Optic Fissure Closure in the Normal Cinnamon Mouse

An Ultrastructural Study

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The purpose of this study was to determine the ultrastructural features of optic fissure closure. Serial coronal sections of fetal eyes from the eleventh to the thirteenth gestational day were examined by light and electron microscopy. Fusion was associated with inversion of the retinal pigment epithelium at the optic fissure and it occurred first between undifferentiated cells at the junction of the retina and retinal pigment epithelium. It later extended to involve the entire thickness of the pigment epithelium and neuroretina, with the inner aspect of the latter being the last area to fuse. There was some evidence that closure does not always start at the center of the fissure as generally described, but may sometimes start near the developing papilla. At an ultrastructural level, there was multifocal disintegration of the basement membrane associated with the formation of cytoplasmic processes at these sites. Simple appositional contacts between processes on either side of the fissure comprised the first stages of fusion. Later intermediate-type junctions were formed between adjacent outer retinal cells (presumptive photoreceptors) and junctional complexes were formed at the apices of pigment epithelial cells at the site of fusion. This suggests an increase in mechanical adherence between cells. While basement membrane disintegrated at the center of the fusion site, there was a continuous layer of basement membrane at the internal and external limits of fusion. Cell death together with two morphological types of phagocytic cells were a constant feature at the fissure margins before, during and after fusion. The possible origins and roles of these cells in the fusion process is discussed. Invest Ophthalmol Vis Sci 31:197–216, 1990

During the early development of the mammalian eye, invagination and differential growth result in the formation of a cleft, the optic fissure, through which the hyaloid artery reaches the interior of the optic cup. Closure of the optic fissure is crucial to normal eye development and also to the development of a normal optic nerve. Failure of this process to occur or to be completed results in various degrees of coloboma, microphthalmia and sometimes orbital cysts.1

Closure of the optic fissure in man has been described previously at the light microscopical level.1,2 There are few reports of closure of the optic fissure in laboratory animals.3,4 Geeraets5 performed the only ultrastructural study to date of this critical process in ocular development. The purpose of the present study was to examine the mechanisms of normal optic fissure closure at an ultrastructural level as well as with light microscopy in order to set up a baseline with which to compare the development of colobomata in microphthalmic mutants.

Materials and Methods

Fetuses were obtained from timed pregnancies of normal cinnamon mice, where the observation of a vaginal plug was taken as day E1. The mothers were killed by carbon dioxide inhalation, and fetuses from the eleventh to the thirteenth gestational day dissected from the uterine tubes. Fetuses were immediately immersed into glutaraldehyde 2.5% and fixed for a minimum of 4 hr, after which they were decapitated. The heads were then bisected and transferred to phosphate buffer and then processed for transmission electron microscopy. The posterior part of the blocks (composed of half a head) were trimmed but in most cases the nose was left attached as a marker for orientation in postmicatied blocks. (All animals were sacrificed by methods which conform to the ARVO Resolution on the Use of Animals in Research).

Serial coronal sections (1 μm) of eyes were taken from anterior lenticular levels down to the developing optic disc. These were mounted on to glass slides and stained with toluidine blue. Several ultrathin sections
Fig. 1. Coronal section of the mouse eye late on the eleventh gestational day. Optic fissure closure is just beginning. Note the inversion of the retinal pigment epithelium (rpe) at the optic fissure. R: retina, fp: folding point, arrow: cell death (scale bar = 10 μm).

Fig. 2. Fissure margins lined by basement membrane (arrows) are approaching each other. Some residual mesenchyme (M) is still present in the optic fissure (OF) (scale bar = 1 μm).
(80–100 nm) were cut from levels of interest (showing the stages of fusion) in each eye. In areas where phagocytic cells were identified within the optic fissure, multiple serial sections (from 40–80 ultrathin sections) were examined to ensure that these cells were definitely within the fissure. Sections were collected onto 200 mesh hexagonal copper grids which were then stained with uranyl acetate and lead citrate. They were then examined in a Hitachi H-500 Transmission Electron Microscope. Fifteen eyes from fetuses in five litters were examined in all.

**Results**

**Light Microscopy**

In the mouse optic fissure closure started late on the eleventh gestational day and was complete by the beginning of the thirteenth gestational day. In some eyes fusion began in the middle of the optic fissure, whereas in others it began at a posterior vitreous level quite close to the developing papilla. In yet another group, fusion in the middle of the optic fissure (posterior lenticular/anterior vitreous level) had reached an equal stage to that in the posterior vitreous near the optic papilla with an intervening area in which fusion was lagging behind.

Initially the optic fissure margins approached each other, but still had a considerable amount of mesenchyme between them. Here hyaloid vessels were usually seen passing through the fissure and connecting with intravitreal vessels. Although melanogenesis had begun within the outer layer of the optic cup, the lack of pigment within the pigment epithelial cells near the fissure was a constant feature.
As the fissure margins approached each other, there was a gradual reduction of the intervening mesenchyme. A striking feature at this stage was the inversion of the retinal pigment epithelium into the optic fissure. A little later fusion was first noted at the junction of the retina and retinal pigment epithelium. It then involved the retinal pigment epithelium completely, gradually followed by the inner retina, which was the last area to fuse at any level. Separation of the retinal layers (neural retina from retinal pigment epithelium) began before fusion had been completed in the inner retina. At this point indentations were seen at the inner and outer aspects of the fusion site. Finally, early on the thirteenth day, separation of the retina and retinal pigment epithelium was complete and the indentations on either side of what had been the optic fissure had disappeared. From this time onwards it was impossible to identify the exact site of the fissure.

Throughout the entire fusion process cell death was a constant feature (Fig. 1). At the light microscopic level this was identified by single pyknotic nuclei or groups of degenerate nuclei. These were seen in both inner and outer layers at the fissure margins and away from them. They were more prominent in the posterior half of the optic cup.

Ultrastructural Study

As the fissure margins approached each other (Fig. 2), there was gradual reduction of the intervening mesenchyme. Initially the basement membranes became apposed multifocally to form a temporary double basement membrane (Fig. 3) that later disintegrated. Cytoplasmic prolongations developed on some of the cells lining the fissure at foci of basement membrane disintegration (Figs. 4–7). Cell contacts of a simple appositional type were first formed between these cytoplasmic processes and thus comprised the first stages of fusion (Figs. 6–7). These were areas where the cell membranes were closely apposed and...
parallel to each other, but with no modification of the cell membrane or the underlying cytoplasm. As more basement membrane disintegrated, more simple appositional contacts were formed until eventually basement membrane disappeared completely within the fusion site. In areas the appositional contacts developed thickening of the cell membrane, a feature suggesting the possible development of a more specialized junction (Fig. 7).

Before fusion, all inner layer cells lining the residual ventricular cavity had intermediate junctions between them. Junctional complexes composed of a combination of tight junctions (usually at the ventricular interface), intermediate junctions and gap junctions were present between adjacent pigment epithelial cells. Where retina joined pigment epithelium at the optic fissure margin, the area Geeraets called the "folding point," a continuous row of intermediate junctions was also present. (Fig. 8) Some of the cells at this point degenerated during fusion.

By the time the retinal layers had separated, a continuous row of intermediate-type cell junctions was seen between adjacent outermost retinal cells (the presumptive photoreceptor cells) across the site of...
fusion. (Figs. 9, 10) Junctional complexes also were seen between adjacent pigment epithelial cells in this area. These were composed of tight junctions at their apices, together with alternating intermediate and gap junctions lower down (Fig. 11). Gap and intermediate-type junctions also were seen between cells of the inner and outer retinal layers.

During the fusion process, although basement membrane was broken down at the center of the fusion zone, its internal (vitreal) and external (choroidal) limits (the areas of indentation seen at light and electron microscopy) were always lined by a continuous layer of basement membrane. This extended from one optic fissure margin, lined the depth of the indentation and then continued onto the opposing fissure margin (Figs. 12, 13). As fusion extended throughout the entire thickness of the retinal pigment epithelium and the inner retina, the indentations gradually flattened, until by the time fusion was complete they had entirely disappeared (Figs. 9, 13-15). The basement membrane had become a flat layer lining the retino-vitreal and retino-choroidal interfaces of the wall of the optic cup. At this point the previous site of fusion was no longer identifiable from the adjacent retina.

Degenerate cells were a constant feature on either side of the fissure in the inner and outer layers of the optic cup. Although both nuclear and cytoplasmic types of cell death were seen, the former was much more frequent. The nucleus frequently broke down into discrete fragments in which there was a characteristic segregation of chromatin. Coincident with the nuclear changes, there was progressive condensation of the cytoplasm. The cells were gradually converted into a number of apoptotic bodies of varying size and composition. Some contained one or more nuclear fragments, others cytoplasmic elements alone.

Debris was seen both extracellularly and within healthy neuroepithelial cells. Immature neuroepithelial cells appeared to have the capacity to phagocytose debris situated in the adjacent extracellular space. Many of these cells were elongated, similar to cells on either side of them, but also contained one or two fragments of rounded, electron-dense debris (apoptotic bodies) which usually indented the nucleus (Fig. 16). Large, round cells packed full of several
clumps of electron-dense debris were also present at or near the fissure margins (Fig. 17). Here again the healthy nucleus was seen to be indented by debris. One could distinguish phagocytic cells from degenerate ones, mainly by the fact that they had a healthy nucleus in which chromatin condensation was absent. Also the borders of degenerate cells tended to shrink and become convoluted before breaking up into fragments; in contrast, phagocytic cells that contained several apoptotic bodies became larger and rounder (Fig. 17). These cells were seen in both the inner and outer retinal layers (Figs. 16–18).

Another type of phagocytic cell also was constantly present near the site of fusion. These cells had several pseudopodia, contained degenerate electron-dense material and also had several electron-lucent vacuoles within their cytoplasm (Fig. 19). They constantly were seen in the vitreous, in the ventricular space away and close to the site of fusion, and within the optic fissure as fusion was taking place. Examination of multiple serial sections through the same phagocytic cell ensured that some of these cells definitely were within the fissure and were not obliquely cut cells that were actually within the fissure margin. Obliquely cut cells at the fissure margin also can be distinguished because they are lined by basement membrane. Although the “amoeboid” cells situated within the fissure were frequently seen to be in contact with the basement membrane on either side of it, they themselves were not lined by basement membrane.

**Discussion**

Although observations at both light microscopic and ultrastructural levels confirm several aspects of previous studies on the optic fissure, this study has raised some questions about certain features in the classic descriptions. Does optic fissure closure always start at the center of the optic fissure? Is there inversion or eversion of the pigment epithelium at the site of fusion?

Optic fissure closure is classically described as starting in the central portion of the fissure and from there proceeding anteriorly and posteriorly, so that the anterior aspect of the fissure and the optic disc region are the last areas to fuse. In some eyes examined at the earliest stage of fusion, this process was first seen to occur at a much more posterior level than generally described in the literature, in fact in the posterior vitreous just anterior to the primitive papilla. In eyes examined at a somewhat later stage, fusion was sometimes most advanced in the area adjacent to the papilla. In others, fusion had reached an equal stage in the middle of the fissure (posterior posterior/lateral/anterior vitreous levels) as it had done in the posterior vitreous very close to the primitive papilla. In these eyes there was an intervening space in which fusion lagged behind.

These findings imply that closure may not always start in the central portion of the optic fissure as previously thought, but may sometimes start much more posteriorly near the optic disc, or may actually start at two or more separate foci at different levels along the fissure. This suggestion is supported by the observations in a recent paper on the light microscopic
mechanisms of optic fissure closure in the mouse. In the latter study, closure began near the primitive papilla in most cases and in others it began at the central portion of the fissure and/or papilla. As the purpose of the present study was to examine the ultrastructural mechanisms of optic fissure closure, the number of eyes examined was insufficient to come to a definite conclusion.

Eversion of the inner layer into the fissure was described previously in the literature. Mann stated that in many vertebrates, among them man and the mouse, the inner layer grows more rapidly and consequently becomes everted. In the present study eversion of the inner layer was not identified; on the contrary, in the first stages of fusion, an inversion of the outer retinal (retinal pigment epithelial) layer into the fissure was noted. At any particular level, these less differentiated outer layer cells that had inverted into the fissure were always the first focus of fusion. Although they have been referred to as pigment epithelial cells, they only develop melanosomes after fusion has occurred.

The reason for this discrepancy is not certain. In Mann's study eversion was demonstrated in horizontal sections, and it is not easy to compare these with the coronal sections of the optic cup used in the present study. Geeraets and Suzuki et al, who also studied coronal sections of the developing eye, also...
Fig. 9. Fusion is nearly complete. A continuous row of intermediate-type junctions has developed between adjacent outer retinal cells (single arrows) across the fusion site (FS). Junctional complexes (double arrows) have developed between adjacent retinal pigment epithelial cells in this area. Note phagocytic cell (PC) in residual ventricular space (scale bar = 10 μm).

noted inversion of the outer layer into the fissure. Another possibility is that different species may have different degrees of inversion or eversion.

Geeraets demonstrated that basal lamina disintegration was essential for this important process in eye development. The observations in the present study confirmed this. The mechanism of basement membrane breakdown is, however, uncertain. The author’s immunocytochemical studies using a polyclonal anti-laminin antibody demonstrated that laminin was present in the basement membrane around the optic cup and that laminin expression first becomes discontinuous and then disappears at the site of fusion (submitted for publication). There is thus good correlation between ultrastructural and immunocytochemical studies. Towards the completion of fusion
restoration of the basement membrane was seen ultrastructurally, and laminin expression was restored at the inner and outer aspects of the optic cup across the fusion site. Presumably all other basement membrane components have to be broken down similarly.

How the basement membrane is restored after fusion is not certain. Although it disintegrates within the central part of the fusion zone at all levels, there is always a continuous basement membrane at the outermost (choroidal) and innermost (vitreal) aspects of the fusion site—the indents shown in Figure 14. As fusion involves the full thickness of the retina at any particular level, the indents become less deep, until they eventually disappear. At all times during fusion, these indents are lined by a basement membrane that is continuous from one margin of the optic fissure, into the deepest part of the indent (the inner and outer limits of fusion) and across to the other side (Figs. 12, 13). This suggests that while basement membrane is being degraded at the central part of the fusion zone, it is being freshly laid down at the inner and outer limits of the fusion process.

Simple appositional contacts between cells on either side of the fissure comprise the first stages of fusion. Thickening of the cell membranes forming some of these contacts suggests that some of them may develop into the intermediate-type junctions and junctional complexes seen between cells lining

Fig. 10. The Fusion Site—intermediate-type junctions (single arrows) are present between adjacent outer retinal cells. Junctional complexes (double arrows) are present between retinal pigment epithelial cells. Note a phagocytic cell (PC) in the residual ventricular space (scale bar = 1 μm).
Fig. 11. Junctional complexes between adjacent retinal pigment epithelial cells are composed of alternating tight junctions (TJ) (usually situated at the apical surface), intermediate-type junctions (IJ) and gap junctions (GJ). R: retina, V: ventricular space (scale bar = 1 μm).

The distinction between tight junctions and gap junctions at transmission electron microscopy was somewhat difficult; however, their distribution within junctional complexes suggested that the junctions present at the apicolateral borders of the outer layer cells (bordering the residual ventricular space), were in fact tight junctions. Similar junctions, considered to be gap junctions, were usually situated further down along the lateral borders of the cells with one or several intermediate-type junctions separating them from the tight junctions. It is only by freeze-fracture techniques or studies using lanthanum compounds that one could determine these junction types with certainty.

As well as involving the rapid development of new cell contacts and junctions, the process of fusion also involves the rapid breakdown of specialized junctions. Long before fusion occurs, there are well developed intermediate junctions at the ventricular aspect...
of the cells at the folding point. (This point has been described by Geeraets as the point where during the invagination of the optic vesicle the single epithelial layer became folded, thus giving rise to a two-layered wall.) In electronmicrographs of the early optic cup, intermediate-type junctions appear to radiate from this point (see Fig. 8). Fusion of the opposing fissure margins and later separation of the fused inner layers and fused outer layers necessitates the breakdown of the intercellular junctions at the folding point. Although cell death in this area may provide one mechanism for the disposal of these junctions, there is
Fig. 13. Early fusion has occurred between cells at the retinal/retinal pigment epithelial junction. Note that the basement membrane (arrows) is present at the inner and outer limits of the fusion site but has disappeared within it. OF: optic fissure (scale bar = 1 μm).
probably an active mechanism for their rapid removal.

Cell death is a constant feature both at the optic fissure margins and away from them during this period. This has been well documented in the past. Several descriptions of degenerating neurons in the developing nervous system have come to the conclusion that there are two distinct types of degenerating neurons on morphological grounds: "nuclear" and "cytoplasmic" types of cell death.

Although cell death in the optic cup is not strictly comparable to that seen in the above studies which were carried out in the neonate, there does appear to be a variation in the type of cell death. A nuclear type of cell death is seen more commonly although cytoplasmic type cell death also occasionally is seen. Cell death presumably has a role in developmental remodelling. Although it could be speculated that enzymes released from dying cells may have a role in the breakdown of basement membrane, there is no definite evidence to support this.

Fig. 14. Fusion has extended to involve cells of the outer and mid-retina. Note the indent (I) at the inner and outer aspects of the area of fusion (F). Note the inversion of the retinal pigment epithelium into the optic fissure. rpe: retinal pigment epithelium; R: retina; FP: folding points (scale bar = 10 μm).
Fig. 15. Separation of the retinal pigment epithelium (rpe) from the retina in the zone of fusion is beginning before fusion has been completed at the inner (vitreal) aspect of the fissure (F). For this to occur some of the intermediate-type junctions at the folding point (FP) have to be broken down. R: retina (scale bar = 10 μm).

epithelial cells with healthy looking nuclei. This phenomenon has been reported previously at both light microscopy in the OR mutant and ultrastructurally in the golden hamster, where Geeraets reports that "a few small dense bodies in the cytoplasm of otherwise normal cells could be seen in the vicinity of the fissure" throughout all the stages of closure.

This finding suggests that cells of neuroepithelial origin have the potential of phagocytosis. The phagocytic function of adult retinal pigment epithelium is a well established feature. The finding of degenerate debris in pigment epithelial cells at this time in development suggests that this property is achieved very early on in pigment epithelial differentiation.

The finding of typical primitive neuroepithelial inner retinal cells phagocytosing adjacent debris came as a surprise. However, similar findings have been described in the parenchymal cells of other tis-
Fig. 16. The neuroretina after fusion has occurred. Note the phagocytic cell (PC) of probable neuroepithelial origin containing ingested cell debris (scale bar = 10 μm).
Fig. 17. Note the phagocytic cells in the neuroretina on either side of the still open fissure (F). Presumably cells similar to those seen in Figure 16 enlarge as they ingest more debris. Arrows demarcate the optic fissure margins (scale bar = 10 μm).

These findings would suggest two sites of origin for the morphological types of phagocytic cell found in the optic cup: a neuroepithelial origin for the first type of cell and a mesenchymal origin for the amoeboïd type found in the vitreous and optic fissure. The presence of amoeboïd-type cells in the ventricular space and retina is more difficult to explain. A mesenchymal origin of these cells would imply that they are migrating from the vitreous or mesenchyme surrounding the optic cup, through the basement membrane at the vitreal or choroidal boundaries of the optic cup, through the retinal layers and into the ventricular space. This would suggest that these cells may have the capacity to break down basement membrane prior to entering the retina. This feature would further support their role in the disintegration of basement membrane which occurs during optic fissure closure. However, it is possible that these amoeboïd cells develop from neuroepithelial cells, in which case their migration into the ventricular space would be much easier to explain.
Fig. 18. Pigment epithelial cells (pe) with healthy nuclei containing ingested debris (scale bar = 10 μm).
Fig. 19. A phagocytic cell (pc) of "amoeboid" type in the ventricular space (V) adjacent to the area of fusion. Note cilium (C) related to the prominent pseudopodia (arrows). R: retina, RPE: retinal pigment epithelium (scale bar = 1 μm).
Key words: optic fissure, fusion, basement membrane, cell junctions, ultrastructure

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