Presumed Retinovitreal Neovascularization in Dystrophic Retinas of Spontaneously Hypertensive Rats

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The authors have observed abnormal blood vessels, strongly suggestive of neovascular proliferation, arising from the retinal circulation and extending through the inner limiting membrane of the retina into the vitreous in five spontaneously hypertensive (SHR) rats with severe retinal dystrophy. The animals in whom these presumptive retinovitreal new vessels occurred were all 15 mo of age or older. The new vessels frequently demonstrated thinned and, rarely, fenestrated endothelium, abnormal intracellular junctions, increased numbers of endocytic vesicles, bizarre appearing pericytes, and highly abnormal basement membranes, features that have been observed in retinovitreal new vessels in proliferative retinopathies in humans. Unlike such new vessels arising from the human retinal circulation, however, those that we observed in dystrophic rat retinas were usually surrounded by proliferating retinal pigment epithelial cells within the retinal substance. Unlike the vessels, the pigment epithelial cells did not break through the inner limiting membrane of the retina to enter the vitreous. The pigment epithelial cells that made contact with the internal limiting membrane of the retina demonstrated apical and basal plasma membrane specializations that are typical of these cells in their normal anatomical location, while pigment epithelial cells migrating in cords through the neural retina lacked such specializations.

This animal model may be of great value in understanding the mechanisms of retinal neovascularization.


Although new vessels derived from the retinal circulation and extending into the vitreous are a feature of several diseases of the human retina, few animal models exist by which such neovascularization can be studied. Among these models, the best known is the retinal neovascularization that follows oxygen administration in neonatal kittens and puppies. New vessels arising from the optic nervehead have been observed following sensitization of monkeys to bovine serum albumin or insulin, followed by an intravitreal “challenge” injection of the same, foreign protein. Finally, at least transient intraretinal neovascularization has been reported in a few rhesus monkeys following retinal branch vein occlusion produced by intense laser photocoagulation.

We report here the entirely unexpected finding of apparent new vessel formation from the retinal circulation, with extension into the vitreous, in spontaneously hypertensive (SHR) rats with retinal dystrophy. Although these presumptive new vessels differ in some respects from the clinical condition found in humans, many features are remarkably similar. Hence, we believe that this animal model may be useful for the study of possible causes and means of prevention of retinovitreal neovascularization in human disease.

Materials and Methods

The animals in this investigation were among those described in the accompanying paper, in which, along with another report from this laboratory, methods for maintenance of the animal colony, for killing the animals, and for fixing, embedding, and sectioning the retinas are described. All of the retinal tissue described in this paper was taken from the posterior retina, within 2 mm of the optic nerve.

This study was performed in accord with the ARVO Resolution on the Use of Animals in Research.

Results

Five of the 46 spontaneously hypertensive rats in our colony demonstrated presumptive retinovitreal new vessels. These included a 15-mo-old streptozotocin diabetic animal, two 15-mo-old diabetic animals that
were receiving the aldose reductase inhibitor Sorbinil (Pfizer Central Research; Groton, CT), a 17-mo-old nondiabetic animal on Sorbinil, and a 17-mo-old animal in the control group. The actual prevalence of neovascularization in these dystrophic animals may be greater, however, since 14 of the 46 animals had received diets containing 30% galactose, which we have found greatly reduces the prevalence and severity of the retinal dystrophy in SHR rats. The animals described here did not differ significantly in age from the other dystrophic SHR rats that we studied, and they were rather evenly distributed among the various experimental groups, suggesting that none of the experimental protocols altered the prevalence of the neovascularization.

On serial thick sectioning, the apparent new vessels were seen to arise from the retinal circulation, or from retinal vessels that had extended outward into the retinal pigment epithelium (RPE), and which then grew inward through the severely dystrophic retina to break through the inner limiting membrane (ILM) of the retina into the vitreous (Figs. 1A, B). The vessels were evidently functional, since formed elements of the blood were nearly always present within their lumina. Pericytes were infrequent in capillary-sized intravitreal vessels, and we did not observe smooth muscle elements in the walls of the larger vessels. The occasional pericytes that we observed often had bizarre shapes (Fig. 1B). Certain observations suggested that the vessels were able to digest the ILM in order to break through into the vitreous. The vessel indicated by the asterisk (*) in Fig. 1B, and shown in complete profile at higher magnification in Fig. 1C, demonstrates attenuation of the overlying ILM, with its ultimate disruption (thin, filled arrow, Fig. 1C). This was observed frequently when intraretinal vessels grew close to the ILM, but was never observed when the ILM lay over ectopic RPE or neural retinal cells. Although we have not demonstrated actual proliferation of these abnormal vessels, since we did not observe them clinically prior to killing and hence, could not document vascular growth, nor did we perform [3H]-thymidine autoradiography to show DNA synthesis in endothelial cell or pericyte nuclei, certain observations strongly suggest neovascular proliferation. For example, in Fig. 1B, a portion of the endothelium of the major vessel that protrudes from the inner surface of the retina appears to be "budding," and a new lumen appears to be forming at the apex of the endothelial "budding zone." This is shown at higher magnification in Figure 1D.

In most cases, the vessels were accompanied, as far as the ILM, but never into the vitreous cavity, by RPE cells that proliferated in cords through the neural retina (Figs. 2A, 2B). The RPE cells often were observed to grow along the inner surface of the neural retina (Figs. 1B, 2D). When they made contact with the ILM, these ectopic RPE cells frequently maintained the plasma membrane specializations of their apical and basal surfaces (Figs. 1B, 2B, 2D). Such specializations were not observed in the RPE cells as they grew in cords through the retina (Figs. 2A, 2B), but only when they contacted the ILM (Fig. 2B). In all locations, however, the RPE cells were observed to form junctional complexes with adjoining RPE cells (Fig. 2C). The plasma membrane infoldings of the basal surface of the ectopic RPE cells produced marked convolutions of the ILM (Figs. 1B, 2D). Junctional complexes between neighboring Müller cells were often found when these cells lay close to the apical surface of an ectopic RPE cell (Fig. 2D). This produced an appearance much like that of the "outer limiting membrane" of the normal retina, save that the junctions were between adjoining Müller cell processes, rather than between these processes and adjoining photoreceptor inner segments. Also, the junctions occurred at the inner ends of the Müller cell processes, rather than at their outer ends, where the "outer limiting membrane" of the retina is normally found.

The endothelial cells of the intravitreal blood vessels varied considerably in thickness. Occasionally, very thin areas were observed, with rare fenestrations (Fig. 3A). The endothelial cell cytoplasm was unusually heavily laden with endocytic vesicles. The intravitreal vessels were surrounded by basement membranes, but they did not have the thick and uniform appearance of basement membranes of the normal retinal vasculature. Often, these basement membranes were multilaminar (Fig. 3A), while in the other areas they were rudimentary (Fig. 1D). Junctional complexes between
endothelial cells of the intravitreal vessels lacked the extensive, pentalaminar plasma membrane fusion zones (zonulae occludentes, or "tight junctions") that typify endothelial junctions of the normal retinal vasculature.\(^7\) The junctions frequently appeared to be "open" (Fig. 3B), but sometimes demonstrated a limited zone of plasma membrane fusion near the apices of the adjoining cells (Fig. 3C). Since we were unaware of the presence of these vessels prior to killing, we did not inject intravascular tracers antemortem to demonstrate breakdown of the blood–retinal barrier, yet observation of the thinned and occasionally fenestrated endothelium, the abnormal junctional complexes, and the increased number of endocytic vesicles,\(^8\) all suggest

Fig. 1 B. Low power electron micrograph of a portion of the inner retina of another SHR animal, age 15 months, illustrating the intravitreal new vessels breaking through the inner limiting membrane of the retina into the vitreous cavity. An ectopic retinal pigment epithelial cell (RPE) has also migrated through the retina and lies on its inner surface, underneath the inner limiting membrane. The RPE cell demonstrates polarization of its apical and basal surfaces, but it now lies "upside down," with its apical microvilli (thick, filled arrows) pointing toward the neural retina, while its extensively invaginated basal plasma membrane faces the inner limiting membrane and the vitreous cavity (thick, open arrows). Note the typical cytoplasmic organelles of the ectopic RPE cell and the plentiful lipofuscin granules. The thin, closed arrow in the vitreous indicates a bizarre pericyte overlying an endothelial cell in a capillary-sized intravitreal vessel. The intra-vitreal cell indicated by an "X" is of an unidentified type. The single asterisk (*) indicates a portion of a vessel shown in its entirety at higher magnification in Figure 1C, while the double asterisk (**) indicates apparent budding of a portion of the endothelial lining of the largest vessel in this micrograph. This area is shown at higher magnification in Figure 1D. The calibration bar = 4 \(\mu\)m.

Fig. 1 C. Enlargement of the vessel seen partially in 1B, and indicated there by an asterisk (*). It is partially surrounded by an ectopic RPE cell. The inner limiting membrane that is intact over the RPE cell (closed arrowheads) is attenuated over the capillary (thin, closed arrow), and beyond its breaking point, only amorphous extracellular matrix material and some fibrillar collagen is present. Pericyte processes (P) and an endothelial cell (E) are indicated. The calibration bar = 1 \(\mu\)m.

Fig. 1 D. An apparent "bud" of proliferating endothelium, indicated by the double asterisk (**) in 1B and shown at higher magnification here. A zone of apparent lumen formation (L) is shown. Regions of thinned, but still intact, basement membrane partially surrounded the endothelial lining of the intravitreal vessels (closed arrowheads), but in other regions, the basement membrane is markedly attenuated (thin, closed arrow). The calibration bar = 1 \(\mu\)m.
that the endothelial lining of these vessels is highly permeable.

**Discussion**

The structure of the vessels that we describe here is strikingly similar to that of the intravitreal new vessels that have been described in various human proliferative retinopathies, and in retinal neovascularization in experimental animals. Anatomical findings that distinguish these vessels from the normal retinal vasculature include rudimentary basement membranes, multilaminar basement membranes, thinned endothelial cell cytoplasm, occasional endothelial fenestrations, and abnormal endothelial junctions.

Because we have not actually observed these vessels to proliferate in our rats by ophthalmoscopic examination in vivo (we did not make ophthalmoscopic examinations on these animals during this series of experiments), and because we did not observe mitotic figures, or perform \[^3\text{H}\]-thymidine autoradiography to demonstrate DNA synthesis in vascular cells, we cannot claim without reservation that these vessels represent true neovascularization. Nevertheless, the anatomic similarities that we have observed between these vessels...
and unequivocal new vessels in human proliferative retinopathies that have been observed by light and electron microscopy strongly suggest this conclusion. Even more strongly suggestive is our observation that these vessels in severely dystrophic retinas of SHR rats are able to break through the ILM into the vitreous, a property that is characteristic of proliferating and migrating vascular endothelial cells. In the course of this study, we noted that only the blood vessels were able to break through the ILM into the vitreous, while we have never observed RPE cells to do so. Kalebic et al.\textsuperscript{14} have recently demonstrated that migrating (and, apparently, simultaneously proliferating) vascular endothelial cells produce a specific collagenase that is capable of degrading basement membrane structures. The presence of such an enzyme in the migrating vascular cells, and its absence in migrating RPE cells, would explain our observations. The report of Kalebic et al.\textsuperscript{14} also suggests that the observation of basement membrane digestion by vascular endothelium is an indicator of neovascularization.

It is possible that intravitreal neovascularization may be a feature of far advanced stages of other retinal dystrophies and degenerations in the rat. For example, intravitreal blood vessels were reported by Lai et al.\textsuperscript{15} in highly disorganized, dystrophic retinas of Wag/Rij rats. These authors did not give an extensive description of the vessels, nor did they report the frequency of this finding in their rat colony.

Despite the similarities in these blood vessels to those in proliferative retinopathies in humans, there is at least one important difference. This is the close association of the proliferating vessels with proliferating cells of the retinal pigment epithelium. This association is also apparent in the electron photomicrograph of intravitreal neovascularization in the Wag/Rij rat shown by Lai et al.\textsuperscript{15} The presence of new blood vessels within the RPE in dystrophic retinas of SHR rats, as well as in other forms of retinal dystrophy and degeneration in the rat\textsuperscript{4,16-19} suggests that the RPE may stimulate the proliferation of vascular endothelial cells. However, other factors must also be able to support neovascular proliferation, since the new vessels evidently continue to grow once they have entered the vitreous, where they are no longer accompanied by RPE cells.

A striking, new observation is the apparent role of
the ILM as an inducer of polarization in ectopic RPE cells. The RPE cells were not observed to develop apical or basal plasma membrane specializations, except where they made contact with the ILM. In the accompanying article, we demonstrate plasma membrane infoldings, like those that are normally present on the basal surface of RPE cells lying on Bruch's membrane, when the RPE cells surround abnormal blood vessels that have elaborated a basement membrane. These observations suggest a function of basement membranes that has previously received little attention, namely, that basement membranes and, perhaps, other types of extracellular matrices, may induce specialized anatomical and physiological properties in the cells that lie upon them. Additional evidence for this proposed function of extracellular matrix may be taken from studies of vascular endothelial cells, as well as various types of epithelial cells in culture, which demonstrate more highly differentiated morphological and biochemical features, either when they are grown on specific extracellular matrices, or when such matrices are laid over the cultured cells.

Fig. 2 C. A higher magnification of a region of such a cord of RPE cells projecting through the retina of an SHR rat, showing junctional complexes between the cells (arrowheads). The calibration bar = 1 μm.

Fig. 3 A. High magnification view of a portion of an intravitreal vessel in a dystrophic SHR rat, showing thinned endothelium and endothelial fenestrations (arrowheads). Note also the copious endocytic vesicles within the cytoplasm, and the thin, multilaminar basement membrane. The calibration bar = 0.2 μm; final magnification = 60,000×.
Fig. 2 D. A portion of another ectopic RPE cell, overlying the neural retina, but beneath the internal limiting membrane. Note the apical microvilli and the basal plasma membrane invaginations, and in particular the junctional complexes between adjoining Müller cells (arrowheads), adjacent to the apical surface of the ectopic RPE cell, and resembling the junctions of the outer limiting membrane of the retina, save for their abnormal location here, and the fact that the junctions are normally between Müller cells and adjacent photoreceptor inner segments. VIT = vitreous. The calibration bar = 1 μm.
Ectopic RPE cells have not been noted in contiguity with intraretinal or intravitreal new blood vessels in any of the proliferative retinopathies in humans, nor have blood vessels been observed within the RPE in any histopathologic studies of human retinal dystrophies. Indeed, in the proliferative retinopathies of humans, the presence of functioning, but hypoxic neural retina, and not abnormalities of the RPE, has
been proposed to be a major stimulus for neovascularization. 33-35 Destruction of cells of the neural retina, for example by photocoagulation, or by various degenerative of dystrophic processes, has been proposed to cause regression of neovascular proliferations by greatly reducing the retina’s enormous metabolic requirements, thereby reducing the production of a vasoproliferative factor which retinal cells are thought to produce under conditions of hypoxic stress. Although we have performed no measurement of any metabolic function in these dystrophic rat retinas, such activity must be profoundly reduced, since the photoreceptors are totally absent, neuronal organization is almost completely gone, and the number of cellular nuclei in all of the retinal layers is profoundly reduced. Hence, the presence of extensive, presumptive retinovitreal new vessels in this animal model seems anomalous, in the light of the major current hypothesis of the cause of such neovascularization. If it is possible to develop a unified concept of retinal neovascularization that applies to all species, then further study of this unexpected model of apparent retinovitreal neovascularization in the rat may provide important clues to the causes of retinal new vessel formation in humans.

Key words: neovascularization, retinal dystrophy, retinal pigment epithelium, spontaneously hypertensive (SHR) rat

References