Immunohistochemical Localization For Aldose Reductase in Diabetic Lenses

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Sugar cataract formation has been demonstrated to result from lenticular sorbitol accumulation. In the lens, the activity of aldose reductase has been observed to increase with the onset of diabetes, while the activity of sorbitol dehydrogenase decreases. This shift in activities of these two Sorbitol Pathway enzymes favors the increased accumulation of sorbitol. Immunohistochemical studies with antibodies prepared against purified rat lens aldose reductase reveal a striking increase in immunoreactive positive staining for aldose reductase in lenses from diabetic rats. Two weeks after the onset of diabetes, increased immunohistochemical staining for aldose reductase appears beneath the epithelial region where water cleft formation occurs, and the intensity of this staining increases with the formation of vacuoles. By 6–8 weeks, the presence of large vacuoles and areas of liquifaction containing dense immunoreactive stain can be observed. Examination of human cataractous lenses with antibodies prepared against purified human placenta aldose reductase suggest similar increases in immunoreactive staining in the human diabetic lens. Cataractous lenses from diabetic patients revealed increased immunoreactive staining for aldose reductase, which was associated with the presence of vacuoles in both the anterior or posterior superficial cortical layers. Examination of similar vacuole containing regions from non-diabetic cataractous lenses revealed no increase in immunoreactive staining for aldose reductase. These results suggest that the enhanced activity of aldose reductase observed in diabetes is due to an increased amount of enzyme, rather than enzyme activation.

Materials and Methods

Animals

Male 50 gram Sprague Dawley rats were injected in the tail vein with a single dose of 100 mg/kg streptozotocin. This resulted in an onset of diabetes, with blood glucose values ranging between 500–600 mg/dl. These values were determined on a Beckman glucose analyzer (Fullerton, CA). These investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Antibodies

Antibodies were raised in goats against human placental aldose reductase and rat lens aldose reductase.

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The enzyme aldose reductase (alditol:NADP⁺ oxidoreductase EC 1.1.1.21) together with sorbitol dehydrogenase (1-iditol dehydrogenase, EC 1.1.1.14) forms the sorbitol pathway through which glucose is converted to fructose. In the first step of this pathway, aldose reductase utilizes NADPH to reduce glucose to sorbitol, while, in the second step, sorbitol dehydrogenase utilizes NAD⁺ to oxidize sorbitol to fructose. Experimental evidence strongly indicates that aldose reductase through the production of sorbitol initiates the cataractous process in diabetic animals. The intracellular accumulation of the polar sorbitol intermediate has been demonstrated to produce a hyperosmotic effect that results in cellular swelling. This swelling is accompanied by an alteration of lenticular electrolyte levels, increased membrane permeability, and the formation of water clefts and vacuoles that result in reversible cortical opacities. As swelling progresses, other lenticular parameters also become altered, and an irreversible nuclear cataract is eventually formed.

Studies with rat lens suggest that diabetes can alter conditions in the lens, so that sorbitol pathway activity is increased. The activity of the key enzyme aldose reductase has been observed to increase with the onset of diabetes, while the activity of sorbitol dehydrogenase decreases. This shift in activities of these two sorbitol pathway enzymes favors the increased accumulation of sorbitol and decreased production of fructose. Immunohistochemical studies with antibodies prepared against purified rat lens aldose reductase and human placental aldose reductase reveal increased immunoreactive positive staining for aldose reductase in lenses from either diabetic rats or diabetic patients.
These antibodies, partially purified by 33% ammonium sulfate precipitation, formed single lines of identity respectively against both crude or purified human placental or rat lens aldose reductase, on either Ouchterloney plates or Laurell immunoelectrophoresis.

**Immunohistochemical Staining**

Rat eyes were fixed for 1–2 days in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4, and then dissected. The excised lenses were washed with graded (10, 15, 20%) sucrose solutions in 0.1 M phosphate-buffered saline (PBS), frozen, and sectioned (ca. 15 μm). The sections were then divided into two groups, and one group of sections was then incubated for 2 hr with PBS containing 2% normal goat serum. This was followed by an overnight incubation at 4°C with antiserum against rat lens aldose reductase (1:4000 dilution in PBS containing 0.3% Triton X-100). The sections were then incubated at 22°C for 2 hr with a 1:400 dilution of linked serum (Miles Laboratories, Elkhart, IN) followed by a 2 hr exposure at 22°C to a 1:800 dilution of the peroxidase-antiperoxidase complex (PAP, Miles Laboratories, Elkhart, IN). The antibody complex was visualized with a solution of 3,3′-diaminobenzidine and hydrogen peroxide (DAB). The second group of sections, which served as control, were subjected to identical experimental conditions, except that antibodies absorbed against rat lens aldose reductase was used in place of the primary antibody.

Human lenses with subcapsular cataracts, surgically extracted from patients between 60 and 82 yr of age, were prepared as previously described. Eight lenses from diabetic patients and nine lenses from non-diabetic patients were fixed for 8–12 hr in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4, and washed with graded (10, 15, 20%) sucrose solutions in 0.1 M PBS. The lenses were then frozen, sectioned (6–10 μm), and divided into two groups. One group of sections was incubated for 2 hr with PBS containing 2% normal goat serum. This was followed by an overnight incubation at 4°C with antiserum against human placental aldose reductase (1:3000 dilution in PBS containing 0.3% Triton X-100). The sections were then
incubated at 22°C for 2 hr with a 1:300 dilution of linked serum, followed by a 2 hr exposure at 22° to a 1:600 dilution of PAP. Finally, the antibody complex was visualized with a solution of DAB. The second group of sections, which served as control, were subjected to identical experimental conditions, except that either normal goat serum or antibodies absorbed against human placental aldose reductase were used in place of the primary antibody.

**Results**

In the normal rat lens, immunoreactive staining for aldose reductase appears primarily in the metabolically active epithelium and bow region. Immunoreactive staining in lenses from young (50 g) rats appeared to be most intense in the epithelial cells (Fig. 1B). Staining was also observed in the bow region where the epithelial cells are differentiating into lens fibers. Staining intensity in the cortical region decreased in the lens fibers as fiber cells progressed toward the lens nucleus. A similar distribution of immunoreactive aldose reductase was observed in the lenses from adult (250 g) rats; however, staining intensity appeared to be less, and the distribution of stain did not extend into the deeper cortical fibers as in the younger lenses (Fig. 2A). No immunoreactive staining was observed in the control (Fig. 2B). The distribution of immunoreactive aldose reductase in the adult rat lens is consistent with results reported previously in rat lens.

A striking increase in immunoreactive staining for aldose reductase was revealed upon examination of lenses from diabetic rats. Compared with the normal, this staining extends deeper into the lens cortex. Two weeks after the onset of diabetes, increased immunohistochemical staining for aldose reductase appears beneath the epithelial region where water cleft formation occurs (Fig. 1C,F). The intensity of this staining increases with the formation of vacuoles after 3 weeks (Fig. 1D), and, after 6–8 weeks, the presence of large vacuoles and areas of liquefaction containing dense immunoreactive stain can be observed (Fig. 1E).

The distribution of immunoreactive aldose reductase in the adult human lens also closely mirrors that observed in the lens from mature (250 g) rats. Immunoreactive staining for aldose reductase in both lenses is mainly confined to the epithelium and superficial cortex (Fig. 2A,C). Examination of eight cataractous lenses from diabetic patients revealed increased immunoreactive staining for aldose reductase, associated with the presence of vacuoles in both the anterior or posterior superficial cortical layers (Fig. 3A,B). Examination of similar vacuole containing regions from nine cataractous lenses from non-diabetics revealed no increase in immunoreactive staining for aldose reductase (Fig. 3C,D). These results suggest that a similar increase in immunoreactive staining for aldose reductase observed in the lenses from diabetic rats occurs in the human diabetic lens.

**Discussion**

Using quantitative histochemical techniques in which the conversion of sorbitol to glucose by aldose reductase was fluorometrically measured in the adult lens, Collins and Corder observed aldose reductase activity to be twofold greater in the epithelium than in the cortex or nucleus. The distribution of aldose reductase presented in Fig. 1b and Fig. 2 qualitatively demonstrates more intense staining in the epithelium than in the superficial cortical fibers. The failure of this technique to clearly demonstrate immunoreactive aldose reductase in the deeper cortical fibers and nucleus, however, may result in part from the detection limits.
Fig. 3. Immunohistochemical localization of aldose reductase in the anterior (A) and posterior (B) regions of a lens extracted from a 60-yr-old female diabetic and in the anterior (C) and posterior (D) regions of a lens extracted from a 69-yr-old female non-diabetic. E = epithelium; PC = posterior capsule; V = vacuoles; arrows illustrate aldose reductase staining associated with vacuoles (×88).

of this immunohistochemical technique. Since immunodetection is proportional to the concentration of aldose reductase antigen present, a decrease in detection could be anticipated with increased cell volume in the elongated lens fiber cells, if one assumes the cellular content of enzyme to be constant.

Varma and Kinoshita have demonstrated that in vitro cultured intact lenses from diabetic rats accumulate polyol to a higher level than those from normal, non-diabetic rats, and that conditions in the diabetic lens favor the increased accumulation of lenticular sorbitol. Enzymatic evaluation also revealed that the activity of aldose reductase in the diabetic rat lens is increased, while the activity of sorbitol dehydrogenase is decreased, and that the lens nucleotide levels of NADPH is increased, while that of NAD⁺ is decreased. Changes in the distribution of aldose reductase activity in the rat lens have also been reported with the onset of diabetes. In the diabetic rat lens, aldose reductase activity increased 44% and 129%, respectively, in the epithelium and cortex, while the activity of aldose reductase decreased in the nucleus by 19%. Although aldose reductase activity is increased, kinetic evaluation of aldose reductase from diabetic lenses reveals no change in the affinity (Kₘ) of enzyme for substrate. This suggests that the enhanced activity of aldose reductase in diabetes is due to an increased amount of enzyme, rather than enzyme activation. The increased immunohistochemical staining for aldose reductase in diabetic rat lens (Fig. 2) supports such a hypothesis, since increased immunoreactive aldose reductase in the cortex qualitatively mirrors the quantitative increases observed in the cortex of diabetic rat lens.

In the adult human lens, the distribution of enzymatic aldose reductase activity is confined primarily to the epithelium and cortex (70% epithelium, 21% cortex, and 9% nucleus). Immunoreactive staining for aldose reductase in adult human lens is consistent with this observation (Fig. 2). Increased aldose reductase activity has also been observed in the lens and kidneys from diabetic patients, and direct correlations between sorbitol levels in extracted diabetic lenses and both Hb A₁ and fasting blood sugar levels have been reported. Moreover, in vitro cultured diabetic lenses have been reported to produce significantly more sorbitol than non-diabetic lenses, and this production can
be reduced by the administration of an aldose reductase inhibitor. Increased immunoreactive staining for aldose reductase also occurs in both the anterior or posterior superficial cortical layers of cataractous lenses extracted from diabetics, but not in those extracted from non-diabetics (Fig. 3). Although there is currently no direct evidence that aldose reductase plays a role in the pathogenesis of human diabetic cataracts, these results suggest that there is an increase in aldose reductase activity similar to that observed in rat. The production of sorbitol confined to localized areas containing increased levels of aldose reductase, as suggested by these immunohistochemical studies, could exert significant osmotic stress, which can result in cataract formation.

Key words: aldose reductase, sorbitol pathway, diabetes, cataract, immunohistochemistry, rat, human

References