Development of retinal vessels. II. Earliest stages of vessel formation

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The initial development of retinal vessels in the rat was studied by light and electron microscopy. In the 15-day-old rat embryo undifferentiated cells derive from the outer wall of the hyaloid vessel, or the immediate surrounding tissue, and migrate into the peripapillary retina beneath the internal limiting membrane. No vascular budding is evident. The cells proliferate, form a network, and then evolve into lumenized vessels. Initially the vascular network is capillary in nature, and there is no evidence that development of veins precedes that of capillaries. Similar observations made by earlier authors are discussed in relation to our work.

In a previous work with retinal digests, whole mounts, and India ink injected material, the development of the retinal vascular system was studied in rats from birth to 5 days of age. It was noted that even at birth abundant vascular tissue was present in the rat retina, and it is this tissue, its origin, and its appearance that is described in this paper.

The transition from nonvascularized to vascularized retina has not been studied previously by electron microscopy, nor by examination of thick sections prepared by embedding tissue in resin. Ink injected preparations and retinal digests were also examined during the course of the present work but proved to be of little additional value. The ink injection technique did not permit observation of the earliest stages of vascular development prior to lumen formation, and retinal digestion, though of great importance in studying later phases of retinal vascular growth, was of little use in studying the initial process. Study of thick sections mounted in resin followed by electron microscopic observation of adjacent thin sections provided the bulk of our present material. In addition, a brief review of the literature on the early stages of retinal vascular development introduces the discussion.

Materials and methods

Albino rats in different stages of development were used. Fetuses were removed from the uterus through hysterotomy at different intervals during the 21 days' gestation period. Several hysterotomies were performed in one pregnancy and the rat was allowed finally to deliver the remaining full-term fetuses.

At the start of this investigation it was necessary to determine the most suitable fixative and the duration of fixation for the immature rat retina. After a series of experiments it was found that osmium tetroxide buffered with dichromate
(Dalton's fixative) was the most satisfactory if the fixation time was half an hour. The eye was enucleated, and immediately after removing the anterior segment under the dissecting microscope, the eye was immersed in the cold fixative. While fixation at 4° C. was continuing, the sclera, choroid, and vitreous were teased away from the retina, and the optic nerve was separated from its sheath. Further processing was achieved by dehydration in graded alcohol and propylene dioxide, and the retinas were embedded in Epon for sectioning. Thick flat sections and thick cross sections (1 μ) were obtained from the whole retina. Serial thick sections were stained with toluidine blue, observed, and photographed with the light microscope, while serial thin sections were cut from selected areas of the blocks, double stained with uranyl acetate in acetic acid and lead citrate, and observed and photographed with the electron microscope (Zeiss EM 9 or Siemens' Elmiskop 1). Preliminary studies showed that the rat retinal vascularization begins at the age of 15 days' intrauterine life; therefore, fetuses of 15, 17, 18, and 20 days after gestation, and newborn rats one to several days old, were studied.

Observation

The first indication of retinal vascularization was observed in the retina of the 15-day-old fetus. Cells which arose from the vessels in the optic nerve invaded the retina and were seen in the nerve fiber layer beneath the internal limiting mem-

brane. In flat thick sections the cells appeared to be arranged in hexagonal patterns immediately around the optic nerve head and usually were placed close to one another to form cords; at the periphery or distal end a few isolated cells could be found among other cellular structures of the retina. When cross sections included the optic nerve head where the hyaloid vessels enter the vitreous, the relationship between these cells and those forming the hyaloid system could be clearly demonstrated (Figs. 1A and 1B). These cells, which are polygonal in shape, preceded the cords and were easily distinguished by light and electron microscope. They were situated at the same level as the cords and lumenized vessels, and their long axis was usually located parallel to the axon cylinder (Fig. 2). In serial sections their connection to the cords and vessels could be traced. By electron microscopy they showed numerous ribosomal particles, free or in association with well-formed endoplasmic reticulum, abundant mitochondria, a Golgi system, and a nucleolus in the nucleus (Fig. 2). A number of mitotic figures were found among these cells, as well as those forming the cords (Fig. 3). The cords were usually formed by a collection of closely

![Fig. 1A. Optic nerve head area showing the hyaloid vessels (H. V.) and the vasculogenic cells of the retina (arrow). Specimen from a 15-day-old fetus. (Photomicrograph x400.)](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933620/)
packed cells which did not show any junctional complexes between them, and their adjacent plasma membranes were separated from one another throughout (Fig. 4). What were classified as cords by light microscopy did not always appear as aggregates of cells by electron microscopy; on the contrary, these so-called cords showed slit or collapsed lumina with well-formed junctional complexes between their endothelial cells (Fig. 5). Sometimes distal to the end of a cord, what appeared to be a cell in thick sections was found to be a vessel with a narrow lumen in thin sections (Fig. 6). At times it was difficult to differentiate between the cords and the lumenized vessels since it might be assumed that a "cord" was actually a lumenized vessel cut obliquely through its wall. Here the presence of basement membrane around the cells was one criterion for the recognition of the vessel wall (Fig. 7). The newly formed blood vessels appeared at first to be of capillary size, and they had one or two layers of cells surrounding a lumen which sometimes contained red cells. Abundant basement membrane material, which was less condensed than that seen in mature capillaries, was often found in the space between the cell wall of the blood vessel and the neighboring tissue. The pattern of the canalized vessels was also hexagonal corresponding to that of the cords (Fig. 8). Junctional complexes between cells were seen to be forming in the transition from the cords to the lumenized vessels.

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Fig. 2. A vasculogenic cell of the retina with long axis parallel to the axon cylinders (N). ILM (internal limiting membrane). Specimen from a 17-day-old fetus. (Electron micrograph x9,000.)

Fig. 3. Thick sections of a cord (C) showing a cell at the distal end (arrow). Mitotic figure (FM). Specimen from a 17-day-old fetus. (Photomicrograph x1,300.)
Fig. 4. Section of a cord of the retinal vascular system showing neither junctional complexes between the cells nor basement membrane material around them. Specimen from a 17-day-old fetus. (Electron micrograph ×20,000.)
Fig. 5. An early blood vessel showing a lumen (L) and well formed junctional complexes (JC) between the endothelial cells. Specimen from a 17-day-old fetus. (Electron micrograph ×27,000.)
Fig. 6. Thin section of the structure seen as a cell in Fig. 3 reveals a cross section of a vessel with a lumen (L) and junctional complexes (JC). Specimen from a 17-day-old fetus. (Electron micrograph ×17,000.)
The cells of the deep capillary bed arose from the superficial vessels near the disc in the retina of 1-day-old rats, and these appeared to pierce the nerve fiber layer. These cells were morphologically similar to those forming the superficial vessels.

Discussion

Observations on the development of the retinal vascular system date back to the Nineteenth Century, and analysis of original source material is particularly enlightening. Kessler, with osmium tetroxide...
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Fig. 8. Early retinal vessels (V) arranged in hexagonal pattern. Specimen from a 1-day-old rat. (Photomicrograph ×820.)

Fig. 9. Sagittal section through the optic nerve head in the rat showing hyaloid and retinal vessels (Kessler, 1877).

for fixation, studied the development of retinal vessels in the rat and concluded that they arose independently of the hyaloid artery, presumably not by vascular budding. On page 77 of his classic work, he wrote concerning developing retinal vessels in the rat,

Die ersten Spuren derselben habe ich bei eben geworfenen Ratten¹ gefunden, Fig. 87; [Fig. 9] zwischen Limitans interna und Nervenfaserschicht schiebt sich eine dünne Schicht einer Zellenmasse vor, welche in der Nähe der leicht convexen Papilla nervi optici aus 2-3 Lagen besteht, je weiter von dieser entfernt desto mehr sich verschmälert, überhaupt aber nur etwa auf das vierfache des Querdurchmessers des Schnenov rings um dessen Eintrittsstelle sich erstreckt; in dieser Zellenschicht, den Nervenfasern dicht aufliegend, verlaufen feinste Capillaren, jedoch nur erst bis auf 1-1½ Opticusquerdurchmesser Entfernung von der Papille, siche Fig. 87. [Fig. 9]. In einem der Schnitte sehe ich ein Gefäßchen von 2-3 Blutkörperchen Durchmesser vom Stamm der Art. central., unmittelbar vor ihrem Austritt in den Glaskörper, abgehen, welches sich dann bald in jene Capillaren auflöst.

Schultze² and Voll,₄ studying the membrana vasculosa retinae in a variety of species, noted that the retinal vessels arose
independently of the hyaloid artery and were preceded by a layer of mesenchymal cells which grew over the surface of the retina. According to Schultze, a layer of mesenchymal cells develops late in embryonic life (90 mm. stage in the pig, third month in man); the cells arrange themselves in a very distinct network and are then hollowed out to form blood vessels.

At the beginning of this century Versari's published several beautifully illustrated articles describing the development of the retinal vascular system in man. He examined injected preparations, as well as serial cross sections, and came to the conclusion that retinal vessels developed from embryonic connective tissue around the hyaloid artery. In a fetus of 10 cm. length he found cell cords in the substance of the cellular mass surrounding the hyaloid artery in the region of the papilla, and these were in continuity with the wall of the hyaloid artery. Patent retinal vessels were found in a fetus measuring 12.5 cm.

Mann, in her classic work, notes that the central retinal artery is just recognizable at the 100 mm. stage (fourth month) of human development. At this time a bulbose swelling appears on the trunk of the hyaloid artery at the point of exit from the disc. From this enlargement, two small buds grow out, one forming the upper and one the lower main branches of the central retinal artery.

More recently, Michaelson's studied the subject of retinal vascular development, and his work is widely quoted. He examined India ink injected specimens of various species, including the rat, and drew a number of conclusions: (1) that vessels grow into the retina by a process of vascular budding from the hyaloid vessel within the head of the optic nerve, and (2) that retinal capillaries grow out from developing veins. Michaelson recognized the fact that vessels do not develop from any in situ cellular component of the retina, but are derived from an outside source and grow into the retina.

Ashton has suggested that undifferentiated spindle cells precede any evidence of cord or vessel formation in the human retina, and his view is strengthened by the observations of Serpell and Nilhausen. Interestingly, he feels that in the cat, normal retinal vascularization takes place by an initial endothelial budding without mesenchymal precursors. Cogan, relying primarily on trypsin digested preparations, has studied retinal vessel development in the human fetus, and he states that solid endothelial cords sprout from the nerve head, apparently from the same vessels that serve the hyaloid system.

Our study indicates that vascular budding does not take place either in the initial or the later stages of normal retinal vascular development in the rat. We can find no evidence of lumenized outpocketings of the hyaloid vessel in this species, but cannot deny that such outgrowths may occur in other animals, or in man. The first indication we have seen of a vascularizing process is the aggregation of cells which seem to derive from the outer coat of the hyaloid vessel. These proliferate into the tissue surrounding the hyaloid system. Initially they do not seem to be joined to one another, but in a short space of time a network of cells or cords can be seen, and soon this evolves into lumenized vessels.

Retinal vascularization appears somewhere around the fifteenth day of fetal life in the rat and is much more extensive than India ink injection can show. Furthermore, there is no evidence that venous development precedes capillary development in the rat, cat, or human being. Indeed the view that there is an initial venous development is not consistent with embryologic evidence from areas other than the eye.

We have found that retinal digestion, at least in the rat, does not provide us with information about the earliest stages of vascular development. In addition, we doubt that study of digest preparations alone could identify the character of cells making up the initial ingrowth, and our
electron microscopic figures cannot establish that they are indeed endothelial in nature. If anything, the cells seen are rather active and appear undifferentiated.

At this stage it appears that Nineteenth Century investigators, limited to routine histologic techniques, were fairly close to describing accurately the sequence of events occurring in early retinal vascularization. They recognized that the retinal vessel precursors do not come from the interior or lumenized portion of the hyaloid artery, but rather from the tissue surrounding this vessel. We interpret our results to indicate that the cells originate from the outer coat of the hyaloid vessel, or from undifferentiated cells closely associated with this vessel. Lumen formation is preceded by development of a cellular meshwork.

It is true that our results run counter to the views expressed by Michaelson, but we must point out that using an inking technique gave results similar to his and might have led to similar conclusions. Indeed it is difficult to account for the appearance of the injected specimens, and it is possible that the vascularizing factor postulated by Michaelson may in some way affect lumenization even if it does not explain the sequence of events conceived at present. The initial cellular ingrowth may be entirely independent of any tissue factor, or of oxygen concentration or gradient. Indeed, the fact that retinal vessel growth is initiated at a time when a patent hyaloid system lies close to the retinal surface would seem to argue against retinal hypoxia being the prime factor in initiating vascular development.

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REFERENCES

Discussion
Dr. Arnall Patz, Baltimore, Md. The authors have presented an interesting concept of retinal vessel development in the rat. Their use of electron microscopy adds a new dimension to previous reports with India ink injections and suggests, at least in the rat, that retinal vascularization does not commence from budding from the lumen of the hyaloid vessel. Instead their data indicate that the vasculogenic cells of the retina originate from the outer coat of the hyaloid vessels or from undifferentiated cells closely associated. The author's observations are in agreement with the studies reported at the turn of the century, and their hypothesis of early retinal vessel development is consistent with general concepts of vascular embryology in other organs.
Dr. Shakib and co-workers' studies may have application to the problem of oxygen and retrolental fibroplasia. We have been interested in the response of these vascologenic cells to oxygen. We found that the response to oxygen of these cells in the newborn rat, however, is very minimal compared with the severe changes in the newborn mouse, kitten, or puppy eye. In these latter species high concentrations of oxygen destroy all vestiges of these cells. We would be interested to learn if future studies by the authors utilizing any of these other species show a similar pattern of vasculogenesis.