Electron microscopic study of the development of retinal Müllerian cells

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The pre- and postnatal development of Müllerian cells of the rabbit retina were studied with light and electron microscopy. The first differentiation of the Müllerian cells appears as the formation of smooth-surfaced endoplasmic reticulum in the innermost (vitread) portion of the retina, on the fourteenth day of gestation. Ganglion and amacrine cells differentiate in the vitread portion of the neuroblastic layer during the eighteenth to twenty-sixth days of gestation, by which time smooth-surfaced endoplasmic reticulum is scattered throughout the inner processes of the Müllerian cells. During the late prenatal period, bipolar and horizontal cells are formed and glycogen particles, characteristic of the mature Müllerian cell, appear scattered throughout their cytoplasm. Mitochondria become concentrated in the apices of these cells, near the external limiting membrane. After birth, the major changes in Müllerian cells are elongation of their microvillous processes, lateral cytoplasmic extensions, and rearrangement of their nuclei. The intercellular extensions of Müllerian cells grow in the outer and inner plexiform layers in these postnatal stages. Their nuclei, which had been located at different levels of the inner nuclear layer at birth, become arranged in the innermost, or second-innermost zone of this layer, external to the amacrine cells. The Müllerian cells are complete by the end of the third postnatal week. Our observations suggest that their synthetic activity occurs in the postnatal period.

The developing retina in mammals has been extensively studied in recent years by electron microscopy. Attention has, however, been focused mainly on the outer region of the retina and its neural organization, and little is yet known about the developmental processes of Müllerian cells during the period when the retina changes from a simple cell to its intricate adult organization. Early light microscopic studies have shown that they are formed early in development. Some histological, histochemical, and tissue culture investigations have briefly reported some aspects of Müllerian cells, suggesting that they mature postnatally.

The present study attempts to describe the sequence of development of retinal Müllerian cells during pre- and postnatal periods in the rabbit.

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Fig. 1. Apical portion of the inner (IL) and outer layers (OL) of the optic cup on the eleventh day of gestation. Terminal bars (t), ciliary processes (ci) and mitosis are seen. Mitochondria (m), Optic ventricle: (OV). (×4,400) Inset: Light photomicrograph of the same preparation. (×120.)

Fig. 2. Innermost portion of the inner layer of the optic cup on the eleventh day of gestation. The inner surface of the cells is covered with a basal lamina (BL). Mitochondria: (m), Nucleus: (N), rough-surfaced endoplasmic reticulum: (rER). (×8,800.)

Fig. 3. Higher magnification of the terminal bar in the apical portion of the inner layer. (Eleventh day of gestation). (×34,000.)

Fig. 4. Higher magnification of the terminal bar in the apical portion of the sensory retina at birth. (×51,000.)
Fig. 5. Innermost portion of the sensory retina on the fourteenth prenatal day. Together with processes of undifferentiated neuroectodermal cells, an elongated process reaching a basal lamina (BL) contains a sparse network of smooth-surfaced endoplasmic reticulum (sER) characteristic of matured Müllerian cells. Increased density of cell membrane is seen in places (arrows). Mitochondria: (m), rough-surfaced endoplasmic reticulum: (rER), Ribosomes: (rib). (x9,000.) Inset: Photomicrograph of the retina on the fourteenth day. (x100.)

Materials and methods

Eyes from fetuses and from postnatal litters of pigmented Dutch rabbits were studied. Ages of the fetuses examined were: 11, 13, 14, 15, 18, 20, 23, 26, 27, 28, and 29 days. Postnatal stages were: newborn, 2, 3, 4, 5, 7, 10, 12, 14, and 21 days.

Some eyes from the eleventh through the eighteenth days of gestation were fixed whole. The others, including postnatal stages, were bisected with a sharp razor blade and the posterior halves fixed. Animals were killed by decapitation, excepting those of 12, 14, and 21 postnatal days.

In the latter, the eyes were removed under anesthesia (Nembutal).

Fixation was carried out by immersion for 2 hours in 3.5 or 4.0 per cent glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at low temperature and continued in cold 1.0 per cent osmium tetroxide in the same buffer solution for two hours. Specimens were then dehydrated with graded alcohol and, when in 70 per cent alcohol, cut into small pieces. Following dehydration, the specimens were transferred to propylene oxide and embedded in Epon.
Sections for study were carefully chosen from the central, or fundic, portions and from the peripheral regions. Thin sections for electron microscopy were cut on a Porter-Blum MT-2 microtome, doubly stained with uranyl acetate and lead citrate, and examined with a Siemens Elmscope 1 electron microscope. Relatively thick sections, for light microscopy, were made on the same microtome, and stained with 0.05 per cent toluidine blue with borax.

Observations

Prenatal stages. At the eleventh day of gestation, the inner and outer layers of the optic cup, which contact each other at their apices, were closely apposed. At this time, the inner layer contained several cells arranged at different levels (Fig. 1, inset). The apical portion of the inner layer facing the optic ventricle had a row of terminal bars which would form the external limiting membrane (Fig. 1). These cell junctions were zonulae adherentes (intermediate junctions), characterized by a relatively wide intercellular space (about 200 Å) and the presence of dense material within the neighboring cytoplasm (Fig. 3). Ciliary processes and mitosis were often found in this region. The innermost portion of the inner layer was covered by a basal lamina, which was continuous with that covering the outer rim and surface of the optic cup (Fig. 2). Some mesenchymal cells and blood vessels were located near the inner surface of the inner layer. The cytoplasm of the neuroectodermal cells contained numerous free ribosomes, mitochondria, rough-surfaced endoplasmic reticulum, and Golgi apparatus. Occasionally, cisternae of rough-surfaced endoplasmic reticulum were seen to be continuous with the outer nuclear membrane. Many dense granules, probably phagocytosed cell debris, were observed in the middle of the inner layer.

By the thirteenth day of gestation, the outer layer of the optic cup had differentiated into a single layer of pigment epithelium. At the fourteenth day, the thickness of the inner layer, which differentiates into the sensory retina, had somewhat increased (Fig. 5, inset). At this time, a sparse network of smooth-surfaced endoplasmic reticulum, characteristic of mature Müllerian cells, was observed in some of the cells in the innermost portion of the retina (Fig. 5). This was the first appearance of Müller cell differentiation. The Müllerian cell nuclei were found in the innermost region of the nuclear zone. The cell surface of the retina near the vitreous was associated with a dense filamentous material, suggestive of basal lamina formation.

Between 18 and 23 days, ganglion cells formed a discrete cell layer (Fig. 8). Smooth-surfaced endoplasmic reticulum was often seen in the inner (vitreous) portion of Müllerian cells in these stages.

By the twenty-sixth day, the differentiation of amacrine cells, which were identified as large, pale-staining cells in the innermost zone of the neuroblastic layer, took place. This was about the time of formation of the inner plexiform layer (Fig. 9), and most somata of the Müllerian cells were found in the zone just sclerad to the amacrine cells. Their inner processes, containing characteristic smooth-surfaced endoplasmic reticulum, could be followed (Fig. 10). On the other hand, the neuroblastic layer sclerad to the Müllerian and amacrine cell layers consisted of undifferentiated cells whose final fate could not be ascertained morphologically (Fig. 6). However, the elongated processes extending to the outermost portions of the retina were often recognizable by electron microscopic observation of subserial sections. These processes invariably contained Golgi apparatus, mitochondria, and rough-surfaced endoplasmic reticulum (Fig. 7).

During the late prenatal period, bipolar and horizontal cells formed in the middle of the outer neuroblastic layer. At 29 days, many dense granules, slightly larger in diameter than the ribosomes, were scattered through the cytoplasm of the Müllerian cells (Fig. 11). These became more evident in newborn animals. It is likely that these were glycogen particles, which...
Fig. 6. Outermost portion of the retina on the twentieth day of gestation. Sensory retina and pigment epithelium (PE) are closely associated with each other. The cells of the neuroblastic layer form terminal bars (t). A cilium (ci) indents the cytoplasm of the pigment epithelium. Mitochondria: (m), nucleus: (N), rough-surfaced endoplasmic reticulum: (rER). (×8,700.)

Fig. 7. Outer portion of the neuroblastic layer consisting of undifferentiated cells (twenty-third day of gestation). Elongated processes of these cells containing Golgi apparatus (G), mitochondria (m), rough-surfaced endoplasmic reticulum (rER), and fine filaments are observed. Some of these processes may be Mullerian. Nucleus: (N). (×5,000.)
Fig. 8. Photomicrograph of the retina on the twenty-third day of gestation. Ganglion cells (GC) are differentiated. Occasionally, elongated processes which are darkly stained are seen in the vitread portion of the retina (arrows). Neuroblastic layer: (Nb). (x230.)

Fig. 9. Photomicrograph of the retina on the twenty-sixth day of gestation. Ganglion (GC) and amacrine cell (A) layers and the inner plexiform layer (IPL) are visible. Darkly-stained processes (arrows) are also seen in this range. Neuroblastic layer: (Nb). (x210.)

Fig. 10. Inner plexiform layer of the retina on the twenty-eighth day of gestation. Two thin processes of Müllerian cells containing smooth-surfaced endoplasmic reticulum (sER) and ribosomal particles (rib) are illustrated. Neural processes: (np). (x14,700.)
Fig. 11. Outermost portion of the retina on the twenty-ninth day of gestation. Many glycogen particles (gl) are scattered in the cytoplasm of the Müllerian cell and are easily distinguished from receptor elements. Short villous processes extend outward from the level of the external limiting membrane (t). Cilium: (ci), mitochondria: (m), nucleus: (N). (×13,000.)

Fig. 12. Outermost portion of the sensory retina on the fifth postnatal day. Immature photoreceptor inner segments (IS) and Müllerian cell microvilli (vi) project into the optic ventricle (OV). Terminal bar: (t). (×12,000.)

Fig. 13. Outermost portion of the sensory retina on the twenty-first postnatal day. Photoreceptor processes (IS and Müllerian cell microvilli (vi) are mature. Terminal bar: (t). (×9,000.)
Fig. 14. Photomicrograph of the newborn retina. Amacrine cells: (A), ganglion cells: (GC), Horizontal cells: (H). (×280.)

Fig. 15: Outer plexiform layer (OPL) of the newborn retina. Lateral extensions of the Müllerian cells are already present (arrows). Golgi apparatus (G) is located at this level. Glycogen: (gl), rough-surfaced endoplasmic reticulum: (rER). (×15,000.)

Fig. 16. Outer plexiform layer (OPL) of the retina on the twenty-first postnatal day. The outer processes of the Müllerian cell become broad, and filamentous material is greatly increased. Bipolar cells: (BC), Golgi apparatus: (G), glycogen (gl), rod spherule: (rs). (×10,000.)
Fig. 17. Photomicrograph of the retina on the fourteenth day (postnatal). Amacrine cells: (A), horizontal cell: (H). (×230.)

Fig. 18. Inner plexiform layer of the retina on the fifth postnatal day. Short lateral extensions of the Müllerian cells are seen (arrows). Glycogen: (gl), neural process: (np), smooth-surfaced endoplasmic reticulum: (sER). (×10,300.)

Fig. 19. Inner plexiform layer of the retina on the tenth postnatal day. Lateral extensions of the Müllerian cells are advanced and more distinct. Smooth-surfaced endoplasmic reticulum (sER), glycogen (gl), microtubules, and fine filaments are distributed in the Müllerian cell process. Neural process: (np). (×12,300.)
the Mullerian cells of the adult retina normally contain. Mitochondria, distributed in the inner cytoplasm of the Mullerian cells vitread to the nucleus, disappeared at this stage.

**Postnatal stages.** At birth, photoreceptor cells were still immature, with no outer segments, but with some development of inner segments. Cells of the sensory retina facing the optic ventricles were united with intermediate junctions, as in embryonic life (Fig. 4). In the zone just scleral to the horizontal cell layer, neurons in the outer and inner regions of the neuroblastic layer began to form processes and synaptic connections, thus creating the outer plexiform layer (Fig. 14). At this time, short lateral extensions of the Mullerian cells were observed in this layer (Fig. 15). Mitotic figures were still numerous in the outermost region of the central portion of the retina.

At the fifth postnatal day, incompletely differentiated inner segments of rods appeared, as well as elongated microvillous processes of the Mullerian cells (Fig. 12). The lateral extensions of these cells became more distinct in both plexiform layers (Fig. 18). The thickness of the inner nuclear layer was reduced from about 10 cells in newborn rabbits to about five at 5 days. Mitosis in the outermost region of the fundus of the sensory retina had almost entirely disappeared by this stage.

The photoreceptor nuclei showed chromat clumping similar to that in adult cell nuclei by the seventh day after birth. Immature outer and inner segments were clearly visible at this stage. Diad synapses were seen in the inner plexiform layer. At 10 days, approximately when the eyelids opened, the retina had differentiated. The developing photoreceptors were elongated, and inner and outer segments had become adult-like in number and form. Synapses in the outer plexiform layer were almost complete, and mature synaptic lamellae were observed in the inner terminals of the photoreceptors. In the inner plexiform layer, diad synapses were frequently ob-
servable. At this time, inner and outer processes of the Mullerian cells became very broad, and fibrous material, as well as glycogen particles, was prominent (Fig. 16 and Fig. 19). Microvillus processes of the Mullerian cells had attained the length found in mature cells and had elongated to the border between ellipsoid and myoid (Fig. 13). Their nuclei in this stage were located in the innermost one-third of the inner nuclear layer, just scleral to the amacrine cells (Fig. 20).

At 14 days, differentiation of the retina was complete, and its morphologic features were similar to those of the adult (Fig. 17). The Mullerian cells were fully differentiated in all layers, excepting the inner nuclear layer, where their nuclei were still arranged in the third innermost zone. At 21 days, they were found in the second innermost zone, but amacrine cell nuclei were still piled up in places (Fig. 21). The inner nuclear layer was slow to mature.

**Discussion**

In the development of the mammalian retina, the pigment epithelium, which is derived from the outer layer of the optic cup, is first to differentiate and forms a single layer of cuboidal cells, as has been shown previously by many investigators. In the retina proper, which is derived from the inner layer of the cup, light microscopic studies with silver impregnation methods, reveal that Mullerian cells are developed at the time of differentiation of the ganglion cell layer, but do not determine whether the Mullerian cells begin to develop smooth-surfaced endoplasmic reticulum, early, at a time when the retina shows no differentiation of any layer. Later, this reticular system is developed in the inner cytoplasm of the cell vitread to the nucleus. The early formation of this organelle suggests that Mullerian cells are the first to differentiate in the retina proper. Ramon y Cajal demonstrated that the Mullerian cell in the prenatal period is of fusiform shape, with external and internal elongated processes,
Fig. 20. Inner nuclear layer of the retina on the tenth postnatal day. The nuclei of the Müllerian cells (M) are arranged at the third innermost zone of this layer. Multiple layers of amacrine cell nuclei (A) are seen. Inner plexiform layer: (IPL). (×3,200.)

Fig. 21. Inner nuclear layer of the retina on the twenty-first postnatal day. The Müllerian cell nuclei (M) are seen in the second innermost zone, but the amacrine cell nuclei (A) are still piled up, differing from the adult retina. Inner plexiform layer: (IPL). (×4,000.)
but is entirely devoid of lateral extensions. This is confirmed by the present study, which shows the embryonic Müllerian cells to be elongated, but columnar, somewhat similar to those reported in the ora serrata of the adult retina in which undifferentiated cells occur.\(^{14}\)

The present study also shows that, following differentiation of the ganglion cells, amacrine cells develop, then bipolar cells, and finally, horizontal cells, in agreement with the description\(^{15}\) in early light microscopic studies. However, autoradiography of the developing retina shows that amacrine and horizontal cells are determined early, at almost the same stage. This discrepancy appears to originate in the difficulty of identifying horizontal cells, which occur in an extremely limited zone in the inner nuclear layer.

It is not until the late prenatal period (about the twenty-ninth day) that the layer of cells destined to form photoreceptors can be identified.

After birth, photoreceptors and their synaptic connections are formed and mature, and the visual pathway is gradually completed. This maturation, especially formation of outer segments of the photoreceptors, has been shown by the developing electroretinogram (ERG).\(^{8,17,18}\) In the case of the rabbit retina, the first response to the ERG has been recorded at the eighth postnatal day.\(^{17}\) In these stages, active growth of Müllerian cells is found to be at its maximum and, from the side walls, expansions are forming at the level of the plexiform layers. However, this growth does not precede, but is parallel to the differentiation of retinal neurons. It is not until the rabbit embryo is in the late period (29 to 31 days) that Müllerian cells are able to synthesize glycogen. Histochemical demonstration indicates that glycolytic activity first occurs early in postnatal life.\(^{13}\) This may suggest that in embryonic life the Müllerian cells function simply as supporting elements of the retina or in passing metabolites, and that, after birth, they begin the synthetic activity required by retinal metabolism.

The external limiting membrane, which is made up of a series of terminal bars, is already present in early prenatal life. The intermediate junction is not changed during retinal development. It is believed that this type of junction binds cells less firmly than do desmosomes or zonula occludens\(^{19}\) and permits the passage of some materials between the cells.\(^{20}\)

The internal limiting membrane between the inner surface of the retina and the vitreous is considered to be a basal lamina produced by the inner processes of Müllerian cells.\(^{15,21,22,23}\) The present study shows (1) that the basal lamina of the inner layer of the optic cup is continuous with that of the outer portion which later differentiates into the pigment epithelium, ciliary body, and iris, and (2) that increased density, similar to formation of the basal lamina of the cornea,\(^{24}\) often can be observed in the vitread surface of the Müllerian cells, strongly supporting the contention that this basal lamina is formed and developed by Müllerian cells. Recent light and electron microscopic examinations of the adult retina suggest that this membrane is divided into two layers.\(^{25-27}\)

In young tissues, intercellular spaces of various dimensions, which may be artifacts in fixation, often occur, especially in the vitread portion of the retina. However, this looseness, if real, may aid cell growth of neural and glial elements.

These intercellular spaces disappear by the fifth day after birth, but functional extracellular spaces, which may play an important role in passage of metabolites, persist in the adult retina.\(^{28,29}\)

REFERENCES


