Original Articles

Development of the prenatal rat retina

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The present observation has demonstrated the developmental order of the rat retina as follows: the pigment epithelium differentiates earlier than the retina cells; Müller's cells and ganglion cells differentiate from the basal portion of the retina within a few days after the optic cup formation; the ganglion cells form a layer on the fourteenth to fifteenth day; the nerve fiber layer is formed on the fifteenth to sixteenth day; the rest of the retina cells differentiate gradually in the postnatal period. The inner limiting membrane is formed at the earliest stage of development. However, the completion of the outer limiting membrane in the adult form is at a much later stage—postnatal tenth day. Development of the nervous processes originates by vesicle formation at the marginal zone of the cytoplasm.

Key words: retina, embryology, prenatal retina, rat, Müller's cell, ganglion cell, growth one.

Electron microscopic studies of the developing retina have been reported by several authors, especially on the development in relatively later stages and on certain specific components of the retina. Details of the cellular differentiation during the period from the earliest optic cup to the formation of the basic layers of the retina have not been well documented. The present study is specifically aimed at describing the earliest sequential differentiation of each retinal cell.

Material and method

Rat fetuses from both Charles River C. D. albino and long-Evans hooded strains were used. Young female rats weighing around 350 grams were mated after their estrous cycles were confirmed by vaginal smear preparations. The day of the first appearance of spermatozoa in the highly cellular vaginal fluid was dated as Day 1. As the ocular tissue was inconspicuous before Day 9, fetuses from ten days to twenty-one days were used in this study. Several pregnant rats were killed at one-day intervals. Many animals were killed at six-hour intervals on Days 13 through 15 in order to observe the very rapid development which occurs during this period. For each study, at least three animals at the same gestation period were used and more than three fetuses in a single pregnancy were examined.

The pregnant rats were anesthetized by intraperitoneal injection of sodium pentobarbital and the fetuses were removed from the amniotic sacs under a dissecting microscope. The whole fetus was immediately placed in 4 per cent glutaraldehyde solution in 0.15 M phosphate buffer (pH 7.2) at room temperature. During the 20 minutes' fixation in the glutaraldehyde solution, the eye tissue was carefully dissected out. By the end of the initial fixations, the tissue was trimmed into pieces not larger than 1 mm, in any dimension. The pieces of tissue were transferred in 1 per cent

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Fig. 1. Light microscopic view of A, 13-day and B, 16-day-old fetus retina. The thick inner layer of the optic cup consists of elongating neuroepithelial cells. Ganglion cell layer becomes distinguishable on the sixteenth day. Thick epon section (0.5 μ), toluidine blue staining. ×280.

Osmium tetroxide solution in the same buffer without washing. The postfixation lasted for 90 minutes at 4° C. The tissue was dehydrated in graded ethyl alcohol (alcohols of lower percentages were at cold temperature), treated with propylene oxide, and embedded in an epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope. Midthoracic sections of the spinal cord of some rat fetuses at the same developmental stage were processed similarly for comparative study. Epoxy sections (0.5 to 1.0 μ thick) were stained with toluidine blue and examined by light microscopy.

Observations

Around the tenth day of gestation, the rat fetus begins to form vesicular protrusions in the forebrain region. The central portion of the extended vesicle invaginates suddenly and forms the optic cup. One-half of the fetuses examined on the eleventh day show optic cups, and all have completed optic cup formation on the thirteenth day. Lens formation by invagination of the ectodermal layer occurs simultaneously. The connective tissue begins to thicken around the optic cup and the outer shell of the globe is formed rapidly. On the fifteenth day, the definite shape of the globe is completed and further differentiation of the retina and other parts of the eye becomes more active (Fig. 1). This investigation is principally a study of the posterior retina, however, the developmental process of the rat retina does not vary greatly by location.

Optic cup formation. The optic vesicle consists of a single layer of the neuroepithelium. The cell has a centrally located large nucleus with homogeneous chromatin and one or two prominent nucleoli, and simple cytoplasm containing free ribosomes and sparse mitochondria (Fig. 2). Cells are loosely packed laterally but prominent apicolateral junctions and a thin basement membrane are present. At the time of optic cup formation, cells of the invaginated inner wall begin to elongate profoundly. Although this inner layer appears to be a multicellular layer, the layer is still one cell thick. The histologic sections give an impression of a multicellular layer because the cell nuclei are found at different positions...
of the thickened layer. Apicolateral junctions and basal attachment to the basement membrane are observed in all cells in this stage. Proliferation of the cells in the inner layer of the cup begins immediately and this layer eventually becomes the retina. The outer layer of the optic cup remains as a single cell layer and becomes the pigment epithelium. Apices of the inner and outer layer cells of the cup are closely attached to each other and the space between the layers disappears (Fig. 3). Although conspicuous apicolateral junctions are present in each cell layer, no junctional structure is observed between cells of the two layers, except for small desmosomal junctions which last only for a short period of time. The cytoplasms of both layers of the optic cup begin to contain mitochondria and endoplasmic reticulum, and the outer layer appears to start off for specific differentiation.

**Differentiation of the pigment epithelium.** The early pigment epithelial cells are cuboidal in shape and are loosely affixed laterally, but their apical portions are joined firmly by prominent apicolateral junctions. The basement membrane, which is formed at the beginning of the development of the optic vesicle is prominent beneath the cell (Figs. 3 and 4). Soon after the optic cup formation, the number of mitochondria and rough-surfaced endoplasmic reticulum increases in the cell body. Melanosomes are the first specific micro-organelles to be developed in the cell (Fig. 4). Minute wavy filamentous materials are formed within small vesicles at the apical portion of the cell at the time immediately following the optic cup formation. These vesicles increase in size and number and become the melanosomes (Fig. 5). Melanosomes of the albino rat remain in the apical portion of the cell until a few weeks after birth and then disappear. In pigmented animals, electron-dense melanin bodies are found on the twelfth to thirteenth day. Both melanosomes and melanin granules increase in number until the eighteenth day (Fig. 6).

Mitotic activity of the pigment epithelium is seen until the fifteenth day and thereafter mitosis is extremely rare. The loosely packed cells become compact and columnal in shape as the cell population increases (Fig. 4). Desmosomes are found on the lateral cell membrane during this period. The pigment epithelial cells on the fifteenth day are slender in shape. Eventually—an increase in size of the globe causes the cells to become shorter again.
On the eighteenth day, the epithelial cells are flat.

The apical cytoplasm of the pigment cell begins to enlarge on around the thirteenth day. The cytoplasm forms infoldings which attach closely to the retinal cells. No junctions are found between these two cells. On the fifteenth day the folded protrusions are similar to microvilli. In addition, the cytoplasm begins to contain smooth endo-

Fig. 3. The pigment epithelium and the apical portion of the retina on the thirteenth day. Active mitosis is seen in both pigment epithelium and neural cells (*). Apicolateral junctions are prominent in both layers (white arrows). x5,000.

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Fig. 4. The pigment epithelium of a 15-day-old fetus. Cells are slender in shape. A few mitochondria and rough endoplasmic reticulum have been formed in the ribosome-rich cytoplasm. Melanosomes are seen in the apical portion. White arrows indicate apicolateral junctions of the pigment epithelium and of the retinal cells. ×4,000.
plasmic reticulum and by the sixteenth day they become the main micro-organelle of the cell. Lysosomes, inclusion bodies are not seen in the prenatal cells. On the eighteenth day, cilia of the retinal cell begin to extend into the space between the microvilli. At the time of formation of the photoreceptor outer segments, the microvilli stretch and the subretinal space is formed.

**Differentiation of Muller's cell and the limiting membranes.** Both the pigment epithelium and the retinal cells have apicolateral junctions and basement membrane from the earliest stage of development. The basement membrane of the pigment epithelium becomes part of Bruch's membrane, and the apicolateral junctions also remain in the mature cells and become Verhoeff's membrane. The cells in the basal zone of the retina proliferate momentarily following the optic cup formation. Many cells in this zone detach from the basement membrane and move toward the outer direction. These cells eventually appear to become ganglion cells. Some cells in this zone appear to keep their original basal attachment and become Müller's cells (Fig. 7). The basement membrane of Müller's cells becomes thicker and fine fibrils begin to develop in the vitreous side. This is the inner limiting membrane. These cells extend the cytoplasm toward the outer layers. Along with the elongation of the cell, the nuclear position also moves outward. The cytoplasm begin to contain large mitochondria and rough endoplasmic reticulum. On the fourteenth day, the cell contains smooth endoplasmic reticulum and Golgi apparatus, whereas the neural cells still contain mainly polysomes and free ribosomes. Although the cytoplasm is in primitive state, the cell appears to begin phagocytotic function (Figs. 7 and 10). Lipid droplets and electron-dense inclusion bodies are found abundantly in these cells on the fourteenth day, especially in the vicinity of the binuclear cells (see below).

Around the fifteenth day, Müller's cells extend cytoplasmic processes horizontally. Intercellular spaces which are abundant in the inner zone of the retina earlier are
Fig. 7. Developing Müller's cell on the fourteenth prenatal day. The cell maintains the basal attachment. The cytoplasm contains mitochondria (m) and rough endoplasmic reticulum (rer). Smooth endoplasmic reticulum are about to appear. The arrow indicates a growth cone of the ganglion cell, $\times18,600$.

Filled with fine processes of Müller's cells within two days. Together with the development of the nerve fiber, the inner layer of the retina becomes compact by the sixteenth day.

Development of the apical portion of Müller's cell is not clearly demonstrated during the prenatal development. Fine extending processes of Müller's cells become recognizable between photoreceptor cells a
Fig. 8. Differentiating ganglion cell of a 14-day-old fetus. The cytoplasm in the inner side is larger. A growth cone is developing at the end of the cell. Müller’s cells (M) have the basal attachment (ILM). ×11,000.

Fig. 9. Growth cone of the axonal process of a ganglion cell of a 15-day-old fetus. The conical protrusion is filled with vesicles. They are not associated with other micro-organelles. ×128,000.
Fig. 10. The nerve fiber layer of a 17-day-old fetus. The basal portions of Müller's cells (M) are attached to the basement membrane. The Müller cell cytoplasm is well-differentiated and contains lipid droplets. The nerve fibers have accumulations of vesicles at one end (arrows). ×12,800.

A few days after the birth of the animal, formation of the apical junctions with the photoreceptor cells and extension of the microvilli are demonstrated after the development of the outer segments at the tenth postnatal day. Although apicolateral junctions have been present since the earliest stage of development, the true outer limiting membrane with the apical ends of the Müller cell is completed around this postnatal stage. Glycogen bodies in the cytoplasm are not seen until the end of the first postnatal week.

**Differentiation of the ganglion cells.** Some cells which have lost the basal attachment stay in the vicinity of the inner limiting membrane instead of migrating toward the apical zone. These cells begin to increase their cytoplasmic volume around the fourteenth or fifteenth day. The enlargement occurs in the cytoplasm toward the basal side of the retina (Fig. 8). The cytoplasm in this area begins to form many mitochondria, rough-surfaced endoplasmic reticulum, and a small conical outpouching. Formation of the outpouching starts with the accumulation of vesicles in a marginal zone of the cytoplasm. The vesicles are in various sizes, 500 to 1,000 Å in diameter, and are highly electron lucent (Fig. 9). The vesiculated site appears to protrude to form the axonal process. Formation of the axons are found to be explosively rapid. On the late fifteenth day, axons are abundant in the inner layer. The newly formed axons show clusters of vesicles in one end and contain regularly arranged but wavy microtubules and small mitochondria (Fig. 10). On the seventeenth day, the inner layer is well packed with bundles of axons and extending Müller's cell cytoplasm.

On around the sixteenth day, the differentiating ganglion cells form a layer separating from the rest of the retinal cells.
Fig. 11. A, ganglion cells of a 16-day-old fetus. The cytoplasm contains abundant micro-organelles. A growth cone is seen at the outer end of the cell (white arrow). x6,200. B, higher magnification of the growth cone. Vesicles (v) are packed in the conical protrusion. The neighboring cytoplasm contains rich rough endoplasmic reticulum (rer) and Golgi apparatus (G). M, Müller cell cytoplasm. x13,000.

(Fig. 1, B). On about the seventeenth day, the nuclear position of the developing ganglion cell is shifted inward and the cytoplasm in the outer side becomes larger and well developed, various micro-organelles start to accumulate in abundance. Rough endoplasmic reticulum is clustered to form Nissl bodies (Fig. 11). Conical protrusions with vesicles, from which dendritic processes develop, are frequently found at the enlarged side of the cell (Fig. 11, B). Other retinal cells still show primitive cytologic appearances at this stage.

**Differentiation of other neural elements.** Cells at the apical zone continue to show active mitosis from the time of the optic cup formation until one week after birth. Most of the dividing cells seem to maintain the apical junctions at all times, even during mitosis (Fig. 12). The proliferating cells at this zone become elongated rapidly. Because of the elongation of cell bodies, their nuclear positions move inward, but many cells are affixed firmly at the apical junctions. The elongating inner side of the neuroblastic cells contain abundant vesicles at their tips on around the fifteenth to the eighteenth day (Fig. 13). From the thick layer of neuroblastic cells the photoreceptor and bipolar cells differentiate. However, the development of the photoreceptor elements and synaptic organs occur postnatally and the processes have been described previously.1

**Necrotic changes in the developing retina.** Many degenerating cells are found in an otherwise normally developing retina at various stages of development (Figs. 14 and 15). Degeneration appears to begin in the nucleus of the cells which have differen-
Fig. 12. Apical portion of the retinal neural cell of a 15-day-old fetus. Cells are firmly joined by apicolateral junctions. The cell on the right is undergoing mitosis (*). Although a large mitochondria is seen in the center, the inner segment has not been formed. ×27,000.

Degeneration is most conspicuous in the area connected to the fissure of the optic stalk at the period immediately following optic cup formation. Sporadic necrosis is seen in the developing neuroblastic cells throughout the embryonal and postnatal development. Degenerating cells in the inner layers disappear when the development of the nerve fiber layer is completed on the sixteenth day. They reappear again in the outer layers when the differentiation of the photoreceptor cells begins at one week postnatal. Also, degenerating cells are found in small numbers in the peripheral zone of the retina until two weeks after birth.

No tissues of the other parts of the eye show extensive degeneration during their development. However, the neural cells of the developing spinal cord show similar degeneration.

Discussion

Since the retina develops from a layer of the neuroepithelium, its polar orientation is determined at the earliest stage of differentiation. The cells have their basement membrane at the basal end. After invagination of the neuroepithelial layer, the basement membrane covers the inner surface of the retina. This is the basic structure of the inner limiting membrane. Early development of this membrane has been pointed out by others.9,9 The first sign of cellular differentiation from the embryonal cell is an increase in the number of mitochondria and rough-surfaced endoplasmic reticulum. Organ-specific micro-organelles develop later. Müller's cell cytoplasm begins to show lipid droplets and debris substance in the early stage of development. They may indicate that the developing Müller's...
Fig. 13. Neuroblastic cells of the outer zone of a 17-day-old fetus. A, a growth cone is out-
pouching in the inner end of the cell. ×40,000. B, the nucleus of the neuroblastic cell is often 
lobulated. Fine process of Müller's cell is seen between neural cells (arrows). ×20,000.

Fig. 14. The posterior pole portion of the optic cup of a 13-day-old fetus. Many cells are 
degenerating in this area. ×280.
cell is already functioning for a certain purpose—phagocytosis.

The outer limiting membrane is a chain of the apicolateral junctions of Müller’s cells and photoreceptor cells. Prominent gap junctions of the neuroepithelial cells which are seen in the earliest stage of differentiation of the retina are the anlage of the outer limiting membrane. However, Müller’s cell cytoplasm reaches this region postnatally when the photoreceptor outer segments develop. After completion of the outer limiting membrane the junction becomes a chain of large desmosomes instead of a zonule adherent, as commonly believed.

Differentiation of the pigment epithelium appears to occur earlier than that of the neural portion of the retina. Mitosis in the pigment epithelium stops on the fourteenth day. On the fifteenth day, the pigment epithelial cells are of high columnal shape but they decrease in height gradually with an increase in the size of the eye. The characteristically rich smooth endoplasmic reticulum is developed in the early stage of differentiation. By the seventeenth day, the development of the basic micro-organelles of the pigment epithelial cells appears to be completed. Lamellar inclusion bodies begin to appear postnatally. Besides the intracellular differentiations, development of microvilli is conspicuous in this cell. Tightly packed microvilli are closely attached to the neural cells of the retina and the photoreceptor outer segments develop into the villi. As shown in the study of regeneration of outer segments following light damage, the pigment epithelial cells and their microvilli appear to have a close relationship, presumably in nutritional support, with the formation of the outer segment.

Mitotic activity of the developing retina shows a specific pattern in distribution.

Fig. 15. The posterior retina of a 15-day-old fetus. Pyknotic and karyorrhectic nuclei are scattered within otherwise normally developing neuroblastic cells. $\times 17,000$. 
Fig. 16. Schematic representation of the prenatal development of the rat retina. A, immediately after the optic cup formation. Mitoses are seen in the inner and outer ends of the retinal tissue and in the pigment epithelium. Müller’s cells begin to differentiate first. B, a 14- to 15-day-old embryo. Ganglion cells (G) begin to develop axon processes. Müller’s cells (M) are elongating. The pigment epithelial cells are slender in shape. C, a 16- to 18-day-old embryo. The nerve fibers (Nf) have been formed. Ganglion cells begin to develop dendritic processes. Neuroblastic cells divide rapidly and elongate their cell bodies. The apical ends of these cells are firmly attached to each other by conspicuous apicolateral junctions. The developing neuroblastic cells show growth cones at the inner ends. The pigment epithelium begin to develop microvilli. D, an 18- to 20-day-old embryo. Ganglion cell layer and nerve fiber layer are separated from other neuroblastic cells. The outer layer of the retina is undergoing active differentiation.

(Fig. 16) at the time of optic cup formation, mitosis is found in both basal and apical portions. Cell division in the basal region lasts for a short period of time. On the other hand, mitotic activity in the apical zone continues until the end of the first postnatal week. Cells which are divided at the basal portion and keep the basal attachment appear to become Müller’s cells and cells detached from the base, but which stay in this region, develop into ganglion cells. Multiplication of both Müller’s cells and ganglion cells occurs early and lasts for only three days.

Dividing cells in the apical region usually maintain the junctions even during mitosis and become photoreceptor cells. Although their nuclei move inward by elongation of the cell body, real migration of the total cell body is observed only in a small number of the neuroblasts, particularly at the later stage of development. One of the migrated cells forms Chievitz’s membrane and eventually separates the bipolar cell layer from the neuroblastic layer. These differentiations occur only after birth.

Neural cells develop vesicles in a marginal portion and form conical protrusions. The outpouched portion develops into axons or dendritic processes. In the ganglion cell, the axons develop two or three days earlier than dendrites. The conical
protrusion was first observed as the growth cone by Ramon y Cajal in 1890, and recently described electron microscopically in the developing cerebellum. Similarly, the vesiculated conical extensions are found abundantly in the neural cells of the developing spinal cord of the rat fetus of the present study, but they are rare in the non-neural cells of eye tissues. Since the vesicles remain at the tips of the growing neural processes, these may be the anlage of the synaptic vesicles. Frequent non-specific vesicular bodies caused by technical artifact are easily distinguished. These vesicles are much larger and regular in shape.

Degenerating cells have commonly been observed in the fetuses, especially in the developing nervous tissue and are called morphogenetic degeneration. Although the occurrence is constant in all developing retinas and in the central nervous system, no specific pathogenic evidence in the surrounding tissue is shown. Also, no correlation with the vascular system of other nutritional systems is found. The degeneration may have occurred in cells which are proliferated excessively or in cells which fail to be matched with other neural cells for future synaptic connections. Because of the limitation of the intraocular space, unnecessary cells may be eliminated as a part of the differentiation. This has been suggested in other embryonal tissue.

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REFERENCES