THE ANATOMICAL RECORD 217:395–401 (1987)

Retinal Cell Death Occurs in the Absence of Retinal Disc Invagination: Experimental Evidence in Papaverine-Treated Chicken Embryos

JUAN A. GARCÍA-PORRERO, ELVIRA COLVEE, AND JOSE L. OJEDA
Department of Anatomy, Faculty of Medicine, University of Cantabria, Santander (Cantabria), Spain

ABSTRACT In an attempt to clarify the relationship between the presence of retinal cell death and the invagination of the optic vesicle, we have tested the occurrence and cytological characteristics of the retinal necrotic areas in the embryonic chicken after the administration in ovo of papaverine. Papaverine, a Ca^2+ antagonist, was found to prevent the invagination of the optic vesicle. All embryonic retinae presented two distinct necrotic areas. However, these areas of cell death appeared abnormally located in the experimental, uninvaginated retina. One area was located at the transition between the retinal disc and the ventral wall of the optic vesicle; a second area was located in the dorsal wall of the optic vesicle, close to the optic stalk. We suggest that these necrotic areas represent the normal necrotic areas, should the invagination of the retinal disc have taken place. Retinal cell death appears to be programmed; it occurs whether the retinal disc invaginates or not. Cell death appears, in this experimental model, as a natural marker giving evidence that the embryonic retinal cells move from the optic stalk into the invagination retinal disc during normal eye cup formation.

In addition to the uninvaginated optic vesicle the lens placode failed to invaginate in 45% of the cases, forming a lens vesicle in 55% of the remaining cases. This suggests that the two processes of invagination are governed by a different set of factors.

During the early development of the vertebrate retina the lateral wall of the optic vesicle (retinal disc) invaginates to form the optic cup. As it occurs with other processes of epithelial invagination, the mechanisms involved in the infolding of the retinal disc are yet not clear. Contraction of the microfilament system and modifications of the apical surface of the retinal cells have been implicated in this process (Hilfer et al., 1981; Yang and Hilfer, 1982; Malloney and Wakely, 1982a). The possible role of other factors such as physical dependence between the lens placode and the retinal disc (Stroeva, 1960; Coulombre, 1965; Zwaan and Hendrix, 1973; Maloney and Wakely, 1982b), regional cell proliferation (Coulombre, 1965; Zwaan and Hendrix 1973) and morphogenetic cell death (Glücksmann, 1951) is still obscure.

Cell death plays an essential role in the morphogenesis of different embryonic organs and structures (Saunders and Fallon, 1967; Hinchliffe, 1981). Cell death has been demonstrated during the formation of the optic cup in several species of vertebrates (Kallen, 1955, 1965; Silver and Hughes, 1973; Schook, 1980). In the embryonic chicken, two areas of cell death appear located at the dorsal and ventral aspects of the invaginating retinal disc (García-Porrero and Ojeda, 1979; García-Porrero et al., 1984a). Cell death in the retina anlage has been related to the process of retinal invagination. However, the possible morphogenetic role of these areas of cell death is still controversial.

Glücksmann (1930, 1951) suggested that the retinal areas of cell death allowed the invagination of the retinal disc by permitting neighboring healthy cells to migrate into the necrotic foci. Silver and Hughes (1973) suggested on the contrary, that the retinal invagination was inhibited in the necrotic areas but permitted in the area adjacent. However, it has recently been reported that, in an anophthalmic strain of mice, the retinal disc invaginates in the absence of cell death (Silver and Hughes, 1974). Although in these cases the optic fissure did not form, these observations suggest that the retinal invagination and the cell death are unrelated processes.

Papaverine, a chemical agent which blocks the entry of Ca^2+ into the cells, inhibits neurulation by disrupting the organization of the microfilament system (Moran and Rice, 1976; Moran, 1976). In the same way, Brady and Hilfer (1982) have shown that the in vitro administration of papaverine to chicken embryos reversibly prevents the invagination of the retinal disc. It should be very interesting to test, under similar conditions, the occurrence and fate of the necrotic areas of the embryonic retina. This paper presents a study of the occurrence and cytological characteristics of retinal cell death

Received August 12, 1986; accepted October 22, 1986.

This paper is dedicated to Prof. Pedro Gómez-Bosque on the occasion of his retirement.

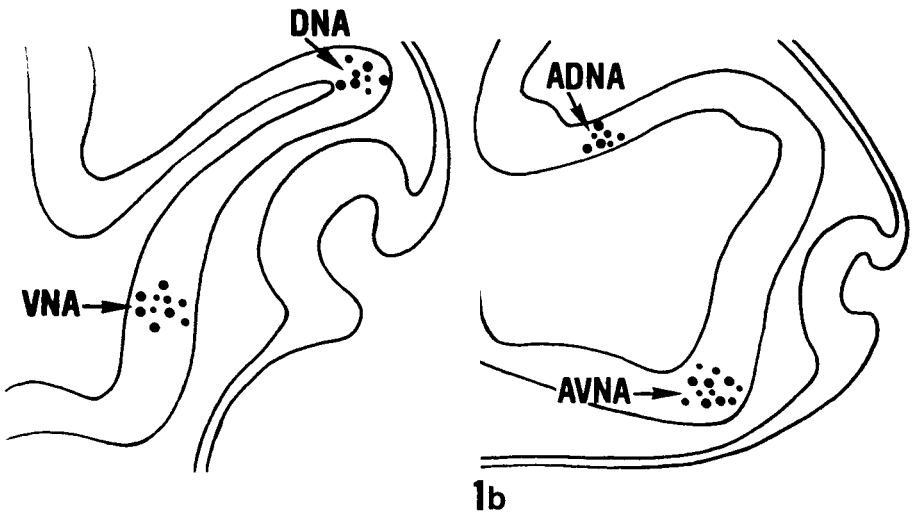


Fig. 1. Camera lucida drawings depicting two frontal sections of the eye anlage. a: Stage-16 control embryo. b: Papaverine-treated embryo 24 hours after the administration of the drug. The retinal disc of the control embryo appears invaginated and shows the normal dorsal (DNA) and ventral (VNA) necrotic areas. The retinal disc of the papaverine-treated embryo appears uninvaginated. However, it also shows two distinct necrotic areas. Note the abnormal location of these areas. (ADNA), abnormally located dorsal necrotic area; (AVNA), abnormally located ventral necrotic area.

in chicken embryos in which the process of invagination of the retinal disc has been prevented by administration in ovo of papaverine.

MATERIALS AND METHODS

Fertile White Leghorn eggs were incubated at 38.5°C in a humidified atmosphere to yield normal embryos of stage 13 (Hamburger and Hamilton, 1951). Just before the beginning of the invagination of the retinal disc (Hilfer et al., 1981), 0.1 ml of a saline solution containing 6×10^{-4} M papaverine (Sigma was dropped on the vitelline membrane over the prospective eye region through a window in the eggshell. This dosage was found, after experimental testing, to prevent the invagination of the retinal disc, permitting the greatest embryo survival. The window was then sealed with cellophane tape to prevent dehydration and the eggs were reincubated for another 24 hours. Control embryos were treated with the same volume of saline and processed simultaneously.

After reincubation the embryos were removed from the eggshell and fixed for 3 hours in 3% glutaraldehyde solution buffered with 0.2 M cacodylate, pH 7.3. Then they were transferred to buffer solution and the head region was dissected free; 110 experimental surviving embryos and 50 controls were used in this study.

A first group of embryos was processed for light and transmission electron microscopy. The specimens were postfixed in 1% osmium tetroxide, dehydrated in acetone and propylene oxide, and embedded in Araldite. To ensure that the specimens would be sectioned frontally they were embedded in flat capsules and carefully oriented under a binocular dissecting microscope. Serial semithin sections (1μm thick) were stained with 1% toluidine blue in 1% sodium borate solution. Camera lucida drawings recording the occurrence and location of the necrotic areas were prepared from selected semithin sections. Ultrathin sections of selected areas were then made, stained with 5% uranyl acetate and lead citrate (Reynolds, 1963), and observed in a Zeiss EM 10 electron microscope.

A second group of embryos was processed for scanning electron microscopy. Frontal sections of the eye rudiment were carefully made under a binocular microscope. The pieces were then dehydrated in graded acetones, dried by the critical-point method, and gold sputter coated. The specimens were observed in a Philips SEM 501.

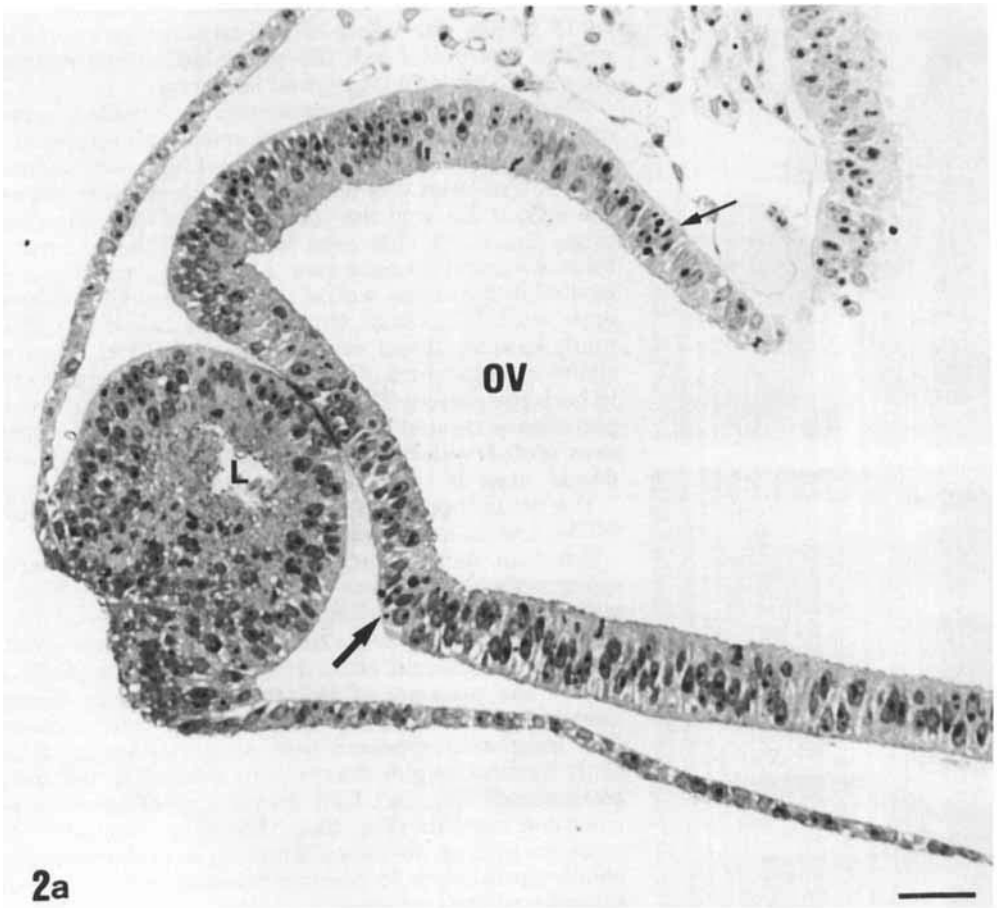
RESULTS

Papaverine administration inhibited the invagination of the optic vesicle in 44 (40%) of the surviving embryos (Fig. 1–4). The uninvagination was unilateral in 29 (66%) of the cases (Fig. 4b) and bilateral in the remaining 15 (34%) (Fig. 2b).

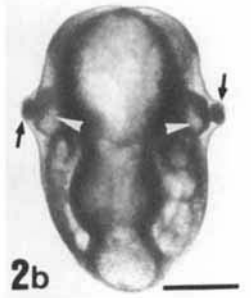
Associated to the inhibition of the infolding of the retinal disc, the lens placode failed to invaginate in 20 (45%) of the cases (Fig. 4), while in the other 24 (55%) embryos the lens vesicle formation remained normal (Fig. 2a,b). In some of these cases, however, the lens vesicle appeared only partially invaginated, showing a

Fig. 2. a: Light micrograph showing a frontal semithin section through the eye anlage of a papaverine-treated embryo. The uninvaginated optic vesicle (OV) shows two distinct necrotic areas: one area is located in the dorsal wall of the optic vesicle (thin arrow); the other area is located at the transition between the retinal disc and the ventral wall of the optic vesicle (thick arrow). The lens rudiment (L) appears invaginated. Bar = 50 μm. b: Light micrograph showing the head of the embryo shown in Figure 2a. The two optic vesicles (arrow heads) remain uninvaginated. The lens rudiments (arrows) appear invaginated. Bar = 400 μm. c: Detailed view of the necrotic area located in the dorsal wall of the uninvaginated optic vesicle. Dark-staining cell fragments (arrows) appear among the healthy neighboring cells. Bar = 25 μm.

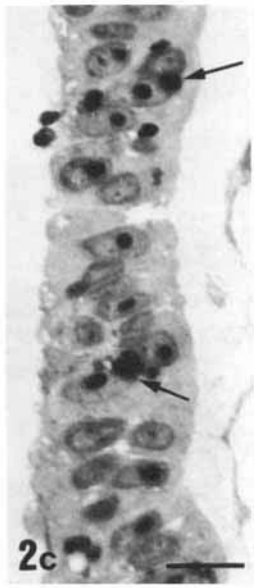
Fig. 3. a: Light micrograph composition of a frontal semithin section through the eye anlage in a paraverine-treated embryo. The optic vesicle (OV) remains uninvaginated. Note the presence of a necrotic area (arrow) located in the zone of transition between the lateral (retinal disc) and the ventral walls of the optic vesicle. The lens cup (L) appears distorted and shows a dorsal profile more angulated than normal. Bar = 50 μm. b: Detailed view of the necrotic area shown in Figure 3a. Note the presence of numerous pyknotic nuclei and phagosomes (arrows) Bar = 10 μm.



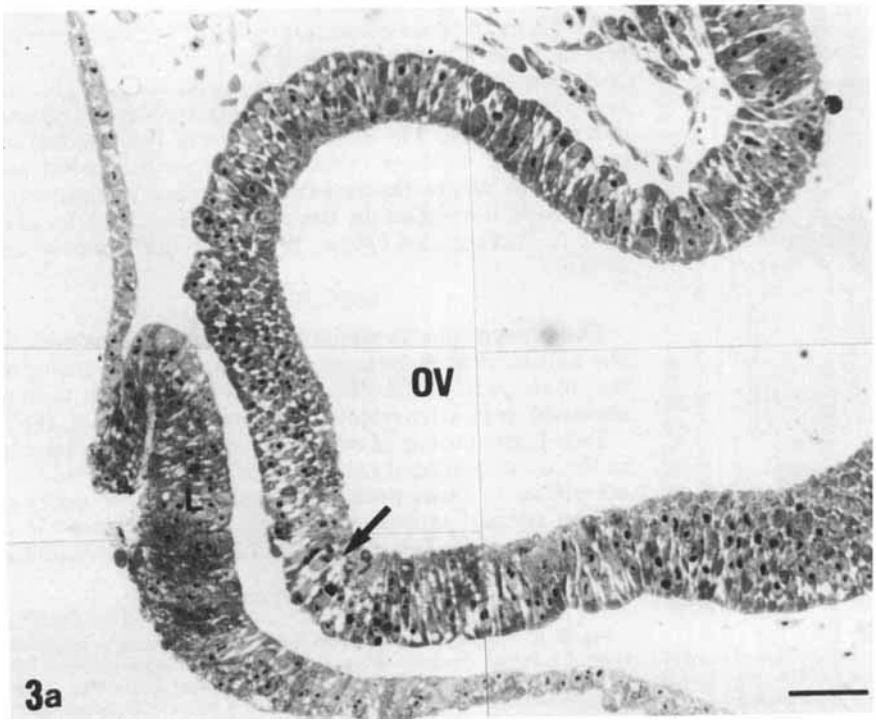
2a



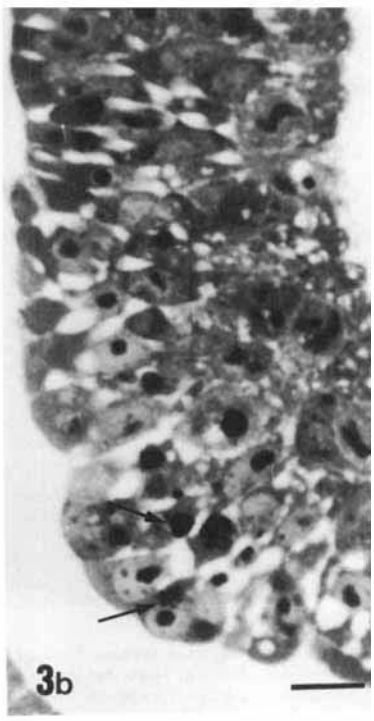
2b



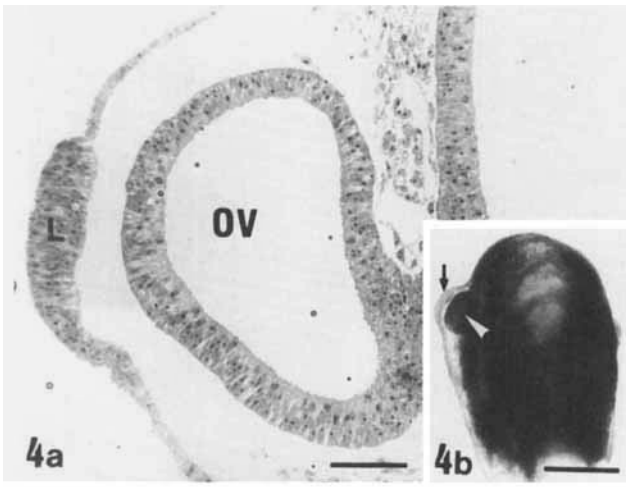
2c

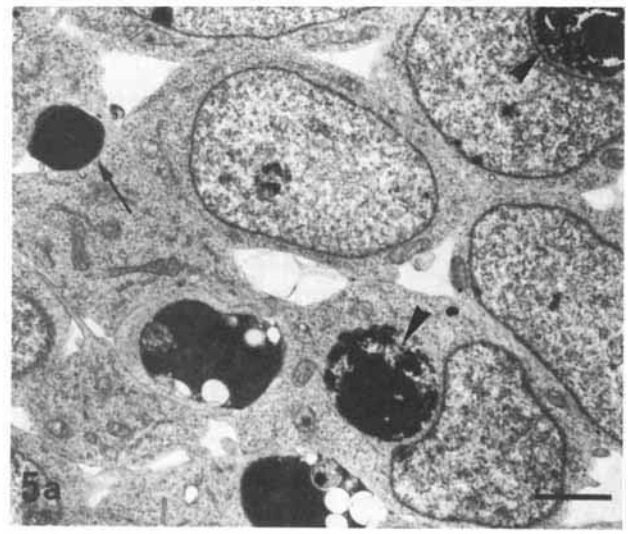


3a



3b





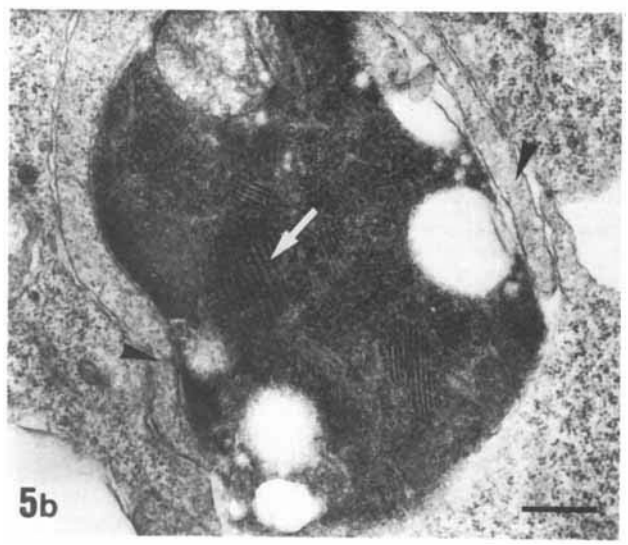


Fig. 4. Frontal semithin section through the eye anlage of a papaverine-treated embryo. **a:** Note the uninvagination of the optic vesicle (OV). The lens rudiment (L) appears in the stage of placode. Bar = 80 μm. **b:** Light micrograph showing the head of the embryo shown in Figure 4a. The left optic vesicle (arrow head) and the lens rudiment (arrow) remain uninvaginated. Bar = 400 μm.

dorsal profile more angulated than normal (Fig. 3a). Control embryos developed normally through stages 16 to 18. When the effect of the papaverine on the eye anlage was unilateral, the contralateral eye rudiment showed an apparently normal optic cup.

All embryonic retinae presented two distinct necrotic areas. However, these areas of cell death appeared abnormally located in the experimental, uninvaginated retina. One area was located at the transition between the retinal disc and the ventral wall of the optic vesicle (Figs. 2a, 3a,b); this area was called the abnormally located ventral necrotic area (AVNA). A second area was located in the dorsal wall of the optic vesicle, close to the optic stalk (Fig. 2a,c); this area was called the abnormally-located dorsal necrotic area (ADNA). Figure 1 shows a comparison of the location of the necrotic areas in both the normal (García-Porrero et al., 1984a) and the papaverine-treated optic vesicles. The normal ventral area is observed through stages 14 to 19 and the normal dorsal area is observed from stage 15 to stage 18.

The areas of cell death were studied by light, scanning (SEM) and transmission electron microscopy (TEM).

Semithin sections in these areas showed numerous dying cells which were identified by the presence of pyknotic nuclei and dark-staining cell material (Figs. 2c, 3b). The amount and extent of cell death were similar in both normal and abnormal necrotic areas. TEM revealed the presence of isolated cells of high electron density and of numerous electron-dense cell fragments. Cell fragments appeared both scattered among apparently healthy neighboring cells and sequestrated within phagosomes (Fig. 5a). Cell debris very often contained ribosome crystals (Fig. 5b). SEM (Fig. 6) showed the presence of large (between 3 and 5 μm in diameter) and small (up to 3 μm in diameter) round bodies that displayed a rough surface and small holes (Fig. 7). Normal columnar epithelial cells intermingled with the dying cells. Many of these columnar cells very often presented one or more surface craters (Fig. 8, 9). The size of the craters was variable (up to 5 μm in diameter). The craters always contained cell fragments similar to the ones described above. The characteristics of this process appear similar to those observed in other epithelial necrotic zones where the cell fragments are phagocytosed by craters developed in the neighboring healthy cells (García-Porrero and Ojeda, 1979; García-Porrero et al., 1984b).

DISCUSSION

The present observations show that in ovo conditions the administration of papaverine inhibits the invagination of the retinal disc. These results are similar to those obtained in in vitro conditions (Brady and Hilfer, 1982).

Two distinct areas of cell death can be clearly observed in the uninvaginated retinal discs. The cytological characteristics of these areas are similar to those observed in the normal embryonic necrotic areas (reviewed by Hinchliffe, 1981; Beaulaton and Lockshin, 1982), includ-

Fig. 5. **a:** Transmission electron micrograph showing a panoramic view of a retinal necrotic area of a papaverine-treated embryo. Electron-dense cell fragments appear either isolated (arrow) or sequestrated into phagosomes (arrow heads). Bar = 2 μm. **b:** Detailed view of the necrotic area shown in Figure 5a. A cell fragment appears partially surrounded by the walls of a phagocytic crater (arrow head). The cell fragment shows ribosome crystals (arrow). Bar = 0.5 μm.

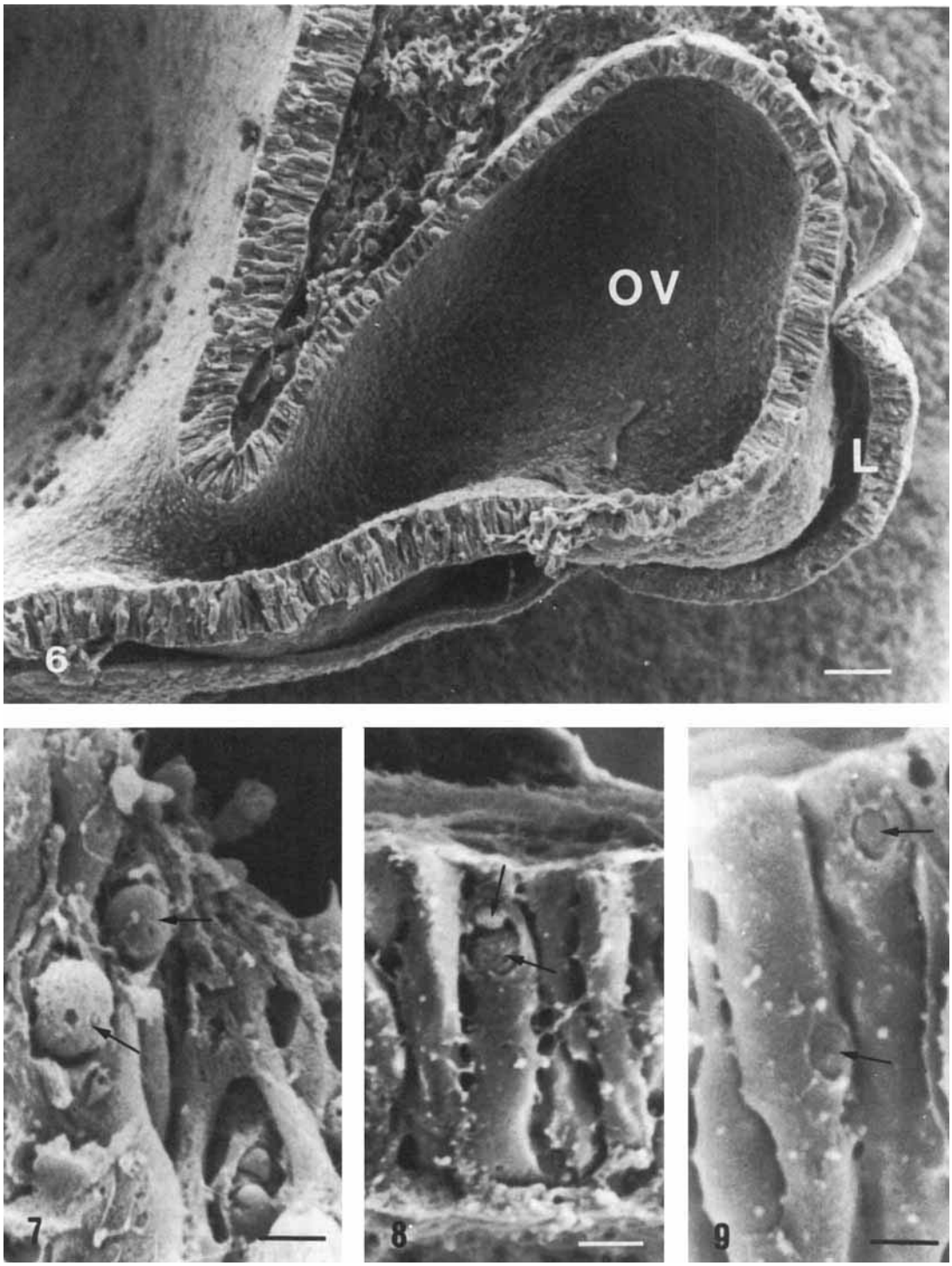


Fig. 6. Scanning electron micrograph showing a frontal section through the eye anlage of a papaverine-treated embryo. The optic vesicle (OV) remains uninvaginated. The lens rudiment (L) appears in the phase of placode. Bar=50 μm.

Figs. 7–9. Scanning electron micrographs depicting several aspects of the retinal necrotic areas of papaverine-treated embryos.

Fig. 7. Large cell fragments (arrows) can clearly be observed in this area. The surface of the fragments is rough and pitted. Bar = 3 μm.

Fig. 8. Two cell fragments (arrows) appear in the course of internalization into a crater developed by a normal neuroepithelial cell. Note the columnar appearance of the retinal cells. Bar = 4 μm.

Fig. 9. In this necrotic area two columnar neuroepithelial cells show craters that contain cell fragments (arrows) in the course of internalization. Bar = 3 μm.

ing those located in the eye rudiment (García-Porrero and Ojeda, 1979; García-Porrero et al., 1979, 1984b). The distinct location of the necrotic areas, their cytological characteristics, and the absence of any sign of tissue damage strongly suggest that these areas of cell death are not due to toxic and/or nonspecific effects of the papaverine. The cell death induced by toxic effects or by tissue damage presents different cytological characteristics (Wyllie, 1981).

The location of the areas of cell death is different in the uninvaginated retinal discs than in the normal developing optic cups. In the uninvaginated retinal disc the necrotic areas appear very close to the optic stalk—one in the ventral side of the retinal rudiment and the other in the dorsal side. Why are the necrotic areas located closer to the optic stalk in the uninvaginated retina? The early shape changes of the optic primordium appear to be due, at least in part, to differentiated additions of cells to the primordium (Hilfer et al., 1981). There is experimental evidence (Holt, 1980) supporting the view that during eye cup formation neuroepithelial cells in the ventral side of the retina move from the optic stalk into the invaginating retinal disc. This movement of cells appears to be necessary for the invagination of the retinal disc. If this process of cell movement occurs not only in the ventral side of the retina but also in the other walls of the retina anlage, the lack of invagination of the retinal disc could produce a delay (or a blockage) of this movement of cells; thus, the prospective necrotic areas could remain in a primitive location. It is suggested here that the abnormally located necrotic areas represent the presumptive normal necrotic areas should the invagination of the retinal disc have taken place. Cell death appears to be in this experimental model, a natural marker for the proximodistal cell movement associated with the normal development of the optic cup.

Retinal cell death occurs in the absence of invagination of the retinal disc. It suggests that both processes are independent of each other. However, we cannot rule out the possibility that cell death is necessary for the normal development of the optic vesicle; in mutant mice of the anophthalmic strain, the absence of cell death has been related to an exaggeration of the process of invagination of the retinal disc (Silver and Hughes, 1974). The present observations also suggest that retinal cells are programmed to die prior to the invagination of the retinal disc and that they do so whether the retinal disc invaginates or not. The existence of programmed cell death has already been established for a number of developing structures (Saunders, 1966; Lockshin and Beaulaton, 1974; Ulshafer and Clavert, 1979; Hinchliffe, 1981; Ojeda and Hurlé, 1981).

Programmed cell death may be triggered by both extrinsic and intrinsic factors (reviewed by Saunders and Fallon, 1967; Hinchliffe, 1981). Among the latter, local diffusion of molecules and microenvironment changes have been held responsible for the triggering of cell death in several embryonic rudiments (Fallon and Saunders, 1968; Hinchliffe et al., 1981; Beaulaton and Lockshin, 1982). However, retinal cell death does not appear to be controlled by the position of the cells within the retina anlage; embryonic retinal cells start dying regardless of their location in the eye rudiment.

It is interesting to note that the lack of invagination of the retinal disc is not always associated with that of the lens placode. This fact emphasizes the idea that the two processes of epithelial invagination appear to be regulated by a different set of factors (Coulombre, 1965; Maloney and Wakely, 1982a,b; Yang and Hilfer, 1982). Dorsoventral asymmetry of the lens cup is a normal feature of the developing chick lens (Wakely, 1976; Bancroft and Bellairs, 1977; García-Porrero et al., 1979). This asymmetry appeared more accentuated in the experimental embryos, the lens cup being, in many cases severely distorted. It has been suggested that the activity of the microfilament system is not essential to the initial invagination of the lens placode; however, it appears to be necessary for the completion of the invagination process (Maloney and Wakely, 1982a). If the activity of the microfilament system is disturbed, the final stages of the invagination of the lens placode will be altered and lens anomalies will result. On the other hand, the possibility that the shape of the optic rudiment may directly influence the shape of the developing lens cannot be discarded (Maloney and Wakely, 1982b).

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. J.M. Icardo for his invaluable help in the preparation of this manuscript, and to Dr. Y.M.T. Berciano for her careful preparation of Figure 1. Thanks are also due to P. Elena-Sinovas for her expert technical assistance.

LITERATURE CITED

Bancroft, M., and R. Bellairs (1977) Placodes of the chick embryo studied by SEM . Anat. Embryol., *151*:97–108.

Beaulaton, J., and R.A. Lockshin (1982) The relation of programmed cell death to development and reproduction: Comparative studies and an attempt at classification. Intl. Rev. Cytol., *79*:215–235.

Brady, R.C., and S.R. Hilfer (1982) Optic cup formation: A calcium-regulated process. Proc. Natl. Acad. Sci. USA, *79*:5587–5591.

Coulombre, A.J. (1965) The eye. In: Organogenesis. R.L. De Haan and H. Ursprung, eds. Holt, Rinehart & Winston, New York, pp. 219–251.

Fallon, J.F., and J.W. Saunders (1968) In vitro analysis of the control of the cell death in a zone of prospective necrosis from the chick wing bud. Devl. Biol., *18*:553–570.

García-Porrero, J.A., and J.L. Ojeda (1979) Cell death and phagocytosis in the neuroepithelium of the developing retina. A TEM and SEM study. Experientia *35*:375–376.

García-Porrero, J.A., J.A. Collado, and J.L. Ojeda (1979) Cell death during detachment of the lens rudiment from ectoderm in the chick embryo. Anat. Rec., *193*:791–804.

García-Porrero, J.A., E. Colvee, and J.L. Ojeda (1984a) Cell death in the dorsal part of the chick optic cup. Evidence for a new necrotic area. J. Embryo. Emp. Morphol., *80*:241–249.

García-Porrero, J.A., E. Colvee, and J.L. Ojeda (1984b) The mechanisms of cell death and phagocytosis in the early chick lens morphogenesis: A scanning electron microscopy and cytochemical approach. Anat. Rec., *208*:123–136.

Glücksmann, A. (1930) Uber die Bedeutung von Zell-vorgangen fur die Formbildung epithelialen Organe. Z Anat Entwgesch., *93*:35–91.

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

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

Hilfer, S.R., R.C. Brady, and J.J.W. Yang (1981) Intracellular and extracellular changes during early ocular development in the chick embryo. In: Ocular Size and Shape. Regulation During Development. S.R. Hilfer and J.B. Sheffield, eds. Springer-Verlag, New York, pp. 47–78.

Hinchliffe, J.R. (1981) Cell death in embryogenesis. In: Cell Death in Biology and Pathology. I.D. Bowen and R.A. Lockshin, eds. Chapman and Hall, London, pp. 35–78.

Hinchliffe, J.R., J.A. García-Porrero, and M. Gumpel-Pinot (1981) The role of the zone of polarising activity in controlling the maintenance and antero-posterior differentiation of the apical mesoderm

of the chick wing bud: Histochemical techniques in the analysis of a developmental problem. Histochem. J., *13:*643–658.

Holt, C. (1980) Cell movements in Xenopus eye development. Nature, *287:*850–852.

Kallen, B. (1955) Cell degeneration during normal ontogenesis of the rabbit brain. J. Anat. *89:*153–161.

Kallen, B. (1965) Degeneration and regeneration in the vertebrate central nervous system during embryogenesis. Prog. Brain. Res., *14:*77–96.

Lockshin R.A., and J. Beaulaton (1974) Programmed cell death. Life Sci., *15:*1549–1566.

Maloney, C., and J. Wakely (1982a) Microfilament patterns in the developing chick eye: Their role in invaginations. Exp. Eye. Res., *34:*877–886.

Maloney, C., and J. Wakely (1982b) Analysis of tissue interactions in chick eye morphogenesis using cytochalasin B. Exp. Eye. Res., *35:*77–87.

Moran, D.J. (1976) A scanning electron microscopic and flame spectrometry study on the role of Ca2+ in Amphibian neurulation using papaverine inhibition and ionophore induction of morphogenetic movement. J. Exp. Zool., *198:*409–416.

Moran, D.J., and R.W. Rice (1976) Action of papaverine and ionophore A23187 on neurulation. Nature *261:*497–499.

Ojeda, J.L., and J.M. Hurlé (1981) Establishment of the tubular heart role of cell death. In: Perspectives in Cardiovascular Research. T. Pexieder, ed. Raven Press., New York, Vol. 5, pp 101–112.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., *17:*208–212.

Saunders, J.W., Jr. (1966) Death in embryonic systems. Science, *154:* 604–612.

Saunders, J.W., and J.F. Fallon (1967) Cell death in embryogenesis. In: Major Problems in Developmental Biology. M. Locke, ed. Academic Press, New York, pp. 289–314.

Schook, P. (1980) Morphogenetic movements during the early development of the chick eye. An ultrastructural and spatial reconstructive study. B. Invagination of the optic vesicle and fusion of its walls. Acta Morphol. Neerl. Scand., *18:*159–180.

Silver, J., and A.F.W. Hughes (1973) The role of cell death during morphogenesis of the mammalian eye. J. Morphol., *140:*159–170.

Silver, J., and A.F.W. Hughes (1974) The relationship between morphogenetic cell death and the development of congenital anophthalmia J. Comp. Neurol *157:*281–302.

Stroeva, O.G. (1960). Experimental analysis of the eye morphogenesis in mammals. J. Embryol. Exp. Morphol *8:*349–368.

Ulshafer, R.J., and A. Clavert (1979) Cell death and optic fiber penetration in the optic stalk of the chick. J. Morphol. *162:*67–76.

Wakely, J. (1979) SEM of lens placode invagination in the chick embryo Exp. Eye. Res., *22:*647–651.

Wyllie, A.H. (1981) Cell Death: A new classification separating apoptosis fron necrosis. In: Cell Death in Biology and Pathology. I.D. Bowen and R.A. Lockshin, eds. Chapman and Hall, London, pp. 9–34.

Yang, J-J.W., and S.R. Hilfer (1982) The effect of inhibitors of glycoconjugate synthesis on optic cup formation in the chick embryo. Dev. Biol., *92:*41–53.

Zwaan, J., and R.W. Hendrix (1973) Changes in cell and organ shape during early development of the ocular lens Am. Zool., *13:*1039–1049.