The Role of Retinal Pigment Epithelium in the Involution of Subretinal Neovascularization
Hedva Miller, Benjamin Miller, and Stephen J. Ryan

The extravascular milieu around laser-induced experimental subretinal neovascularization (SRN) was studied during the evolution of the neovascular membrane from its early leaky stage to its late involuted stage. When the first signs of visible leakage appeared on angiography, newly formed vessels were spread in the subretinal space around the break in Bruch’s membrane, fluid was accumulating in the subretinal space, and retinal pigment epithelial (RPE) cells were proliferating in a papillary pattern around the newly formed vessels; the RPE proliferation began with the undamaged cells at the edges of the laser injury. With further maturation, the RPE continued to envelope the subretinal vessels. This RPE proliferation was associated with the disappearance of fluid between the enveloped vessels and the sensory retina, and the gradual cessation of fluorescein leakage during angiography. At the end of the involution process, when the neovascular membrane no longer demonstrated any leakage, the subretinal vessels were found to be tightly enveloped by RPE cells, and no fluid separated them from the sensory retina. The authors’ results suggest that involution of the neovascular membrane with maturation, as demonstrated by the cessation of visible fluorescein leakage, is the result of RPE proliferation that tightly envelopes the newly formed vessels and probably resorbs the previously accumulated subretinal fluid, as well as preventing its further accumulation in the subretinal space. Invest Ophthalmol Vis Sci 27:1644–1652, 1986

Subretinal neovascularization (SRN) is the common denominator of a number of different disease processes, and is the determinant of the disciform response. The pathogenesis of SRN and the disciform process remains poorly understood. Since disciform macular degeneration is the leading cause of central or reading vision loss in the United States, there is a need for extensive studies of this disease to understand its pathogenesis. This can be done properly only in an animal model, such as in the primate eye. In our model, choroidal SRN is induced by high-intensity laser photocoagulation. The model closely correlates with the development of subretinal vessels in man after therapeutic laser photocoagulation, and also has features in common with many diseases characterized by the disciform response. As in the clinical situation, the experimental neovascularization shows many variations and may lead to serous detachment, spontaneous hemorrhage, and ultimately cicatrization.

In both humans and our animal model, the newly formed blood vessels that proliferate from the choroidal vessels into the subretinal space demonstrate leakage and pooling of fluorescein during angiography. This leakage persists for a limited period of time, after which it diminishes gradually until it disappears completely, and staining of the scar only is seen on the angiogram. This process is defined as involution of the subretinal neovascular membrane.

In previous studies of our model, we showed that the involution process is not a result of degeneration and disappearance of the subretinal vessels, but, rather, results from disappearance of fluid pooling in the subretinal space between the vessels and the sensory retina, in which the dye previously collected.

We also showed that the disappearance of this subretinal fluid could not be attributed to changes in the ultrastructural features of the vessels with maturation, as both young and mature newly formed vessels had fenestrated endothelial walls and permeable interendothelial cell junctions. We thus concluded that non-vascular elements must regulate the presence of fluid in the subretinal space.

In the present study, changes in the subretinal milieu around experimental newly formed vessels were investigated by light and electron microscopy during the evolution of the neovascular membrane from its leaky stage to its involuted stage. Our results suggest that the RPE cells play an important role in regulating the
Fig. 1. Late phase fluorescein angiograms demonstrating the various stages of development and involution of laser-induced neovascular lesions. A, Three weeks after photocoagulation: lesions 1, 2, and 3 demonstrating for the first time leakage and pooling of dye in the subretinal space; they are defined as leaky lesions. B, Five weeks after photocoagulation: lesions 1 and 2 are in the involuting stage; they still demonstrate fluorescein leakage and pooling, albeit in a smaller amount than demonstrated 2 weeks earlier. Lesion 3 has completely involuted and shows staining of the scar only. Lesions 4 through 8 did not demonstrate any leakage at any time after photocoagulation; they are defined as non-leaky lesions.

amount of fluid overlying the newly formed subretinal vessels.

Materials and Methods

Choroidal neovascularization was induced in cynomolgus monkeys by intense laser photocoagulation. Nine monkeys were used in a manner conforming to the ARVO Resolution on the Use of Animals in Research. Eight high-intensity laser burns were applied at and around the macula of one eye of each monkey, as previously described. The lesions were monitored once a week by fluorescein angiography for up to 10 weeks; one monkey was followed for 10 months.

On the basis of the angiographic findings, the laser lesions were divided into three groups: 1) leaky lesions, those that leak fluorescein profusely and pool dye in the subretinal space; 2) involuting lesions, in which fluorescein leakage starts to diminish; and 3) involuted lesions, those that no longer leak and pool dye in the subretinal space but show staining of the scar only. Thirteen leaky, three involuting, and seven involuted lesions were studied. Of the leaky lesions, five were studied by light microscopy (LM) and eight by transmission electron microscopy (TEM); of the involuting lesions, two were studied by LM and one by TEM; of the involuted lesions, five were studied by LM and two by TEM. The eyes were enucleated at 2, 3, 7, and 10 weeks 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, so that orientation of the lesion with respect to the fovea was maintained. The lesions to be studied by LM were dehydrated and embedded in glycolmethacrylate; 3 μ serial sections were cut and stained by periodic acid-Schiff. The lesions to be studied by TEM were postfixed with 1% osmium tetroxide for 2 hr at room temperature, dehydrated in a series of graded alcohols followed by propylene oxide, and embedded in plastic. Thin sections were taken at various planes of the laser lesions (e.g., the periphery of the scar, middle of the scar), stained by uranyl acetate and lead citrate, and viewed by a Zeiss (Thornwood, NY) 10 TEM at 60 kV.

Results

Of the 72 laser lesions investigated in this study, 23 (32%) demonstrated leakage on angiography. The first signs of leakage could be detected between 2 and 3 weeks after laser photocoagulation. The duration of the leakage varied among the laser lesions. In the current study, 13 lesions were studied 2–7 weeks after photocoagulation, while still actively leaking dye; and 3 lesions were studied during the involution process, which was 4 weeks after the first signs of leakage appeared on angiography (7 weeks after laser photocoagulation). Among the seven lesions that were allowed to completely involute, the duration of leakage varied between 1 and 5 weeks.

The typical fluorescein angiographic picture of the laser-induced neovascular lesions during the various stages of their development and involution is shown in Figure 1. Three weeks after photocoagulation, three of the eight lesions that had been applied at and around the macula of the monkey eye demonstrated, for the first time, various amounts of fluorescein leakage and pooling in the subretinal space; these lesions are defined as leaky lesions. Five weeks after photocoagulation, lesions 1 and 2 were in the involuting stage; they still demonstrated leakage and pooling, albeit in a smaller
Serial sectioning of the involuting lesions showed that, at this stage of development, the RPE cells surrounded the newly formed subretinal vessels throughout the lesion. Figure 3 is a typical cross-section at the periphery of an involuting lesion. The newly formed subretinal vessels are enveloped by RPE cells, while the fluid that has been accumulating during the neovascular membrane development7 is still present between the RPE cells and the sensory retina. Electron microscopy of the same area revealed that the RPE cells have proliferated in a papillary pattern around the newly formed subretinal vessels. Thus, between Bruch’s membrane and the newly formed subretinal vessels, a double layer of RPE cells was arranged, apices toward apices, with the basal side of the second layer facing the subretinal vessels (Fig. 4), while the monolayer of RPE cells between the vessels and the sensory retina was arranged with its apical villi towards the sensory retina and its basal portion facing the subretinal vessels (Fig. 5).

The RPE cells in both layers between Bruch’s membrane and the sensory retina had a normal appearance, with their mitochondria at the basal zone of the cell, the nucleus at the intermediate zone, and the microvilli on their apical surfaces; however, the cells comprising the second layer contained fewer pigmented granules.

Serial sectioning of the leaky lesions revealed that, when the first signs of leakage appeared on angiography, the newly formed fibrovascular tissue had already proliferated into the subretinal space around the center of the scar (defined by the break in Bruch’s membrane), and fluid, assumed to come from the newly formed subretinal vessels, had accumulated in the subretinal space (Fig. 2). At this early stage of the neovascular membrane development, proliferating RPE cells were also found at the periphery of the scar around the break in Bruch’s membrane. Serial sectioning of the early leaky lesions showed that, on most of the cross-sections, the proliferating RPE cells were found only between Bruch’s membrane and the newly formed subretinal vessels (Fig. 2A); however, on a few cross-sections, a monolayer of RPE cells was present also in some areas between the newly formed subretinal vessels and the sensory retina (Fig. 2B).

With further maturation, the neovascular lesions gradually demonstrated smaller amounts of fluorescein leakage and pooling during angiography. At this stage of development, they were regarded as involuting lesions (Fig. 1).
Fig. 3. A light micrograph of the periphery of an involuting lesion. The RPE cells (curved arrows) are now enveloping the subretinal vessels (straight arrows). Note the fluid (open arrows) that is separating the enveloped subretinal vessels from the sensory retina (R). b = Bruch's membrane. (X970)

Fig. 4. Electron micrograph of the cross-section in Figure 3; the area between Bruch's membrane and the subretinal vessels. A continuous double layer of RPE cells is separating Bruch's membrane (b) from the subretinal vessels (V). The RPE cells are arranged in a papillary pattern, apices toward apices, with the basal side of the second layer facing the subretinal vessels; the cells in both layers have a normal appearance with their mitochondria (m) at the basal zone, the nucleus (N) at the intermediate zone, and the microvilli (MV) on their apical surfaces. Note the fewer pigmented granules (P) in the second layer. (X2,500)
Fig. 5. Electron micrograph of the cross-section in Figure 3; the area between the subretinal vessels and the sensory retina. A monolayer of RPE cells separates the subretinal vessels (V) from the overlying fluid (F). In this area the monolayer is continuous, with junctional complexes (arrows) between adjacent cells. The RPE cells have normal zonal organization of their cytoplasmic organelles; however, their lateral surfaces contain many microvilli (long arrow). m = mitochondria; N = nucleus. (×4,200)

than did the RPE cells adjacent to Bruch's membrane (Fig. 4).

While the double layer of RPE between Bruch's membrane and the subretinal vessels was continuous (Fig. 4), the RPE layer between the subretinal vessels and the sensory retina was not continuous during the involuting stage. In some areas, the cells were joined by normal junctional complexes and appeared to have zonal organization of their cytoplasmic organelles, although their lateral surfaces contained many microvilli (which is not the normal pattern) (Fig. 5). In other areas, the cells did not have apical villi or a zonal organization of their cytoplasmic organelles, and appeared to form plaques, between which were fluid-filled spaces (Fig. 6).

At the end of the involution process, the lesions stopped demonstrating fluorescein leakage and showed staining of the scar only (Fig. 1); at this stage of development, they were regarded as involuted lesions. Serial sections of lesions at the involuted stage revealed a tube of RPE cells completely ensheathing the newly formed subretinal vessels (Fig. 7). These vessels were connected to the choroidal vasculature through the center of the scar (where the break in Bruch's membrane occurred) (Fig. 7B), and were spread in the subretinal space around the break in Bruch's membrane. The RPE cells were arranged around the vessels in a papillary pattern (Fig. 7A). The RPE envelope seemed to end at the edges of the break in Bruch's membrane; the center of the lesion consisted of a chorioretinal scar (Fig. 7B). No fluid was present between the enveloped vessels and the sensory retina. At this stage of development, all RPE cells surrounding the subretinal vessels had a normal appearance with zonal organization of their cytoplasmic organelles; however, the cells comprising the monolayer between the vessels and the retina still contained increased numbers of lateral microvilli (Fig. 8). This monolayer was now continuous throughout the lesion, with tight junctions between adjacent RPE cells (Fig. 9).

Discussion

The results of this study show that laser-induced choroidal vessel proliferation into the subretinal space is accompanied by RPE proliferation around the newly formed subretinal vessels that eventually totally en-
**Fig. 6.** Electron micrograph of the cross-section in Figure 3; the area between the subretinal vessels and the sensory retina. The RPE cells that separate the subretinal vessels (V) from the overlying fluid (F) are not continuous in this area, and appear to form plaques, between which are fluid-filled spaces (arrow). Note the absence of zonal organization of the cytoplasmic organelles of the RPE cells. m = mitochondria. (X4250)

**Fig. 7.** Two representative serial sections of an involuted lesion that had leaked for 4 weeks. The eye was enucleated 8 months after involution was completed. A, Light micrograph of the periphery of the lesion. The RPE cells (white arrows) have formed a tight envelope around the subretinal vessel (arrowheads). Note the papillary arrangement of RPE cells and the absence of subretinal fluid. R = retina. B, One hundred and forty microns further; center of the scar. The RPE envelope (long arrows) on each side seems to end at the edge of the break in Bruch's membrane (short arrows). The subretinal vessels (arrowheads) are connected to the choroidal vasculature through the center of the lesion where a chorioretinal scar (S) is formed.
velopes the vessels. As the RPE proliferation progresses, the subretinal fluid diminishes, and, at the end of the involution process, no fluid separates the vessels from the sensory retina.

Previous studies have shown that laser photocoagulation triggers RPE proliferation. The high intensity laser burns that we applied also involved the choroid. As a result of this, fluid that escaped from damaged choroidal vessels could cause retinal detachment, which has also been found to trigger the proliferation of RPE cells. The exact cause of RPE and choroidal vessel proliferation in our model may be a combination of these factors.

Figure 10 demonstrates schematically the progression of RPE proliferation around the newly formed subretinal vessels with maturation of the neovascular membrane. When the first signs of leakage appear on angiography (between 2 and 3 weeks after laser photocoagulation), both choroidal vessel proliferation and RPE proliferation are underway. The newly formed blood vessels have already invaded the subretinal space, and, since they have fenestrated endothelial walls and open interendothelial junctions, serum leaks from them and starts to accumulate in the subretinal space. At the same time, RPE cells are proliferating around the newly formed fibrovascular membrane. It appears that the proliferation starts at the periphery of the injury, probably with the RPE cells that have not been severely damaged, and proceeds in a papillary pattern around the neovascular membrane (Fig. 10). This papillary arrangement of RPE cells has been described in many other pathological conditions in which RPE is triggered to proliferate.

With further maturation of the neovascular membrane, the RPE cells continue to envelop the newly formed subretinal vessels. From our previous studies,
we know that the subretinal vessels have fenestrated endothelial walls and permeable interendothelial junctions throughout their development; thus, as long as the RPE does not form a tight barrier around the neovascular membrane, fluid can leak from the vessels and accumulate in the subretinal space (Fig. 10).

At the end of the involution process, the RPE forms a continuous tube of cells joined by tight junctions around the subretinal vessels, which are connected to the choroidal vasculature through the center of the scar where the break in Bruch's membrane occurred. At this stage of development, no fluid is present between the subretinal vessels and the retina (Fig. 10).

As RPE cells are known to actively transport fluid from the subretinal space into the choroid, the cells that envelop the subretinal vessels are probably responsible for the absorption of the accumulated subretinal fluid during involution. This would explain the abnormally increased number of microvilli on the lateral sides of the proliferated RPE cells. Furthermore, Glaser and colleagues have recently shown that RPE cells cause regression of new blood vessels in vivo and inhibit proliferation of fetal bovine aortic endothelial cells. Thus, the proliferation of RPE cells around the subretinal vessels probably causes not only the absorption of the subretinal fluid, but also may inhibit further proliferation of subretinal vessels by releasing substances that inhibit endothelial cell proliferation.

It seems, therefore, that, in our experimental model, and perhaps also in human cases of SRN with leakage of fluid into the subretinal space, there is a normal healing process that involves RPE proliferation around the newly formed subretinal vessels, with subsequent total envelopment of the vessels. The newly formed RPE barrier may be responsible not only for pumping out the previously accumulated subretinal fluid, but also for blocking further passage of fluid from the vessels into the subretinal space. Thus, once a tight barrier of RPE cells is formed around them, the potential of the subretinal vessels to leak fluorescein cannot be demonstrated, as there is no space in which the dye can accumulate and, therein, be visible on angiography. This could partially explain the differences in the involution periods between the various laser lesions, as the envelopment of the subretinal vessels by the RPE cells occurs more rapidly in some lesions than in others.
Fig. 10. Schematic drawings presenting the progression of RPE proliferation around the newly formed subretinal vessels with maturation of the neovascular membrane. A, Early leakage stage. Newly formed subretinal vessels (V) have proliferated into the subretinal space. Fluid (F) leaks from the vessels and accumulates in the subretinal space. Retinal pigment epithelial (RPE) cells are proliferating in a papillary pattern around the newly formed subretinal vessels. The proliferation begins at the edges of the scar with the undamaged cells (heavy line). The finer line represents the newly formed RPE cells. B, Involuting stage. The retinal pigment epithelial (RPE) cells partially envelope the subretinal vessels (V). Less fluid is seen between the enveloped vessels and the sensory retina. C, Involute stage. The retinal pigment epithelial (RPE) cells have formed a tight envelope around the subretinal vessels (V). No fluid is present in the subretinal space. b = Bruch’s membrane; s = chorioretinal scar.

Key words: laser-induced subretinal neovascularization, monkey, fluorescein leakage, retinal pigment epithelium

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