Ocular vascular and epithelial barriers to microperoxidase. Richard S. Smith and Linda A. Rudt.

Microperoxidase (MP) is an ultrastructural tracer of small molecular weight (1,900) derived from horse heart cytochrome c. Within the central nervous system, it is capable of entering the periaxonal space which is not open to horseradish peroxidase (HRP). Because of its small size and unique behavior, MP was used to probe ocular vascular and epithelial barriers. MP did not enter any ocular space from which HRP was excluded. This tracer establishes a new lower level of permeability in these ocular barrier systems.

Horseradish peroxidase (HRP) has been used in the eye and other structures as an ultrastructural tracer. While the molecular weight of HRP (40,000) is less than most serum proteins, the need for a tracer of smaller molecular weight led Feder to develop microperoxidase (MP). MP is a digest of horse heart cytochrome c with a molecular weight of 1,900. It retains peroxidase activity and is demonstrated by the method used for HRP. MP was used to determine the permeability of the blood-aqueous, blood-retinal, and choroidal barriers. The presence or absence of MP transport was studied in an effort to establish a new lower level of permeability for these ocular barrier systems.

Methods. Four vervet monkeys (Cercopithecus aethiops) were anesthetized with sodium pentobarbital and received an intravenous injection of 250 mg of MP (prepared by Miles-Seravac). Eyes were enucleated at ten, twenty, and thirty minutes after tracer injection. The eyes were fixed as previously described. Tissue blocks from iris, ciliary body, choroid, and retina were thoroughly rinsed in 0.2 M phosphate buffer and incubated in a solution of diaminobenzidine and hydrogen peroxide for three hours followed by postfixation in osmium. Control tissues incubated in a similar fashion showed no reaction product. Lead-stained thin sections were examined in a Siemens 1A electron microscope.

Results. Iris. At all times following injection of MP, the iris capillaries were filled with tracer. The zonulae occludentes which seal adjoining endothelial cells were impermeable to MP. An occasional pinocytotic vesicle was filled with MP, but did not open on the basement membrane of the endothelial cell (Fig. 1). No tracer was seen in pericytes or in iris stroma.

Ciliary body. The fenestrated ciliary capillaries were filled with MP and the endothelial basement membrane was heavily stained. Reaction product filled the extracellular space between pigmented epithelial cells and the apical extracellular space (AES) between the two layers of ciliary epithelium (Fig. 2). High magnification (Fig. 2, inset) showed reaction product in the gap junctions of the AES. As with HRP, the MP was stopped by the zonula occludent at the apex of the non-pigmented epithelial cells (Fig. 2, inset). Vesicles containing reaction product were seen in the non-pigmented epithelial cell adjacent to the basement membrane (Fig. 3).

Choroid-retinal pigment epithelium. The fenestrated choriocapillaris was outlined by MP (Fig. 4) which also stained Bruch's membrane. Very little MP was detected beyond Bruch's membrane. MP was found in the extracellular space of retinal pigment epithelium. Zonulae occludentes at the apex of the pigment epithelium were free of reaction product. There was no uptake in phagosomes or pinocytotic vesicles. No MP deposits were seen in the outer retina. This picture remained constant for thirty minutes after injection.

Retina. MP was confined to the lumen of the retinal capillaries by zonula occludentes between adjacent endothelial cells (Fig. 4, inset). Pinocytotic activity was absent. The endothelial cell basement membrane was free of reaction product. No MP was detected in the perivascular retina.

Discussion. Vascular and epithelial barriers throughout the body show different degrees of permeability. Peroxidase tracers of varying size are useful differential probes of these barriers. Recognizing the need for smaller tracers, Karnovsky utilized cytochrome c. MP was stopped by the zonula occludent at the apex of the non-pigmented epithelial cells (Fig. 2, inset). Vesicles containing reaction product were seen in the non-pigmented epithelial cell adjacent to the basement membrane (Fig. 3).

Choroid-retinal pigment epithelium. The fenestrated choriocapillaris was outlined by MP (Fig. 4) which also stained Bruch's membrane. Very little MP was detected beyond Bruch's membrane. MP was found in the extracellular space of retinal pigment epithelium. Zonulae occludentes at the apex of the pigment epithelium were free of reaction product. There was no uptake in phagosomes or pinocytotic vesicles. No MP deposits were seen in the outer retina. This picture remained constant for thirty minutes after injection.

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Fig. 1. Granular MP reaction product fills the lumen (L) of an iris capillary. A few pinocytic vesicles containing MP are present (arrows). The endothelial basement membrane (B) and the nearby iris stroma show no evidence of the tracer (x16,100).

Fig. 2. The lateral and apical extracellular spaces (arrows) are filled with reaction product. Gap junctions (arrowheads) do not show MP at this magnification (x12,000). Inset A shows the barrier to free diffusion of MP produced by the zonula occludens (curved arrow) at the apex of the nonpigmented epithelial cell (x48,000). Inset B shows deposits of reaction product penetrating the narrow extracellular space of two gap junctions (C) (x60,000).
Fig. 3. Two pinocytic vesicles (arrows) lie close to the posterior chamber (PC) surface of a nonpigmented epithelial cell (NPE). Fragments of zonular material (Z) are present (x20,000).

that these zonulae occludentes are continuous.\textsuperscript{5, 8} The distribution of MP and HRP is identical in the iris capillaries.\textsuperscript{5} Pinocytic activity was insignificant. Reaction product was not seen in the iris stroma.

In primates, HRP\textsuperscript{1} and MP rapidly escape from the fenestrated ciliary capillaries and stain the endothelial basement membrane. Both tracers enter the extracellular space between adjacent pigmented epithelial cells and penetrate the AES. As with HRP,\textsuperscript{1, 8} MP is stopped by zonulae occludentes at the apex of adjacent nonpigmented epithelial cells. Pinocytic transport of MP to the posterior chamber resembles that seen with HRP.\textsuperscript{1, 8} Since both MP and HRP act as enzymes,\textsuperscript{2, 7} a single molecule of tracer may produce multiple molecules of reaction product. Thus, we are dealing only with qualitative tracer movement and the absolute quantity of protein transported is unknown.

Retinal capillaries\textsuperscript{1} are sealed by zonulae occludentes and are impermeable to HRP. During the thirty minute duration of our experiments, MP did not penetrate this tight junction. No tracer was seen in retinal tissue adjacent to the capillary.

Most ultrastructural tracers readily escape from the lumen of fenestrated capillaries.\textsuperscript{5, 6} MP filled the fenestrated choriocapillaris and produced heavy staining of Bruchs' membrane. The bulk of the tracer did not move beyond this point. A small amount could be identified in the basal portion of the extracellular space between adjacent retinal pigment epithelial cells. Tracer was not unequivocally identified in the apical portion of this space and did not penetrate the tight junction at the apex of the cell. MP was not found in the subretinal space around the photoreceptor outer segments or in the outer retina. The apparent lack of filling of the lateral extracellular space between retinal pigment epithelial cells contrasts with the pattern observed by Peymen and Bok\textsuperscript{1} for HRP. Feder\textsuperscript{7} suggests that MP may form aggregates, increasing its effective size. A portion may also bind to a component of Bruchs' membrane accounting for its apparent inability to penetrate the lateral extracellular space as compared to HRP.

Although MP can enter spaces from which HRP is excluded\textsuperscript{1} in the brain, the tight junctions of the ocular barrier systems exclude MP from adjacent tissue compartments. The molecular weight (1,900) of MP thus establishes a new lower limit of permeability for these barriers, based on molecular weight. This limit may be of interest in evaluating the possibilities of entry into the eye of substances of similar size. No conclusions can be drawn from these experiments regarding the ability of smaller molecules of different physical and biochemical properties to enter the aqueous humor or retina. The possible opening of these barriers under altered physiologic conditions or in disease remains to be studied.
Fig. 4. Granular reaction product fills a choroidal capillary (CC) and stains Bruchs' membrane (B). A small amount of MP is seen in the basal invaginations (arrows) of a retinal pigment epithelial cell (RPE). The tracer is not identified in the lateral extracellular space (curved arrow) or zonula occludens (ZO) (×16,000). Inset shows granular reaction product in the lumen (L) of a retinal capillary. No MP is seen outside the vessel (×18,000).

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Basement membrane thickness was measured in two groups of spiny mice (Acomys cahirinus): one with spontaneous diabetes, the other with streptozotocin-induced diabetes. A statistical analysis of the morphometric results of the two groups showed a significant basement membrane thickening in the group with induced diabetes when compared to the spontaneously diabetic group.

Capillary basement membrane thickening is a frequent observation in diabetic microangiopathy. However, the question whether basement membrane thickening has to be considered as a direct manifestation of the hereditary diabetic trait, or if it results from long-standing hyperglycemia and subsequent metabolic disorders has yet to be resolved.

The study of microvasculature in human diabetic retinopathy is rendered difficult by the fact that material for ultrastructural studies can only be obtained after enucleation (mostly for r tumors) and not from biopsy. The material obtained at autopsy does not give, in general, satisfactory ultrastructural preservation due to the long delay between death and fixation of the tissue. Therefore, it appears necessary to study possible microvascular changes in diabetic animals. Retinal lesions comparable to some extent to the ones observed in human diabetes have been reported in streptozotocin-diabetic rats.

In this study, retinal capillary basement membrane thickness of a group of spontaneously diabetic Acomys was compared with a group of streptozotocin-induced diabetic animals. The results seem to indicate that experimentally induced hyperglycemia may induce or enhance capillary basement membrane thickening in the retina.

Material and methods. Twelve spiny mice (A. cahirinus) were used in this study and raised under normal laboratory conditions. A group of normoglycemic animals had intraperitoneal injections of 100 mg per 100 grams of body weight of streptozotocin in order to induce, experimentally, a diabetic state (= induced group). Two months after the injection, they were weighed and then killed by injections of nembutal. Of these animals, two were 20 months old, three were 24 months old, and two were nine months old, the average age being 19 months. Another group consisted of five spontaneously diabetic animals (= spontaneous group). At the time of death, three animals were 19 months old and two were 21 months old, the average age of this group was 20 months. No hypoglycemic therapy was administered to either group.

The metabolic state of the animals was checked periodically with tests for urine glucose. In the spontaneous group, urine sugar was over 6 Gm. per liter in three animals and between 1 and 4 Gm. per liter in two animals, none had ketonuria; glycemia at the time of death was about 320 mg. per cent (range 130 to 529 mg. per cent). Immunoreactive insulin (IRI) was 22 mU. per milligram (range 15 to 28 mU. per milligram) in the pancreas and 134 μU per milliliter (range 48 to 296 μU per milliliter) in serum. In the induced group, urine sugar was over 6 Gm. per liter in six animals and between 1 and 4 Gm. in one animal, five animals had ketonuria; glycemia was about 440 mg. per cent (range 292 to 627 mg. per cent). IRI was 5.7 mU. per milligram (range 2.8 to 10.5 mU. per milligram) in the pancreas, and 76 μU per milliliter (range 14 to 237 μU per milliliter) in serum.

The eyes were enucleated, opened at the equator, fixed in 4 per cent phosphate-buffered glutaraldehyde, and postfixed in 1 per cent osmium tetroxide. They were then dehydrated in a graded series of alcohol and embedded in Epon 812. Semithin and thin sections were cut on a Porter-Blum MT-2 ultramicrotome. Thin sections were stained with lead citrate and uranyl acetate and observed in a Philips 300 electron microscope.