Morphologic Observations on Experimental Subretinal Neovascularization in the Monkey

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Subretinal neovascularization is a poorly understood and potentially disastrous feature of many eye diseases. We used light and electron microscopy to study the sequence of events that lead to the formation of new vessels after laser photocoagulation of the retina and choroid of primates. In this animal model there is a rapid development of new blood vessels; one day after photocoagulation, endothelial cell degeneration and thrombus formation were observed in the capillaries, venules and arterioles of the choroid around the center of the lesion. Re-endothelialization began in some thrombosed choroidal vessels, with migration of the activated endothelial cells within the old basement membrane. Two days after photocoagulation, re-endothelialization was observed in almost all thrombosed choroidal vessels, and the initial stage of the endothelial cell budding was observed in the pre-existing choroidal vessels; this was especially prominent in venules with pericytes. Three days after photocoagulation, not only the endothelial cells in pre-existing vessels but also those in re-endothelialized vessels showed budding and lumen formation. The lumen of vessels was formed by the budding of adjacent endothelial cells that were coupled by transient intercellular junctions. Mitotic figures were frequently found in the endothelial cells distal to the growing tip. Five to eight days after photocoagulation, many new vessels extended into the subretinal space.

In our study, we observed a rapid development of new blood vessels after laser photocoagulation. One day after photocoagulation, endothelial cell degeneration and thrombus formation were observed in the capillaries, venules, and arterioles of the choroid around the center of the lesion. Re-endothelialization began in some thrombosed choroidal vessels, with migration of the activated endothelial cells within the old basement membrane. Two days after photocoagulation, re-endothelialization was observed in almost all thrombosed choroidal vessels, and the initial stage of the endothelial cell budding was observed in the pre-existing choroidal vessels; this was especially prominent in venules with pericytes. Three days after photocoagulation, not only the endothelial cells in pre-existing vessels but also those in re-endothelialized vessels showed budding and lumen formation. The lumen of vessels was formed by the budding of adjacent endothelial cells that were coupled by transient intercellular junctions. Mitotic figures were frequently found in the endothelial cells distal to the growing tip. Five to eight days after photocoagulation, many new vessels extended into the subretinal space.

Vascular endothelial cells normally have a very low basal rate of replication,1 but various physiologic or pathologic states can stimulate replication of endothelial cells and result in new vessel formation.

Subretinal neovascularization (SRN) is a common pathologic feature of a number of different eye diseases.2 Hyman et al3 found that SRN is responsible for 90% of the cases of severe visual loss secondary to macular degeneration, which is the leading cause of loss of central or reading vision. In SRN, the newly formed choroidal vessels proliferate from the choroid into the subretinal space where they are diagnosed by fluorescein leakage during angiography.4,5 However, the mechanisms of new vessel formation in SRN are unknown, as are those operative in normal and other abnormal neovascularization.

We have developed a primate model in which SRN is induced by high-intensity laser photocoagulation.6,7 New vessel formation in this experimental model is similar to that which occurs in the wound healing process. Clinico-pathologic studies of these new vessels in the late stage have been reported previously.8–12 In the present study we used light and electron microscopy to study the sequence of events that lead to the formation of new vessels during healing of the lesions produced by laser photocoagulation.

Materials and Methods

Five adult cynomolgus monkeys of both sexes were used in this study; all experiments conformed to the ARVO Resolution on the Use of Animals in Research. Laser photocoagulation was performed as previously described.6,7 Eight high-intensity laser burns were applied around the macula of one eye of each monkey; lesions were produced with 700-mW power, 0.1-sec duration, and 100-μm spot size exposures. Specimens were prepared for light and electron microscopy at 1, 2, 3, 5, and 8 days after laser photocoagulation; one animal was used for each time point studied. At sacrifice, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (10 mg/kg) and the treated eye enucleated. Eyes were opened by cutting a corneoscleral window and were fixed by immersion in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr. For light microscopy, three

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Fig. 1. Laser lesion one day after photocoagulation. At the center of the lesion the choroid, Bruch's membrane and RPE have been destroyed. The coagulated outer retina evaginates into the destroyed choroidal tissue; thrombi (arrows) are present in the choroidal vessels in the area adjacent to the center of the lesion. PMNs are present throughout the lesion (Richardson's, ×340).

Fig. 2. Thrombus in choroidal venule one day after photocoagulation. The thrombus consists primarily of platelets. The cellular components of the vessel are degenerated; however, remnants of the basement membrane are still present, to which partially degraded, aggregated platelets are attached (×7,930).
Fig. 3. Partially re-endothelialized choroidal vessel 1 day after photocoagulation. The lumen of this choroidal venule is occluded by a thrombus that consists primarily of fibrin strands (F). The endothelial cells (E), which have many ribosomes and abundant rough endoplasmic reticulum, are migrating into the thrombotic area. Intercellular junctions (arrows) are present between these endothelial cells. Red blood cells (R) and leukocytes (L) are entrapped among endothelial cells. Remnants of basement membrane (BM) are also observed in the degenerated vessel wall (X15,360).

Fig. 4. Re-endothelialized choroidal vessel two days after photocoagulation. New lumen (L) covered by endothelial cells is established within the old basement membrane (BM). The endothelial cells (E) have many ribosomes and rough endoplasmic reticulum, but do not have a basement membrane of their own. Platelets (P) and cellular debris are present between the endothelial cells and the old basement membrane (X6,500).

Fig. 5. Initial stages of endothelial cell budding in a pre-existing pericytic venule 2 days after photocoagulation. (a) The cytoplasmic extension of the endothelial cell (arrow) projects into the perivascular connective tissue. Mitotic figures were never observed in a budding cell. Macrophages (M) containing phagocytic material and degenerated collagen fibers (CF) are present (X8,250).
laser lesions from each eye were dehydrated in a graded series of alcohol and embedded in glycol methacrylate; 2.5-μm serial sections through the entire block were cut and stained by periodic acid-Schiff reagent. Five laser lesions from each eye to be studied by transmission electron microscopy were washed in 0.1 M phosphate buffer, postfixed in 2% phosphate-buffered osmium tetroxide for 2 hr at room temperature, dehydrated in a series of graded alcohol followed by propylene oxide, and embedded in an epoxy resin. One-micron-thick sections were stained with Richardson's stain and viewed by light microscopy for orientation; thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with an electron microscope. Some lesions were also serially thin-sectioned.

Results

One Day After Laser Photocoagulation

The high-intensity laser photocoagulation resulted in

lium (RPE); at the center of the lesion many of the cellular elements of these structures were completely destroyed (Fig. 1). Red blood cells, proteinaceous material containing fibrin strands, and degenerated collagen fibers were present in and around the laser lesion. Polymorphonuclear leukocytes (PMNs) constituted the predominant inflammatory cell type, although some macrophages were also present. Through the break in Bruch's membrane and RPE, the outer retina evaginated into the destroyed choroidal tissue. Cells comprising the outer retina showed coagulation necrosis. Capillaries (choriocapillaris), venules and arterioles of the choroid were absent in the center of the lesion. In the area adjacent to the center of the lesion, various cellular components of chorio capillaris, venules and arterioles of the choroid had disappeared while others appeared degenerated; remnants of the basement membrane, however, were still present. These basement membrane remnants showed structural changes, including thickening and folding, and were more osmiophilic than normal.
Fig. 6. Budding endothelial cell 3 days after photocoagulation. (a) Two processes (arrows) of a budding endothelial cell from a pre-existing venule (V) extend into the extracellular space. Macrophages (M) are present around the vessel (×4,000).

composed primarily of platelets, fibrin strands and unidentifiable cellular fragments. Aggregates of platelets showing partial or complete degranulation were attached to the basement membrane remnants.

At the periphery of the areas where thrombus formation was seen, endothelial cells in pre-existing choroidal vessels contained many ribosomes and were rich in rough endoplasmic reticulum. In some locations between thrombotic and non-thrombotic areas, re-endothelialization began to occur, with migration of endothelial cells within the old basement membrane (Fig. 3).

At one day after laser injury no mitotic figures were observed in the endothelial cells of the three lesions.
that were examined by serial sections at the light microscopic level.

Two Days After Laser Photocoagulation

Re-endothelialization of most choroidal vessels was completed by 2 days after injury (Fig. 4); only a few vessels containing thrombi were still present. New lumens were established within the old vascular basement membrane, and platelets, fibrin strands, red blood cells and cellular debris were often present between the endothelial cells and the old basement membrane. The endothelial cells lining these recanalized...
Three Days After Laser Photocoagulation

At this time, endothelial cell cytoplasmic processes of various shapes and lengths were present in the pre-existing venules with pericytes (Figs. 6, 7) as well as in pre-existing and re-endothelialized vessels, and were never observed in the budding endothelial cells.

At 2 days, the number of PMNs decreased, whereas macrophages increased in number. Macrophages showing phagocytic activity were frequently observed in the area of the budding endothelial cells (Fig. 5a). Fibrin strands, red blood cells and degenerated collagen fibers were also present throughout the laser lesions.

Fig. 7. Longitudinal section of a budding endothelial cell from a pre-existing venule 3 days after photocoagulation. A long gap (G) in the intercellular junction is present between two budding endothelial cells (E1, E2). The intercellular junction (arrow) still has an adherent region at the tip of the cell process. These endothelial cells (E1, E2) have many ribosomes, microfilament bundles and Weibel-Palade bodies (arrowheads) (X12,800).
the re-endothelialized vessels (Figs. 8, 9) at the periphery of the laser lesions. These cytoplasmic processes were rich in ribosomes, rough endoplasmic reticulum, mitochondria, Golgi apparatuses, and microfilament bundles. The budding portion of endothelial cells derived from re-endothelialized vessels projected into the perivascular tissue through breaks in the old basement membrane (Figs. 8, 9). The basal portion of the budding endothelial cell had a thin, finely granular basement membrane, possibly representing newly-formed basement membrane, but no basement membrane was present around the tip of the cytoplasmic process (Fig. 6b, c). The interendothelial cell junctions between the budding cells and adjacent cells were frequently open and short, and also showed the formation of wide gaps (Fig. 6c).

Newly-formed vascular lumens were observed in the budding portion of endothelial cells of both pre-existing (Figs. 6, 7) and re-endothelialized vessels (Figs. 8, 9). The new lumens were composed of at least two budding endothelial cells that were connected by intercellular junctions. Progressive stages
of lumen formation were observed (Figs. 6–8). The initial stage consisted of budding of two adjacent endothelial cells coupled by junction (Fig. 8). The junction became longer with a number of short adherent regions in which the junctional membranes showed increased electron density, the cytoplasm immediately adjacent to the membranes was relatively dense, and the intercellular matrix showed tenuous condensation (Fig. 8, inset). In the next stage the adherent regions disappeared on the luminal side of the junction, resulting in the formation of a small gap between the two budding endothelial cells (Fig. 6d). As the disappearance of the adherent regions took place in succession, the gap, which was apparently connected to the lumen of the parent vessel, became longer and larger (Fig. 7). The endothelial cells that covered the newly formed lumen appeared metabolically active and lacked fenestrations (Figs. 6–8).

Budding pericytes were also observed in the pre-existing and re-endothelialized vessels (Fig. 9). The
pericyte cytoplasm that exhibited budding had many ribosomes and an abundant rough endoplasmic reticulum.

At this stage mitotic figures of endothelial cells were frequently found in pre-existing as well as re-endothelialized vessels (Fig. 10); the budding endothelial cells themselves, however, did not show mitotic figures. Pericytes also showed mitotic activity.

At 3 days, macrophages, which were actively phagocytic, were distributed throughout the laser lesion but were especially prominent around the budding endothelial cells (Fig. 6a); some macrophages showed mitotic figures. Only a few PMNs were observed. Fibrin strands, red blood cells and degenerated collagen fibers were still present although in lesser amounts than at 1 or 2 days after laser treatment.

Five to Eight Days After Laser Photocoagulation

At this time, the budding and mitosis of many endothelial cells and pericytes continued. Established newly-formed vessels, which were derived from choroidal vessels, extended into the subretinal space (Fig. 11). Many macrophages were evident around and beyond the neovascular sprouts (Fig. 11).

Discussion

The mechanisms involved in the development of new blood vessels have not yet been defined precisely, but it is likely that both migration and proliferation of endothelial cells, and perhaps pericytes, are operative. From studies on corneal neovascularization in the rat and rabbit, it is becoming clear that new vessel growth takes place as a series of sequential steps that are similar regardless of the type of angiogenic stimulus. The first morphologic changes are the hypertrophy or activation of vascular endothelial cells and the local degradation of the basement membrane in the parent pericorneal vessels (usually venules), from which the new capillaries will arise. Through the break in the basement membrane, activated endothelial cells initially extend pseudopods and then migrate toward the angiogenic stimulus to form a solid cord of cells that eventually canalizes. Mitosis is confined to endothelial cells distal to the leading edge of migration. Pericytes emerge along the length of the capillary sprout, followed by synthesis of new basement membrane. The processes of new vessel formation seen in the present study are quite similar to those described above, but with some new and important findings.

In our studies the newly-formed vessels were derived from two types of vessels: pre-existing vessels and re-endothelialized vessels. Neovascularization arising from pre-existing vessels has been described by a number of investigators, but however, to our knowledge there have been no reports of new vessels arising from re-endothelialized vessels. Re-endothelialization is a common phenomenon in injured vascular walls. In our study, 1 or 2 days after laser photocoagulation, re-endothelialization by migrating activated endothelial cells occurred within the old basement membrane, which formed a scaffold and pathway for the orderly reconstitution of the vessel, as previously described.

This process is not true neovascularization but, more properly, is recanalization; in fact, mitosis of endothelial cells was not observed 1 day after laser photocoagulation and was only rarely observed 2 days after photocoagulation. This suggests that migration of cells is the major event in restoring the small endothelial defects and in recanalization of pre-existing vessels, as has been reported by Sholley et al and Reidy and Schwartz.

Pre-existing vessels which showed neovascularization were mainly venules in the upper portion of the choroidal stroma. These vessels were larger than the choriocapillary vessels and lined by a single layer of endothelial cells lying on a basement membrane. They did not have smooth muscle cells or an internal elastic lamina, but rather an outer discontinuous coat of pericytes. New vessels arising from venules have been reported in the model of the corneal neovascularization.

In the formation of true neovascularization, endothelial cell budding of various shapes and lengths was seen in our primate model. The budding endothelial cells appeared metabolically very active, as suggested by the abundance of ribosomes, rough endoplasmic reticulum, mitochondria, Golgi apparatuses and microfilament bundles. As microfilament bundles are thought to be important in providing the force of contraction for cell migration, their presence in the budding cytoplasm of endothelial cells would suggest that these cells were in the process of migration. The inter-endothelial cell junctions between the budding cells and adjacent cells were frequently open and short; gap formation between cells or disappearance of junctional complexes has been shown to occur either by internalization and subsequent intercellular breakdown or by the dissolution of junctional components. Lane and Swales postulated that breakdown of the junctions permits the cell movement and rearrangement.

In some cases we have observed lumen formation...
Fig. 10. Light micrograph of endothelial cell mitosis 3 days after photocoagulation. This mitotic endothelial cell (arrow) is present in a pre-existing choroidal venucle (PAS, X1,200).

Fig. 11. Light micrograph of newly-formed vessel 8 days after photocoagulation. A newly-formed vessel (long arrow) originating from a choroidal venucle has reached the subretinal space. Many macrophages (short arrows) are present around and beyond the newly formed vessel (PAS, X280).
as early as 3 days after laser photocoagulation. A number of hypotheses have been advanced to account for lumen formation in new vessels\textsuperscript{13,17,28,29}; these have been categorized by Wagner\textsuperscript{30} as being of two basic mechanisms: an intracellular mechanism whereby vacuolization that occurs in the cytoplasm of endothelial cells leads to the lumen formation, and an intercellular mechanism whereby two adjacent endothelial cell processes delimit a narrow lumen. Using cultured capillary endothelial cells, Folkman and Haudenschild\textsuperscript{28} showed the vacuole, which seems to become a vascular lumen surrounded by an extremely thin wall of cytoplasm. In their study, basement membrane-like material was observed inside the lumen rather than on the outer surface of the endothelial cells, which suggests that the structures described were, in fact, inverted endothelial tubes. Recently, Montesano et al.\textsuperscript{29} in an in vitro study using cloned capillary endothelial cells from bovine adrenal cortex, showed that capillary-like tubes were formed by two or more cells delimiting a narrow lumen.

In our studies the newly formed vascular lumens were always composed of at least two budding endothelial cells; we found no evidence that lumen formation resulted from vacuolization within the bodies of the endothelial cells themselves.

The initial stage of lumen formation consisted of budding of adjacent endothelial cells, which exhibited a junctional complex. The intercellular junction gradually became longer and more open with short adherent regions. Although it is difficult to define the type of junctions using transmission electron microscopy, junctions between the budding endothelial cells could be classified as zonula adherens or intermediate junctions, which are the first junctional contacts to form both in vivo\textsuperscript{31} and in vitro\textsuperscript{32}; with time, the adherent region on the luminal side of the junction disappeared, as has been reported by other investigators.\textsuperscript{24-27} A small gap was then formed between two budding endothelial cells; this gap was apparently connected to the lumen of the parent vessel.

The role of pericytes in the genesis of new vessels is not clear.\textsuperscript{13,14,17} Archer and Gardiner\textsuperscript{9} speculated that pericytes play an important role in choroidal angiogenesis, as these cells were present in large numbers at and beyond the advancing tip of neovascular sprouts. These investigators could not, however, show a definite relationship between vascular sprouts and budding or mitosis of pericytes in the early stage of angiogenesis. In our study, endothelial cells as well as pericytes showed budding and mitosis; however, the initial stage of vascular sprout and lumen formation was not always accompanied by migration and proliferation of pericytes. Our findings support in vitro studies\textsuperscript{28,29} which show that all information necessary to develop a capillary tube can be expressed by a single cell type, the endothelial cell. Therefore, pericytes may not be involved in the initial vascular sprout and lumen formation.

Many substances, including tumor extracts, PMNs and lymphocyte extracts, vasoactive substances and growth factors, have been found to stimulate angiogenesis in model systems, but their relevance to spontaneous events in vivo remains obscure.\textsuperscript{33} Recently, several investigators\textsuperscript{34-38} demonstrated a role for macrophages in the induction of new vessels. Macrophages have the ability to stimulate vascular ingrowth, and in culture have been shown to secrete a potent endothelial cell growth factor, termed the "macrophage-derived growth factor". The presence of large numbers of macrophages around the tip of newly formed vessels in our study suggests that these cells may play a role in the pathogenesis of SRN, although the precise role of these cells in the neovascularization process per se remains unknown.

Our findings in this experimental model suggest that SRN is the result of endothelial cell budding and mitosis from re-endothelialized and pre-existing vessels, and that the lumen is formed by gap formation in the intercellular junction between budding endothelial cells. Additional studies are required to elucidate the precise factors that induce the migration and proliferation of vascular endothelial cells.

**Key words:** subretinal, neovascularization, monkey, endothelial cell, angiogenesis

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