Newly-Formed Subretinal Vessels

Fine Structure and Fluorescein Leakage

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The ultrastructure of experimentally induced newly formed subretinal vessels was correlated with the amount of fluorescein leakage demonstrated by the neovascular membranes during angiography. The membranes that demonstrated leakage contained subretinal vessels with a fenestrated endothelial wall and intermediate interendothelial cell junctions. As these subretinal plexi matured, they stopped demonstrating leakage. This involution process was accompanied by the formation of focal interendothelial tight junctions; however, loss of fenestrations was not observed. The membranes that never demonstrated fluorescein leakage also contained fenestrated subretinal vessels at both early and late stages of development; and their interendothelial junctions showed similar maturation from open to focal tight junctions. Thus all subretinal vessels had “leaky” morphology strongly resembling that of the normal choriocapillaris, whether they demonstrated fluorescein leakage or not. The authors conclude that newly formed subretinal vessels retain the characteristics of the choriocapillaris from which they are believed to proliferate; they have the potential to leak fluorescein at all stages of their development. The absence of fluorescein leakage during angiography cannot always be correlated with the absence of “leaky” morphology. Invest Ophthalmol Vis Sci 27:204–213, 1986

Subretinal neovascularization (SRN) is a pathologic feature of many eye diseases. The newly formed vessels proliferate from the choroid into the subretinal space and are diagnosed by fluorescein leakage during angiography. Fenestrations have been observed in the endothelial wall of both human and experimental newly formed subretinal vessels and were suggested to account for this leakiness. Clinicopathological correlations showed, however, that blood vessels can be present in the subretinal space without demonstrating leakage during angiography. These clinicopathological observations were confirmed by us in an experimental model of SRN. We used high intensity laser photocoagulation to induce subretinal neovascular lesions in the macular region of a primate eye. While all lesions developed newly formed subretinal vessels, only 40% of them demonstrated fluorescein leakage. The lesions were thus divided into two groups: a) leaky, those that demonstrated leaking and pooling of fluorescein, and b) “nonleaky,” those that never demonstrated leaking and pooling of dye.

In the leaky lesions, the newly formed vessels proliferated from the choroid through a break in Bruch’s membrane and coursed peripherally into the subretinal space around that break; they were separated from the sensory retina by an overlying fluid-filled space. With maturation, these lesions gradually stopped leaking fluorescein, ie, involuted. This involution was accompanied by disappearance of the subretinal fluid while the blood vessels remained present in the subretinal space.

In the “nonleaky” lesions, the vessels were concentrated at the center of the scar and, similar to the involuted lesions, were not associated with overlying fluid.

We concluded that detection of the subretinal vessels during angiography was dependent upon the presence of a fluid-filled space between the vessels and the sensory retina, as this provided a place for the dye to pool and thereby be apparent. The aim of this study was to find whether differences in the ultrastructural features of the subretinal vessels could be responsible for the accumulation of fluid in the subretinal space of the leaky lesions and for its absence in the involuted and “nonleaky” lesions.

Materials and Methods

Choroidal subretinal neovascularization was induced in the macular region of cynomolgus monkeys by intense laser photocoagulation, as previously described. The animals were treated in a manner that conforms
Fig. 1. Late phase fluorescein angiograms of retina with eight laser lesions (1 to 8) in the macular area of a cynomolgus monkey. All lesions were lasered with identical intensity of 700 mW 100µM spot size for 0.1 sec. A. One week after laser photocoagulation. All lesions show staining of the scar only. B. Two weeks after laser photocoagulation. Lesions 1, 2 and 3 are leaking and pooling fluorescein in the subretinal space; they are defined as leaky lesions. The other five lesions (only four are shown here) show staining of the scar only and are defined as nonleaky lesions at this stage of development of the neovascular membrane.

with the ARVO Resolution on the Use of Animals in Research. The laser lesions were followed up routinely once a week by fluorescein angiography, and on the basis of angiographic findings were divided into three groups: (1.) Leaky lesions at the early stage: Laser lesions that actively leak and pool fluorescein in the subretinal space; (2.) Leaky lesions at the involuted stage: Laser lesions that previously leaked and pooled fluorescein but then stopped leaking. Fluorescein angiography shows staining of the scar only; (3.) “Nonleaky” lesions: Lesions that never leaked and pooled fluorescein during angiography.

The eyes were enucleated at 2, 3, 7, or 10 wk and at 10 months after photocoagulation, opened and fixed overnight by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Each laser lesion was resected in a triangular block as previously described so that orientation of the lesion with respect to the fovea was known. Each block of tissue was post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 2 hr, dehydrated in a series of graded alcohols followed by propylene-oxide, and embedded in epoxy resin. One-micron thick sections were stained with Richardson’s stain and viewed by light microscopy for orientation; thin sections of each interesting area were stained with uranyl acetate and lead citrate and viewed with a Zeiss EM-10B Transmission Electron Microscope (Carl Zeiss, Inc.; Oberkochen, West Germany) at 60 kV. Five leaky, five nonleaky and four involuted lesions were investigated. The leaky and nonleaky lesions were from five different monkeys; the involuted lesions were from two different monkeys. Each lesion was investigated at several sectioning planes through both the periphery and the center of the lesion. On each cross section of the leaky and involuted lesions, 6 to 10 different profiles of subretinal vessels were studied. Since the nonleaky lesions contained fewer subretinal vessels, only two to five different profiles of the vessels were studied on each cross section of these lesions. Each blood vessel profile was studied in several thin cross sections.

Results

Fenestrations in the Endothelial Walls of the Subretinal Vessels

Leaky laser lesions: The eight standard laser lesions are demonstrated by fluorescein angiography in Figure 1A. Two weeks after photocoagulation, three of the eight lesions showed for the first time leaking and pooling of fluorescein in the subretinal space (Fig. 1B); according to our definition these were leaky lesions. Light microscopy revealed that at this early stage of development of the leaky lesion, established newly formed blood vessels were present already at the periphery of the scar internal to proliferating retinal pigment epi-
Fig. 2. Photomicrograph of the periphery of lesion 1 in Figure 1, examined 2 wk after photocoagulation. Established, newly formed, subretinal vessels (short arrows) are present in the subretinal space at the periphery of the scar, where Bruch's membrane (B) is intact. The vessels are lying on top of proliferating retinal pigment epithelial cells (RPE) and are separated from the degenerated and folded outer retina (OR) by a fluid-filled space (open arrows). The areas noted with asterisks contained many vascular sprouts (see Fig. 3) (×200).

Fig. 3. Electron micrograph of a capillary from the area in Figure 2 marked with arrows. Interendothelial junctions (1–5) and the diaphragmed fenestrations (short arrow and inset). Multiple layers of basal lamina (BL) surround the capillary. N = nucleus (×14,500; inset ×40,000).
Fig. 4. A newly formed subretinal vessel from the periphery of a leaky lesion. The lesion began to leak fluorescein 3 wk after photocoagulation. The eye was enucleated 7 wk after photocoagulation when the lesion was still actively leaking and pooling fluorescein in the subretinal space. Note the wide lumen (L), the thin endothelial cells (E) and the diaphragmed fenestrations (arrows). Ery = erythrocyte (×56,000).

The endothelial cells (Fig. 2). Electron microscopy of the same area showed many vessels with a slit-like lumen characteristic of the terminal vessels of the neovascular complex (Fig. 3). Fenestrations closed by a diaphragm could be observed in the more established capillaries of the neovascular complex and also in the endothelial walls of the terminal vascular sprouts (Fig. 3, inset).

At longer periods after photocoagulation the actively

Fig. 5. A newly formed subretinal vessel from an involuted lesion. The eye was enucleated 10 months after laser photocoagulation, long after fluorescein leakage stopped; staining only of the scar was seen. The lumen (L) is wide, the endothelial cells (E) are thin and contain many diaphragmed fenestrations (short arrows). Multitubular lattices (TL) are frequently found in endothelial cells of the subretinal vessels. Ery = erythrocytes (×16,500).
leaking subretinal vessels had wide lumens formed by thin endothelial cells with many diaphragmed fenestrations (Fig. 4). As the leaky lesions involuted, ie, stopped leaking fluorescein, their subretinal vessels did not degenerate and disappear. Furthermore, electron microscopy revealed that the subretinal vessels of the involuted lesions retained the ultrastructural features of the actively leaking subretinal vessels; they had wide lumens formed by thin endothelial cells that contained many diaphragmed fenestrations (Fig. 5).

"Nonleaky" lesions: The "nonleaky" laser lesions demonstrated staining only of the scar at all time points studied after laser photocoagulation (Fig. 1). The subretinal vessels of these clinically "nonleaky" lesions were similar to those of the leaky lesions. The younger "nonleaky" neovascularizations contained many relatively primitive subretinal vessels that were comprised of endothelial cells rich in cytoplasmic inclusions and closely ensheathed by many large pericytes showing similar endowment of cellular inclusions (Fig. 6). The endothelial walls of these vessels contained diaphragmed fenestrations; however, these were not present on every cross section. (This applies to vessels of both the leaky and "nonleaky" neovascularizations at all stages of development.) At longer periods after photocoagulation, the subretinal vessels closely resembled those of the actively leaking lesions and were characterized by fenestrated endothelial walls (Fig. 7).

Interendothelial Junctions of the Subretinal Vessels

Leaky laser lesions: The subretinal vessels of the leaky lesions had open interendothelial clefts with short adherens regions characterized by a finely filamentous material on the inner surface of the junctional membranes, densification of the junctional plasma membranes, and various degrees of condensation of the intercellular matrix in this region (Fig. 8). Fusion points between adjacent endothelial cells were rarely observed.

At the involuted stage of development of the neovascular membrane, the junctional complexes were highly convoluted and contained longer adherens regions (Fig. 9A). Fusion points between outer leaflets

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**Fig. 6.** Cross section of a nonfenestrated, newly formed subretinal vessel from a "nonleaky" lesion 2 wk after laser photocoagulation. On angiography the lesion showed staining of the scar only. The endothelial cells (E) of the blood vessel are closely ensheathed by a large pericyte (P), and both contain many dilated cisternae of rough endoplasmic reticulum (short arrows). The basal lamina (BL) surrounds both the endothelial cells of the capillary and the pericyte. N = nucleus; Ery = erythrocyte. Long arrow, an interendothelial junction that will be described in Figure 10 (×14,500).
of plasma membranes of opposing cells were often observed (Fig. 9A, inset); examination of the same junction on several thin sections revealed the fusion points to be focal (Fig. 9B).

"Nonleaky" laser lesions: At early stages of development of the clinically "nonleaky" neovascular membranes, most of the intercellular junctions were open with short adherent regions similar to those of the leaky lesions (Fig. 10). At longer durations after photocoagulation (7 wk), approximately half of the junctions were open, while in the others focal fusion points were seen between opposing plasma membranes (Fig. 11).

Discussion

The results of this study show clearly that in our experimental model the ultrastructure of the subretinal vessels that leak and pool fluorescein in the subretinal space is identical to that of the subretinal vessels that do not demonstrate any leakage during angiography. The newly formed subretinal vessels of the leaky lesions had fenestrated endothelial walls when they were first detected by angiography (2 wk after laser photocoagulation); this was true of both the terminal vessels of the neovascular complex and the more established capillaries. The fenestrations did not disappear with maturation of the neovascular complex, and the subretinal vessels were equally fenestrated at the leaky stage and involuted stage of development. Lesions that never demonstrated fluorescein leakage also contained fenestrated subretinal vessels at both short (2 wk) and longer (7 wk) periods after laser photocoagulation.

In a previous study we failed to identify fenestrations in the endothelial walls of the subretinal vessels at the involuted stage of development. We believe that this discrepancy can be explained in part on the basis of a sampling error, since not every cross section of the subretinal vessels of both the leaky and "nonleaky" lesions contains fenestrations. In the present study we examined each blood vessel on more than one cross...
Fig. 8. An interendothelial junction of a subretinal vessel from a leaky lesion. The eye was enucleated 7 wk after laser photocoagulation when the lesion was still actively leaking and pooling fluorescein in the subretinal space. Note the short adherens regions (arrows and inset) in which the junctional membranes show increased electron density. The cytoplasm immediately adjacent to the membranes has finely filamental material and the interendothelial matrix a tenuous condensation; the amount of both varies between the regions. E = endothelial cell; L = lumen; Ery = erythrocyte (×34,000; inset ×85,000).

section; each vessel, regardless of the type of lesion, was found to contain fenestrations.

Quantitative analysis, however, might reveal a reduction in the number of fenestrations throughout maturation and will provide the final answer to this question. If such a reduction occurs, it should be more apparent in a longer-term study. In the previous report the involuted lesions were studied approximately 100 wk after laser photocoagulation (70 wk after involution was documented by angiography), while in the present study the longest follow-up was 45 wk after photocoagulation (38 wk after documentation of involution), a difference of time which could further partially explain the discrepancy between the two studies. Such reduction in the number of fenestrations however, even if it occurs, can not explain the total absence of leakage demonstrated by both the involuted and "nonleaky" lesions.

The interendothelial junctions of the actively leaking subretinal vessels were open with short adherens regions in which the junctional membranes showed increased electron density, the cytoplasm immediately subjacent to the membranes was relatively dense, and the intercellular matrix showed tenuous condensation. These types of junctions are classified as intermediate junctions. At the involuted stage, the adherens regions were longer, and focal fusion points between opposing plasma membranes were frequently observed. This suggests progression from open to focal tight junctions throughout maturation of the neovascular membrane. Previous reports have shown that intermediate junctions are the first junctional contacts to form, both in vivo and in vitro. Our results thus further support the hypothesis that intermediate junctions are a prerequisite for tight junction formation as they initially determine the region of intercellular contact at which...
Fig. 9. Two cross sections of the same interendothelial junction of a newly formed blood vessel from an involuted lesion. The eye was enucleated 10 wk after laser photocoagulation when the lesion was no longer demonstrating fluorescein leakage. Note the long adherens regions (long arrows) of the junction. A. The endothelial cell process (*) of one endothelial cell (E1) is forming a tight junction (short arrow) with another endothelial cell (E2). N = nucleus (×32,000). Inset: higher magnification of the tight junction. The outer leaflets of the opposing plasma membrane form two fusion points (arrows) (×88,000). B. The same junction several thin sections apart. The endothelial cell process noted with asterisk (*) is now continuous to the one endothelial cell (E1) while the junctional region that was closed in Figure A is open on this cross section (short arrow). TL = multitudinal lattice. N = nucleus (×32,000).

fusion points between adjacent endothelial cells will subsequently form.13,16

Maturation of the interendothelial junctions, however, does not explain the decrease in the amount of demonstrated leakage during involution since open junctions were found in lesions that never demonstrated fluorescein leakage. These clinically “nonleaky” vessels showed the same pattern of interendothelial
Fig. 10. An interendothelial junction of a newly formed subretinal vessel from a "nonleaky" lesion 2 wk after photocoagulation (the junction noted with long arrow in Fig. 6). The interendothelial cleft (long arrows) is open. A short adherent region (short arrow) is present on the luminal side of the junction. Note the increased electron density of the junctional membranes in this region, the finely filamentous material in the cytoplasm adjacent to the membranes, and the condensation of the intercellular matrix. E = Endothelial cell; P = pericyte; Ery = erythrocyte; N = nucleus; BL = basal membrane (X72,500).

Fig. 11. An interendothelial junction of a newly formed blood vessel of a "nonleaky" lesion 7 wk after laser photocoagulation. The lesion did not demonstrate fluorescein leakage during the entire period. The interendothelial cleft (long arrow) is open except for a relatively long adherent region (open arrow and inset) on the luminal side, in which a fusion point between the outer leaflets of the opposing plasma membranes can be observed (short arrow in inset). L = lumen; E = endothelial cell; TL = multitubular lattice (X46,000; inset X100,000).
junctions as did the leaky vessels. It seems, therefore, that they follow the same pathway of maturation from open to tight.

The choriocapillaries are also known to have fenestrated endothelial walls in all species investigated.\textsuperscript{17-19} As for their intercellular junctions, experiments in monkeys and humans showed fusion points between adjacent endothelial cells on transmission electron microscopy; freeze fracture experiments revealed that these tight junctions are of the leaky type, ie, macula oculudens (Greg S. Hageman, personal communication). Permeability studies of rat choriocapillaris showed the presence of fusion points between adjacent endothelial cells that blocked the passage of hemoglobin (Einstein Stoke's radius (ESR) 32Å).\textsuperscript{20} Similarly, we previously found that mature, newly formed subretinal vessels block the passage of horseradish peroxidase (ESR 30Å).\textsuperscript{7} Thus the ultrastructural characteristics of the choriocapillaris are very similar to those of mature newly formed subretinal vessels. It seems, therefore, that once the newly formed subretinal vessels mature, they retain the characteristics of the choriocapillaris from which they are believed to proliferate.

Archer and Gardiner\textsuperscript{8} based on their studies of experimental subretinal neovascularizations suggested that the new vessels have ultrastructural characteristics similar to those of the choriocapillaris; however, they did not investigate involuted or "nonleaky" subretinal vessels. We show that all mature, newly formed subretinal vessels, whether or not they have ever demonstrated fluorescein leakage, have the morphological appearance of the normal choriocapillaris. Since normal choriocapillaries are known to be highly permeable to fluorescein,\textsuperscript{21} the newly formed subretinal vessels would also be expected to be highly permeable to this dye.

We thus conclude that absence of fluorescein leakage on angiography cannot always be correlated with absence of "leaky" morphology of the blood vessels investigated. The accumulation of fluid over some neovascular membranes and its absence over others is probably regulated by the extravascular constituents of the subretinal milieu. The mechanism of this regulation remains to be elucidated.

**Key words:** subretinal neovascularization, monkey, fluorescein leakage, fenestrations, interendothelial junctions

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