Breakdown in the blood-retinal barrier occurs in retinal neovascularization in a number of diseases. To study the anatomic basis of this breakdown, we examined retinal neovascularization induced by injection of 250,000 homologous fibroblasts into the vitreous cavity of pigmented rabbits. Neovascularization is evident by electron microscopy in this model 3 days after fibroblast injection. Fluorescein angiography followed by intravenous horseradish peroxidase (HRP) injection was performed prior to enucleation on 2, 3, 5, 7, and 14 days after fibroblast injection. Fluorescein leakage from retinal vessels occurs early (at day 1) and persists as the neovascularization progresses. The leakage in the early stages is concentrated near puckers from the medullary wings. In the later stages, fluorescein leakage is most prominent in the developing tips of the new vessels. Horseradish peroxidase was not observed to leak from the lumen of new vessels. "Gaps" or separations in the endothelial cell junctions were not observed in developing vessels. The breakdown of the blood-retinal barrier in this model of retinal neovascularization is therefore selective, (ie, fluorescein leaks but not HRP) and it is not due to gaps or fenestrations between endothelial cells in developing vessels. Invest Ophthalmol Vis Sci 28:1108-1115, 1987

Breakdown in the inner blood-retinal barrier occurs in retinal neovascularization in a number of diseases. In diabetes mellitus, this breakdown precedes the development of retinal neovascularization. Electron microscopic studies of membranes removed during vitreous surgery for proliferative diabetic retinopathy have indicated fenestrations (endothelial cell membrane specialization) and gaps between endothelial cells as the basis of the breakdown in the blood-retinal barrier evident by fluorescein angiography. However, recent studies demonstrated that fenestrations are not sufficiently numerous to explain the degree of breakdown. To better understand the anatomic basis for fluorescein leakage, we investigated retinal and optic disc neovascularization induced by the injection of fibroblasts into the vitreous cavity of rabbits.

Materials and Methods

Pigmented rabbits of both sexes weighing 2.5-3.5 kilograms were used in the experiments. The animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research. The eyes were examined by slit-lamp biomicroscopy and fundus ophthalmoscopy, and those that were judged to be normal were selected for experimental use. Four eyes received injection of phosphate buffered saline (PBS) as controls. Fourteen eyes were injected with 250,000 homologous fibroblasts (in 0.1 ml of PBS) into their vitreous cavity. The injection was made with a 27-gauge needle 4 mm behind the limbus, under stereomicroscopic control. The cells were placed near the vascularized medullary wings which extend temporally and nasally from the optic disc, and the cells were placed near the vascularized medullary wings which extend temporally and nasally from the optic disc as well as over the optic disc itself. After injection of the cells, the rabbits were placed on their backs for 30 min to allow the cells to settle on the posterior retina. The eyes were examined on days 1, 2, 3, 5, 7, 14, and 28 after injection. Fundus photography was performed on these days using a Zeiss fundus camera with a 30° field (Carl Zeiss, West Germany). Fluorescein angiography was performed on selected eyes of anesthetized animals (ketamine and Rompun [Xylazine], Mobay Corporation, Shawnee, KS) between days 1 and 28 by injecting the marginal ear vein with 0.5 cc of a 10% solution of Na fluorescein. Preinjection photography was performed to determine the degree of auto- and pseudofluorescence.

Enucleations were performed on days 2, 3, 5, 7, and 14 after fibroblast injection. On the day of enucleation, each animal had a fluorescein angiogram performed to demonstrate the degree of fluorescein leakage from affected areas of the fundus, with em...
phasis on the macular puckers. Following the fluorescein angiogram, horseradish peroxidase (HRP, type II, Sigma Chemical Company, St. Louis, MO) at 0.25 g HRP/kg body weight was dissolved in 2 ml of PBS adjusted to pH 7.3 and injected into the marginal ear vein using a Harvard pump (Harvard Apparatus, Millis, MA) at 0.68 cc/min and allowed to circulate for 30 min prior to enucleation. The enucleated eyes were placed in 4% paraformaldehyde and 5% glutaraldehyde in 0.05 M phosphate buffer. A 4-mm slit was made in the inferior portion of each eye to allow better access for the fixative. After 5 hr, the eyes were placed in 0.05 M phosphate buffer for two 10 min rinses, and placed overnight in fresh 0.05 M phosphate buffer at 26°C.

Eyes were dissected and placed into 0.05 M phosphate buffer until placed in 3,3′ diaminobenzidine-Tris Buffer (DMB) (Sigma Chemical Company). While under low light conditions, tissue sections were placed in small petri dishes and covered with DMB. These dishes were placed in total darkness and incubated with DMB for 1 hr. Tissue was then incubated in fresh DMB containing 0.005% H2O2 in darkness for 1 hr. Tissues were washed three times with distilled water. After washing, they were placed in 1.3% osmium tetroxide, 1.5% ferrocyanide, 5% sucrose in 0.05 M phosphate buffer and incubated for 90 min. The tissue was washed three times for 10 min each in 0.05 M phosphate buffer containing 5% sucrose.

After the third wash, the tissues were dehydrated in graded concentrations of ethanol and embedded in a low-viscosity epoxy resin. The blocks were trimmed and sectioned for light (1 µm) and electron microscopy (60-90 nm), using an LKB ultramicrotome (LKB Instruments Inc., Rockville, MD). Thin sections were stained with lead citrate and uranyl acetate and examined with a JEOL 100 transmission electron microscope (JEOL Inc., Peabody, MA).

**Results**

**Fluorescein Leakage**

In the control eyes (sham injection of PBS), no fluorescein was seen to leak from the retinal vasculature (Fig. 1). Additionally, the media remained clear and no inflammation was observed during any stage of the experiment. In experimental eyes, fluorescein leakage was prominent one day after fibroblast injections, and was concentrated near areas of retinal folding due to the presence of the overlying clumps of fibroblasts (pucker). Sometimes the media were hazy. The underlying retinal vessels were dilated (Fig. 2a). Moderate fluorescein leakage occurred in the underlying retinal vasculature and vessels immediately adjacent to the injected fibroblasts (Figs. 2b and 2c), but was not apparent in vessels distant from the fibroblast-induced puckers.

Fluorescein leakage could be documented throughout the 28-day observation period, during which time new vessels continued to develop. Dilatation of vessels continued to be prominent in the areas of retinal puckering. In the later phases of the pathology in fibroblast-injected eyes, retinal detachment began to develop, but fluorescein leakage and clinically apparent neovascularization also developed in the absence of retinal detachment. The developing vasculature could first be distinguished clinically at approximately 7 days by the appearance of a reddish blush surrounding the fibrous strand over the medul-
Fig. 2. (a) Fundus photograph one day after fibroblast injection showing fibroblasts causing pucker and adjacent dilated vasculature.

lary wings (Figs. 3a and 3b), and in the later phases (day 28) by definite loops of developing vessels.

Electron Microscopy and HRP Injection

Electron microscopy of stained and unstained sections of eyes removed during days 2, 3, and 5 showed similar results. In these eyes, neovascular vessels could be identified by examining serial step sections to demonstrate vessel budding changes. New blood vessels had thickened walls (Fig. 4), and multiple finger-like extensions of the endothelial cells into the surrounding extracellular matrix (Fig. 5). The basal lamina was fragmented with multiple layers of basement membrane. In the earliest capillary buds, the lumens were quite narrow and devoid of blood cells (Fig. 6).

HRP was present in the lumens of the new blood vessels (Figs. 5–9). No gaps or fenestrations were evident, but HRP was present in vesicles within the endothelial cells (Figs. 6–9). No extravasated HRP was observed by light microscopy of thick sections or by electron microscopy (Figs. 5–9).

Unstained thin sections (no lead citrate or uranyl acetate) allowed clear delineation of the location of HRP within the vessel lumens. Capillary buds were also evident, characterized by narrow lumens that contained no blood cells. Even in the most immature developing buds, HRP did not leak (Fig. 7). Close examination of the vessels containing HRP demonstrated well-formed intercellular junctions. In a few, the junctions showed cystic dilatation between points of attachment, indicating abnormality but not total separation (Fig. 8).

Stained thin sections of developing cells showed well-developed intercellular junctions between endothelial cells (Fig. 9). No cellular separations, fenestrations, or leakage of HRP were found at any of the times examined.

Discussion

Breakdown of the inner blood-retinal barrier occurs in many diseases associated with retinal neovascularization, and is most prominent clinically during fluorescein angiography of elevated extraretin-
Fig. 3. (a) (left) Fundus photograph; and (b) (right) Midphase angiogram demonstrating capillary blush apparent 7 days after injection of fibroblasts.

Fig. 4. Electron micrograph showing thick-walled new vessels (arrows) and early endothelial cell bud (asterisk) on day 3 after fibroblast injection (bar = 10 μm).

Retinal neovascularization, which results from such diverse causes as diabetes mellitus, branch vein occlusion, sickle retinopathy, and retinopathy of prematurity. Histopathologic examination of such vessels has demonstrated fenestrations; these have been postulated to explain, in part, the leakage of fluores-
Fig. 5. Electron micrograph showing finger-like projection of endothelial cell (arrow) into surrounding extracellular matrix. Note in area basal lamina is fragmented, but with an increased amount of multilayered basement membrane-like material (asterisk) (bar = 5 μm).

Fig. 6. Electron micrograph of unstained tissue containing immature developing vessel (day 3) with extremely narrow lumen devoid of cellular components. Lumen stains darkly because of intravascular HRP reaction product. Note HRP does not leak out of lumen (bar μm).
Fig. 7. Unstained section showing developing capillary bud at edge of venule. No leakage of HRP despite moderate vesicular uptake (bar = 1 μm).

Fig. 8. Higher magnification of electron micrograph of unstained tissue complex showing dilatation of space between points of cell-cell attachment (arrows). No leakage of HRP evident (bar = 1 μm).
In experimental models of neovascularization in the cornea and other tissues, gaps have been demonstrated in the developing tips of new vessels. However, since retinal vessels normally do not leak fluorescein, in contrast with such vascular systems as conjunctival vessels and the choriocapillaris, we hypothesized that the developing aspects of retinal neovascularization might differ from those of other vascular systems that are normally leaky.

Indeed, in our study we demonstrated important differences in the development of retinal neovascularization compared to corneal neovascularization in the rabbit, including absence of fenestrations and gaps in the developing capillary buds. However, it is apparent that fluorescein leakage is still a prominent feature of the experimentally-elicited retinal neovascularization, with early and prominent leakage of fluorescein in the areas of retinal puckering. This leakage is not found distant from the puckering sites, indicating a localized as opposed to a diffuse reaction to the pucker and fibroblasts. The fluorescein leakage continues throughout the observation period of the induced neovascularization and appears to be most prominent at developing tips of new vessels.

Electron microscopy using the tracer protein HRP demonstrated several interesting aspects of the developing vasculature. First, no obvious physical gaps were seen between cells, from the stages of earliest budding to well-developed and elevated neovascularization. Additionally, even though prominent leakage occurs after 1 day, no neovascular vessels could be identified at this stage. In this model, fluorescein leakage precedes the development of neovascularization, which was evident on day 2 after fibroblast injection. Electron microscopy revealed no leakage of HRP either after endothelial vesicular uptake or due to gaps or fenestrations in the developing vessels. Higher magnification showed well-formed intercellular junctions; however, in several areas there was ballooning of intracellular spaces between junctions, but these did not leak HRP.

Although fenestrations are typically present in vessels which normally leak fluorescein profusely, such as the choriocapillaris, we believe that fenestrations indicate a relatively mature and differentiated expression of vessel wall function. There is now evidence that fenestrations in the choriocapillaris are induced by proximity to retinal pigment epithe-
This indicates that fenestrations represent a differentiated feature of endothelial cells as opposed to an immature stage of developing vasculature. This is consistent with other studies demonstrating that fenestrations were not sufficient to explain leakage of fluorescein dye from human diabetic developing vessels.

The breakdown of the blood-retinal barrier in our model of neovascularization appears to be selective, that is, fluorescein does leak from the vessels but the leakage is not due to gaps or fenestrations in the developing vessel. The barrier does not leak HRP and thus is not open to all proteinaceous contents of the vascular lumen. This selectivity is probably based on charge and size limitations. HRP has an Einstein-Stokes radius of approximately 30 Å and is positively charged. Fluorescein bound to albumin is similar in dimension (36 Å) but is negatively charged. Free fluorescein is about 5 Å and is more lipid-soluble. Thus, free fluorescein could leak from the developing new vessels, whereas the larger and more positively-charged HRP does not. Further studies are indicated to define more specific aspects of the inner retinal barrier characteristics in developing vessels, using tracers of different sizes and charges.

Key words: blood-retinal barrier, retinal neovascularization, horseradish peroxidase, electron microscopy, rabbit

References