Expression of the IGF System in Normal and Diabetic Transgenic (mRen-2)27 Rat Eye

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PURPOSE. In the present study, a recently described model of diabetic eye disease was used to investigate the distribution of the insulin-like growth factor (IGF) system in the eyes of transgenic (mRen-2)27 rats (exhibiting hypertension and elevated serum and ocular renin levels) with streptozotocin-induced diabetes.

METHODS. Female transgenic (mRen-2)27 rats were randomized to receive either streptozotocin (diabetic) or citrate buffer (control). After 10 months, the rats were killed and the eyes fixed and embedded in paraffin. In situ hybridization (ISH) was used to document the cellular distribution of mRNAs for components of the IGF system (IGF-I, IGF-I receptor [IGFIR] and IGF binding proteins [IGFBP]1 to -6) in the eyes.

RESULTS. In nondiabetic rats, mRNA for IGFBP-1, -5, and -6; IGF-I, and IGFIR were detected in the retina. In addition, IGF-I mRNA was present in the cornea, IGFBP-1 mRNA was observed in the cornea and iris, and IGFBP-5 and -6 mRNAs were identified in the ciliary body, iris, and cornea. mRNAs for IGFBP-2, -3, and -4 were not found in the eyes. In diabetic rats, reduced levels of IGFBP-6 mRNA were detectable, whereas levels of IGFBP-5 mRNA were increased in the inner and outer retina, rods and cones, iris, cornea, and ciliary body. Other components of the IGF system in the eye were unchanged with diabetes.

CONCLUSIONS. In the diabetic (mRen-2)27 rat, IGFBP-6 is downregulated and IGFBP-5 is upregulated by induction of diabetes. Because these IGFBPs may respectively have IGF-enhancing and IGF-inhibitory effects, these findings suggest a possible net IGF-enhancing effect induced by diabetes, providing further evidence for a role of the IGF system in the development of diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2005;46: 2708–2715) DOI:10.1167/iovs.04-0921

Diabetic retinopathy (DR) is a sight-threatening complication of diabetes mellitus. Together with retinopathy of prematurity (ROP) and age-related macular degeneration (AMD), it is one of several retinal diseases characterized by neovascularization that share similar pathophysiological.1 The renin-angiotensin system (RAS) has a key role in the development of diabetic microvascular complications, including retinopathy.2,3 The insulin-like growth factor (IGF) system has also been shown to play a role in the etiology of diabetic retinopathy.4 Other evidence points toward interactions between IGF-I and the key pathogenic cytokine vascular endothelial growth factor (VEGF) in the hypoxic mouse model of retinopathy, in which VEGF enhancement of proliferative retinopathy is blunted by inhibition of IGF’s action at the level of the IGF-I receptor (IGFIR).5

The transgenic rat line TGR(mREN-2)27 was established by inserting the murine Ren-2 gene into the rat genome,6 resulting in fulminant hypertension that develops by 4 weeks of age, with mean blood pressure being approximately twice normal.6 In the eyes of (mRen-2)27 rats, renin and prorenin expression is localized to the macroglial Müller cells7,8 and nonpigmented ciliary epithelium, at levels of intensity much greater than in humans or nontransgenic rats.8 Prorenin is present in the posterior part of the Müller cells, whereas active renin is present throughout but particularly in end feet adjacent to retinal vasculature, suggesting a directional processing of renin in these cells.8

A recently described model of diabetic eye disease incorporates the induction of diabetes in the (mRen-2)27 rat and has revealed induction of endothelial cell proliferation in the retina and iris.9 This proliferation is associated with an elevation of VEGF and VEGF receptor 2 mRNA, as well as an increase in activated ocular renin in diabetic (mRen-2)27 rats when compared with nondiabetic (mRen-2)27 rats. These changes are reversible with angiotensin converting enzyme (ACE) inhibition.9 It is possible that the pathogenic systems of retinopathy are further linked by the RAS, resulting in enhanced activity of the IGF system, as well as VEGF.

Most IGF-I and -II are bound to one of six IGF-binding proteins (IGFBPs), which are structurally similar proteins displaying high affinity for the IGFs. The IGFBPs vary in their tissue location, affinity for the IGFs, and role in IGF function, either potentiating or inhibiting, often in a cell- or tissue-specific manner.10,11 The IGFs act in endocrine, autocrine, and paracrine manners, and the IGFBPs regulate all these actions.12

We hypothesize that, just as interactions between VEGF and both the RAS and IGF system are important in pathogenesis of DR, interactions between the IGF system and RAS may also contribute to the development of DR. In keeping with previously observed effects of elevated IGF-I levels being associated with DR and decreased levels being protective,13,14 a change in the IGFBP profile in the eye15 leading to a net IGF-I-enhancing effect has been hypothesized, regardless of a change in abundance of either IGF-I itself or of its receptor. The recently described diabetic m(Ren-2)27 rat15,16 offers a unique opportunity to study changes in expression patterns induced by diabetes using in situ hybridization.
Table 1. Distribution of IGF System mRNA in the Eyes of Transgenic Ren-2 Rats

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Distribution of mRNA for all of the IGF system except IGFBP-5 and -6 were identical in both the diabetic and nondiabetic state. IRL, inner retinal layer; ORL, outer retinal layer; LRC, layer of rods and cones; Cil Body, ciliary body; ND, nondiabetic; D, diabetic; THGFR, type I IGF receptor. Symbols represent relative silver grain intensity: −, absent; +, present; +++, present in significantly greater quantity.

Methods

Animals

All experimental procedures adhered to the code for the care and use of animals for scientific purposes of the National Health and Medical Research Council of Australia and were authorized by the University of Melbourne bioethics committee. All protocols adhered to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

As previously described, female transgenic Ren-2 rats were randomized at 6 weeks of age to receive either 55 mg/kg streptozotocin (STZ; diabetic) or 0.1 M citrate buffer (control) injected into a tail vein.

Animals were maintained in a standardized environment with free access to tap water and rat chow (GR2; Clark-King & Co., Gladesville, New South Wales, Australia), and blood glucose and blood pressure were monitored at weekly and monthly intervals, respectively. Diabetic rats received daily doses of intraperitoneal insulin (2–4 IU, Ultratard; Ultra North Dakota, Bagswart, Denmark) to maintain survival with elevated blood glucose levels. After 10 months of age, all rats were killed, their eyes fixed and embedded in paraffin, and 3-μm sections prepared.

In Situ Hybridization

Complementary (anti-sense; AS) and noncomplementary (sense; S) RNA probes were synthesized from cDNA clones encoding the 3′ end of IGF-I,17 Type 1 IGF receptor18 (both courtesy Charles T. Roberts, Jr, Oregon Health Science University, Portland, OR) and IGFBP-1 to -617-22 (courtesy Shunichi Shimasaki, Whittier Institute, La Jolla, CA) using a method previously described.19 The RNA polymerases T3, T7, and SP6 were used for linearization of the probes. The purified RNA probes were then alkaline hydrolyzed to approximately 200 bpi in length. The 35S-labeled cRNA probes (5 × 105 cpm/μl) were added to the hybridization buffer (300 mM NaCl, 10 mM Tris-HCl [pH 7.5], 10 mM Na2HPO4 [pH 6.8], 5 mM EDTA [pH 8.0], 1× Denhardt’s solution, 50 mg/ml yeast RNA, 50% deionized formamide, and 10% [wt/vol] dextran sulfate), and 30 μl of the 35S-labeled cRNA probe mixture was added to each pretreated slide. Hybridization was performed at 60°C in a humidified (50% formamide), darkened chamber overnight. Slides were washed in 2× SSC and 50% formamide, and treated with RNase A before exposure to x-ray film (XAR film; Eastman Kodak, Rochester, NY) for 1 to 3 days. After autoradiography, slides were dipped in premelted emulsion (LM-1; GE Healthcare, Sydney, Australia) and incubated at 4°C for between 2 and 4 weeks. Slides were then developed in (D19 developer; Kodak) and fixed (Hypam; Ilford; Basildon, UK). The slides were then counterstained with hematoxylin and cosin.

Quantification of In Situ Hybridization

Tissue sections were assessed and scored based on silver grain intensity in sections on dark-field microscopy with a light microscope (Eclipse 600; Nikon, Tokyo, Japan) with a video camera (5CCD; Sony, Tokyo, Japan). Microcomputer image device software (MCID, ver. 3; St. Catharines, Ontario, Canada) was used to capture and analyze silver grain intensity for image downloading into a computer for statistical analysis (Prism; GraphPad, San Diego, CA).

All eye samples were treated with both antisense (complementary) and sense (noncomplementary) 35S-labeled cRNA, with the latter acting as a control for nonspecific hybridization. A mean of six eyes from six different rats were assessed for each group.

Statistics

All data analyses were performed with commercial software (Prism 3.02; GraphPad; and StatXact 4; StatC, College Station, TX). P < 0.05 was used to demonstrate statistical significance. Data were summarized as medians and ranges, and group comparisons were performed with the Kruskal-Wallis equality of populations test, because data were non-normally distributed.

Either one or two sections each from the eyes of between three and four animals were analyzed for each of the RNA probes, apart from those for IGFBP-5 and -6, for which a total of between 9 and 11 animals were analyzed.

Results

Body Weight, Blood Pressure, Glycemia, and Ocular Renin

As expected, the diabetic Ren-2 rats had a lower body weight (304.6 ± 10.9 g) than did the nondiabetic control animals (390.0 ± 15.4 g) at 10 months of age and had higher HbA1c (5.9% ± 0.5% vs. 3.3% ± 0.2%). The systolic blood pressure was not different between the two groups (diabetic 176.3 ± 6.0; nondiabetic 172.3 ± 6.0), nor were overall ocular renin levels, although in the diabetic animals, more of the renin was in the active form. These results have been reported.

In Situ Hybridization for the IGF System

Messenger RNAs for most components of the IGF system were detected; however, only IGFBP-5 and -6 mRNAs clearly showed different expression between the diabetic and nondiabetic animals. Detailed assessment of each component of the IGF system follows and is summarized in Table 1.
IGF-I. In nondiabetic animals, IGF-I mRNA was detected in inner and outer retinal layers and the rods and cones, as well as in the choroid. There was no difference in distribution or intensity in the diabetic state. IGF-I mRNA was also detected in the cornea in both the nondiabetic and diabetic animals.

Type 1 IGF Receptor. The type 1 IGF receptor mRNA was localized to only the retina in both nondiabetic and diabetic animals, and no change was observed with diabetes between the distribution and abundance of mRNA at any location. Specifically, areas displaying mRNA for the receptor were the inner and outer retinas, rods and cones, and choroid.

IGFBP-1. In both the nondiabetic and diabetic states, IGFBP-1 mRNA was detected in the inner and outer retinal layers and the rods and cones, without any difference in distribution or intensity in the diabetic state. The IGFBP-1 mRNA was also present in the cornea and iris in both the nondiabetic and diabetic animals, with no difference between them.

IGFBP-2, -3, and -4 mRNAs. There was no detectable mRNA observed in any part of the eye in either of the nondiabetic or diabetic animals for IGFBPs-2, -3, or -4.

IGFBP-5. In the nondiabetic animals, low levels of IGFBP-5 mRNA were observed in the retina, cornea, iris, and ciliary body. In the diabetic animals, IGFBP-5 mRNA was present in much greater abundance in the cornea and iris (Fig. 1), the anterior and posterior retinas and the layers of rods and cones (Fig. 1) and the ciliary body (Fig. 2). No changes were seen in any other areas of the eye. These changes are shown graphically in Figure 3.

IGFBP-6. In the nondiabetic animals, IGFBP-6 mRNA was present in several retinal layers, specifically the inner and outer...
retinas, the rods and cones, and the choroid (Fig. 4). It was also present in the iris and cornea (Fig. 4) and the ciliary body (Fig. 5). In the diabetic animals, mRNA for IGFBP-6 was observed at much lower abundance than in the nondiabetic animals. These changes are shown graphically in Figure 6.

**DISCUSSION**

The RAS plays a key role in microvascular diabetic complications including retinopathy and nephropathy, as shown in animal models and clinically. In the (mRen-2)27 rats, fulminant hypertension occurs in association with elevated tissue renin levels, including ocular renin. When diabetes is induced in (mRen-2)27 rats, neovascularization of the iris and retinal endothelial cell proliferation occur, both of which are attenuated by RAS blockade using an ACE inhibitor, providing further evidence of the role of the RAS in diabetic ocular disease. Clinical evidence of the role of RAS in diabetes includes the EUCLID study (EURODIAB Controlled Trial of Lisinopril in Insulin Dependent Diabetes) which reported that the ACE inhibitor, lisinopril, may decrease retinopathy's progression in nonhypertensive patients who have diabetes. A local RAS has been identified in the eyes of various species including humans, but the way in which the RAS interacts to influence the development of retinopathy is still to be fully determined. Interactions are seen between the RAS and various hormonal systems in the body, including transforming growth factor β, endothelin-1, and VEGF. It is possible that part of the mechanism of the effect of the RAS on retinopathy pathogenesis lies in interactions between it and the IGF system.

In this study, we identified alterations in the IGF system in a model of diabetic eye disease that has already been shown to be associated with raised VEGF and VEGFR-2 mRNA and elevated active ocular renin. We report changes in the gene expression of IGFBP-5 and -6 mRNA. In eyes of the (mRen-2)27 rats, IGFBP6 mRNA expression was downregulated, and IGFBP5 mRNA expression was upregulated in the retina, ciliary body, cornea, and iris on induction of diabetes. Transcripts for IGF-I, type 1 IGF receptor, and IGFBP-1 were detected in the eyes, but no change in the mRNA level was induced by diabetes. Messenger RNA for IGFBPs-2, -3, and -4 were not detected in the (mRen-2)27 rats' eyes, either with or without diabetes.

As only (mRen-2)27 rats were used in these experiments, this precludes a specific analysis of the effect of the elevated renin alone on ocular distribution of the IGF system. There are previous data, however, on the expression of the IGF system mRNA in the Sprague-Dawley (SD) rat without diabetes. In
the nondiabetic state, IGF-I mRNA was identified in the retina and choroid, similar to the (mRen-2)27 rats. In comparison, IGF-I mRNA was not present in the cornea but instead was found in the ciliary processes. Type 1 receptor mRNA was similarly present in the retina and ciliary processes, but, in contrast to the (mRen-2)27 rats, was present in the cornea, conjunctiva, and lens. In the SD eyes, IGFBP-1 mRNA was not identified; IGFBP-2, -3, and -4 were present, but were not identified in the retina; and IGFBP-5 and -6 mRNAs were both expressed in the retina, choroid, and ciliary processes. After 6 months of STZ-induced diabetes in SD rats, after which there was little evidence of retinopathy, no changes in mRNA for components of the IGF system were seen (Burren C, personal communication, July 1998). Although the differences between these findings and those of the present study may reflect an effect of an activated RAS, they may also be indicative of the presence of retinopathic changes in the present study. Further evaluation of these possibilities would require a direct comparison between the two models.

IGFBP-1 mRNA expression in the retinas of the (mRen-2)27 rats appears unique. IGFBP-1 has not been identified in newborn33 or adult rat eyes,15 whereas in humans, vitreous IGFBP-1 appears to be of systemic rather than local origin.4 The exact explanation for the appearance of IGFBP-1 mRNA in this model is not clear, but due to its absence in both normal and diabetic SD rats and presence in both nondiabetic and diabetic (mRen-2)27 rats, suggests an effect of excess renin levels and hypertension rather than diabetes and hyperglycemia.

In contrast, mRNAs for IGFBP-2 and -3 have been identified in both newborn rat35 and bovine36 retinas but not in the adult
SD rat retina. The differences suggest possible species and developmental differences in IGFBP expression. In particular, retinal expression of IGFBP-2 and -3 mRNA was not found in adult SD or (mRen-2)27 rats, irrespective of whether they were nondiabetic or diabetic. Alternatively, these adult studies were performed with in situ hybridization, which may have lacked the sensitivity to detect low-level expression of these mRNAs.

Induction of diabetes combined with the overexpression of renin in the (mRen-2)27 rats did not result in alteration of IGF-I and IGF receptor mRNA expression. However, an overall increase in IGF-I activity may occur in the retina in this state as IGFBP-5 has been shown to have a net IGF-I-enhancing effect. IGFBP-6 has a net IGF inhibiting effect, although uniquely among the IGFBPs, IGFBP-6 has a much greater IGF-II-binding affinity and reduced IGF-I-binding affinity. Given that both VEGF and VEGF receptor mRNA levels are also increased in these animals, and part of the effect of VEGF on induction of neovascularization in the retina appears to be due to interactions with the IGF system, it may be that some of the observed effects on IGFBP levels are due to the VEGF system changes. VEGF has also been shown to increase IGFBP-5 mRNA expression in diabetic bovine endothelial cells. These findings suggest a potential shared pathway by which various pathogenic systems, including IGFs, the RAS, and VEGF may interact to result in the vascular changes of diabetic retinopathy.

Changes in the IGF system in diabetic retinopathy and other neovascular states are complex. In human studies of diabetic retinopathy, serum and vitreous levels of IGF-I protein are elevated, compared with levels in nondiabetic control animals, whereas vitreous levels of IGFBP-1 and -3 protein are also increased, but due to other systemic factors. In contrast, in the retina of humans with diabetes, there are lower levels of IGF-I mRNA, but elevated levels of IGF-I receptor mRNA, when compared with the nondiabetic state. In vitro analyses comparing human retinal endothelial cells (HRECs) of diabetic origin with HRECs of nondiabetic origin have shown that in the diabetic state, HRECs produce more IGF-I receptor and IGFBP-1, -2, and -5 protein and mRNA; less IGF-I protein and mRNA; and similar levels of other IGF system components. Therefore, careful analysis of changes in the IGF system in any new model of diabetic retinopathy is warranted.

This study did not examine IGF system protein levels to confirm that mRNA alterations led to changes in protein expression. Cytokine levels in the eye not only reflect local production but also reflect systemic production, which may also be altered in these animals. Our study was designed to examine local contributions of the IGF system, whereas examination of IGF proteins by immunohistochemistry would not distinguish between locally expressed and circulation-derived peptides.

The changes in levels of mRNA of the IGFBPs that were observed occurred in both the retina and in the anterior eye structures: cornea, ciliary body, and iris. These were the same areas of the eye where reproducible pathologic changes have been identified in the diabetic animals: iris neovascularization and retinal endothelial cell proliferation. Although no change in IGF-I or IGF receptor mRNA

**FIGURE 5.** Bright-field 3-μm paraffin-embedded sections showing IGFBP mRNA localization in the ciliary body from nondiabetic and STZ diabetic transgenic (mRen-2)27 rats. IGFBP6 labeling (arrows) in ciliary body appears more intense in nondiabetic (A) than diabetic (B) (mRen-2)27 rats. Counterstain, hematoxylin and eosin. Original magnification, ×40.

**FIGURE 6.** IGFBP6 mRNA localization in the m(Ren-2)27 rat eye. Results are expressed as arbitrary units of silver grain intensity in each tissue section studied and represent the mean ± SEM of results in four to six rats per group. The differences between nondiabetic and diabetic animals are all significant at P < 0.05. Abbreviations are as in Figure 4.
abundance were identified, the location of the IGFBP mRNA changes suggest a potential change in bioavailable IGF-I to the IGF receptor with a possible net IGF-enhancing effect induced by diabetes. This ocular regulation may have functional effects in the induction of diabetic eye disease. Our studies provide further support for the role of the IGF system in the development of diabetic retinopathy and new evidence for an interaction between the IGF system and RAS. This newly established model of diabetic eye disease offers the possibility of assessment of specific targeting of the IGF system to alter neovascular eye disease in diabetes.

References


