crystallins and eventually lose their nuclei and stop making protein (Goss, 1978; McAvoy, 1980). The cells in the more anterior epithelium proliferate, migrate or get displaced rearwards, and differentiate into lens fibers (Goss, 1978; McAvoy, 1980). Thus, the lens is an unusual organ in that it contains only one cell type. In its mature form, it is neither vascularized nor innervated and contains no lymphatic vessels (Goss, 1978). If there are any mammalian cells, other than blastomeres, that can survive without signals from other cell types, lens epithelial cells should be among them. We show here that although lens epithelial cells can survive for weeks in high density cultures in the absence of signals from other cell types, they undergo apoptosis when they are cultured at low density, suggesting that they depend on other lens epithelial cells for their survival. We provide evidence that lens cells support one another's survival by secreting survival factors. We also show for the first time that normal cell death occurs in the lens epithelium, not only during development, but also in the adult; this death is strictly confined to the region of the anterior suture (Davson, 1990), except at the time of lens vesicle formation.

Materials and Methods

Animals and Materials

Sprague-Dawley rats were obtained from the breeding colony of the University College London Animal Facility. Recombinant human PDGF-AA, PDGF-BB, TGF-α, TGF-β2, interleukin (IL)[1]-1β, and basic FGF were purchased from Peprotech, Inc. (Rocky Hill, NJ). Insulin, propidium iodide, RNase A, poly-D-lysine, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cysteine, cystine, soybean trypsin inhibitor, trypsin, BSA, 2-mercaptoethanol (ME), staurosporine, aurintricarboxylic acid (ATA) and laminin were purchased from Sigma Chemical Co. (St. Louis, MO). FCS, high melting point (Tm) agarose, and low Tm agarose were purchased from GIBCO-BRL (Gaithersburg, MD). EDTA was purchased from BDH Laboratory Supplies, Merck, Ltd. (Poole, UK). DME and Ham's F-12 medium (F-12) were purchased from Flow Laboratories, (Irvine, UK). NGF was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Insulinlike growth factor (IGF)-1 was provided by Kabigen AB (Stockholm, Sweden). Interleukin (IL)-6 was purchased from R & D Systems, Inc. (Minneapolis, MN).

Frozen Sections and Propidium Iodide Labeling

Heads or eyes obtained from rat embryos (embryonic day 12 [E12] to E20) and lenses obtained from postnatal rats (up to 38 days postnatal[P38]) were incubated in 4% paraformaldehyde at 4°C overnight and transferred to 30% sucrose in PBS until equilibrated. Lenses were frozen in Tissue-Tek O.C.T. compound (Miles, Inc., West Haven, CT) and 7 μm tangential frozen sections were cut with a Bright cryostat. The sections were collected onto gelatinized glass microscope slides, air dried, post-fixed in 70% ethanol for 10 min at −20°C, and stained with a propidium iodide solution (4 μg/ml), containing DNase-free RNase A (100 μg/ml) in DME for 30 min at 37°C (Rodriguez-Tarduchy et al., 1990; Barres et al., 1992). The slides were washed three times in PBS, mounted in Citifluor (City University, London, England), examined in a Zeiss Universal fluorescence microscope and photographed with Tri-X film rated at ASA 400. In some cases, the slides were examined in a Bio-Rad MRC 600 (Bio-Rad Laboratories, Richmond, CA) scanning confocal microscope equipped with an argon-xenon laser, and the images were printed from the computer using a Mitsubishi color video copy processor.

Whole Mount Preparations of Lens Epithelium

Lenses from P4 to adult rats were rinsed in PBS. In some experiments, the anterior suture was marked with fine particles of charcoal, using a needle. The lenses were fixed in ethanol/acetic acid (3:1) for 24 h and then stored in 70% ethanol in DME until used (Howard, 1952; McAvoy, 1978). After a rinse in PBS, lenses were stained with a propidium iodide solution as above, and then washed three times in PBS. At this stage, the equatorial zone could be identified as a pink line. A cut was made just below this line and the posterior half of the lens was discarded and the anterior lens capsule, with attached epithelial cells, was peeled off with a needle. Several incisions were made at the edge of the isolated capsule, which was then mounted in Citifluor on a gelatinized glass slide (Stone, 1981).

Explant Cultures

Lens epithelial explant cultures were prepared according to McAvoy and Fernon (1984), except that the explanted tissue was not trimmed. Lenses were removed from P11 rats and gently rolled on sterilized filter paper to remove any cells adhering to the lens capsule. The lens capsule was then torn at the posterior pole and separated from the lens fibers, using two pairs of forceps. The capsule, with adhering epithelial cells, was pinned down around its periphery to the surface of the culture dish (35 mm; Falcon Labware, Oxnard, CA), with the epithelial cells facing upward. In some experiments, the explants were pinned down with the epithelial cells facing downward; in these cases the culture dish was first coated with either poly-D-lysine (10 μg/ml, 130 K) or laminin (0.1 mg/ml) (Parmigiani and McAvoy, 1991). The explants were incubated in a mixture (1:1) of DME and F-12 (DME/F-12).

Trypsinization of Cells in Explant Cultures

Lens explants in 35 mm tissue culture dish were washed once with Ca^{2+}- and Mg^{2+}-free DME, and incubated with a mixture of trypsin (0.05%) and EDTA (0.025%) in Ca^{2+}- and Mg^{2+}-free DME for 8 min at 37°C. The incubation was stopped by the addition of 0.1% soybean trypsin inhibitor in DME/F-12, and the debris of the lens capsules was removed by filtering through Nylon gauze. The cells were washed once in DME/F-12 containing 0.5% BSA (highly purified, crystalline grade) and resuspended in an appropriate volume of either DME/F-12 for microcultures or F-12 for agarose gel cultures.

Microcultures

Trypsinized lens epithelial cells suspended in DME/F-12 were seeded into the 10-μl wells of a Terasaki plate (Nunc, Roskilde, Denmark). 30 min after plating, 1 μl of DME/F-12 or of DME/F-12 containing 10 mM cysteine, was added to each well, and plates were transferred to a 5% CO_2 incubator. In most experiments uncoated plates were used, but in some the wells were coated with either poly-D-lysine (10 μg/ml) or laminin (0.1 mg/ml) before use. No significant difference was observed whether coated or uncoated plates were used.

Agarose Gel Cultures

Agarose gel cultures were set up in 96-well tissue culture plates (Falcon Labware) as described by Benya and Schaffer (1982), with some modifications. High Tm agarose (1% in water) was autoclaved for 20 min and used while above 90°C. Each well was rapidly coated with agarose and the excess

1. *Abbreviations used in this paper*: ATA, aurintricarboxylic acid; DME/F-12, mixture (1:1) of DME and F-12; F-12, Ham's F-12 medium; IGF, insulin-like growth factor; IL, interleukin; ME, 2-mercaptoethanol; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; Tm, melting point.

agarose was removed by aspiration. Plates were placed on a level surface at room temperature for the agarose to solidify and were then transferred to a CO_2 incubator until used. Cell suspensions were prepared in low Tm agarose (2% in water), which was autoclaved for 20 min and then cooled to 37°C before being mixed with an equal volume of 2× concentrated DME at 37°C to give a final concentration of 1% agarose in DME.

Trypsinized lens epithelial cells suspended in F-12 were mixed with an equal volume of 1% agarose in DME at 37°C to give the appropriate cell densities in 0.5% agarose in DME/F-12. 100 μl of the cell suspension was added to each well of the tissue culture plate that had been previously coated with a thin layer of high Tm agarose. The plates were held at 37°C in a CO_2 incubator for 15 min before the agarose was allowed to gel at 4°C for 15 min. After gelation, an additional 100 μl of DME/F-12 was added to each well, and the plates were transferred to a CO_2 incubator.

Preparation of Conditioned Medium

Lens epithelial cells were cultured in agarose gel as described above, at a density of 2×10^6 cells/ml. 100 μl of medium was removed and replaced by fresh medium containing 1 mM cysteine every 12 h. The conditioned medium harvested in this way was immediately transferred to low density (10^5 cells/ml) agarose gel cultures, which were fed with conditioned medium every 12 h.

Cell Survival Assay

Cell survival was assessed by an MTT assay, which measures mitochondrial function (Mosmann, 1983). MTT was dissolved in DME/F-12 at 5 mg/ml and sterilized by passage through a filter (0.22 μm) (Millipore Corp., Bedford, MA). This stock solution was added (one part to 10 parts of medium) to each well of the Terasaki plates or 96-well tissue culture plates, and the plates were incubated at 37°C for 1 h. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazin reaction product.

In Terasaki plates, all cells, both live or dead, adhered to the plastic. The total numbers of live and dead cells were counted in an inverted microscope. In agarose gel cultures, four fields were randomly selected per well and the numbers of live and dead cells were counted.

Time-lapse Video Recording

Lens epithelial explants were pinned down with cells facing downward in the center of a laminin-coated 35-mm tissue culture dish in 2 ml of DME/F-12 medium, as described above. After 24 h in culture, the dish was placed on the stage of an inverted Zeiss microscope and maintained at 37°C in a humid 95%, 5% CO_2 environment, and the cells were viewed using phase-contrast microscopy. Time-lapse recordings were made using a CCD video camera coupled to a time-lapse video tape recorder (Panasonic model AG6720A, Matsushita Electronic Industrial Co., Ltd., Osaka, Japan), which acquired images at a rate of one image every 6 s. At the same time, images were also captured once every 4 min by a computer-controlled image processor (Matrox MVP-AT, Matrox [UK] Electronic Systems, Ltd., Swindon, UK); these images were signal averaged and enhanced and then later transferred to videotape to make a high speed summary of the recording period. After 24 h of recording, the process of death was observed from the videotapes, after the fate of cells as they migrated from the edge of the explant. For observation of the fate of dissociated cells, the cells in the lens explant were trypsinized, plated at the density of 10^5 cells/ml in a 35-mm tissue culture dish in 2 ml of DME/F-12 medium, and studied by time-lapse video recording, as described above.

Electron Microscopy

Eyes from P7 rats were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 60 min. Lenses were taken out and placed in fresh fixative overnight at room temperature. After rinsing with 0.1 M phosphate buffer (pH 7.2), the lenses were postfixed with 1% osmium tetroxide in the buffer for 2 h, rinsed in distilled water, and stained en bloc with 1.0% uranyl acetate, all at 4°C in the dark. Lenses were then washed in distilled water, dehydrated with a graded series of alcohol, and embedded in Epon. Thin sections were cut on an LKB ultratome (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), counterstained with uranyl acetate, and examined using an electron microscope (model 100 CX II, Jeol Ltd., Tokyo, Japan) at 80 kV.

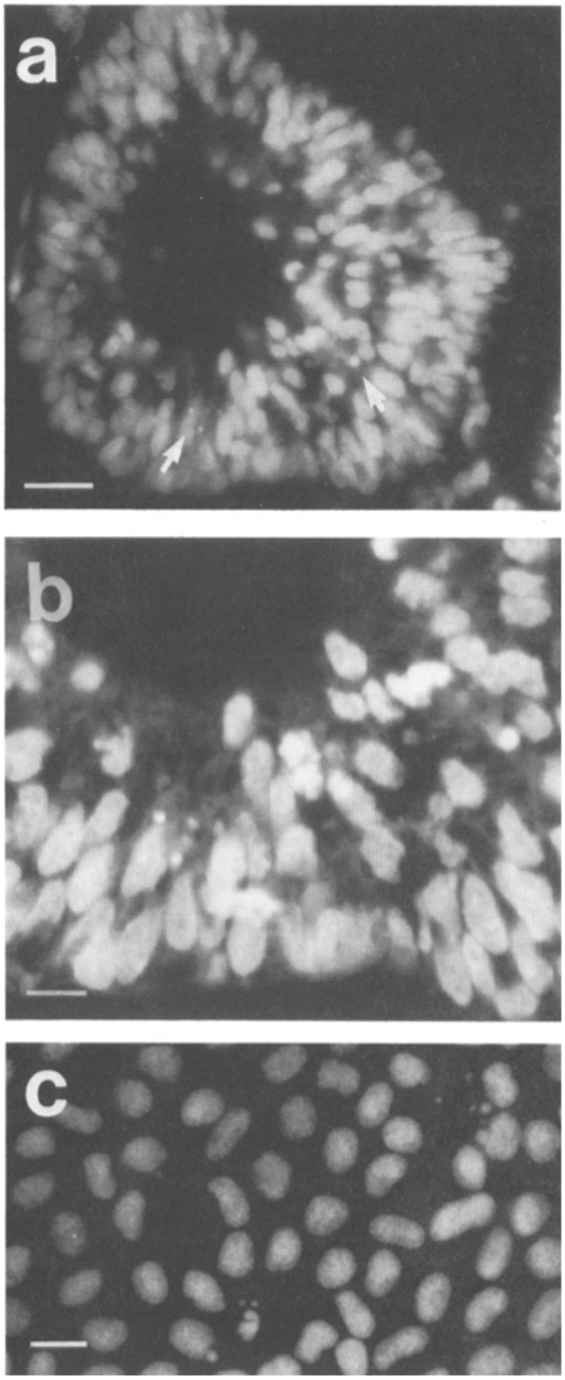

Figure 1. Normal cell death in the developing lens epithelium. (*a*) A fixed and frozen E12 head was sectioned coronally and labeled with propidium iodide to stain the nuclei of normal and dead lens epithelium cells (*arrows*). (*b*) Higher magnification of dead lens cells in E12 lens. (*c*) A lens from P21 rat was fixed and labeled with propidium iodide, and the anterior epithelium in the region of the anterior suture was examined as a wholemount; note the two dead cells with fragmented nuclei. Bars, 25 μm in *a*, 10 μm in *b*, and 10 μm in *c*.

Results

Normal Cell Death in the Developing Lens

To determine whether lens epithelial cells die during normal

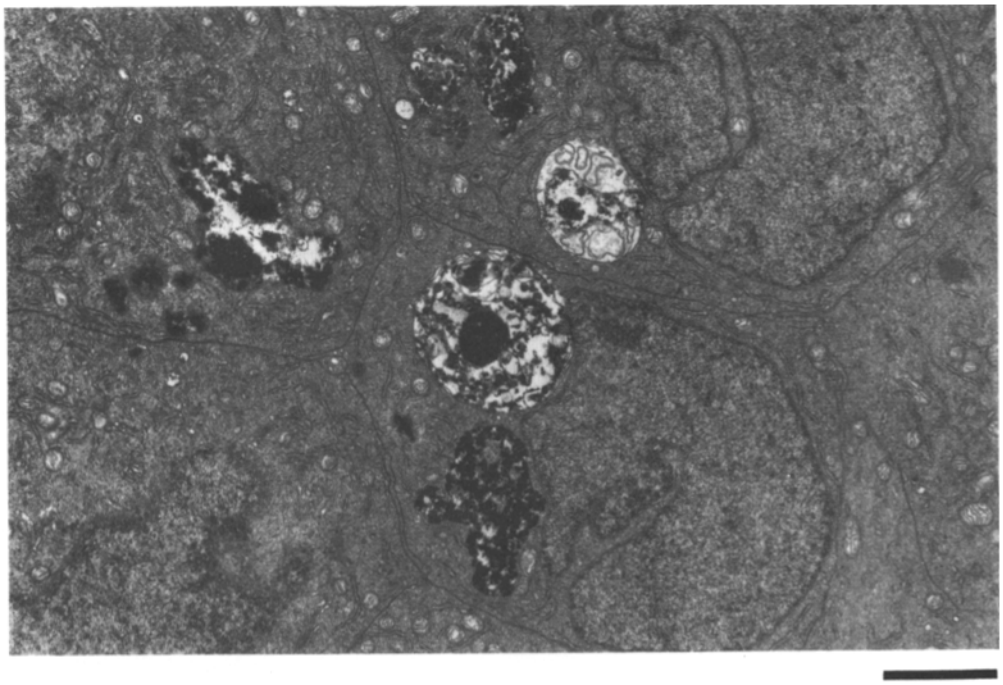

Figure 2. Ultrastructure of apoptotic bodies in the anterior suture of the lens. A lens from P7 rat was fixed and processed for electron microscopy. The apoptotic bodies have been phagocytosed by neighboring lens epithelial cells. Bar, 2.5 μm.

development, we looked for pyknotic cells in frozen sections and whole mount preparations of lenses, after staining with the fluorescent nuclear stain propidium iodide. Pyknotic cells were identified with phase-contrast optics by their shrunken, phase-dark appearance, and with fluorescence optics by their condensed, sometimes fragmented nucleus. Such cells were frequently observed in lenses of 12-d-old embryo (Fig. 1, *a* and *b*), which is the time when the lens pit deepens and breaks away from the overlying ectoderm to form the lens vesicle (Goss, 1978; McAvoy, 1980). Pyknotic cells were rarely seen, however, in lenses from older embryos or from postnatal rats up to 38 d postnatal (P38), except in the region of the anterior suture, where the anterior ends of lens fibers converge (Davson, 1990). In this region, ~5% of cells were pyknotic, and usually fragmented, in lenses from P4 to P21 rats (Fig. 1 *c*). When this region was examined by electron microscopy, apoptotic bodies were found phagocytosed by neighboring lens epithelial cells (Fig. 2). Pyknotic cells were also observed in this region in older animals (body wt >250 g), although their frequency (~1%) was lower than in younger animals.

Cell Survival in Explant Cultures of Lens Epithelium Does Not Require Exogenous Protein

To determine the requirements for lens epithelial cells to survive in culture, we explanted the lens capsule, with attached epithelial cells facing upward, on a plastic culture dish in serum-free DME/F-12 medium containing no added protein. The lens epithelial cells survived for more than 1 mo under these conditions, suggesting that lens cells do not require signals from other cell types to survive in culture. Cell viability was assessed by phase-contrast microscopy (Fig. 3 *a*), propidium iodide staining and fluorescence microscopy (Fig. 3 *b*) and by the MTT assay (not shown).

To exclude the possibility that the survival of the lens epi-

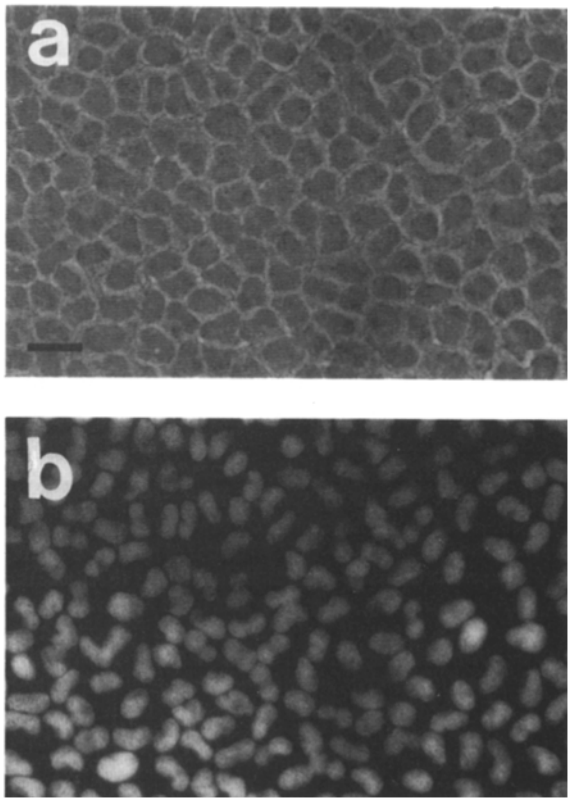

Figure 3. Long-term survival of lens epithelial cells attached to the lens capsule in protein-free explant cultures. The anterior half of the lens capsule from a P11 rat, with attached epithelial cells upward, was pinned down to the bottom of a tissue culture dish and cultured in serum-free medium containing no added protein. After one month, the cells were examined by phase-contrast (*a*) or by fluorescence microscopy after propidium iodide staining (*b*). Bar, 25 μm.

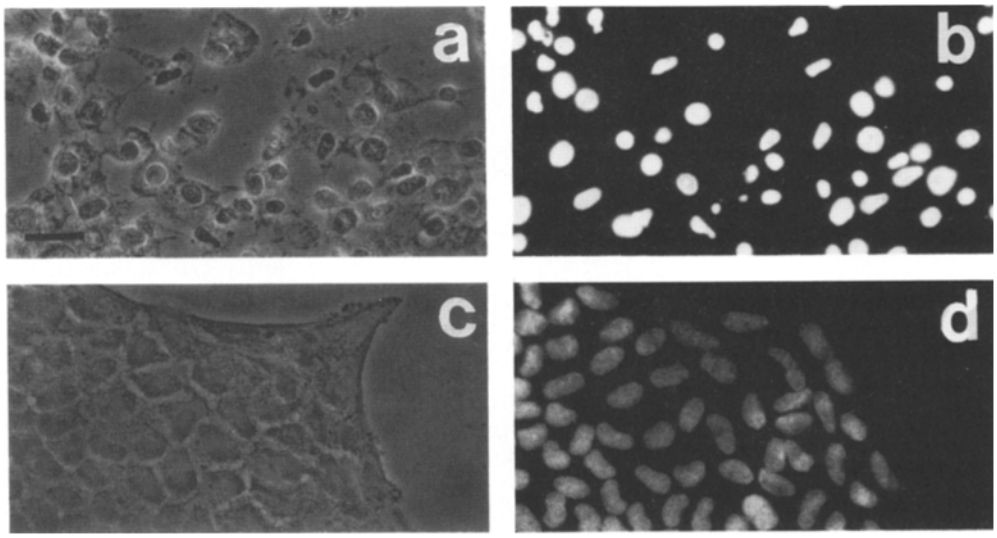

Figure 4. Apoptosis in cells growing out from the edge of a lens epithelium explant. The anterior half of the lens capsule from a P11 rat, with attached epithelial cells facing downward, was cultured on a laminin-coated culture dish in serum-free medium containing no added protein. Many of the cells that migrated from the edge of the capsule died on the second day after explanting, as shown here by phase microscopy (*a*) or or by fluorescence microscopy after propidium iodide staining (*b*). Cysteine (1 mM) completely prevented this death, as shown by phase microscopy (*c*) or by fluorescence microscopy after propidium iodide staining (*d*). Bar, 25 μm.

thelial cells in these explants depended on extracellular proteins adsorbed onto the lens capsule, we wished to test cultures in which the lens cells would migrate off the capsule so that the capsule could be removed. We therefore cultured the anterior lens capsule with its attached epithelial cells facing downward. In uncoated culture dishes, the cells remained attached to the capsule and did not migrate onto the plastic dish; as before, explants under these conditions survived for more than 1 mo in protein-free medium. If the culture dish was coated with laminin, however, the epithelial cells migrated out from under the lens capsule as a monolayer and formed a halo of cells around the capsule (Parmigiani and McAvoy, 1991). Frequently, the cells at the edge of the halo began to die on the second day after explanting. In some cases, cells stopped dying and migrated further, but, in other cases, death continued until most of the cells that had migrated from the explant died, usually within a few days. When examined by phase-contrast microscopy (Fig. 4 *a*) or by fluorescence microscopy after staining with propidium iodide (Fig. 4 *b*), the cells that died in the outgrowth area had the typical morphological features of apoptosis. Time-lapse video recording confirmed that these cells died by typical apoptosis: the cells showed active surface blebbing and then shrank (not shown). The time-lapse studies also showed that some lens epithelial cells could divide in the absence of serum or added proteins (other than laminin).

Cysteine Prevents Cell Death in the Outgrowth Zone of Explant Cultures

As cysteine and pyruvate have been reported to prevent the death of chondrocytes in protein-free cultures (Tschan et al., 1990), we tested the ability of these molecules to prevent the death of lens epithelial cells in the outgrowth areas from explant cultures on laminin. Pyruvate, even at concentrations as high as 5 mM, did not prevent cell death at the edge of these explants. Neither did the reduced or oxidized form of glutathione (1 mM). Cysteine (1 mM), however, completely prevented the death (Fig. 4, *c* and *d*), as did cystine (1 mM), although not as completely as cysteine. ME, at a concentration of 50 μM, also completely prevented the death (not shown). Whereas both basic FGF (10 ng/ml) and 10% FCS stimulated migration and proliferation of lens cells, neither prevented the cell death at the edge of explants; again cysteine completely prevented the death that occurred in the presence of basic FGF or FCS (not shown).

When explants on laminin were cultured in the presence of cysteine, the monolayer of cells that grew out from the explant survived for many weeks in protein-free medium, even after the lens capsule was removed from the culture with a pair of forceps. Thus, lens cells can survive in monolayer cultures in protein-free medium in the absence of the lens capsule.

Cell Survival in Dissociated-Cell Microcultures Is Density Dependent

The results obtained with explant cultures suggested that either a lens epithelial cell does not require signals from other cells to survive or the signals it requires can be provided by other lens epithelial cells.

To try to distinguish between these two possibilities, we cultured dissociated cells at low density. We dissociated lens epithelial cells from explant cultures with trypsin and EDTA and then plated the cells into the 10 μl wells of a Terasaki microculture plate. All of the cells, both live or dead, adhered to the plastic, even in the absence of FCS, poly-D-lysine, and laminin. Although the initial viability of the cells was low, with a survival rate at 1 h of 49 ± 8% (mean ± SD of four cultures), when plated at high density (≥1,000 cells/well), either in FCS on the plastic substratum or in protein-free medium on laminin, the viable cells formed a confluent monolayer and survived for many weeks. Cell survival in the microwells, however, was strikingly cell-density dependent. The survival rate in protein-free medium at 1 d was 32 ± 3% at 200 cells/well, 14 ± 4% at 100 cells/well, and 1 ± 2% at 30 cells/well; no cells survived for more than a day when cultured at 5 cells/well. At 5 cells/well, cysteine

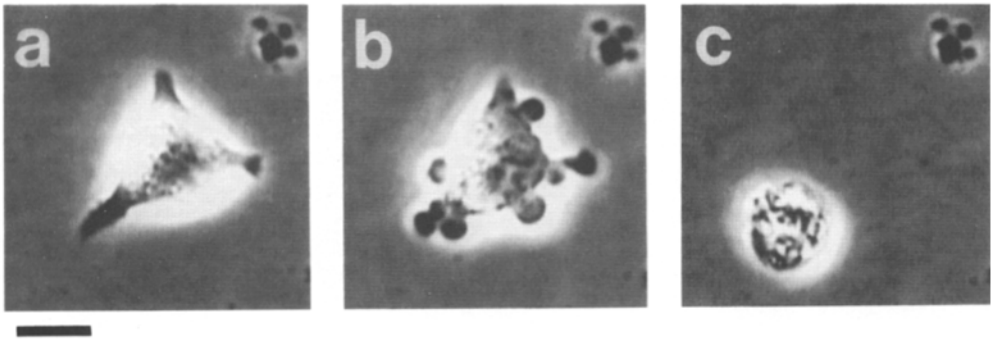

Figure 5. Apoptotic death of a dissociated lens cell revealed by time-lapse video recording. The epithelial cells in a lens explant were trypsinized, plated at the density of 10^5 cells/ml on a plastic dish, and studied by time-lapse video recording. The cell depicted in this figure showed two episodes of vigorous surface membrane blebbing before it died. After the first episode, which began 27 h after plating and lasted for 1 h, it looked apparently healthy for 4 h (*a*). But, in the second episode, after the vigorous surface membrane blebbing of 1.5 h duration (*b*), it eventually stopped all its movements and shrank (*c*). Bar, 10 μm.

(1 mM) greatly improved survival at 1 d, but not longer: 46 ± 4% of the cells survived at 1 d, but none survived for 2 d. Time-lapse video recording showed that cells plated at low density exhibited two or three episodes of vigorous blebbing of their plasma membrane before they finally died with the characteristic features of apoptosis. Each episode of blebbing lasted for ~1 h and was separated from the next episode of blebbing by an apparently healthy, quiescent phase of 3–5 h duration. In the final episode, a cell eventually stopped all of its motions and shrank (Fig. 5).

We tested a variety of other substances to see if they could improve survival in the low density cultures (5 cells/well) in the presence or absence of cysteine, and none of them did. These included 10–20% FCS, basic FGF (10 ng/ml), insulin (5 μg/ml), IGF-1 (10 ng/ml), TGF-α (10 ng/ml), TGF-β2 (10 ng/ml), PDGF-AA (10 ng/ml), PDGF-BB (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml), and NGF (10 ng/ml), alone and in various combinations (not shown). Coating the wells with poly-D-lysine or laminin did not improve survival. These findings suggested that individual lens cells require help from other lens cells to survive in culture, but it was unclear whether this help required cell–cell contact or could operate at a distance. To find out, we cultured the cells in agarose gels, where cells could be maintained at various densities under conditions where they could not contact one another.

Lens Cells in Agarose Gel Cultures Promote One Another's Survival by Secreting Survival Factors

As was the case in microcultures, lens epithelial cell survival in agarose gel culture was strikingly density dependent (Fig. 6). When cultured at a density of 10^6 cells/ml, most of the cells that survived for 2 d were still alive after 7 d, even though the cells were not in direct contact with one another (Fig. 7). By contrast, when cells were cultured at $\leq 10^5$ cells/ml, none survived for 1 d (Fig. 6). Thus, lens epithelial cells can promote one another's survival without cell–cell contact. In these low density cultures, cysteine improved survival for 3 d but not for longer (Fig. 6).

When conditioned medium from high density (2×10^6 cells/ml) agarose gel cultures was added to low density (10^5 cells/ml) agarose gel cultures, it increased the survival of cells in the low density cultures up to the level of survival seen in high density cultures: 26 ± 5% survived for 3 d in control low density cultures, whereas 46 ± 8% survived for 3 d in 50% conditioned medium, but only if fresh conditioned medium was added every 12 h (all of these experiments were carried out in the presence of 1 mM cysteine). Thus, lens epithelial cells can help other lens epithelial cells to survive by secreting soluble factors.

When added to low density agarose gel cultures, insulin,

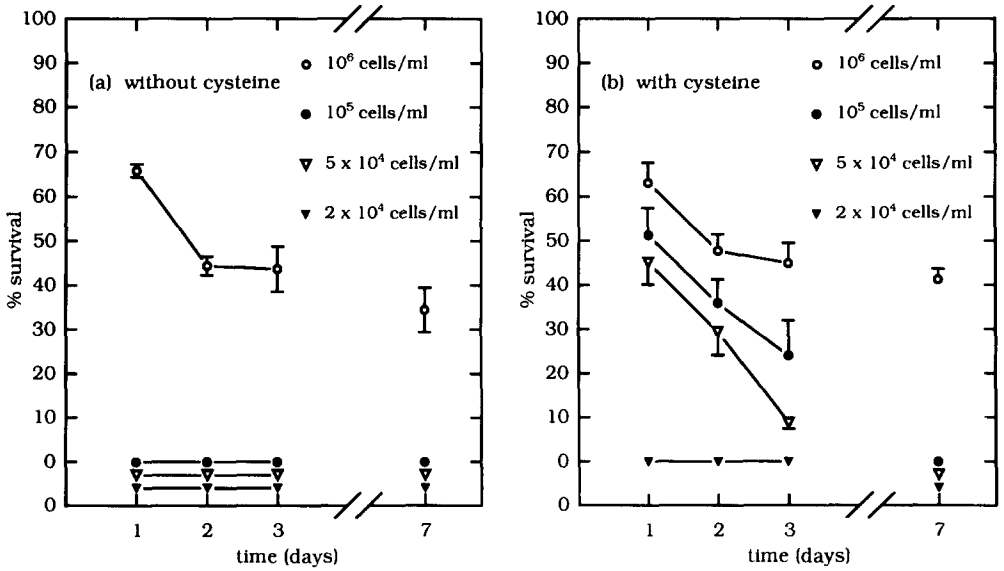

Figure 6. Survival of dissociated lens epithelial cells in agarose gel cultures. Lens epithelial cells were dissociated from explant cultures with trypsin and cultured in agarose in DME/F-12 at various densities in 96-well tissue culture plates. Half of the wells received 1 mM cysteine. MTT assays were performed on the days indicated, and the results are expressed as means ± SD of four experiments. Survival at 1 h after plating was 62 ± 13%.

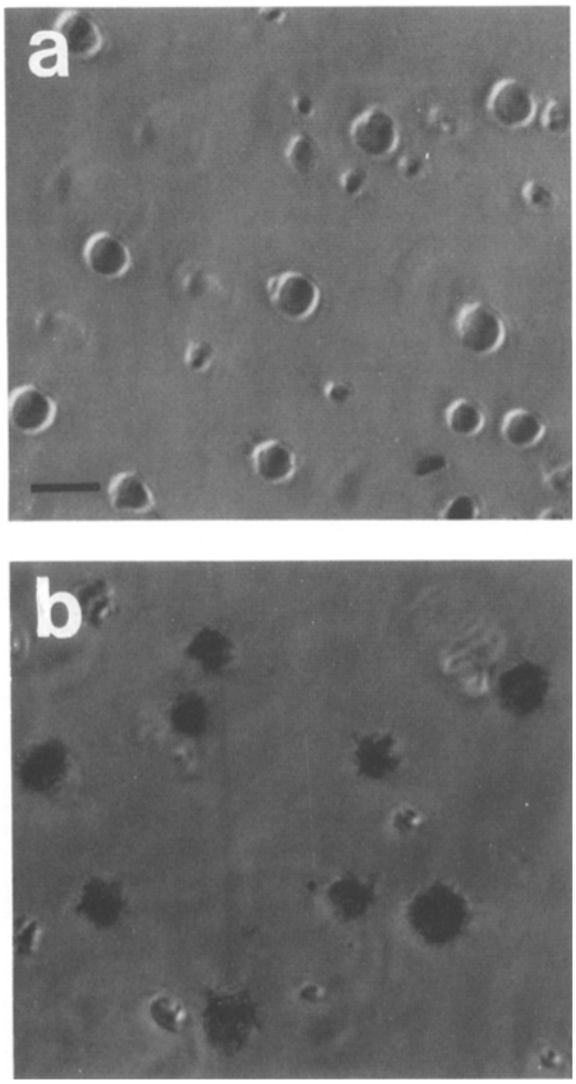

Figure 7. Survival of lens epithelial cells in agarose gel culture examined by phase-contrast microscopy (*a*) or MTT assay (*b*). The cells were dissociated from lens explant cultures and cultured for 1 wk at high density (10^6 cells/ml) in an agarose gel in serum-free medium containing no added protein. Different cultures are shown in *a* and *b*, and the live cells are black in (*b*). Bar, 10 μm.

IGF-1, basic FGF, TGF-α, TGF-β2, PDGF-AA, PDGF-BB, IL-1β, IL-6, NGF, alone or in combination, did not improve cell survival (not shown). FCS, however, promoted both cell survival and proliferation, so that about 25% of the cells were still alive after 7 d.

Staurosporine Induces Lens Epithelial Cells to Undergo Apoptosis

If lens cells promote the survival of other cells by secreting survival signals, one would predict that inhibiting intracellular signaling pathways might abolish these cell–cell interactions and cause lens cells, even at high density, to die by apoptosis. Treatment of lens explants with a high concentration (1 μM) of the protein kinase inhibitor staurosporine induced almost all of the cells to die within 48 h with the morphological features of apoptosis (Fig. 8, *c* and *d*). ATA (100 μM), an inhibitor of endonucleases, largely prevented the cell death induced by staurosporine (Fig. 8, *e* and *f*).

Discussion

McAvoy and Fernon (1984) showed previously that lens epithelial cells can survive on their capsule in culture in the absence of serum or added proteins. We have extended their findings and show that these cells can survive in explants or high density dissociated cell cultures for weeks in serum-free and protein-free culture, even in the absence of their capsule, indicating that they do not require signals from other cell types to survive. This has also been shown to be the case for chondrocytes (Bruckner et al., 1989; Tschan et al., 1990). Lens and cartilage are unique in that they both contain only one cell type and are not penetrated by blood vessels, lymphatic vessels or nerves. It is perhaps not surprising, therefore, that these cells do not depend on signals from other cell types for their survival, although, in principle, there was no good a priori reason to expect that their survival does not depend on hormones or paracrine signals from neighboring tissues.

Although lens epithelial cells do not require signals from other types of cells to survive in culture, they do depend on other lens epithelial cells for survival: they die with the morphological features of apoptosis when cultured at low cell density in the absence of serum or added proteins. How do lens cells promote the survival of other lens cells in culture? Three lines of evidence suggest that they do so by secreting survival factors. First, lens epithelial cells can promote one another's survival when they are maintained in high density agarose gel cultures, where the cells are not in direct contact with one another. Second, conditioned medium from high density lens cell cultures promotes the survival of lens cells in low density cultures; the finding that the conditioned medium has to be replenished every 12 h to see the effect suggests that the survival factors are either labile or rapidly consumed. Third, when lens cells in explants are treated with a high concentration of staurosporine, a protein kinase inhibitor, almost all of the cells died with the morphological features of apoptosis, as one might expect of cells that require continuous signaling from other cells to avoid programmed cell death. Moreover, the staurosporine-induced cell death is prevented by ATA, an endonuclease inhibitor that has been shown to suppress apoptosis in hemopoietic cells deprived of survival factors (McConkey et al., 1989; Shi et al., 1990; Batistatou and Greene, 1991; Crompton, 1991). We showed previously that staurosporine-induced apoptosis in human fibroblasts in culture is suppressed by the over-expression of the *bcl-2* proto-oncogene (Jacobson et al., 1993), which has been shown to suppress programmed cell death in a variety of hemopoietic cells (Vaux et al., 1988; Nunez et al., 1990; Strasser et al., 1991) and in sympathetic neurons (Garcia et al., 1992).

We have not identified the survival signals secreted by lens epithelial cells in culture. Although FCS promotes the survival of lens cells in low density agarose gel cultures, growth factors that have been shown to be made by, or act on, lens cells (reviewed in McAvoy and Chamberlain, 1990), including IGF-1, basic FGF, and PDGF, do not. Neither does insulin, TGF-α, TGF-β2, IL-1β, IL-6, nor NGF. Even a combination of all of these growth factors is ineffective. Whatever the

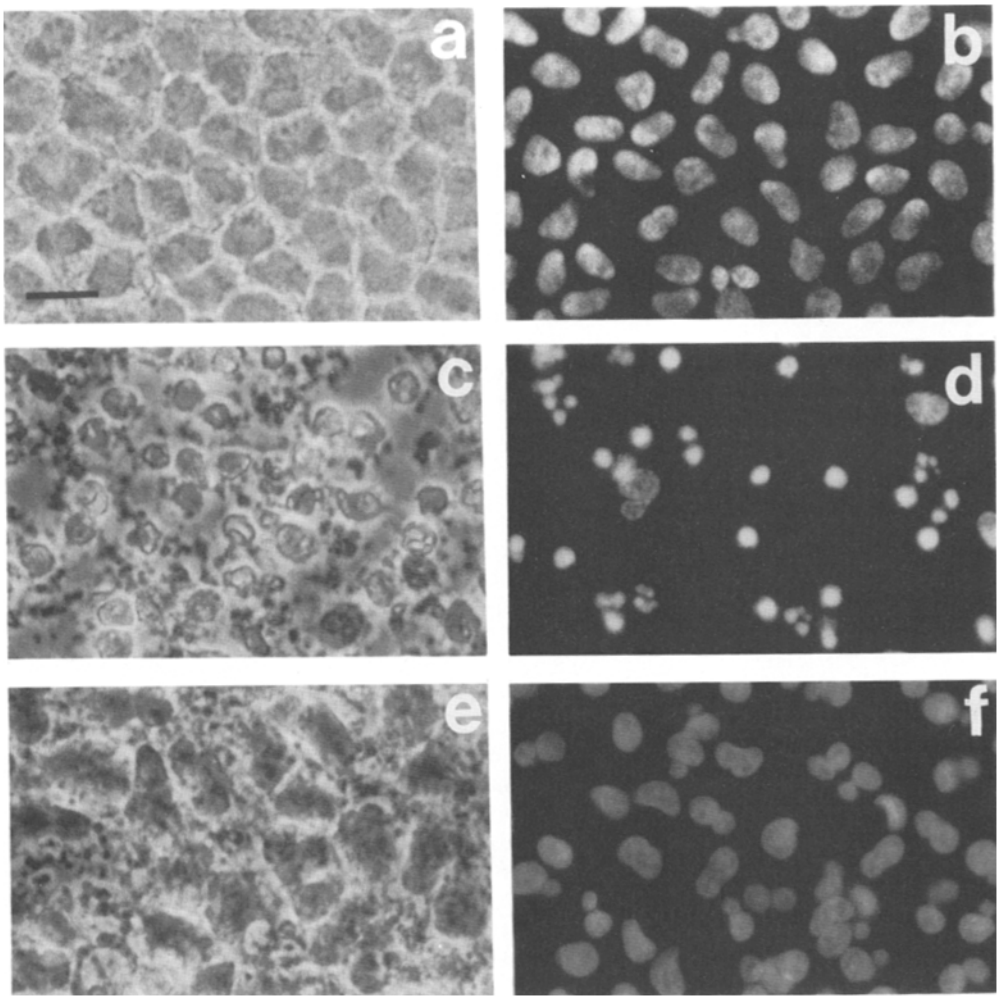

Figure 8. Apoptotic death of lens cells treated with staurosporine and its prevention by ATA. Lens epithelial explants were left untreated (*a* and *b*), or were treated with either staurosporine (1 μM) (*c* and *d*) or staurosporine and ATA (100 μM) (*e* and *f*) for 48 h. They were then fixed and stained with propidium iodide and examined by phase-contrast (*a*, *c*, and *e*) and fluorescence (*b*, *d*, and *f*) microscopy. Bar, 15 μm.

nature of the survival factor(s), the minimum effective concentration of it is apparently only reached in high density cultures.

It has been reported that chondrocytes in high-density, serum-free cultures promote the survival of chondrocytes in lower density cultures by secreting a cysteine-like small molecule that seems to function as a scavenger of free radicals (Tschan et al., 1990). Whereas we find that cysteine promotes the survival of lens cells in low density cultures for 2 or 3 d, it does not mimic the effect of growing the cells at high density, in which case lens cells survive for weeks. Moreover, medium conditioned by high density cultures of lens cells increases the survival of lens cells in low density cultures well beyond the increase seen with cysteine. Although the chondrocytes and lens cells were cultured in agarose gels in our experiments and those of Tschan et al. (1990), the low density chondrocyte cultures (Tschan et al., 1990) were 10-fold higher in density than the low density cultures used in the present study (10^6 cells/ml vs 10^5 cells/ml). Thus, it is possible that chondrocytes, like lens cells, require more than just free-radical scavengers from their neighbors to survive in culture.

We find that when lens cells on their capsule are explanted onto a laminin-coated culture dish, many of the cells die with the morphological features of apoptosis as they migrate off the capsule onto the dish from the edge of the explant. This result contrasts with the results of Parmigiani and McAvoy (1991), who did not observe cell death in similar cultures. The reason for this discrepancy remains to be determined. Although the cause of death in the cell outgrowth regions of our cultures is unclear, it is possible that the cells that migrate from the explant die because they become deprived of short-range survival signals secreted by the bulk of lens cells on the capsule. Remarkably, this death is completely prevented by the addition of cysteine or ME to the culture medium. The effect of cysteine and ME is unlikely to be due to their acting as a scavenger of noxious free radicals outside cells, as cystine is also effective and glutathione, which cannot enter cells except when degraded, is not. How cysteine, cystine and ME prevent apoptosis in these circumstances is a mystery, but it is possible that they act by reducing the redox potential in cells (Zimmerman et al., 1989; Roederer et al., 1990).

Normal cell death is a feature of many developing mammalian tissues (reviewed in Glucksmann, 1951). As reported by others (see references in Glucksmann, 1951), we find cell death in the lens epithelium at the stage of lens vesicle formation, where it is probably related to the pinching off of the vesicle from the overlying ectoderm, which would be an example of Glucksmann's "morphogenetic" cell death (Glucksmann, 1951). It is conceivable that the death of lens cells in the outgrowth region of an explant is mechanistically

related to the death seen at the time of lens vesicle formation. At later stages of lens development, we find a substantial amount of apoptotic cell death in the lens epithelium in the region of the anterior suture, where the anterior ends of lens fibers converge, and this cell death persists throughout life. The reason why lens cells die in the anterior suture region is unclear, but it may be related to mechanical stress exerted by the lens fibers on the epithelium in this area. Interestingly, McAvoy (1978) reported a significantly higher mitotic index in this region compared to neighboring regions; the increased proliferation may be a response to the cell deaths and may compensate for them. The lack of cell death elsewhere in the developing lens epithelium after the period of lens vesicle formation suggests that developing lens epithelial cells do not compete with one another for limiting amounts of survival factors in the way that developing neurons and oligodendrocytes are thought to (see Introduction). If such a competition is normally part of a mechanism to match the numbers of different types of cells in an organ (Raff, 1992), then perhaps it is not surprising that it does not occur in the developing lens epithelium, which contains only one cell type.

It is an interesting but unanswered question whether the gradual denucleation of lens fiber cells, which occurs as these cells differentiate during lens development (McAvoy, 1980; Davson, 1990) and is accompanied by DNA fragmentation (Appleby and Modak, 1977), is related in molecular mechanism to normal apoptotic death. In neither case is the molecular mechanism known.

Whereas the present study demonstrates that lens cells, like chondrocytes, can survive in culture without signals from other types of cells, it remains possible that all mammalian cells, other than blastomeres (Biggers et al., 1971), require signals from other cells to survive. Whether or not this extreme death-by-default hypothesis turns out to be valid, it would seem a worthy enterprise to determine which cell types require signals from other cells to survive, what these signals are, and how they promote cell survival.

We thank John McAvoy for many helpful suggestions and for pointing us to the anterior suture region of the lens to look for cell death, Barbara A. Barres for helpful comments on the manuscript, and Mats Lake at Kabigen for providing the IGF-1.

Y. Ishizaki is supported by the Uehara Memorial Foundation and J. T. Voyvodic was supported by a postdoctoral fellowship from the National Institute for Neurological Diseases and Stroke of the National Institutes of Health.

Received for publication 19 January 1993 and in revised form 22 February 1993.

References

Appleby, D. W., and S. P. Modak. 1977. DNA degradation in terminally differentiating lens fiber cells from chick embryos. *Proc. Natl. Acad. Sci. USA.* 74:5579–5583.

Barde, Y.-A. 1989. Trophic factors and neuronal survival. *Neuron.* 2:1525–1534.

Barres, B. A., I. K. Hart, H. S. R. Coles, J. F. Burne, J. T. Voyvodic, W. D. Richardson, and M. C. Raff. 1992. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell.* 70:31–46.

Basserga, R. 1985. The Biology of Cell Reproduction. Harvard University Press, Cambridge, MA. 256 pp.

Batistatou, A., and L. A. Greene. 1991. Aurintricarboxylic acid rescues PC12 cells and sympathetic neurons from cell death caused by nerve growth factor deprivation: correlation with suppression of endonuclease activity. *J. Cell Biol.* 115:461–471.

Benya, P. D., and J. D. Shaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 30:215–224.

Biggers, J. D., W. K. Whitten, and D. G. Whittingham. 1971. The culture of mouse embryos in vitro. *In* Methods in Mammalian Embryology. J. C. Daniel, editor. Freeman Publications, San Francisco. 86–116.

Bruckner, P., I. Horler, M. Mendler, Y. Houze, K. H. Winterhalter, S. G. Eich-Bender, and M. A. Spycher. 1989. Induction and prevention of chondrocyte hypertrophy in culture. *J. Cell Biol.* 109:2537–2545.

Cowan, W. M., J. W. Fawcett, D. D. M. O'Leary, and B. B. Stanfield. 1984. Regressive events in neurogenesis. *Science (Wash. DC).* 225:1258–1265.

Crompton, T. 1991. IL-3 dependent cells die by apoptosis on removal of their growth factor. *Growth Factors.* 4:109–116.

Davson, H. 1990. Physiology of the Eye. 5th ed. Macmillan Press, London. 140 pp.

Garcia, I., I. Martinou, Y. Tsujimoto, and J.-C. Martinou. 1992. Prevention of programmed cell death of sympathetic neurons by the *bcl-2* proto-oncogene. *Science (Wash. DC).* 258:302–304.

Glucksmann, A. 1951. Cell death in normal vertebrate ontogeny. *Biol. Rev.* 26:59–86.

Goss, R. J. 1978. The Physiology of Growth. Academic Press, New York. 210–223.

Ham, R. G. 1981. Survival and growth requirements of nontransformed cells. *In* Tissue Growth Factors. R. Basserga, editor. Springer Verlag, Heidelberg. 13–88.

Hamburger, V., and R. Levi-Montalcini. 1949. Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. Exp. Zool.* 111:457–502.

Hamburger, V., J. K. Brunso-Bechtold, and J. Yip. 1981. Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* 1:60–71.

Hofer, M., and Y.-A. Barde. 1988. Brain-derived neurotrophic factor prevents neuronal death *in vivo*. *Nature (Lond.).* 331:261–262.

Howard, A. 1952. Whole mounts of rabbit lens epithelium for cytological study. *Stain Technol.* 27:313–315.

Jacobson, M. D., J. F. Burne, M. P. King, T. Miyashita, J. C. Reed, and M. Raff. 1993. Bcl-2 protects cells lacking mitochondrial DNA from undergoing apoptosis. *Nature (Lond.).* 361:365–369.

Kerr, J. F. R., and J. Searle. 1973. Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch. B. Cell Pathol.* 13:87–92.

Kerr, J. F. R., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Brit. J. Cancer.* 25:239–257.

Korsching, S., and H. Thoenen. 1983. Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc. Natl. Acad. Sci. USA.* 80:3513–3516.

Koury, M. J., and M. C. Bondurant. 1990. Erythropoietin retards DNA breakdown and prevents programmed cell death in erythroid progenitor cells. *Science (Wash. DC).* 248:378–381.

Krypianou, N., and J. T. Issacs. 1988. Activation of programmed cell death in the ventral prostate after castration. *Endocrinology.* 122:552–562.

Levi-Montalcini, R. 1987. The nerve growth factor: 35 years later. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1145–1154.

McAvoy, J. W. 1978. Cell division, cell elongation and distribution of α-, β-, and γ-crystallins in the rat lens. *J. Embryol. Exp. Morphol.* 44:149–165.

McAvoy, J. W. 1980. Induction of the eye lens. *Differentiation.* 17:137–149.

McAvoy, J. W., and C. G. Chamberlain. 1990. Growth factors in the eye. *Prog. Growth Factor Res.* 2:29–43.

McAvoy, J. W., and V. T. P. Fernon. 1984. Neural retinas promote cell division and fibre differentiation in lens epithelial explants. *Curr. Eye Res.* 3:827–834.

McConkey, D. J., P. Hartzell, P. Nicotera, and S. Orrenius. 1989. Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:1843–1849.

Metcalf, D. 1989. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature (Lond.).* 339:27–30.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55–63.

Nunez, G., L. London, D. Hockenbery, M. Alexander, J. P. McKearn, and S. J. Korsmeyer. 1990. Deregulated *bcl-2* gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol.* 144:3602–3610.

Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453–501.

Oppenheim, R. W., L. J. Haverkamp, D. Prevette, J. L. McManaman, and S. H. Appel. 1988. Reduction of naturally occurring motor neuron death *in vivo* by a target-derived neurotrophic factor. *Science (Wash. DC).* 240: 919–922.

Parmigiani, C. M., and J. W. McAvoy. 1991. The roles of laminin and fibronectin in the development of the lens capsule. *Curr. Eye Res.* 10:501–511.

Purves, D. 1988. Body and Brain. A Trophic Theory of Neural Connections. Harvard University Press, Cambridge, MA. 231 pp.

Raff, M. C. 1992. Social controls on cell survival and cell death. *Nature (Lond.).* 356:397–400.

Rodriguez-Tarduchy, G., M. Collins, and A. Lopez-Rivas. 1990. Regulation of apoptosis in IL-3 dependent hemopoietic cells by IL-3 and calcium ionophores. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2997–3002.

Roederer, M., F. J. T. Staal, P. A. Raju, S. W. Ela, L. A. Herzenberg, and L. A. Herzenberg. 1990. Cytokine-stimulated human immunodeficiency virus replication is inhibited by N-acetyl-L-cysteine. *Proc. Natl. Acad. Sci. USA.* 87:4884–4888.

Sato, G., editor. 1979. Hormones and Cell Culture. Cold Spring Harbor Laboratory Press, New York. 982 pp.

Searle, J., J. F. R. Kerr, and C. J. Bishop. 1982. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. *Pathol. Annu.* 17:229–259.

Shi, Y. F., M. G. Szalay, L. Paskar, B. M. Sahai, M. Boyer, B. Singh, and D. R. Green. 1990. Activation-induced cell death in T cell hybridomas is due to apoptosis. Morphological aspects and DNA fragmentation. *J. Immunol.* 144:3326–3333.

Stone, J. 1981. The Wholemount Handbook. Maitland Publications, Gordon, New South Wales. 128 pp.

Strasser, A., A. W. Harris, and S. Cory. 1991. Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell.* 67:889–899.

Tschan, T., I. Hoerler, Y. Houze, K. H. Winterhalter, C. Richter, and P. Bruckner. 1990. Resting chondrocytes in culture survive without growth factors, but are sensitive to toxic oxygen metabolites. *J. Cell Biol.* 111: 257–260.

Vaux, D. L., S. Cory, and J. M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature (Lond.).* 335:440–442.

Williams, G. T., C. A. Smith, E. Spooncer, T. M. Dexter, D. R. Taylor. 1990. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature (Lond.).* 343:76–78.

Wyllie, A. H., J. F. R. Kerr, I. A. M. Macaskill, and A. R. Currie. 1973. Adrenocortical cell deletion: the role of ACTH. *J. Pathol.* 111:85–94.

Wyllie, A. H., J. F. R. Kerr, and A. R. Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251–307.

Zimmerman, R. J., B. J. Marafino, Jr., A. Chan, P. Landre, and J. L. Winkelhake. 1989. The role of oxidant injury in tumor cell sensitivity to recombinant human tumor necrosis factor in vivo. *J. Immunol.* 142:1405–1409.