The Prenatal Development of the Optic Fissure in Colobomatous Microphthalmia

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The coloboma in the cinnamon mouse homozygous for the microphthalmia gene is caused when optic fissure closure, which normally occurs between the 11th and the 13th gestational day, does not occur. This study sought to determine the cause of this fusion failure, and to identify any foci of fusion that occur later in gestation. Microphthalmic fetuses from the 11th–20th gestational day were obtained by datemating cinnamon mice heterozygous for the microphthalmia gene. Coronal serial sections of the eyes were examined at light and electron microscopy. Initially, the fissure margins became apposed only in the posterior aspects of the eye. A failure of basement membrane disintegration at the fissure margins prevented fusion at the 12th and 13th days. On the 14th day, small foci of basement membrane disintegration were identified in the area of the developing optic disc. Although the fusion zone enlarged later in gestation, it was limited to the area of the optic disc and showed that the two retinal layers did not separate. This study has shown that abnormal growth and invagination lead to delayed apposition of the optic fissure margins. These features together with a failure of basement membrane disintegration appear to be the main factors involved in coloboma formation. It is suggested that the excessive number of outer-layer cells that are inverted into the fissure, as well as abnormal or reduced numbers of phagocytic cells, may affect the persistence of the basement membrane. Alternatively, a primary defect of the pigment epithelial cell may lead to the development of the hypercellular and nonpigmented outer layer associated with the lack of basement membrane disintegration and nonfusion in this mutant.

Materials and Methods

Mutant fetuses (mi/mi) were obtained from datemated cinnamon mice heterozygous for the microphthalmia gene (mi/-). They were identified on the 11th gestational day due to the lack of ocular pigment development. All animals were killed with methods that conformed with the ARVO Resolution on the Use of Animals in Research. The mothers were killed either by carbon dioxide inhalation or cervic dislocation. Fetuses from the 11th to the 20th gestational day (E11–E20) were immediately dissected from the fallopian tubes and were fixed in a variety of fixatives: glutaraldehyde 2.5%, paraformaldehyde 4%, paraformaldehyde 4% and glutaraldehyde 2%, and paraformaldehyde 2% and glutaraldehyde 1%. The younger fetuses (E11–E12) were fixed whole. After a minimum of 4-hr fixation, they were decapitated and the heads were bisected. From the 13th gestational day, the heads were bisected fresh and their posterior aspects were removed. The nose was left as a marker for orientation of postosmicated specimens. To achieve fix good fixation, most of the tissue around the eye in fetuses was removed from the 18th to the 20th gestational day. All tissue was left in fixative for a minimum of 12 hr and then processed routinely for electron microscopy. Coronal serial 1-μm sections were cut from anterior lenticular levels down to the anterior aspects of the optic nerve. Sections were stained with toluidine blue and 80–100 millimicron sections were taken at selected levels of interest. These were collected on 200-mesh hexagonal copper grids and were stained with uranyl acetate and lead citrate.
They were examined with a Hitachi 500 electronmicroscope. Best results were obtained with tissue fixed in paraformaldehyde 4% and glutaraldehyde 2%.

Results

Light Microscopy

A definition of the terms used in this study is necessary before we describe our findings. Apposition implies that the opposing fissure margins have made contact, but that the basement membranes that line them persist. Fusion refers to contact of the fissure margins with basement membrane disintegration. The folding 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.

On the 11th gestational day, microphthalmic fetuses could be identified by the absence of development of melanin pigment in the outer layer of the optic cup. At this stage, thickening of the dorsal aspects of the outer layer also distinguished the mutant optic cup (Figs. 1A–C). In a normal eye, the pigment epithelium develops from a pseudostratified nonpigmented epithelium to a pigmented monolayer of cuboidal cells at about this time.

As in the normal mouse, the optic fissure developed in the mutant on the 11th day. However, although the

Fig. 1. Coronal sections of microphthalmic fetus at gestational day 12 (E12). Thickening of the outer layer (OL) of optic cup in all sections occurs. L, lens; M, mesenchyme; IL, inner layer of optic cup. (A) Section at anterior lenticular level shows that the fissure margins (FM) are far apart anteriorly. Invagination is not as well developed anteriorly as it is posteriorly. Bar = 100 μm. (B) Section at posterior lenticular level. Fissure margins (FM) are much closer, but mesenchyme is prominent in the optic fissure. Bar = 100 μm. (C) Section at the level of the developing optic disc. Posteriorly, the fissure margins (FM) are becoming apposed. Note the pyknotic cells (arrows) in the fissure margins and elsewhere in the optic cup. Bar = 100 μm.
fissure margins began to approach each other on the 11th day, this process was somewhat delayed, so that by the 12th gestational day, the fissure margins became apposed only in retrolenticular levels, especially in the region of the developing optic papilla (Fig. 1C). Here, the folding points became symmetrically apposed and foci of cell death were seen in the optic fissure margins (Fig. 1C). In spite of the similarity to the situation in the normal fusion process, no evidence of fusion in this area at this gestational age (E12) exists. In contrast to the normal mouse, where the outer layer at the area of imminent fusion is composed of one layer of cells, in the mutant, the outer layer adjacent to the fissure is either pseudostratified or composed of two to three layers of cells.

A delay occurred in the development of the anterior aspects of the optic fissure so that the fissure margins remained far apart and much mesenchyme was seen between them (Fig. 1A). Vessels connecting with the hyaloid vessels were frequently seen in the fissure at all levels. Clumps of pyknotic nuclei and single pyknotic nuclei were seen in the fissure margins, especially in retrolenticular levels (Fig. 1C).

By the 14th gestational day (Fig. 2A-C), the fissure margins approached each other at midlenticular levels. At anterior vitreous levels, rolling of one of the fissure margins occurred (Fig. 2B). This rolling became more prominent at E15 (Fig. 3A). No evidence of increased mesenchymal tissue, in relation to the rolled fissure margin, exists.

At E14, the first focus of fusion was identified in the optic disc region (Fig. 2C). This effect occurred between the outer-layer cells of the slightly rolled margin and the inner-layer cells of the opposite margin. All cells in the fusion area appeared poorly differentiated at this stage. Outer-layer cells were defined as cells external to the folding point and inner-layer cells as those internal to it.

From the 15th to the 20th day, the area of fusion enlarged but remained limited to the region of the

Fig. 2. Microphthalmic fetus at gestational day 14 (E14). (A) Coronal section taken at anterior lenticular level. Although the fissure margins (FM) have approached each other, they are far apart. Marked thickening of the dorsal outer layer (OL) occurs. L, lens; IL, inner layer of optic cup. Bar = 100 μm. (B) Coronal section at retrolenticular level. The fissure margins are approaching each other, and there is early rolling of one of the fissure margins (arrow). HV, hyaloid vessels. Bar = 100 μm. (C) Coronal section taken at the level of the developing optic disc. Inferior aspect of optic cup. Note area of fusion (arrow). FP, folding points; OL, outer layer; IL, inner layer. Bar = 100 μm.
Fig. 3. Mutant optic cup at gestational day 15 (E15).
(A) Coronal section at posterior lenticular level. Note proximity of fissure margins and marked rolling of one of the fissure margins (arrow). OL, outer layer; IL, inner layer; L, lens. Bar = 100 μm. (B) Coronal section at the level of the developing optic disc. The area of fusion (F) has enlarged. Note the persistent folding points (FP) and the failure of separation of the retinal layers. OL, outer layer; IL, inner layer; Arrowheads, unfused fissure. Bar = 10 μm.
developing optic disc. In a few eyes, it extended a little more anteriorly into vitreous/posterior lenticular levels. From the 18th to the 20th gestational day, the lens often appeared to fill the entire eye, and often its posterior surface was situated just anterior to the developing papilla.

After the 15th day, in spite of the attempt at fusion in the region of the optic disc, the folding points persisted (Fig. 3B). Thus, the retinal layers in the limited fusion zone did not separate. In more anterior levels, although the fissure margins were tightly apposed, no evidence of fusion and vessels of various sizes were frequently seen in the intervening space (Fig. 4). At anterior lenticular levels, the fissure margins were widely separated with much intervening mesenchyme.

From the 15th day, the nerve fiber layer extended into the fissure margins. By the 20th day the ganglion cell layer and nerve fiber layer extended into the fissure margins and into the outer layer adjacent to the optic fissure. Retinal differentiation was most prominent in retrolenticular levels and was not seen at anterior lenticular levels late in gestation. Here the fissure margins were lined by pale cuboidal cells. Axons first infiltrated the area of fusion at E16 and late in gestation ganglion cell differentiation was seen within the area of fusion (Fig. 5).

Electron Microscopy

At the 12th gestational day, although the fissure margins approached each other, they only became apposed in posterior levels. Late on the 12th day and especially on the 13th day, small foci of basement membrane apposition were seen in the area of the developing papilla (Fig. 6). However, no evidence of basement membrane disintegration at this stage was seen. More anteriorly, several mesenchymal cells and vessels were seen within the fissure. Degenerate cells, similar to those seen in the normal process of fusion, were identified in the posterior aspects of the mutant optic fissure margins from the 12th to the 15th gestational day. In contrast to the normal mouse, it was unusual to see degenerate cells in lenticular levels of the mutant optic fissure. The nuclei of degenerate cells frequently broke down into discrete fragments in which there was characteristic segregation of chromatin. Coincident with nuclear changes, there was progressive condensation of cytoplasm.

Amoeboid phagocytic cells were seen within the fissure (7A-C), at its vitreal and choroidal aspects, as well as within the rest of the vitreous and the mesenchyme surrounding the optic cup. They were also seen within the retina and ventricular space. These amoeboid phagocytic cells were similar to those identified in the normal mouse during and after optic fissure closure. They contained electron-dense debris, electron-lucent vacuoles, and also had several pseudopodia.

A second type of phagocytic cell was seen within the fissure margins in both the inner and outer layers (Fig. 8). These were cells similar to the surrounding neuroepithelial cells; however, they differed in that they contained rounded bodies of electron dense debris. This debris was usually indenting a healthy nucleus, hence, differentiating these cells from degenerate cells.

Both these types of phagocytic cells were seen from late on the 11th to the 15th gestational day. They were seen mainly in the region of the developing optic papilla. It was unusual to see them in the fusion zone after the 15th day, although they were seen in other areas of the optic cup. After the 15th day, amoeboid type phagocytic cells were most frequently seen in the
vitreous and ventricular space; they were also seen in various parts of the retina.

On the 14th gestational day, foci of basement membrane disintegration began to appear in the region of the optic papilla. These were associated with the development of cytoplasmic prolongations (Fig. 9A-B) that later made simple appositional contacts\(^\text{10}\) with similar processes from the opposite fissure margin (Fig. 10A-B).
Fig. 7. Amoeboid-type phagocytic cells characterized by pseudopodia, electron dense debris, and electron lucent vacuoles. (7A) Section at the level of the optic disc. E12 mutant optic cup shows the choroidal aspect of the optic fissure. Note the proximity of the fissure margins (FM) and the amoeboid-type phagocytic cell (APC) in the optic fissure. Bar = 1 μm. (7B) E13 mutant optic cup. Vitreal aspect of the optic fissure shows an amoeboid phagocytic cell (APC) and a vessel (V) entering the optic fissure (OF). Bar = 1 μm. (7C) Posterior aspect of E14 mutant optic cup. Outer (choroidal) aspect of the optic fissure. The amoeboid phagocytic cell (APC) in the optic fissure is closely related to a cytoplasmic prolongation (arrow) that develops at a focus of basement membrane disintegration (higher magnification shown in Fig. 9B). Bar = 1 μm.

B). As more basement membrane disintegrated, more appositional contacts were formed, thus resulting in enlargement of the area of fusion. At this time, amoeboid type phagocytic cells were frequently seen at the outer (choroidal) aspects of the fissure (Fig. 7A,C).

From the 15th to the 20th day, although the area of fusion enlarged, it was limited to the area of the developing papilla. Throughout this time only simple appositional contacts were seen between cells in the fusion zone (Fig. 11A-B). There was no evidence of more specialized junction formation and breakdown of the intermediate type junctions that form the folding points was not seen (Fig. 12). This occurrence prevented the separation of the retinal layers at the fusion zone. From day 16, axons began to infiltrate the area of fusion. Later in gestation, this area was completely infiltrated by axons and ganglion cells could also be identified (Fig. 13). More anteriorly, where the fissure margins were not fused, differentiation into nerve fiber layer and ganglion cell layer was seen to extend right around the fissure margins and into the outer cell layer just external to the fissure.
Anterior to the fusion area, the fissure margins were often separated only by two basement membranes (similar picture to that seen in Fig. 6). In other areas, vessels were seen within the fissure (Fig. 14). From midlenticular levels, the margins became more widely separated and on day twenty, immature collagenous fibrils, as well as mesenchymal cells, were sometimes seen within the fissure.

Discussion
In this study, a detailed examination of the development of the microphthalmic optic fissure throughout gestation has been performed at the light and electron microscopal levels. We confirmed the observations made by Muller and Packer that the hypercellularity of the dorsal outer layer of the optic cup is associated with a delay in the invaginative process. However, the mechanism by which this delay in invagination occurs remains unclear. This study has identified that abnormal invagination causes lack of apposition of the entire length of the fissure margins at the correct time and a relative delay in the development of the infero-anterior aspect of the microphthalmic optic cup throughout gestation. Where the fissure margins
do come into apposition, persistence of the basement membrane is a major factor in preventing fusion from occurring. The study has also identified a small area of partial fusion that occurs at the 14th gestational day at least 24 hr after the completion of fusion in the normal mouse. Although this area enlarges in the later stages of gestation, it remains limited to the area of the developing papilla.

On the 12th day (E12), in addition to the absence of development of ocular pigment and thickening of the outer layer, the mutant showed that the entire length of the fissure margins did not appose. Although in the area of the developing optic disc the fissure margins became apposed, they did so only late on the 12th gestational day (at least 12 hr after this happens in a normal mouse). Before this although the fissure mar-

Fig. 9. Development of cytoplasmic prolongations in E14 mutant optic fissure at the level of the developing optic disc. (9A) Note cytoplasmic prolongations (arrows) developing at the optic fissure margins. Bar = 1 μm. (9B) Cytoplasmic prolongations (arrowheads) develop at foci of basement membrane disintegration. Basement membrane, arrows. Bar = 1 μm.
gins approached each other, intervening mesenchymal cells were still seen within the fissure. In the normal mouse opposing basement membranes first become apposed late on the 11th and early on the 12th gestational day. It may be that the timing of fissure margin apposition is critical to the normal process of fusion.

It is difficult to explain why the basement membrane does not disintegrate where apposition of the fissure margins has occurred in the posterior levels of
the mutant eye late on the 12th day. As in the normal, the basement membranes become focally apposed, and cell death and phagocytic cells are seen. The timing of events may be critical or the extent of cell death and the number of phagocytic cells could be reduced and could lead to a reduction of the enzymes that may affect basement membrane disintegration. The phagocytes may lack the enzymes necessary for basement membrane breakdown. This suggestion is supported by the fact that abnormal macrophage and osteoclas-
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Fig. 12. E16 mutant optic cup. Note persistence of intermediate type junctions (arrows) at the folding point (FP) adjacent to the area of fusion. IL, inner layer of optic cup; OL, outer layer of optic cup. Bar = 1 μm.

Fig. 13. E17 mutant optic cup. Area of fusion at the level of the developing optic papilla. Axons (A) infiltrate the area of fusion. The cells (G) that contain paler nuclei probably represent ganglion cell differentiation within the area of fusion. Note the amoeboid phagocytic cell (APC) adjacent to the developing choroidal vessel (V). Bar = 10 μm.

tic activity has been reported in these mice, thus leading to the development of osteopetrosis and lack of tooth eruption.11,12

Dying cells and two types of phagocytic cells are seen throughout the entire length of the fissure margin in the normal mouse,9 but mainly posteriorly in the mutant. Although in the normal mouse these cells are also more prominent in the posterior aspects of the eye,7,9 similar cells are frequently seen anteriorly. In contrast, in the mutant, these cells are only occasionally seen in the anterior. Interestingly, the posterior aspect of the mutant fissure is the area that is to show some attempt at fusion later in gestation. This finding further supports the suggestion that enzymes released from dying cells and/or phagocytic cells may affect basement membrane disintegration during the normal fusion process.9

In the normal fusion process, there is inversion of the outer layer into the fissure; the outer layer is at this stage one-cell thick. In the normal mouse, disintegration of the basement membrane is associated with death of at least some of these outer-layer cells that have become inverted into the optic fissure. In contrast, the mutant outer layer in this area is thickened to form a pseudostratified outer layer, or one that is two to three cell layers thick in the immediate vicinity of the fissure. Although some cell death occurs in the outer layer-cells that are inverted into the fissure, there always appears to be an excess of these cells when compared with the situation in the normal fusion process. Thus, although there is inversion of the outer layer into the fissure, the excess of outer-layer cells in this region may further disturb the fusion pro-
cess. It may be that this excess of cells forming the fissure margin is responsible for maintaining the basement membrane at this site and so in preventing its disintegration.

A primary defect of the retinal pigment epithelium that causes the lack of differentiation may be the reason for the absence of pigment and the hypercellularity of the outer layer in this mutant.

Rolling of the fissure margins and consequent asymmetry of the folding points is a feature noticed on the 14th day. No excess of mesenchyme, which may cause traction, is seen at the vitreal aspects of these rolled margins. Before this, (on E12) the folding points are symmetrically positioned and apposed in the area of the developing papilla (Fig. 1C). More anteriorly, the folding points are symmetric but the fissure margins are separated by mesenchyme (Fig. 1B). It is only on the 14th day (E14), 24 hr after the completion of fusion in the normal mouse, that rolling of the fissure margins begins as a consequence of this lack of fusion at the correct time. The rolling of the unfused margins and the consequent asymmetry of the folding points therefore occurs secondarily to a failure of fusion and has no role in its causation.

This study has identified a focus of fusion posteriorly in the area of the developing optic disc. However, this appears to be a primitive form of fusion as no specialized junctions develop in this area. Only simple appositional contacts are seen (Fig. 11A-B). There is also persistence of the preexisting intermediate type junctions seen at the two folding points (Fig. 12). These features thus lead to a lack of separation of the retinal layers. The formation of intermediate junctions between the outermost cells of the inner-layer (presumptive photoreceptor cells), and the formation of junctional complexes between adjacent outer-layer cells which occur at the normal fusion site, may only be possible at a certain time in development. If breakdown of basement membrane and the development of simple appositional contacts occur later than is normally scheduled, a primitive form of fusion persists. Breakdown of junctions at the folding points may only be possible if fusion occurs between the 11th and 13th gestational day. This finding would
explain the persistence of intermediate type junctions at the folding points in the mutant. Although it starts at the 14th day and enlarges throughout gestation, the area of fusion appears limited to the area of the developing papilla. In contrast, foci of fusion have also been reported to occur anteriorly in the microphthalmic golden hamster.6

In the normal mouse, the nerve fiber layer is restricted to the vitreal aspect of the wall of the optic cup. In contrast, in the mutant, as most of the fissure margins remain unfused, retinal differentiation proceeds along the fissure margins to extend to the outer layer on either side of the optic fissure. Von Szily1 suggested that in the rabbit, retinal differentiation at the fissure margins was the reason why fusion did not occur and the reason for coloboma formation. The findings in this study would suggest that this lack of fusion is primarily due to delayed apposition of the fissure margins and persistence of the basement membrane. Retinal differentiation at the unfused fissure margins is a secondary event which occurs around the 14th to 15th day, at least 24 hr after the process of fusion would have been completed in a normal mouse.

In conclusion, abnormal growth and invagination, together with a lack of basement membrane disintegration, appear to be the main factors involved in coloboma formation. It is suggested that the excessive numbers of outer-layer cells that are inverted into the fissure, as well as abnormal or reduced numbers of phagocytic cells, may affect the persistence of basement membrane. An alternative explanation is that the above abnormalities are due to a primary defect of the pigment epithelial cell that may lead to the development of the nonpigmented and hypercellular outer layer. A primitive area of fusion has been identified in the area of the developing optic disc in the later stages of gestation.

Key words: optic fissure, development, coloboma, microphthalmia, basement membrane

References