Tissue culture studies of the embryonal chicken retina

L. Barr-Nea and R. Y. Barishak

Pure neuroectodermal growth was obtained from the retina of the chicken embryo by removing the pecten before preparation of the cultures. Retinal tissue was cultured either as explants in plasma clots or as isolated cells after trypsinization. Epithelial cells formed sheets on which the other retinal cells grew. Horizontal cells maintained the associational characteristics. Development of photoreceptors with incomplete outer segments was observed.

Key words: tissue culture, chick embryo, neural retina, differentiation, retinal rods, retinal cones, retinal horizontal cells, retinal pigment epithelium, retinal Müller glial cell, epithelial growth, photoreceptors.

The embryologic development of the retina has been studied histologically and experimentally by various methods including tissue culture. Tissue culture affords the possibility to follow the differentiation of undifferentiated neuroepithelial cells into the different types of cells forming the mature retina and to observe all the stages of their development. Moreover, the cellular characteristics of developing cells, as well as their associational patterns, can be studied better in vitro than in histologic sections of the developing eye.

The retina of the chick embryo was selected for this study because the retina of birds lacks vessels. The pecten, a vascular structure attached to the optic disc and protruding into the vitreous, could be removed before the preparation of cultures and in this manner retinal tissue free of mesodermal components could be obtained. Tissue culture experiments were performed on explants and on isolated retinal cells obtained by trypsinization. The growth within the explant resembles the normal growth in vivo; the cells continue to grow and differentiate under the influence of induction exercised by the surrounding milieu, whereas isolated cells are free of the inductive effect and show different association patterns.

Materials and methods

Chick embryos were removed from fertile eggs at ages ranging from 4 to 17 days, and the eyes were put in sterile Petri dishes containing Earle's balanced salts solution (BSS). The globe was opened at the limbus with a Graefe knife under the dissecting microscope. The section was completed with scissors. The lens and vitreous were removed, and the pecten was cut out surrounded by a wide rim of retina. The remaining retina was detached gently with a spatula. For plasma clot cultures the retina was cut into fragments (1 by 1 mm.), and for trypsinization it was re-
moved in toto. The plasma clot and the trypsinization techniques were both used for each embryonal stage studied.

The plasma clot was prepared on a 18 by 18 mm. coverslip and placed within a 35 by 10 mm. Petri dish with the retina maintained flat in the plasma clot. The explant was covered with nutrient medium made of 20 per cent fetal calf serum and 80 per cent Eagle basal medium. For histologic studies the clot was prepared on the top of a thin layer of agar-agar (1 per cent in BSS) spread on the bottom of the Petri dish. The cultures were incubated at 38° C. in an atmosphere of CO₂ in air (5 per cent) and 98 per cent humidity. The medium was changed 3 times a week, and the cultures were examined daily under an inverted microscope. The cultures were discontinued after 3 weeks.

In each experiment, explants were fixed and treated in one of two ways: (1) fixation in either alcohol-formalin-acetic acid or neutral formalin diluted in BSS, followed by staining in toto by any of the following procedures: hematoxylin and eosin, Bielschowsky, Holzer, periodic acid-Schiff (PAS) or Alcian blue; (2) fixation in alcohol-formalin-acetic acid, embedding in paraffin, and serial sectioning at 5 μ, followed by staining with either hematoxylin and eosin, PAS, Alcian blue, or Luxol fast blue.

For trypsinization, the retina was rinsed in warm BSS free of calcium and magnesium and then incubated in a 1 per cent solution of trypsin in the same BSS for 30 minutes. The suspension obtained was rinsed in several changes of BSS free of calcium and magnesium, and the cells were sedimented by centrifugation (280 g for 3 minutes). The sediment was resuspended in 1 ml. of Eagle's medium by repeated flushing with a 1 ml. pipette. The suspension was then diluted with 9 ml. of Eagle's basal medium containing 20 per cent fetal calf serum; 1 ml. aliquots were transferred to 10 Leighton tubes, and the cells were allowed to settle on a coverslip at 38° C. The nutrient medium was changed on the next day and then three times a week. The coverslips were removed at regular intervals, and the cultures were examined and photographed under a phase contrast microscope or after staining with the hematoxylin and eosin, Alcian blue, Holzer, Bielschowsky, or the PAS procedures.

**Results**

**General aspects of growth.**

*Plasma clot technique.* During the first 24 hours, large cells with clear cytoplasm and an oval nucleus and small round cells with scanty cytoplasm and dense nucleus started to migrate (Fig. 1). At the same time the cells which remained within the explant sent out processes, some of which were fine and brushlike, others thick and in bundles (Fig. 2).

Within a few days, a pattern of growth was discernible: The large clear cells grew rapidly and profusely in a sheetlike pattern resembling epithelial cells. The round cells were usually present on the surface of the epithelial sheets. Some were arranged in cords, clumps, or rosettes and were identified as the precursors of photoreceptors. Others had fine processes and developed into glial cells and neurons. The thick fibers arranged in bundles contained neurofibrils which could be impregnated with the Bielschowsky method and were identified as axons of ganglion cells. The fine ones belonged to neurons or glial cells and were impregnated, either by the Bielschowsky stain for neurons or the Holzer stain for glia.

The pattern of growth was dependent upon the age of the embryo. Up to 8 days photoreceptor growth and neuron migration were predominant, between 8 and 11 days photoreceptor and glial growth were still active, but migration of neurons was less apparent; after 11 days the epithelial growth became predominant, the glial

---

**Fig. 1.** Fourteen day embryo (E-14), 4 days in tissue culture (T. C.-4). Round dark cells on top of epithelial sheet at border of explant. (Bielschowsky; x100.)

**Fig. 2.** E-7, T. C.-2. Fibrillar growth. (Phase contrast; x300.)

**Fig. 3.** E-7, T. C.-6. Juxtaposition and curling of edges of explant. e, Edge of explant; J, juxtaposition; r, rosette. (Hematoxylin and eosin; x200.)

**Fig. 4.** E-14, T. C.-4. Fusion of edges of explant. F, Site of fusion; L, lumen. (Hematoxylin and eosin; x400.)

**Fig. 5.** E-7, T. C.-4. Rosettes at edge of explant. (Hematoxylin and eosin; x400.)

**Fig. 6.** E-11, T. C.-1. Reaggregation of trypsinized cells. (Hematoxylin and eosin; x200.)
Figs. 1 to 6. For legends, see opposite page.
growth continued, but growth of photoreceptors and rosette formation markedly decreased.

Serial sections of the explant cultured in plasma clots showed the presence of a marked proliferative activity within the explant itself. Although the retina was placed flat on the plasma clot, it frequently showed a tendency to curl. Either juxtaposition (Fig. 3) or fusion (Fig. 4) could be observed between the two growing edges of the explant. The newly formed tissue at the edge of the explant was particularly rich in rosettes, especially in cultures of young embryos (7 to 8 days) (Fig. 5).

Trypsinization technique. During the first 24 hours, the trypsinized cells reggregated and formed scattered islands on the coverslip (Fig. 6). The pattern of growth depended upon the ages of the embryos. Until the age of 11 days, photoreceptor and glial growth and isolated neurons were observed on the epithelial sheets; after the age of 11 days epithelial growth was preponderant from the first days of culture, whereas the glial and the photoreceptor growth were markedly reduced with only a few neurons visible.

Epithelial cells. Epithelial cells multiplied so rapidly that they covered the whole coverslip in 2 to 3 weeks, with numerous mitoses and presence of occasional multinucleated cells (Fig. 7).

Newly formed cells were round with a clear nucleus and a moderate amount of homogeneous cytoplasm. Later, they flattened out and increased in size, their nuclei becoming oval and showing two conspicuous nucleoli. Their cytoplasm was abundant, vacuolated, and occasionally granular, with some PAS-positive granules. The cells were well delimited and their membrane well brought out by silver impregnation.

Neurons, photoreceptors, and glial cells were usually in contact with the epithelial cells. This phenomenon was even better observed on islands of epithelial cells in cultures of trypsinized material (Fig. 8).

Epithelial cells originated from clusters of retinal pigment epithelium put accidentally in the cultures. The cells coming out of the clusters lost their pigmentation progressively as they moved farther away. Those at the periphery were nonpigmented and appeared similar to the other epithelial cells seen in our cultures (Fig. 9).

Serial sections of the retinal explants showed that epithelial cells originated in the inner nuclear layer, migrated along the Müller fibers, crossed the outer nuclear layer, and once outside the external limiting membrane, spread over the external surface (Fig. 10).

The epithelium proliferating inside the explant showed a peculiar concentric arrangement with formation of amorphous eosinophilic masses in the center. These masses were stained by nuclear fast red, Alcian blue, and PAS but not by Congo red. The formation of these masses followed a definite pattern: first, granules showing staining characteristics similar to

![Fig. 7. E-10, T. C.-21. Epithelial sheet. b, Binucleated cell; m, mitosis. (Hematoxylin and eosin; ×400.)(Fig. 8. E-11, T. C.-4. Island of epithelial cells. Neuroectodermal cells on top, ep, Epithelial sheet (Hematoxylin and eosin ×400.)](http://iovs.arvojournals.org/article-pdf-access.ashx?url=data/journals/iovs/933613/)

![Fig. 9. E-7, T. C.-10. Epithelial growth around retinal pigment epithelium. RPE, retinal pigment epithelium. (Osmic acid–dichromate; ×200.)](http://iovs.arvojournals.org/article-pdf-access.ashx?url=data/journals/iovs/933613/)

![Fig. 10. E-17, T. C.-3. Cells from the inner nuclear layer migrating out of the explant. Arrow, external limiting membrane; c, migrating cells; In, inner nuclear layer; On, outer nuclear layer; p, photoreceptor. (Hematoxylin and eosin; ×800.)](http://iovs.arvojournals.org/article-pdf-access.ashx?url=data/journals/iovs/933613/)

![Fig. 11. E-14, T. C.-4. Concentric arrangement of epithelial cells. Arrow, granules; as, amorphous substance; d, degenerated epithelial cell. (PAS; ×800.)](http://iovs.arvojournals.org/article-pdf-access.ashx?url=data/journals/iovs/933613/)

![Fig. 12. E-14, T. C.-4. Epithelial cell arrangement reminiscent of psammoma body. (Hematoxylin and eosin; ×800.)](http://iovs.arvojournals.org/article-pdf-access.ashx?url=data/journals/iovs/933613/)
Figs. 7 to 12. For legends, see opposite page.
those of the masses appeared in the cytoplasm of some epithelial cells; later, the granules increased in number and size and filled the cytoplasm of cells which lost their nuclei. The degenerated cells conglomerated to form masses of amorphous material around which other epithelial cells arranged themselves in concentric layers (Fig. 11). In some sections the contours of the cells and the granular appearance of the cytoplasm could still be discerned in the degenerated cells. This pattern of formation is reminiscent of the structure of psammoma bodies (Fig. 12) as seen in meningiomas.11

Glial cells. The glial cells could be identified tentatively according to their morphology as astroglial, oligodendroglial, or Müller cells.

The astroglial cells had a large cell body with a relatively clear nucleus surrounded by a moderate amount of cytoplasm. Their thin and long processes divided by dichotomy and were attached to each other (Fig. 13).

The oligodendroglial cells had a hyperchromatic nucleus with a thin rim of cytoplasm. Their processes were short and thin. The cells were arranged in chains or cuffs along nerve fibers. No myelin formation could be detected in these cultures (Fig. 14).

Müller cells were characterized by a fusiform nucleus, a moderate amount of eosinophilic cytoplasm, and a fine and long process at each pole (Fig. 15). To differentiate these glial cells from bipolar neurons, the Holzer stain for glia and the silver impregnation method of Bielschowsky were used.

Astroglial as well as oligodendroglial cells were often connected together forming nets or were found isolated inside dense tufts of glial fibers.

A type of cell frequently encountered and thought to be of glial origin was a tiny cell, smaller than an oligodendrocyte, with a round hyperchromatic nucleus and scanty cytoplasm, occasionally extending in comma shape. These cells were found singly, dispersed, or in groups and they showed no special arrangement and no connections with other cells. They were observed in embryos of all ages, around the explant as well as within it, and scattered on the epithelial sheet (Fig. 16).

Neurons. The identification of neurons in tissue culture had to be based on their morphology, arrangement and staining properties, although neither criterion was considered as specific per se.

In plasma clot cultures some ganglion cells remained within the explant and were identified by their axons extending from the explant. Others were scattered on the epithelial sheet. They were also observed in cultures of trypsinized material (Fig. 17) but were less numerous than in the plasma clot cultures.

Bipolar cells were found individually dispersed in cultures of trypsinized material and were more numerous around rosettes in the plasma clot cultures (Fig. 18).

---

Fig. 13. E–8, T. C.–4. ag, astrocyte. (Phase contrast; ×800.)
Fig. 14. E–8, T. C.–4. og, oligodendrocyte. (Phase contrast; ×800.)
Fig. 15. E–7, T. C.–4. Arrow, Müller cells. (Hematoxylin and eosin; ×400.)
Fig. 16. E–10, T. C.–21. Arrow, round tiny cell. (Hematoxylin and eosin; ×800.)
Fig. 17. E–8, T. C.–4. gc, ganglion cell. (Phase contrast; ×800.)
Fig. 18. E–8, T. C.–4. bc, bipolar cell. (Phase contrast; ×800.)
Fig. 19. E–7, T. C.–8. Chain of horizontal cells along the explant. Arrow, chain. (Bielschowsky; ×200.)
Fig. 20. E–10, T. C.–4. Net of horizontal cells along the explant. Arrow, net. (Bielschowsky; ×200.)
Fig. 21. E–7, T. C.–3. Net of horizontal cells on rosette. (Bielschowsky; ×200.)
Figs. 13 to 21. For legends, see opposite page.
Amacrine cells were rarely identified, whereas cells presumed to be horizontal cells were frequently encountered. These cells had a long and flat perikaryon with processes extending from both poles and from one side of the cell body. They were connected together forming chains along the explant (Fig. 19) and nets away from it (Fig. 20). Such nets were also seen above rosettes in a different plane than the photoreceptor and glial cells (Fig. 21). Some of their processes terminated between the cells forming the rosettes.

Photoreceptors. The development of photoreceptors depended on the method of culture. In plasma clot cultures the interdependence of the cells was maintained and photoreceptors grew out of the explant in clumps and cords (Fig. 22) which eventually formed rosettes (Fig. 23). In cultures of trypsinized material, isolated precursors of photoreceptors reaggregated forming clumps or cords curling into rosettes. As a result of multiplication, the rosette acquired new peripheral layers of cells (Fig. 24). In such a rosette glial cells, neurons, and superimposed nets of horizontal cells were also sometimes found.

In cultures of trypsinized material the transformation of single, round, undifferentiated neuroepithelial cells into photoreceptors could be observed. At first, the round cells became elongated, the nucleus acquired a triangular shape, and moved toward the side from which the axon emerged (Fig. 25). At the opposite side the cytoplasm contained a vacuole. At a later stage, the cytoplasm increased in size and the three types of photoreceptor cells normally present in the chicken retina could be distinguished: the single cone with a wide triangular inner segment, an eccentric nucleus, and an axon on the one side and a cilium on the other; the accessory cone, with a broad cell body almost completely filled by a large vacuole, a flat peripheral nucleus (Fig. 26), and occasionally a cilium; the rod with a slender, long inner segment (Fig. 27).

No fully developed outer segments of cones and rods were found in our cultures.

Photoreceptors showed a parallel arrangement similar to their in vivo pattern (Fig. 28) or were oriented radially (Fig. 29).

Connections between photoreceptors and neurons such as bipolar or horizontal cells and with glial cells were occasionally observed.

Discussion

In cultures of the retina, mesenchymal constituents derived from the capillaries are usually the most profuse elements and impede the growth of the other elements. Most authors used the retina of newborn rats which is immature at birth and the retina of human and mouse fetuses, all of which contain capillaries. In the chicken the retina lacks vessels and as the pecten, which is a vascular structure, was completely removed during the preparation of explant, our cultures were free of mesodermal elements.

Hansson and Sourander described a monolayer sheet of large, thin cells with
Figs. 22 to 29. For legends, see opposite page.
frequent mitoses and claimed that these cells were mesodermal in origin and that different types of neuroectodermal cells grew on the surface of these sheets. Monolayer sheets which showed a characteristic epithelial arrangement were observed also in our cultures. As our cultures were free of mesodermal elements, it is obvious that these cells were of ectodermal origin.

The origin of the epithelial cells could be followed in our cultures. They were seen migrating out of the inner nuclear layer and extending outside the edges of the explants. They were derived from undifferentiated retinal cells present in the inner nuclear layer, and their sheets resembled the ependymal cell growth described by Hild. Similar cells were seen around clusters of retinal pigment epithelium. It is concluded that also the cells of the retinal pigment epithelium have the possibility to differentiate into ependymal cells.

The epithelial cells arranged themselves in concentric layers, in the center of which a mass of amorphous substance appeared as a result of degeneration. This was reminiscent of the psammoma bodies in meningiomas and in arachnoidal nests. This phenomenon can therefore be considered as an expression of a tendency of these epithelial cells to differentiate into arachnoidal-like cells.

Thus, the retinoblasts may not only de-differentiate into the most primitive form of neuroectodermal cells but also can maintain the capacity to differentiate into other types of neuroectodermal cells, for instance into arachnoidal cells.

The role of the epithelial cells is not clear, but in our cultures neurons, photoreceptors, and glial cells grew usually on the surface of the epithelial sheets, and their processes often terminated within the cytoplasm of epithelial cells. It seems that in tissue culture the epithelial cells play a role in the metabolism of the other neuroectodermal cells of the retina.

In our cultures, neurons were difficult to identify by histologic methods. However, horizontal cells with their associational properties were easy to identify. Such an association was demonstrated in histologic sections by Vrabec. This author observed nets of horizontal cells at the periphery of the retina after peripheral cystoid degeneration caused the disappearance of all the other neurons and many glial cells. It appeared that horizontal cells could survive and maintain their associational characteristics while other neurons disappeared with the process of aging.

The precursors of the photoreceptors, unlike the neurons, showed a high proliferative activity in tissue culture. This activity was more pronounced in cultures of young embryos and provoked a marked infolding within the explants themselves and fusion of the edges. The fusion implied curling of the retina and new formation of a retinal tissue rich in rosettes. When fusion did not take place each edge continued to grow in its own direction and curled around itself.

As a result of the high proliferative activity of the precursors of photoreceptors, rosettes formed outside the explants. Such a rosette, obtained from a 4-day-old culture of a 9-day-old chick embryo was examined by electron microscopy. Desmosomes at the site of the external limiting membrane, mitochondria, and glycogen in the inner segment, and microvilli and short cilia in the lumen were observed. The rosette with its photoreceptor, glial, and nervous elements can be considered as a model of primitive retina.

Hild and Callas and Hansson and Sourander obtained growth of photoreceptors in tissue cultures of the retina of newborn rats and of mouse and human fetuses and came to different conclusions regarding the development of the outer segment. Hansson and Sourander claimed that they obtained fully developed receptors, whereas Hild and Callas claimed that only the inner segment was well developed. In our experiments performed on chicken
embryo retinas, we did not observe a complete formation of the outer segment of the photoreceptors.

The incomplete formation of the outer segment suggested that some factor responsible for the maturation of the photoreceptors is missing in vitro. Yamada and Ishikawa\(^1\) studied the development of the outer segment and the pigment epithelium in the human fetal eye by electron microscopy and concluded that the pigment epithelium played an important role in the development of the membrane of the outer segment. They observed an increase in the smooth endoplasmic reticulum in the cells of the pigment epithelium parallel to the development of the double membranes in the photoreceptors and, therefore, suggested that the material necessary for the membrane formation is synthesized within the smooth endoplasmic reticulum and transferred to the photoreceptors which are in intimate contact with the pigment epithelium.

It is believed that the tissue culture approach might provide answers to the following outstanding questions, as it allows a step-by-step follow-up of the dynamics of differentiation: the nature of the cytoplasmic organelles which are responsible for the synthesis and formation of the outer segment membranes on the one side and the axon on the other side of the receptor cell; the formation of connections and the nature of the synapses between the horizontal cells and other structural elements of the retina; and the functional significance of the various synapses in the explants and in the retina.

We wish to thank Mrs. Naomi Papo for her skillful assistance and Mr. M. Azury for the preparation of the photographs.

REFERENCES