Retinal microvascular patency in the diabetic rat

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Abstract

Purpose: To study whether the patency to erythrocytes in retinal microvessels of diabetic rats is reduced or blocked before the vessels lose their patency to plasma flow. Methods: We used recognized techniques to induce diabetic and galactose related microvascular retinal lesions in rats: (1) alloxan induction (2) streptozotocin induction (3) galactose-containing diet. The rats were followed up to 17 months. We used our vascular trichrome technique to observe the effects of the ongoing diabetes on the retinal microcirculation.

Results: A focal leakage of a plasma-borne fluorescent dye was noted around the junction of the deep retinal capillaries and the ascending venules to the superficial retinal circulation in the streptozotocin and alloxan diabetic rats by the 14th month, and, by the 16th month, retinal capillary non-perfusion and retinal vascular malformations were present. The affected vessels showed patency to microspheres (0.2 μm in diameter) but no perfusion of erythrocytes. No such changes were seen in the galactose-fed rats.

Conclusions: (1) The location between the deep retinal capillary net and the ascending venules may be the site of early vascular leakage in the diabetic rat model, (2) the erythrocytes’ passage in the affected retinal microcirculation was blocked before the development of complete blockage to plasma in diabetic rats. The logical assumption that during the development stage of retinal capillary occlusion there may be a transient stage of microvascular insufficiency was examined. The lathyrogen, imino-diproprionitrile (IDPN), had previously been effective for creating a fast-developing model of retinal vasculopathy. Using that model, we demonstrated a stage in which the retinal microvasculature was blocked to erythrocytes but not to plasma [1]. However, we questioned the applicability of our findings to more slowly developing microvasculopathies, such as diabetic retinopathy. We designed the current study to examine the presence of such stage in slowly developing microvasculopathy. Animal models that are known to induce “diabetic retinopathy-like” changes used [2–4]. The diabetic animals were followed for a period of 17 months. Starting at the 12th month, a few animals of each group were killed and the retinas were examined with our trichrome method [1] for relative capillary patency to erythrocytes and plasma, for functionality of endothelial cells, and for disturbances in the blood–retinal barrier. The results of this study support the hypothesis that retinal microvascular insufficiency does exist as a temporary stage that precedes the development of complete capillary blockage in long-term developing rat models of diabetic retinopathy.

Materials and methods

Diabetes was induced in albino, male Wistar rats (10–12 weeks old, 0.25–0.3 kg body weight). Four models were used to produce diabetic retinopathy-like changes, with the diabetes being induced by: a subcutaneous injection of 2% alloxan (Sigma) solution in 0.2 M phosphate–citrate buffer (group 1; n = 22, pH 4.5, 200 mg/kg body weight), an intraperitoneal injection of 10% streptozotocin (Sigma) in 0.2 M phosphate–citrate buffer (group 2; n = 22, pH 4.5, 110 mg/kg body weight), a diet
containing 30% galactose (group 3; n = 12) and a diet containing 40% galactose (group 4, n = 12). In the 30 and 40% galactose diets, the galactose replaced a proportionate part of the carbohydrate content of the diet and was supplied pelleted (Glen Forrest Stockfeeders, WA, Australia). Twelve rats on a standard diet comprised the control group (group 5). All animals had access to water and food ad libitum. In the chemically induced diabetes groups, a combination of polydipsia, polyuria and hyperglycemia confirmed the development of diabetes mellitus. Polydipsia and polyuria were assessed by the increased consumption of water and the frequent replacement of the wet sawdust in the diabetic rats cages, compared to controls. A drop of blood was obtained from the tip of the tail for glucose level measurement (Accutrend, Boehringer–Mannheim, Germany). The blood glucose level was measured 24 hours after induction, weekly during the first month and monthly afterwards. Rats were monitored daily for general appearance and behavior and weekly for weight. The experiment was conducted throughout a period of 17 months. Starting at 12 months from induction, animals were subjected to the experiment at monthly intervals. Each time, the retinas of three rats of groups 1 and 2 and two rats of groups 3, 4 and 5 were examined and the findings were documented. At 17 months, all the remaining rats were killed.

The vascular trichrome technique was used to demonstrate three components of the retinal microcirculation, the erythrocytes, plasma, and endothelial/pericyte cell nuclei, as well as the integrity of the blood–retinal barrier [1]. The rat was anesthetized by spontaneous respiration of halothane (3%; ICI, Australia) through a mask. The femoral vein was cannulated, and 250 U (1000 U/ml) of heparin sulfate (David Bull Mulgrave, Victoria, Australia) was injected. The lathogen, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate (bis-benzimide, Hoechst 33342: Sigma), was made up to a solution of 5 mg/ml in 0.9% NaCl and was perfused through the vein over 30 seconds to add up to a total dose of 10 mg/kg body weight. Fluorescent polystyrene microspheres (Duke Scientific, Palo Alto, CA), 0.2 μm in diameter, were then perfused at 0.2 ml/minute for 5 minutes. Loops were placed around the optic nerves of both eyes, and both ocular cir-

Calculations were clamped abruptly to cause instant arterial occlusion. Using this technique, the blood flow was assumed to be arrested instantly, such that the components of the blood remained in the same location as they were at the time of occlusion. The eyes were enucleated and placed in 10% buffered formol–saline solution (pH 7); immediately afterwards, the animal was killed by barbiturate overdose. Fifteen minutes after enucleation, the anterior segments and lenses were removed, and the retinas were washed directly with a gentle flow of 10% buffered formol saline while being dissected free from the underlying choroid. They were then whole-mounted by viscous transparent solution (Gurr, BDH, England) and coverslipped. Within 20 minutes after enucleation, the retinas could be observed with normal and fluorescence microscopy.

Specific elements in the third dimension could be pinpointed by focusing the microscope into deeper and superficial layers of the retina [1]. The erythrocytes were observable under white light microscopy since the brown hemoglobin can be visualized easily [1]. The 0.2-μm microspheres were observed under fluorescence microscopy (excitation filter 470 nm, barrier filter 530 nm) and appeared green in color. The endothelial cells were observed under fluorescence microscopy (excitation filter 360 nm, barrier filter 455 nm) and were blue in color. Each individual retinal area could be observed and photographed sequentially with the appropriate illumination to reveal each of the three views of the same retinal area at any depth of the observed specimen.

The whole procedure, from initial bis-benzimide injection to retinal photography, could be easily performed within 45 minutes. Since bis-benzimide is not specific for endothelial cell nuclei, it gradually diffused to cells more distant from the vessel lumen. Endothelial and pericyte nuclei were first to be observed [1] and finally, after several hours, the nuclei of all retinal cells were stained, giving the retina a diffuse blue fluorescence. This typical diffusion pattern was noted in groups 3 and 4 and the controls of each experiment, with the diffusion pattern of the bis-benzimide in the control group providing a measure of the integrity of the blood-retinal barrier. The retina was in a good state for photographing endothelial cells and pericytes for 2–3 hours before the stained nuclei