A hallmark of early diabetic retinopathy is the selective loss of the retinal mural cells (pericytes) from vessels. Using antibodies prepared against purified human placental aldose reductase, the presence of the enzyme aldose reductase can be demonstrated immunohistochemically in the cytoplasm of retinal mural cells of trypsin-digested human retinal vessels. This enzyme, which reduces various hexose sugars to their sugar alcohols, has been implicated in the pathogenesis of several diabetic complications. Invest Ophthalmol Vis Sci 24:1516-1519, 1983

Diabetic retinopathy, one of the leading causes of blindness in the United States, generally is divided into non-proliferative and proliferative forms. Non-proliferative retinopathy is characterized by vascular changes in the retinal capillary bed with formation of microaneurysms, exudates, and intraretinal hemorrhages, while the formation of new vessels and fibrous tissues is seen in proliferative retinopathy. The vascular changes appear to begin with the capillaries which, as seen in Figure 1A, are made up of two types of cells—endothelial cells and mural cells. One of the hallmarks of early diabetic retinopathy is the selective loss of the retinal mural cells, leaving only portions of basement membrane (mural cell ghosts, Fig. 1B) which precedes the appearance of microaneurysms. 1 This selective loss strongly contrasts to the relative persistence of the endothelial cells in diabetic retinas.

While the pathogenesis of diabetic retinopathy remains unknown, we have been exploring the possibility that adverse effects resulting from the aldose reductase catalyzed accumulation of sorbitol may be related to the observed degeneration of retinal mural cells. Aldose reductase (alditol:NADPH oxidoreductase E.C. 1.1.1.21), an enzyme in the sorbitol pathway, has been implicated in the pathogenesis of several diabetic complications. Under hyperglycemic conditions, this enzyme, which utilizes the cofactor NADPH to reduce hexose sugars to their respective sugar alcohols, has been shown to play a significant physiologic role in the production of sorbitol. In diabetic or galactosemic rats, the intracellular accumulation of sugar alcohols...
has been shown to initiate cataract formation, result in delays in the re-epithelialization of denuded corneas, and decrease the motor nerve conduction velocity of sciatic nerves. Moreover, all of these diabetic complications can be either prevented or reversed through the use of potent aldose reductase inhibitors.1-4

Aldose reductase has been localized by radioimmunoassay in isolated cultured mural cells of rhesus monkeys.5 Moreover, these cells, when grown in high glucose medium, displayed increased levels of sorbitol and eventually lysed, reflecting the possible adverse effects of the sorbitol pathway in cultured mural cells. The presence of immunoreactive aldose reductase, however, could not be demonstrated in freshly isolated monkey retinal capillaries. Similarly, investigators reporting on the histochemical localization of aldose reductase in the sectioned retinas of rats6 and dogs7 have failed to find evidence for the presence of this enzyme in retinal vessels. However, we have detected the presence of aldose reductase in retinal vessels of humans. Here we report its histochemical detection in the mural cells of retinal vessels isolated through trypsin digestion.8

Materials and Methods. For these studies, antibodies against purified human placental aldose reductase, produced in either goats or rabbits, were used.9 These antibodies, partially purified by 33% ammonium sulfate precipitation, formed a single line of identity against either purified or crude human placental aldose reductase on either Ouchterloney plates or Laurell immunoelectrophoresis.

Eleven human retinas of less than 7 hours postmortem were fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for 8 hours at 4°C. Each retina was divided into quarters and the fixed retinal quarters were washed with phosphate buffer and then subjected to digestion with 3% trypsin (Difco 1:250) in 0.1 M tris buffer, pH 7.8 for 5-10 minutes at 37°C, followed by incubation for 30 minutes with 0.1% trypsin inhibitor (Sigma from soybean) in phosphate buffer pH 7.4. Following washing with phosphate-buffered saline, the retinal vessels were divided into two groups and mounted with air drying on microscope slides. One group was incubated with 2% normal serum (rabbit or goat) for 2 hours at 22°C. The primary antibody, diluted 2,000-fold in phosphate-buffered saline containing 0.3% Triton X 100, then was incubated with the vessels at 4°C for up to 48 hours. This was followed by a 2-hour incubation at 22°C with 200-fold diluted linked antibody, and finally with the PAP complex, diluted 500-fold, for 2 hours at 22°C. The antibodies then were visualized by the DAB-PAP method.10

The other group, used as control, was prepared simultaneously under identical conditions, with the expectation that either a preadsorbed antibody solution or normal goat or rabbit serum were substituted for the specific aldose reductase antibodies. The preabsorbed antibody solution was prepared by titrating the antibody solution with purified human placental aldose reductase antigen until enzyme activity could no longer be inhibited. This mixture then was centrifuged at 20,000 rpm to remove the formed precipitate and the supernatant was employed.

Results and Discussion. The results of immunohistochemical localization, shown in Figure 1C, indicate that human retinal mural cells specifically stain for the presence of immunoreactive aldose reductase. In all cases, no staining could be observed in the endothelial cells. The immunoreactive enzyme appears to be localized in the cytoplasm of the mural cells. This can be observed both by selective focusing through the vessels and by the incomplete staining of some cells. Staining is limited to the perinuclear cytoplasm (→) and some cytoplasmic processes (●) leaving the nucleus unstained. No specific staining could be observed in the controls (Fig. 1D) in which serum or aldose reductase antibodies preadsorbed with purified human placental aldose reductase were used in place of the primary antibody. In PAP staining and controls, sporadic nonspecific staining of peroxide-containing erythrocytes also could be observed; however, their staining intensity appeared dependent on their location.

Since the immunohistochemical localization of cultured monkey mural cells also employed human placental aldose reductase, we also have investigated the localization of this enzyme in monkey (Macaca fascicularis) retinal preparations. Unlike these investigators, however, our antibodies failed to cross-react with monkey aldose reductase as observed by either immunodiffusion on Ouchterloney plates or the more sensitive PAP method. No specific staining could be observed in either the antibody-incubated monkey vessels or their controls.

This is the first direct demonstration that aldose reductase is localized in the mural cells of human retinal vessels. This successful localization is, in part, due to the use of short-term (5- to 10-minute) trypsin digestion that can result in the clean preparation of morphologically, well-preserved retinal vessels with intact basement membranes. Specific mural cell localization of the enzyme is difficult to observe in frozen retinal cross-sections because of the size and distribution of these mural cells. This may partially explain the previous failure of investigators to find aldose reductase in the retinal vessels of either rat or dog retinal cross-sections that were prepared from paraffin sections.

The selective presence of aldose reductase in human retinal mural cells, coupled with the previous bio-
Fig. 1. Human trypsin-digested retinal capillaries stained with PAS and hematoxylin. The normal distribution of endothelial cells (e) and mural cells (m) are illustrated in A, while in B, the selective loss of these mural cells (mural cell ghosts, mg) in the diabetic retina can be observed. Human trypsin-digested retinal capillaries subjected to immunohistochemical staining for aldose reductase using antibodies prepared against human placental aldose reductase is shown in C. The perinuclear cytoplasm of mural cells are mainly stained. For controls (D) normal serum or aldose reductase antibodies preabsorbed with purified human placental aldose reductase were used in place of the primary antibody. In the controls, * indicates faint nonspecific staining of erythrocytes while mural cells (m) remain unstained.
chemical results of cultured monkey mural cells, provides evidence for the attractive hypothesis that the aldose reductase initiated accumulation of sorbitol may be involved in the selective degeneration of human mural cells in non-proliferative diabetic retinopathy.

Key words: aldose reductase, diabetes, retinopathy, mural cell, pericytes, microangiopathy, immunohistochemistry

From the Laboratory of Vision Research, National Eye Institute, National Institutes of Health, Bethesda, Maryland. Submitted for publication March 25, 1983. Reprint requests: Peter F. Kador, Ph.D., Bldg. 6 Rm. 237, National Institutes of Health, Bethesda, MD 20205.

References

Galactose-induced Retinal Capillary Basement Membrane Thickening: Prevention by Sorbinil

Robert N. Frank, Richard J. Keirn, Alexander Kennedy, and Korni W. Frank

Normotensive and spontaneously hypertensive rats fed a 30% galactose diet until 15–21 months of age developed significant (P < 0.05) retinal capillary basement membrane thickening, compared with animals fed a standard test diet. Animals on the high galactose diet containing 250 mg/kg of the aldose reductase inhibitor, Sorbinil, did not develop basement membrane thickening. No cytologic abnormalities of pericytes or endothelial cells were noted, and pericyte:endothelial cell nuclear ratios did not differ in the various experimental groups. This model reproduces a characteristic lesion of diabetes mellitus in non-diabetic animals, and should facilitate study of the biochemical mechanisms of basement membrane thickening. Invest Ophthalmol Vis Sci 24:1519–1524, 1983

Thickening of capillary basement membranes is characteristic of diabetes mellitus.1,2 Its pathogenesis, however, remains unexplained. We have been attempting to develop a model of diabetic retinopathy in the laboratory rat by a number of methods, including the production of galactosemia by long-term feeding of a diet enriched in galactose, and the induction of diabetes or galactosemia in spontaneously hypertensive (SHR) rats. In the course of these experiments, we discovered that the prolonged (15–21 months) feeding of a high galactose diet produced significant thickening of retinal capillary basement membranes in both normotensive and hypertensive rats. The basement membranes not only were thickened, but also frequently demonstrated focal abnormalities that have been described previously in human subjects with diabetic retinopathy. Simultaneous administration of the aldose reductase inhibitor, Sorbinil (d-6-fluoro-spiro[chroman-4,4':imidazoline]-2',5'-dione; CP-45,634, Pfizer), prevented the abnormality. This animal model should be useful in the study of the mechanism of capillary basement membrane thickening, and may have relevance to the pathogenesis of one of the most common vascular lesions of diabetes mellitus.

Materials and Methods. Normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) female rats were entered into the study at 6 weeks of age. Control animals were fed the standard Ralston Purina test diet, in pellet form. Other rats were placed on a diet in which a portion of the carbohydrate (30% of the total weight of the diet) was replaced by galactose,