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Apoptosis and lens vesicle development

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PURPOSE. *To study the development of the rat lens vesicle in relation to apoptosis.*
MATERIALS AND METHODS. *Fetuses of Wistar Kyoto rats were removed by laparotomy on day 10-15 of gestation. Some fetuses were fixed in 2% paraformaldehyde and embedded in paraffin for a TUNEL technique examination of DNA fragmentation. Macrophages were stained immunohistochemically with antibody. Some fetuses were fixed in 4% glutaraldehyde and 1% osmic acid and embedded in Luveak 812, then examined with a transmission electron microscope (TEM).*
RESULTS. *On day 11 of gestation (E11) before the start of lens invagination, apoptotic changes were noted in the cells between the surface ectoderm and optic vesicle, with the appearance of phagocytic cells. Apoptotic cells were present at the junction of the surface ectoderm and the lens placode, in the ventral and dorsal thirds of the lens placode and in the outer layer of the optic vesicle in the same axes on E12. Apoptotic changes appeared in the lens stalk, surface ectoderm and the anterior lens epithelium on E12.5. The lens vesicle was detached completely from the surface ectoderm by E13 and some cells had the typical characteristics of macrophages in the extracellular space between the surface ectoderm and the anterior lens epithelium. Apoptotic changes were confirmed by the TUNEL method, and macrophages were stained immunohistochemically.*
CONCLUSIONS. *Apoptosis may have a major role during the whole process of lens vesicle development. Apoptosis may eliminate the cells between the surface ectoderm and the optic vesicle, help trigger invagination and facilitate separation from the ectoderm. Apoptosis might aid in the bowing of the optic vesicle during lens invagination. (Eur J Ophthalmol 2003; 13: 1-10)*

KEY WORDS. *Lens vesicle, Transmission electron microscope, TUNEL method, Rat*

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INTRODUCTION

Tissue regression, remodeling and accompanying cell death are essential components of the development of species throughout the animal kingdom (1). The roles and mechanisms of cell death during development have become an increasingly active area of research over the last 30 years (2).

The eye is formed from neural ectoderm, surface ectoderm and mesenchyme. Normal ocular development depends on dynamic interactions between embryonic tissues, as well as between individual cells within each tissue. These include the death of cells in predictable locations and at precise stages of development by a process known as programed cell death (PCD), or apoptosis (3). A cell that undergoes PCD in animal development is usually degraded so rapidly – often disappearing in an hour or less – that even when there is large-scale PCD, surprisingly few dead cells are to be seen. This suggests that the extent of PCD has been underestimated. As it is still not possible to measure clearance times in most animal tissues, the

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quantitation of PCD remains an unsolved problem (4).

The location of PCD in developing tissues is known or conjectured but its function is unknown. Important examples are seen during folding, pinching off, and fusion of epithelial sheets during vertebrate morphogenesis, and in the formation of the neural tube and lens vesicle (5).

Our research has two aims: first, to determine when and where apoptosis occurs during the development of the lens vesicle; second, to try to explain the role of apoptosis during that process.

MATERIALS AND METHODS

Specimens

Fetal Wistar Kyoto rats were used. A female and a male rat were caged together overnight. A vaginal plug the next morning was considered to indicate the first half-day of gestation (E0.5). Specimens were taken from day 10 (E10) to day 15 (E15) of gestation. The process of development from days 10-15 of gestation was examined in the litters from one dam. The experiment was repeated in 20 litters (a litter consisted of 14-15 fetuses) from 20 dams. Pregnant females were killed under ether anesthesia and fetuses were removed from the uterus by caesarian section. All fetuses from E10-14 were fixed *in toto*, E15 fetuses were decapitated, and hemisection and fixation of the heads were done.

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In situ *DNA end-labeling*

For *in situ* DNA end-labeling, tissues were fixed in 2% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 6 h at 4°C, embedded in paraffin at a melting point of 56°C, and cut into 5 µm sections. The sections were dried on silane-treated microscope slides then deparaffinized with xylene and a graded alcohol series. A non-radioactive detection kit (Apop Tag; Oncor, Gaithersburg, MD, USA) was used to examine DNA fragmentation in the sections. We used the terminal transferase-mediated nick end-labelling (TUNEL) technique described by Gavrieli et al (6) to identify cells undergoing apoptosis. Endogenous peroxidase was quenched with 3% hydrogen peroxide in 0.1 M phosphate buffered saline (PBS) for 5 min. The sections were incubated at 37°C for 1 h in a working buffer containing terminal deoxynucleotidyl transferase (TdT), digoxygenin-dideoxy-uridine triphosphate, and dideoxy-adenosine triphosphate, and incubated with blocking buffer containing sodium chloride and sodium citrate at 37°C for 10 min before the addition of anti-digoxygenin antibody conjugated to peroxidase. After incubation with the antibody at room temperature for 30 min peroxidase activity was examined by exposing the sections to a solution containing 0.05% 3,3'-diaminobenzidine tetrahydroxychloride and 0.01% hydrogen peroxide in Tris buffer at pH 7.6 at room temperature for 3-6 min. For the negative control, distilled water was used in place of TdT enzyme. DNase was used to generate the positive control.

Immunohistochemistry for macrophages

The monoclonal antibody used for the labeling of rat macrophage was ED-1 (Serotec, Oxford, UK, macrophage/monocyte marker). Immunohistochemical staining was done according to the manufacturer's protocol. After the tissue sections had been deparaffinized, 3% hydrogen peroxide was applied to block endogenous peroxidase activity. The slides were incubated at 4°C with primary antibody overnight. ED-1 was used in a 1:100 dilution and then anti-mouse lgG antibody was applied for 60 min. Peroxidase-labeled streptavidin was applied for 20 min, and the slides were visualized with 3,3'-diamino-benzidine tetrahydro-chloride (DAB, Dotite).

Light and transmission electron microscopy

Some specimens were fixed with 4% glutaraldehyde in 0.05M cacodylate buffer for 1 h, washed in the same buffer overnight, postfixed with osmium tetroxide in veronal acetate buffer for 1 h, dehydrated in a series of ethanols and embedded in Luveak 812. For light microscopy, semi-thin sections were cut with a Porter-Blum MT2 microtome and stained with 1% toluidine blue for selection of the site for electron mi-

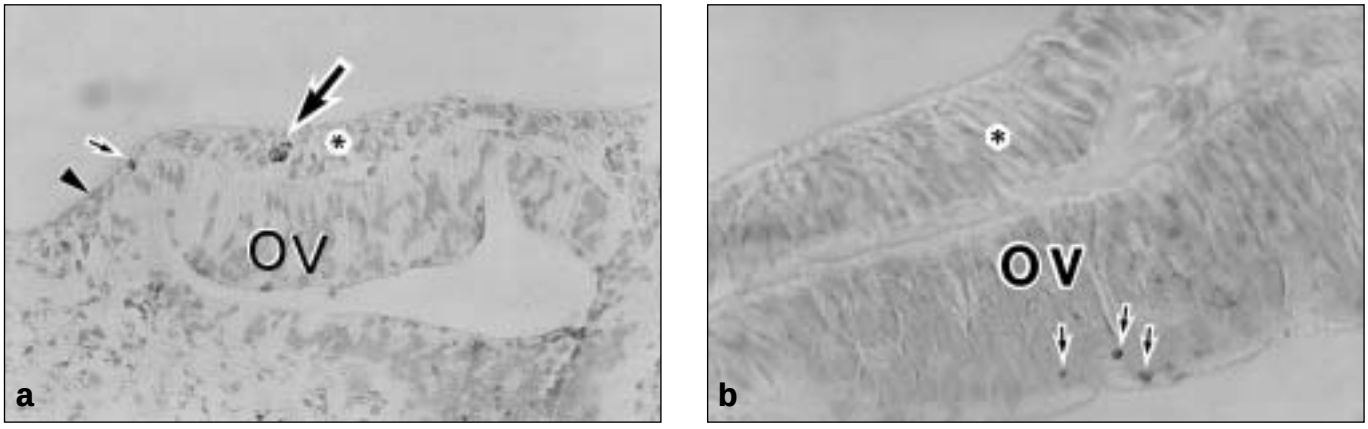


Fig. 1 - a) *Normal eye of an E12 rat with TUNEL-staining. One TUNEL-positive cell (small arrow) is seen at the junction between the surface ectoderm (arrowhead) and lens placode (asterisk). TUNEL-positive cells (large arrow) are seen in the proximal part of the ventral third of the lens placode. OV = outer layer of the optic vesicle. Original magnification x 400.*
b) *Normal eye of an E12 rat with TUNEL-staining. TUNEL-positive cells (arrows) are seen in the proximal part of the outer layer of the optic vesicle (OV). Asterisk = lens placode. Original magnification x 1,000.*

croscopy. Ultrathin sections were cut with a Porter-Blum MT2 microtome, stained with uranyl acetate and lead citrate and examined with a Hitachi H300 electron microscope.

RESULTS

In situ *DNA end-labeling and immunohistochemistry*

On E12 one TUNEL-positive cell was seen at the junction between the lens placode and surface ectoderm (Fig.1a). TUNEL-positive cells were also detected in the proximal part of the ventral third of lens placode (Fig. 1a) and in the proximal part of the outer layer of optic vesicle (Fig. 1b). We did not detect any TUNEL-positive cells in the distal part of the lens placode or the outer layer of the optic vesicle at this stage.

On E12.5 a TUNEL-positive cell was seen in the regressing lens stalk connecting the surface ectoderm and lens vesicle, and another in the anterior lens epithelium (Fig. 2a). After complete separation of the surface ectoderm and lens vesicle on E13, a TUNEL-positive cell was seen in the surface ectoderm adjacent to the lens vesicle (Fig. 2b). Negative controls could not be stained. An ED-1 positive cell was seen in the space between the surface ectoderm and the anterior lens epithelium on E13 (Fig. 2c).

Electron microscopic findings

The optic vesicle began as an outgrowth of the forebrain and grew laterally to reach the surface ectoderm. On E11 the presumptive lens epithelium consisted of two layers: an outer flattened periderm and a basal layer of cuboidal to low columnar cells (Figs. 3a, b). The cells of the optic vesicle stretched from the apical to the basal surface of the cell layer. The widest part of the cell contained the nucleus, which was situated apically, basally, or in an intermediate position in adjacent cells. This distribution of nuclei gave the epithelium a stratified appearance (Figs. 3a, b). Many free flat cells were seen wandering in the space between the surface ectoderm and the optic vesicle (Figs. 3a, b).

At this stage, apoptotic changes were seen in the cells between the surface ectoderm and the optic vesicle (Fig. 3b). Some cells showed condensation of chromatin forming one or more electron-dense chromatin masses (apoptotic bodies) with shrinkage of the cytoplasm. Apoptotic bodies and cell debris were seen in neighboring cells of the surface ectoderm (Fig. 3a) or optic vesicle cells, or in cells between them (Fig. 3b). Sometimes, we noted free fragments of dead cells in the extracellular space (Fig. 3b). Mitotic figures were frequent in the cells of the optic vesicle (Fig. 3b).

On E 11.5, the outer layer of the optic vesicle started to thicken, and mitotic figures were seen frequently in this layer (Fig. 4). A few cells were identified in the

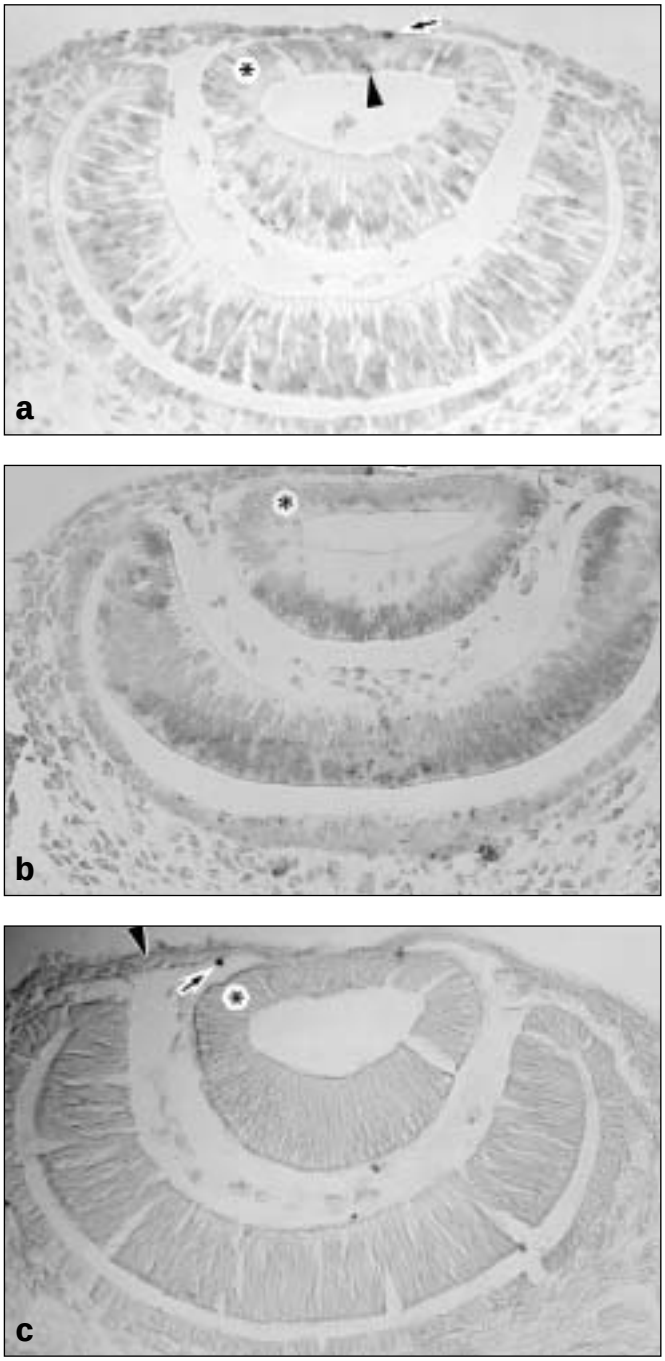


Fig. 2 - a) *Normal eye of an E12.5 rat with TUNEL-staining. A TUNEL-positive cell (arrow) is seen in the regressing lens stalk. Another positive cell (arrowhead) is seen in the anterior lens epithelium (asterisk). Original magnification x 400.*
b) *Normal eye of an E13 rat with TUNEL-staining. A TUNEL-positive cell (arrow) is seen in the surface ectoderm adjacent to the lens vesicle (asterisk). Original magnification x 400.*
c) *Normal eye of an E13 rat with ED-I staining. An ED-I positive cell (arrow) is seen in the space between the surface ectoderm (arrowhead) and lens vesicle (asterisk). Original magnification x 400.*

extracellular space between the surface ectoderm and the optic vesicle, and apoptotic changes continued to occur in these cells (Fig. 4). These changes took the form of shrinkage and condensation of cellular nuclei and cytoplasm or of apoptotic bodies or cellular debris in adjacent cells. The opposing cells of the surface ectoderm and the outer layer of the optic vesicle started to form protrusions towards each other (Fig. 4).

By E12, the surface ectoderm overlying the optic vesicle had thickened to form the lens placode, and the outer layer of the optic vesicle had become multilayered. The protrusions between the opposing cells of the lens placode and the optic vesicle had become more prominent, with the appearance of filamentary deposits (Fig. 5a). In some regions, these filaments organized to form bundles connecting the cells of the lens placode with those of the optic vesicle (Fig. 5a). Lens invagination was started by the inward bowing of the lens placode and the outer layer of the optic vesicle. Apoptotic changes were seen at this stage in the following places: cells surrounding the lens placode and connecting it with the surface ectoderm (Fig. 5b); in the proximal cells of the lens placode at the junction between the ventral and dorsal thirds of the lens placode (Figs. 6a, b, c); in the proximal cells of the outer layer of the optic vesicle along the same axes as the apoptotic cells of the lens placode (Figs. 7a, b); and in the extracellular space between the lens placode and the optic vesicle (Fig. 6a). The inner layer of the optic vesicle had thinned to a simple cuboidal epithelium forming the primordium of the retinal pigment epithelium (Figs. 7a, b). Some cells showed early apoptotic changes with crescent-shaped margination of the nuclear chromatin along the inner nuclear membrane, and shrinkage of the cytoplasm (Fig. 7b). Some cells showed condensation of both the nucleus and the cytoplasm and were separated from their attachments with neighboring cells (Figs. 6a, 7a). In some cells condensation of chromatin formed one or more electron-dense chromatin masses (apoptotic bodies) with vacuolation of organelles (Figs. 6a, b, c, 7b). These apoptotic cells were eliminated in two ways: cell remnants were engulfed by neighboring cells and destroyed intracellularly (Figs. 5b, 6a) or else they underwent extracellular degeneration (Fig. 6a, b). In the latter pathway, both cytoplasmic and nuclear compartments could still be recognized, and the material became fi-

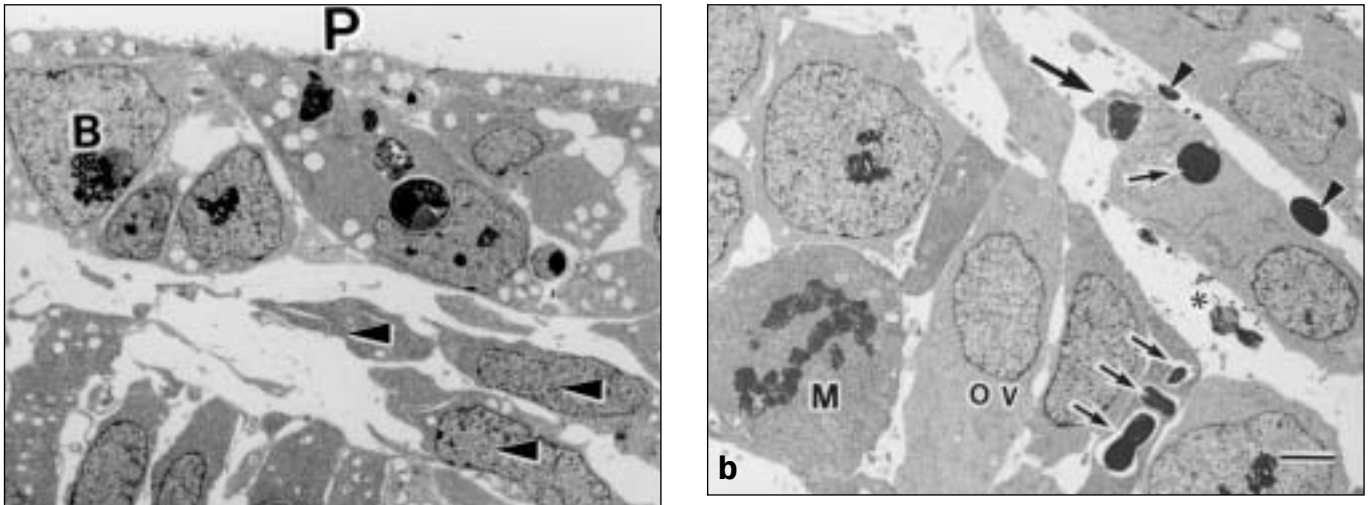


Fig. 3 - a) *Electron micrograph of the distal part of the eye of an E11 rat. The presumptive lens epithelium consists of a basal cuboidal layer (B) and an outer flat layer (P). Some apoptotic bodies are engulfed by the lens epithelium. Free flat cells (arrowheads) are seen in the space between the lens and the optic vesicle (asterisk). Bar = 5µm.*
b) *Electron micrograph of the distal part of the eye of an E11 rat. Apoptotic bodies (small arrows) are engulfed by optic vesicle cells (OV) and wandering cells in the extracellular space. One apoptotic cell (large arrow) shows condensation of chromatin with shrinkage of its cytoplasm. Two apoptotic bodies (arrowheads) and cell debris (asterisk) can be seen in the extracellular space. Mitotic figures (M) are present in the cells of the optic vesicle. Bar = 2 µm.*

brogranular, mostly with moderate electron density. Sometimes very few cells were trapped in the extracellular space between the lens placode and the optic vesicle (Fig. 6a).

As the lens invagination progressed, the marginal borders of the lens placode fused and the lens vesicle remained connected to the ectoderm by a small regressing epithelial stalk on E12.5. Most of the apoptotic changes found at this stage appeared in cells in the epithelial stalk, adjacent ectoderm and anterior epithelium of the lens vesicle (Fig. 8). Most of the apoptotic cells underwent degeneration (secondary necrosis) with disintegration of their nuclear and cytoplasmic components. Some cells still contained masses of chromatin condensations (apoptotic bodies), and some cytoplasmic organelles like mitochondria could be identified (Fig. 8). Sometimes lysosomal bodies were seen adjacent to masses of cell debris (Fig. 8).

The lens vesicle was detached completely from the surface ectoderm by E13. The apoptotic changes were similar to those noted in the preceding stage (in the surface ectoderm, the anterior epithelium of the lens vesicle and in the intervening space). Some cells showed the typical features of macrophages in the extracellular space between the surface ectoderm and the anterior lens epithelium (Fig. 9). These cells contained large masses of cell debris and lysosomes. By this stage the organized filament bundles and the cytoplasmic protrusions had disappeared completely from the surface ectoderm facing the lens vesicle.

By El4 the posterior lens epithelium had elongated rapidly to fill the lumen of the lens vesicle and had been transformed into the primary lens fibers.

DISCUSSION

The early steps in eye development involve extensive programed cell death associated with morphogenesis (7, 8). Most studies of death during early development of the lens of the eye in mammals used only light microscopy (3, 8-13). The present electron microscopic observations agree with previous studies that assume contact is necessary between the op-

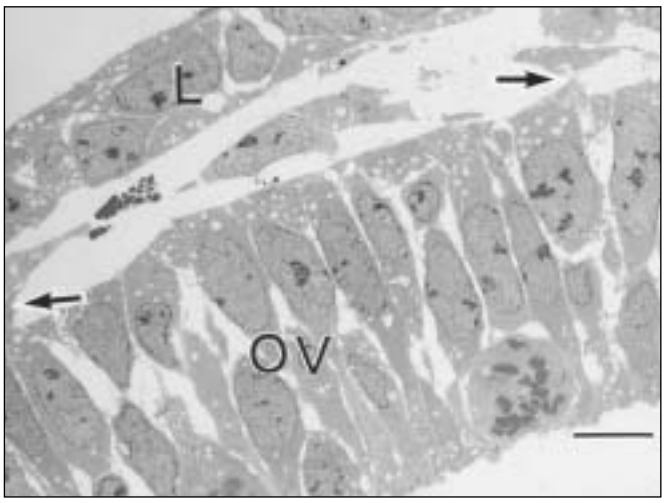


Fig. 4 - *Electron micrograph of the distal part of the eye of an E11.5 rat. Many apoptotic cells and apoptotic bodies are seen in the space between the lens epithelium (L) and the optic vesicle (OV). The opposing cells of the lens epithelium and the OV have protrusions towards each other (arrows). Mitotic figures are seen in the cells of the OV. Bar = 10 µm.*

tic vesicle and the surface ectoderm for lens induction to occur (3, 10, 11, 14). We found that most of these mesenchymal cells were eliminated by apoptosis on E11 and E11.5 before the start of lens invagination.

Our assumption was that most mesenchymal cells between the surface ectoderm and the optic vesicle have to be eliminated to provide free space facilitating contact between the two tissues and a chance for the development of cellular extensions with bundles of filaments connecting the surface ectoderm and the optic vesicle. These bundles of filaments connecting the cellular extensions may be needed to establish inward mechanical traction on the lens placode and consequently the optic vesicle. At the same time (E12), there were two sites in the lens placode and the outer layer of the optic vesicle that showed apoptotic changes concurrently with the start of lens invagination. These were at the junction between the ventral and dorsal thirds of the lens placode and along the same axes as in the outer layer of the optic vesicle. These apoptotic changes were confined to the proximal parts of the lens placode and the outer layer of the optic vesicle and were not found in the distal parts of these tissues.

We can speculate that these apoptotic centers are weak *foci*, and the inward mechanical traction between

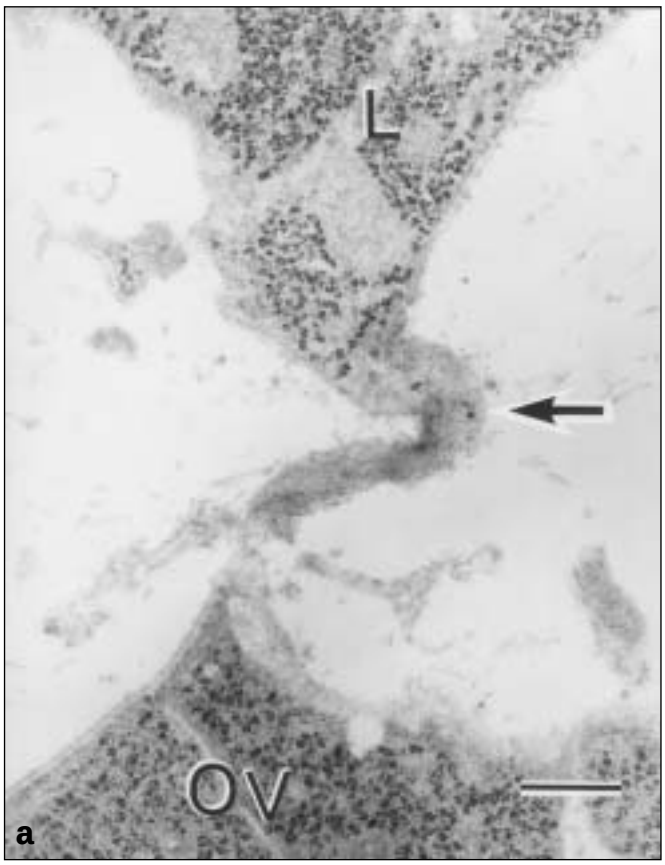


Fig. 5 - a) *High magnification of the cellular protrusions between the lens epithelial cells and the cells of the optic vesicle (OV) of an E12 rat. Note the organization of the filaments into a thick bundle (arrow) connecting one cell of the OV with one epithelial cell of the lens (L). Note the filamentary deposits in the extracellular space. Bar = 1 µm.*
b) *Electron micrograph of the junctional zone (arrowheads) between the surface ectoderm (S) and lens placode (L) of an E12 rat. Many apoptotic bodies are engulfed by cells at the junctional zone between the S and the L. Bar = 2 µm.*

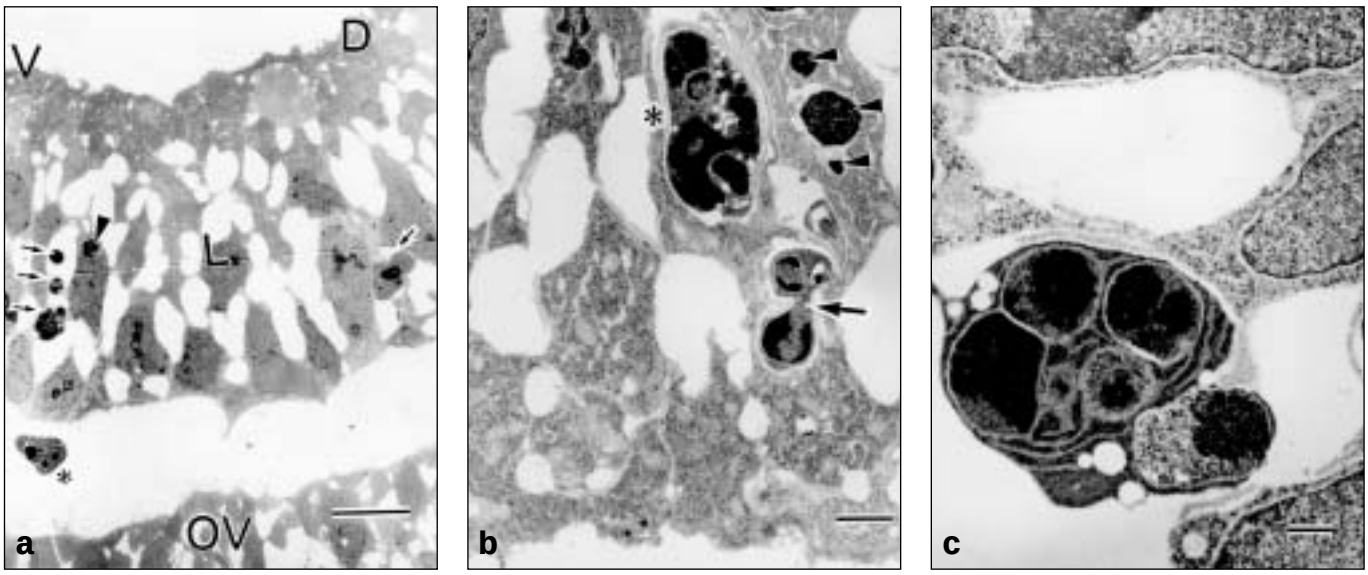
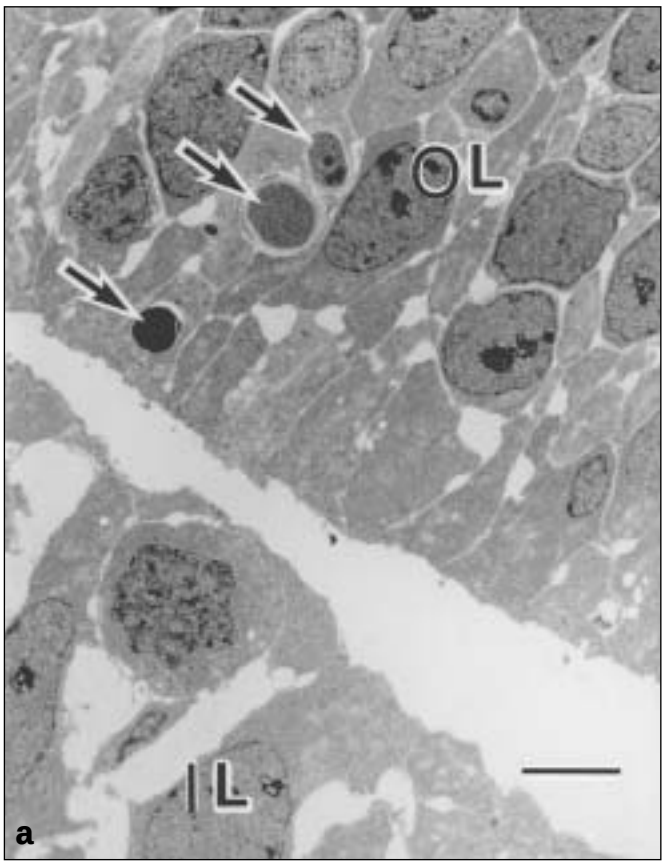


Fig. 6 - a) *Electron micrograph of the lens placode of an E12 rat. Apoptotic changes are seen in the proximal part of the lens placode at the junction between the ventral (V) and the dorsal (D) thirds of the lens placode. Apoptotic cells (arrows) show condensation of chromatin and shrinkage of cytoplasm and loss of their attachments with neighboring cells. An apoptotic body (arrowhead) is engulfed by a neighboring cell. An apoptotic cell at a late stage (asterisk) is starting to disintegrate in the extracellular space between the lens placode (L) and the optic vesicle (OV). Bar = 10 µm.*
b) *Electron micrograph of the proximal part of the lens placode of an E12 rat. One apoptotic cell shows crescentic margination of its chromatin with characteristic blebbing of the cell membrane and condensation of its cytoplasm (arrow). Another shows condensation of chromatin forming multiple apoptotic bodies with vacuolation of the cytoplasm (asterisk). Note cell debris (arrowheads) in the neighboring cell. Bar = 2 µm.*
c) *Electron micrograph of the proximal part of the lens placode of an E12 rat. One apoptotic cell shows condensation of chromatin and vacuolation of its cytoplasm. The apoptotic cell is losing its attachment to the surrounding cells. Bar = 2 µm.*

the surface ectoderm and the optic vesicle is helped by their presence in the lens placode and the optic vesicle. Thus, apoptosis facilitates invagination of the lens placode and optic vesicle. This means that if mechanical traction is present and both lens vesicle and optic vesicle are tough cellular structures without weak points, invagination might not occur or might take an abnormal direction. In addition, the apoptotic changes in the lens placode and optic vesicle in the same axis facilitate the harmonious invagination of the two tissues. The fibrillar network that builds up between the lens placode and the optic vesicle may also be needed to maintain strong adhesion between these tissues, which may be important for coordinated and simultaneous morphogenetic movements during the process of invagination.

The apoptotic changes in the surface ectoderm encircling the lens placode may play an important role in loosening the lens placode itself from its attachment with the surface ectoderm, making it easier for the mechanical traction to mould the placode, thus facilitating invagination. We noticed that the cellular extensions and the filamentary bundles found during invagination disappeared after the completion of lens vesicle development, suggesting that they had served their purpose. It is logical to believe in the inward traction force which makes the lens placode and subsequently the outer layer of the optic vesicle invaginate; otherwise the replicated tissues of the lens placode and the optic vesicle would continue their outgrowth.

Lens formative cells are restricted to a portion of the ectoderm in immediate contact with the presumptive retina, which thus coordinates the initial number of cells in the early lens primordium (15). Cells at the periphery of the lens placode may lack sufficient impetus for differentiation, and degenerate, forming the marginal pyknotic zone of the lens, while those cells completely removed from the circle of the retinal influence re-establish continuity of the surface ecto-



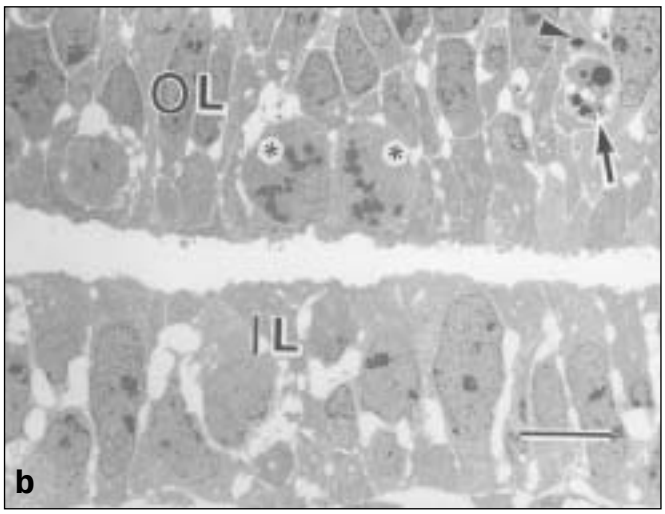


Fig. 7 - a) *Electron micrograph of the ventral third of the proximal part of the optic vesicle of an E12 rat. Apoptotic cells (arrows) are seen in the proximal part of the outer layer (OL) of the optic vesicle. The inner layer (IL) of the optic vesicle thins to a single layer of cells forming the primordium of the retinal pigment epithelium. Bar = 5 µm.*
b) *Electron micrograph of the dorsal half of the proximal part of the optic vesicle of an E12 rat. An apoptotic cell forming multiple apoptotic bodies with vacuolation of its cytoplasm (arrow) is seen in the proximal part of the outer layer (OL) of the optic vesicle. Note cell debris in the neighboring cell (arrowhead). Mitotic figures are seen in the proximal part of the OL. The inner layer (IL) of the optic vesicle thins to a single layer of cells. Bar = 10 µm.*

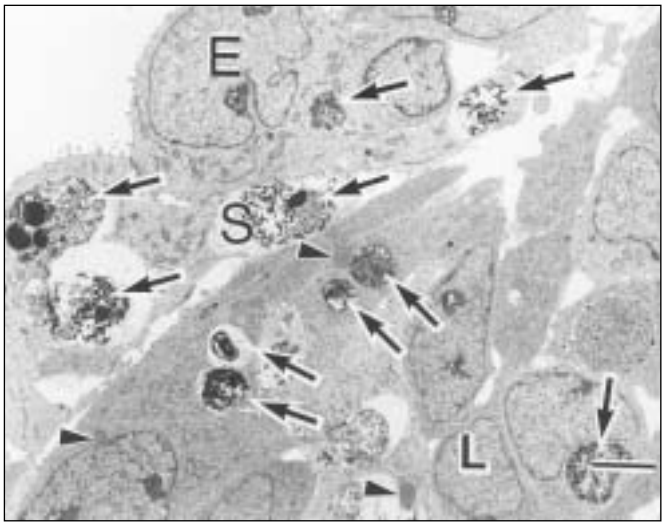


Fig. 8 - *Electron micrograph of the surface ectoderm and the anterior lens epithelium of an E12.5 rat. Apoptotic changes (arrows) are seen in the surface ectoderm (E), epithelial stalk (S) and anterior lens epithelium (L). Lysosomal bodies (arrowheads) can be seen adjacent to the masses of cell debris and there are mitochondria in the cell debris. Bar = 2 µm.*

Fig. 9 - *Electron micrograph of the distal part of the eye of an E13 rat. Note the complete separation between the surface ectoderm (E) and the anterior lens epithelium (L). One cell with the typical features of macrophages is seen in the extracellular space, containing large masses of cell debris (arrows) and lysosomal bodies (arrowheads). Bar = 2 µm.*

derm to form the prospective cornea (15). If this is true, why do apoptotic changes appear in the lens placode itself although it faces the presumptive retina and is even in contact with it in some parts?

Jacobson et al (4) showed that dead cells or their fragments during normal development are rapidly phagocytized by neighboring cells or macrophages before there is any leakage of their contents, so they do not induce an inflammatory response. They confirmed that apoptotic cells in developing tissues are almost always inside other cells, suggesting that dying cells are usually phagocytized before they display the morphological changes of apoptosis. Phagocytosis of apoptotic cells or of the apoptotic bodies, the product of cellular disintegration, is without doubt an integral feature of apoptosis. Uptake and degradation of apoptotic cells *in vivo* is rapid and can be achieved by either professional phagocytes, such as macrophages, or non-specialized phagocytes, for example epithelial cells (7). Eventually, apoptotic cells will be digested by a lysosomal pathway. In most cases, at least *in vivo*, this is probably accomplished inside the phagocytizing cell (16).

TEM findings suggest that lens invagination occurs with the active contraction of the terminal web of fine filaments below the apex of each cell. This causes the cell apex to become narrower than the base and the whole sheet of cells to curve when many cells contract together (17). Scanning electron microscopy of the chick placode supports the view that invagination involves a reduction in the diameter of the cells in and around the concavity (18). Wrenn and Wessels (17) themselves, in the same study, suggested that it is not justified to say that the filaments, if they are contracting, are the sole cause of invagination. They may be aided in the process by other unknown factors.

We agree with most of the previous studies that apoptotic changes appearing in the lens stalk before separation of the lens vesicle from the surface ectoderm help to release the lens vesicle from its attachment with the surface ectoderm (8, 9, 11, 13, 17, 19). After complete separation of the lens vesicle from the surface ectoderm on E13, apoptotic changes were seen in the surface ectoderm and anterior epithelium of the lens vesicle, and phagocytic cells (macrophages) appeared in the space between the two tissues to engulf the debridement. There have been many contradictory opinions about the mechanism of lens vesicle development and especially the role of cell death during this process. In this study we tried to explain some speculations about the role of apoptosis in the process.

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