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Diabetic Neovascularization: Permeability and Ultrastructure

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The permeability of newly formed blood vessels in proliferative diabetic retinopathy at different stages of development was correlated with their ultrastructural features. The young proliferating vessels were much more permeable to fluorescein than were the older vessels. Transmission electron microscopy revealed that the blood vessels within the young intravitreal proliferating fronds had intermediate interendothelial junctions, whereas the blood vessels within the old neovascular preretinal membranes had tight interendothelial junctions of the macula occludens type. Fenestrations were observed only rarely in the endothelial wall of either the young or the older proliferating vessels. We conclude that the principal route of fluorescein leakage from the newly formed blood vessels in proliferative diabetic retinopathy is via the interendothelial clefts. *Invest Ophthalmol Vis Sci* 25:1338-1342, 1984

In proliferative diabetic retinopathy, newly formed blood vessels are proliferating from the retinal vessels, first on the surface of the retina and, later on, into the vitreous cavity.

The proliferation begins with the formation of new vessels having very few glial and fibrous tissue components. As the fibrovascular fronds mature, the glial and fibrous components in the tissue increase.

Normal retinal vessels form a blood-retinal barrier which retains fluorescein dye during angiography.¹ The proliferating retinal vessels do not share the impermeability characteristics of normal retinal vessels; during fluorescein angiography they show profuse leakage of dye into the vitreous cavity. The newest capillaries at the edge of the neovascular frond demonstrate a greater leakage of fluorescein than does long-standing neovascularization associated with fibrosis.²

In the present study, the permeability characteristics of the proliferating retinal vessels at different stages of development were correlated with their ultrastructural features in order to elucidate the morphologic basis for their increased permeability.

Our results indicate that the ultrastructural features of the interendothelial junctions account for the different permeability of the newly formed vessels at different stages of their growth.

Materials and Methods. Preretinal neovascular membranes were excised from nine diabetic patients, four males and five females. Ages ranged from 20 to 55 years old with a median of 41 years. All of the patients were diabetic for at least 15 years and had advanced diabetic eye disease. Human informed consent was obtained prior to undertaking this study. The preretinal membranes were divided into two groups: (1) young neovascular intravitreal proliferating fronds that contained "naked," newly formed blood vessels (three membranes) and (2) old fibrovascular preretinal membranes that contained older newly formed blood vessels involuted in fibrous tissue (six membranes). The membranes were excised during pars plana vitrectomy using the Ocutome system and fine vitreous scissors and collected in the Ocutome collecting bottle in the infusion fluid (lactated Ringer's solution). The samples were centrifuged for 3 min in model CL International clinical centrifuge and the membranes were fixed in 2.5% glutaraldehyde in 0.15 M phosphate buffer, postfixated for 1 hr at room temperature with 1% osmium tetroxide in 0.15 M phosphate buffer, dehydrated with a graded alcohol series followed by propylene oxide and embedded in Epon 812. Sections were cut and stained with toluidine blue for light microscopic orientation. Thin sections (50-70 nm) were cut for electron microscopy, stained with uranyl acetate and lead citrate and viewed with a JEOL 100B transmission electron microscope at 80 kv.

Ten different capillaries were studied within each of the three young intravitreal proliferating fronds. Only three of the six old preretinal membranes contained blood vessels, each of which was encased in a large amount of collagen. Therefore, only seven, five, and four different capillaries could be investigated in

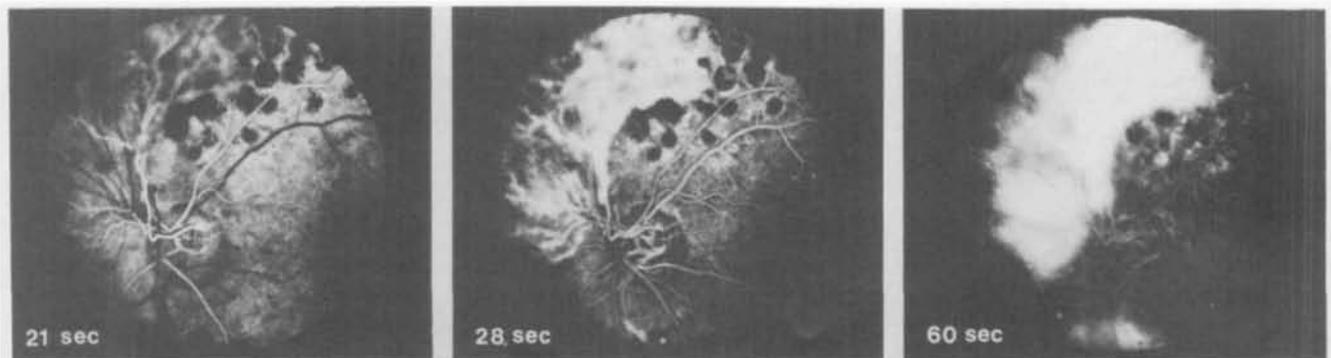


Fig. 1. A, Fluorescein angiogram of a young neovascular intravitreal frond demonstrates an early and extensive leakage.

each sample, respectively. Each of the newly formed blood vessels was studied in numerous cross-sections.

Results. On comparing the fluorescein angiogram of a young neovascular intravitreal frond with that of an old fibrovascular preretinal membrane (Fig. 1), the immature neovascularization clearly demonstrated a much faster and greater leakage of fluorescein than did the mature neovascularization.

Electron microscopy of the fibrovascular membranes revealed that both the young and the older neovascularization consisted of a continuous type nonfenestrated endothelial wall surrounded by multilaminated basal lamina typically seen in diabetics. However, the young vessels were comprised of plump endothelial cells, rich in cytoplasmic inclusions, forming a narrow lumen (Fig. 2A); the older vessels consisted of thin endothelial cells with fewer cytoplasmic organelles and has widely patent lumens (Fig. 2B). Only rarely were fenestrations observed in either the old or the young newly formed blood vessels. A few pinocytotic vesicles were found in both the young and the old vessels (Fig. 2).

The interendothelial junctions of both the young and the older vessels were difficult to visualize throughout the entire junction. However, numerous cross-sections of each blood vessel revealed a marked difference between these junctions of the young versus

the older vessels. The young newly formed blood vessels had open interendothelial clefts with short adherent regions characterized by a symmetrical layer of fluffy material on the inner surface of the junctional membranes and by tenuous condensation of the intercellular matrix (Fig. 3); no fusion points between adjacent endothelial cells could be identified.

In the older newly formed blood vessels, very few open interendothelial clefts could be observed and the number of adherent junctions also was reduced greatly. Instead, many interendothelial junctions showed fusion points between the adjacent endothelial cells (Fig. 4).

Discussion. The morphologic basis for the increased permeability of the newly formed blood vessels in proliferative diabetic retinopathy is not quite clear. It has been suggested^{3,4} that fenestrations in the endothelial wall of the proliferating retinal vessels account for their characteristic "leakiness."^{3,4} However, in our study we found very few blood vessels containing fenestrated walls, either within the young, highly permeable proliferating fronds or the older, less permeable preretinal membranes.

In a recent study of 30 preretinal membranes excised from diabetic patients, Hamilton et al⁵ also reported that only 5 of the 21 membranes containing blood vessels had fenestrated capillaries. Furthermore,



Fig. 1. B, Fluorescein angiogram of an old fibrovascular preretinal membrane shows relatively late and less extensive leakage.

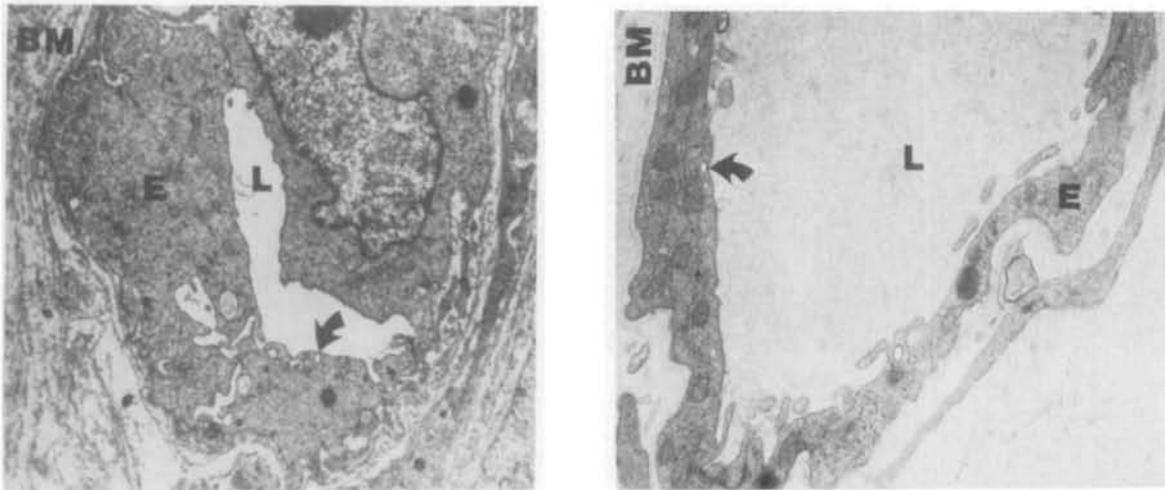


Fig. 2. Low-power electron micrographs of newly formed blood vessels reveal a continuous type nonfenestrated endothelial wall surrounded by multilaminated basal membrane (BM). A few pinocytotic vesicles can be seen (arrow). **A, left.** Young vessel within a neovascular intravitreal proliferating frond. Note the plump endothelial cells (E) and the narrow lumen (L) ($\times 6000$). **B, right.** Older vessel within a fibrovascular preretinal membrane. Note the thin endothelial cells (E) and the widely open lumen (L) ($\times 14,000$).

since fluorescein is an anion, it should be discriminated against passage via diaphragmed fenestrations, which were found to contain a high concentration of high-affinity anionic sites on their luminal surface.⁶ We conclude that the presence of fenestrations in the endothelial wall of the proliferating retinal vessels cannot account for their increased permeability to

fluorescein. Similarly, the presence of fenestrations in brain tumor capillaries could not be correlated with the degree of Perchnetate uptake by the tumor.⁷

Pinocytotic vesicles were seen in both the young and the older newly formed blood vessels; however, they were not present in an amount sufficient to suggest that they are responsible for the increased permeability (Fig. 2).

While the presence of fenestrations or increased pinocytotic activity does not provide an explanation for the increased permeability of the proliferating retinal blood vessels, the ultrastructural features of their interendothelial junctions not only could account for their increased permeability, but could also explain the different amount of fluorescein leakage from the young and older newly formed vessels.

Previous reports on the nature of interendothelial junctions of newly formed blood vessels within diabetic preretinal membranes gave conflicting data. Hamilton et al⁵ reported that all the capillary endothelial cells were joined by well-formed intermediate-type junctions. In their study, they do not distinguish between young and old neovascular membranes. On the other hand, in a study of old neovascular preretinal membranes, Wallow and Geldner³ found that the endothelial cells of most blood vessels were connected by tight junctions showing periodic fusions of the outer leaflets of their plasma membranes; only in some instances were the outer leaflets merely approximated, without periodic fusions.

Our results confirm that the diversity of the junctions does indeed exist among the newly formed blood vessels. However, we have defined precisely the type of junctional complexes and have shown that

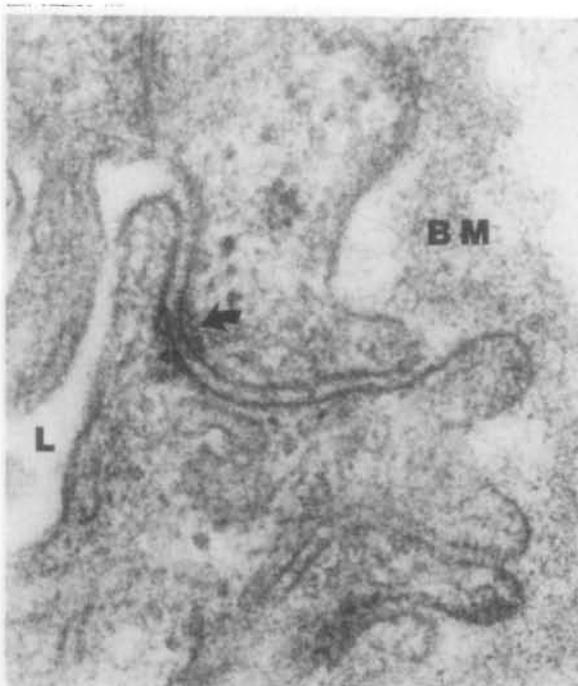
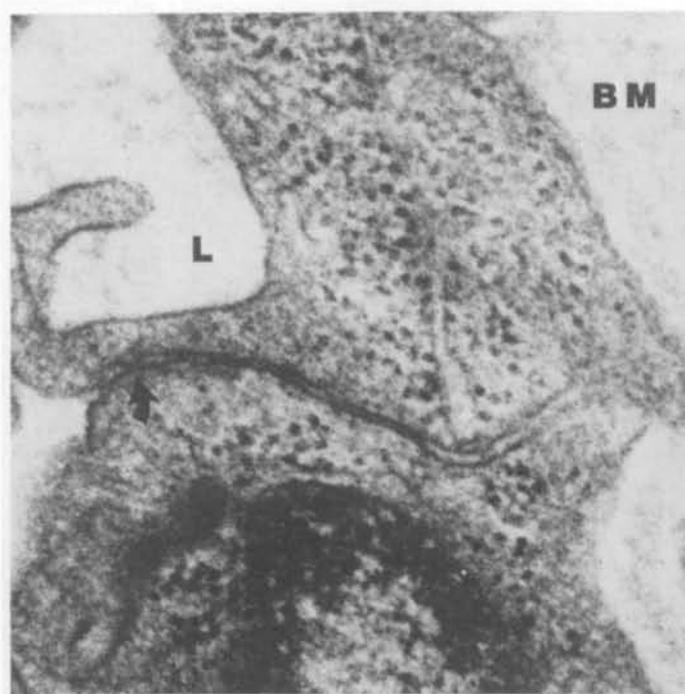


Fig. 3. Open interendothelial junction of a young newly formed blood vessel. Note the short adherent region (arrow) with a symmetrical layer of fluffy material on the inner surface of the junctional membranes and a tenuous condensation of the intercellular matrix ($\times 96,000$).

Fig. 4. Interendothelial junction of an old newly formed blood vessel. Fusion points between adjacent endothelial cells can be observed (arrow) ($\times 99,000$).



there is a marked difference between the junctions connecting endothelial cells of vessels within the intravitreal proliferating fronds and those within older neovascular preretinal membranes.

The recently proliferated blood vessels had open interendothelial junctions with one to several short adherent regions in which the junctional membranes had a layer of finely filamentous material on their inner surfaces (Fig. 3). These junctions are classified as intermediate junctions⁸ and are known to present no effective barrier to the penetration of the intercellular cleft.⁹

In the more mature newly formed vessels, the number of open intercellular junctions decreases and tight junctions between adjacent endothelial cells can be observed (Fig. 4); these tight junctions did not block completely the passage of fluorescein from the blood vessels into the vitreous (Fig. 1). We therefore assume that they did not form a complete belt around the adjacent endothelial cells and therefore should be classified as macula occludens.¹ While the macula occludens of the old neovascular channels allow passage of fluorescein from the blood to the vitreous, this is obviously a much smaller amount than allowed by the intermediate junctions of the young newly formed blood vessels.

It has been suggested⁸ that intermediate junctions are a prerequisite for tight junction formation. It is tempting to speculate that the intermediate junctions of the young retinal neovascularization represent the first step in the development of the maculae occludentes found in older newly formed blood vessels.

As normal retinal blood vessels have zonular tight junctions from the outset of their development,¹⁰ it is reasonable to assume that vessels proliferating from the retinal net also would develop zonular tight junctions. The presence of zonulae occludentes in newly formed blood vessels of some brain tumors⁷ suggests that the incomplete formation of the tight junctions is not an intrinsic property of newly formed blood vessels but, rather, a result of the diabetic condition.

Key words: proliferative diabetic retinopathy, newly formed blood vessels, fluorescein angiography, ultrastructure, interendothelial junctions

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Sialic Acid on the Surface of Photoreceptors and Pigment Epithelium in RCS Rats

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Incubation of isolated retinas and pigment epithelium (PE) from normal and dystrophic RCS rats with colloidal iron (CI) at pH 1.8 resulted in dense labeling of rod outer segments (ROS) and PE surfaces. Pretreatment with neuraminidase reduced subsequent binding of CI. The authors conclude, therefore, that sialic acid residues are localized on the ROS and PE surfaces. In dystrophic rats, uningested ROS tips, which accumulate in the subretinal space, did not bind CI. Invest Ophthalmol Vis Sci 25:1342-1345, 1984

In dystrophic RCS rats, the failure of the pigment epithelium (PE) to phagocytize shed rod outer segments (ROS) tips leads to an accumulation of ROS debris and, eventually, to the degeneration of photoreceptors.¹ La Vail et al² have observed that in dystrophic rats, interphotoreceptor-matrix staining is virtually absent in the ROS debris zone. Visualization of the interphotoreceptor-matrix components was attained with light microscopic histochemical procedures employing colloidal iron and other cationic dyes.

Colloidal iron (CI), by virtue of its electron density, is a useful cytochemical marker for anionic determinants at the ultrastructural level. In some cellular systems, CI has been shown to bind specifically to cell-surface sialic acid.³ Recently, Cohen and Nir⁴ have used CI at pH 1.8 for the detection of sialic acid residues on the surface of cultured PE cells. In view of the role of surface sialic acid in intercellular recognition,⁵ it was of interest to determine if sialic acid could be localized also on the surface of ROS and to establish whether differences in CI staining could be visualized on the surface of normal and dystrophic retina and PE tissues.

Materials and Methods. Tissue: Pigmented dystrophic RCS rats (RCS-P⁺) and the genetic control (RCS-P⁺rdy⁺) were used in this study. The animals

were maintained on a 12:12-hr light:dark cycle. Sixty minutes after the onset of light, 16-21-day-old rats were euthanized with chloroform. Following enucleation, the retina was separated from the PE layer, which remained attached to the posterior eyecup. The isolated retina and eyecup were rinsed in phosphate buffer (0.15 M, pH 7.0) for 15 min at 4°C with gentle shaking in order to remove soluble components of the interphotoreceptor matrix. The tissues then were fixed with 1% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0) for 60 min at room temperature and rinsed in phosphate buffer.

Cytochemical reactions: Colloidal iron solution (pH 1.8) was prepared according to Gasic et al.⁶ Fixed tissue was rinsed in distilled water and incubated with colloidal iron for 5-10 min at room temperature. The tissue then was rinsed in acetic acid (pH 1.8) followed by distilled water. The specificity of the CI staining was determined by neuraminidase digestion prior to the cytochemical incubation. Unfixed tissue was incubated for 30 min at 37°C with 100 units/ml *Vibrio cholerae* neuraminidase (free of protease, aldolase, and lecithinase C; Behring Institute; Marburg, West Germany) in a medium containing 3 mM calcium, 0.145 M NaCl, and 4 mM NaHCO₃ (pH 6.5).⁷ The tissue then was fixed and incubated with CI.

Electron microscopy: Following the cytochemical reactions, the tissue specimens were rinsed and post-fixed with 1% OsO₄ in phosphate buffer (pH 7.0), then dehydrated in a graded ethanol series and embedded in Araldite. Thin sections were stained with uranyl and lead salts and viewed in a Jeol 100B electron microscope (Tokyo, Japan).

Results. In dystrophic RCS rats, 13 days and older, uningested shed ROS tips accumulate in the subretinal space. During the separation of the PE from the