Insulin-like growth factor-I expression is not increased in the retina of diabetic BB/W-rats*

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Summary

A combination of immunocytochemistry, in situ hybridization and ligand binding were used to investigate the localization of IGF-I and its receptor in the retina of diabetic and non-diabetic BB/W-rats. Immunocytochemical localization revealed the presence of IGF-I in retinal pigment epithelium, ganglion cells, Muller cell processes and in microvessels. In most sites immunoreactivity was increased in the diabetic retina compared to that of non-diabetic BB/W-rats. In microvessels, however, immunoreactivity was decreased in diabetes. In situ hybridization using an antisense IGF-I riboprobe provided evidence of IGF-I synthesis in all retinal layers with a similar grain density in diabetic and non-diabetic rats. Autoradiographic localization of IGF-I receptors, using $[^{125}I]$-IGF-I binding, demonstrated a diffuse localization in all retinal layers, with an increase in diabetic animals. These findings suggest that IGF-I synthesis is not altered in the diabetic retina, and that the increased immunoreactivity of IGF-I detectable in the various layers of the retina from diabetic rats may be due to an increased uptake of blood-derived IGF-I suggested by increased receptor density in diabetic rats.

Introduction

Trophic factors probably play a role in the pathogenesis of diabetic complications [1]. In the diabetic retina, several trophic factors released into the extracellular space have been identified and may be important in promoting neovascularization [1]. A number of investigators have reported that insulin-like growth factor-I (IGF-I) expression is diminished in various tissues in experimental diabetic rats, whereas systemic hyperinsulinism induced by exogenous insulin administration augments IGF-I expression in certain tissues [2–4]. In clinical diabetes, however, conflicting results have been obtained with respect to IGF-I levels in vitreous and serum from diabetic patients [5,6].

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Insulin, IGF-I and -II are present in the central nervous system and exhibit important effects on neuronal tissue growth, survival and regeneration [7]. Although the IGF-I gene is expressed by the retina [8], it is not known to what extent retinal IGF-I is affected by diabetes. IGF-I receptors have been demonstrated in the retinal pigment epithelium (RPE), retinal rod cells, and the neural retina [9–11]. Moreover IGF-I binds to specific receptors on retina derived endothelial cells and pericytes and stimulates their DNA synthesis in vitro [12], which may suggest a potential role for IGF-I in retinal neovascularization. It is, however, not clear to what extent retinal IGF-I represents localized synthesis or uptake of exogenous blood-derived IGF-I.

In the present communication we report on the presence and synthesis of IGF-I and the localization of IGF-I receptors in the retina. Further, we attempt to compare the abundance of retinal IGF-I and its receptors in diabetic and non-diabetic rats.

Materials and Methods

Animals
Five spontaneously diabetic male BB/W-rats with a diabetes duration of 3 months, and age- and sex-matched non-diabetic BB/W-rats were used in this study. (NIH Colony, Department of Pathology, University of Massachusetts, Worcester, MA, U.S.A.). Animals were maintained in air-filtered metabolic cages with a 12 h off and on light cycle and given commercial rat chow (Wayne Lab Blox-F-6, Wayne Feed Division, Chicago, IL, U.S.A.) and water ad libitum. The animals were monitored with respect to body weight and blood glucose levels as previously described [13,14].

Tissue collection
Animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg of body weight) and killed by whole body perfusion with 4% paraformaldehyde in 50 mmol/l phosphate buffer (pH 7.4). The eyes were dissected and processed for paraffin embedding. Five micron thick sections on aminoalkylsialane coated slides were used for immunocytochemistry, in situ hybridization and autoradiographic localization of IGF-I receptors.

Immunocytochemistry
Rabbit anti-IGF-I antibody (kindly provided by Drs. J.J. von Wyk and L. Underwood) at a dilution of 1:100 was used for overnight incubation at 4°C. Equally thick tissue sections were stained as previously described [4] by employing a biotin-streptavidin amplified system (Stravigen kit, Biogenex Laboratories, San Ramon, CA, U.S.A.). Negative controls included sections incubated with non-immune serum, or preabsorbed antiserum incubated for 24 h in 4°C with excess IGF-I. The experimental conditions were identical for diabetic and control animals.

Immunodot analysis
Individual retinal samples from five diabetic and five non-diabetic animals were homogenized in 3 vol of 5 mmol/l Tris-HCl (pH 7.4), centrifuged at 45,000 × g for 40 min, and the supernatants were collected. Two dilutions of the homogenate containing 10 and 40 μg of protein were applied to a nitrocellulose membrane with a 96-well manifold. All samples were analyzed in one assay using previously described techniques [15]. Briefly, after blocking nonspecific proteins by incubation with 5% skim milk, the membranes were incubated with rabbit anti-IGF-I antibody (1:100 in Tris buffered saline, pH 8.0 containing 0.02% Tween 20). Membranes were washed, and the presence of IGF-I was revealed by incubation with anti-IgG alkaline phosphate substrate (Promega, Mississauga, ONT, Canada). The slope of the line of best fit for the relationship between the optical density area (independent variable, determined by densitometry), and protein concentration, (dependent variable), was determined for each sample and designated arbitrary units of IGF-I immunoreactivity.

In situ hybridization

Sections were deparaffinized and processed for in situ hybridization as previously described [16]. Hybridization was performed at 45°C in a solution containing 50% formamide, 0.3 mmol/l NaCl, 20 mmol/l Tris-HCl (pH 8.0), 1 × Denhardt's solution, 1.0 nmol/l EDTA, 0.005% ssDNA, 0.005% poly (A), 20 mmol/l mercaptoethanol, 0.05% yeast tRNA and 10% dextran sulphate. Riboprobes were prepared from a template constructed by subcloning an IGF-I cDNA into the pGEM-3Z vector. T7 and Sp6 polymerases were used for the generation of sense and antisense riboprobes [17]. Autoradiography was performed using Kodak NTB2 tracking emulsion. Slides were exposed for 5–10 days prior to development of the photographic emulsion.

[^{125}I]-IGF-I binding to retinal sections

Retinal sections were deparaffinized and receptor localization was carried out as previously described [18] using [^{125}I]-IGF-I. Binding was achieved by the addition of 10 mmol/l HEPES buffer (pH 7.6) containing 0.5% bovine serum albumin, 0.025% bacitracin, 0.0125% N-ethylmaleimide, 100 Kallikrein inhibitor unit/ml aprotinin and 0.64 nmol/l (4 × 10^5 cpm) [^{125}I]-IGF-I. Excess unlabelled IGF-I (300 ng) was added to
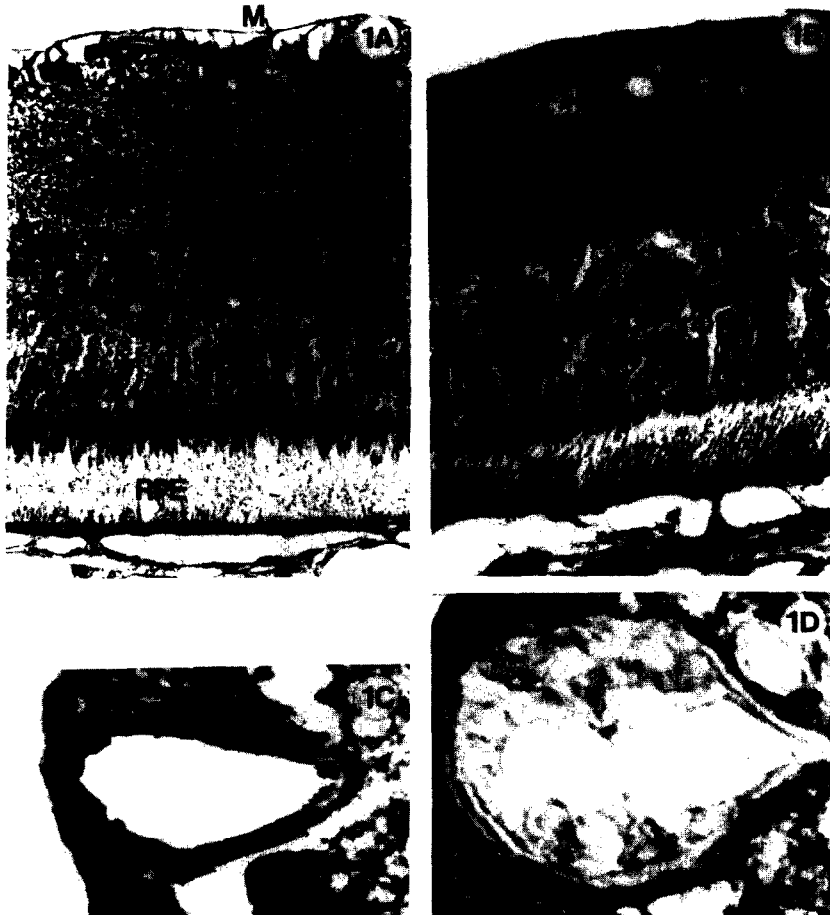

Fig. 1. Retinal micrographs showing IGF-I immunoreactivity in (A) non-diabetic BB-rat and (B) diabetic BB-rat. Microvessels from 'A' and 'B' are shown in 'C' and 'D', respectively. Note diminished immunostaining of endothelial cells in 'D' compared to 'C'. RPE = retinal pigment epithelium, G = ganglion cell, M = Muller cell process.

TABLE 1

Retinal IGF-I and IGF-I receptors

	IGF-I immunoreactivity (Arbitrary units)	IGF-I mRNA*	IGF-I receptors*
Control	0.005 ± 0.002	26.2 ± 1.8	52.5 ± 1.9
(n = 5)	ns	ns	P < 0.01
Diabetic	0.010 ± 0.003	27.6 ± 0.4	75.3 ± 5.5
(n = 5)			

* Expressed as number of grains in a circle of 1 cm radius drawn on micrographs with a total magnification of 1600 ×.

determine nonspecific binding. After 20 h of incubation the slides were exposed to Kodak NTB 2 emulsion and autoradiography was performed.

Autoradiographic sections were photographed with a final magnification of 1600 times for quantification of grain density. Random circles of 1 cm radius were drawn on various retinal layers, and the number of grains within each circle was counted. Mean numbers of grains/circle from each layer were determined and the mean number of grains/circle/retina calculated.

Statistical analysis

Data were expressed as mean ± SEM and analyzed by Student's t-test.

Results

Diabetic animals showed marked hyperglycemia (23.4 ± 2.4 mmol/l vs 5.1 ± 0.2 mmol/l in controls), polyuria, glycosuria and occasional ketonuria. At death, diabetic rats weighed 418.8 ± 21.1 g vs control rats who weighed 516.2 ± 7.6 g.

Immunocytochemistry

In non-diabetic retinas IGF-I was localized to the retinal pigment epithelium (RPE), ganglion cells, Muller cell processes and areas opposing the inner and outer limiting membranes. The outer plexiform layer and some cells of the inner nuclear layer showed positive immunoreactivity. The
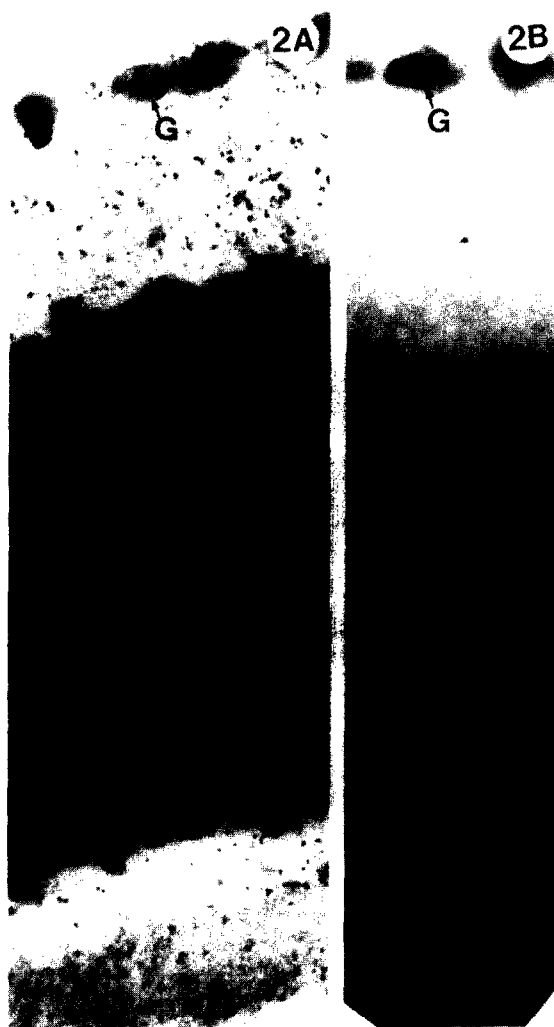

Fig. 2. Autoradiographs following in situ hybridization of retina with IGF-I riboprobes from a normal rat with antisense probe (A) and with sense probe in (B). G = ganglion cell.

outer nuclear layer showed no staining (Fig. 1). Positive staining was observed in the walls of retinal microvessels including both endothelial cells and pericytes (Fig. 1). In diabetic retinas the distribution of IGF-I appeared to be similar, however, the staining intensity was increased compared to controls (Fig. 1). Retinal glial and neuronal components as well as the RPE showed increased immunoreactivity in diabetic rats, whereas in retinal microvessels the immunoreactivity of endothelial cells was diminished (Fig. 1). No staining was observed in sections incubated with non-immune serum or with preabsorbed antiserum (data not shown).

Immunodot analysis showed a nonsignificant doubling of IGF-I immunoreactivity in the retinal extracts of diabetic rats compared to that of the controls (Table 1).

In non-diabetic retinas, autoradiographic signals following antisense IGF-I riboprobe hybridization showed a diffuse distribution in all retinal layers (Fig. 2). A similar distribution was observed in diabetic retinas. Only minimal hybridization was detected with the sense riboprobe (Fig. 2). Quantification of grain density revealed no difference between diabetic and control animals, (Table 1).

Specific binding of [^{125}I]-IGF-I was found in all retinal layers suggesting a widespread distribution of IGF-I receptors in the retina (Fig. 3). The density of grains, was significantly higher in retinas of diabetic rats compared to control retinas (Fig. 3, Table 1).
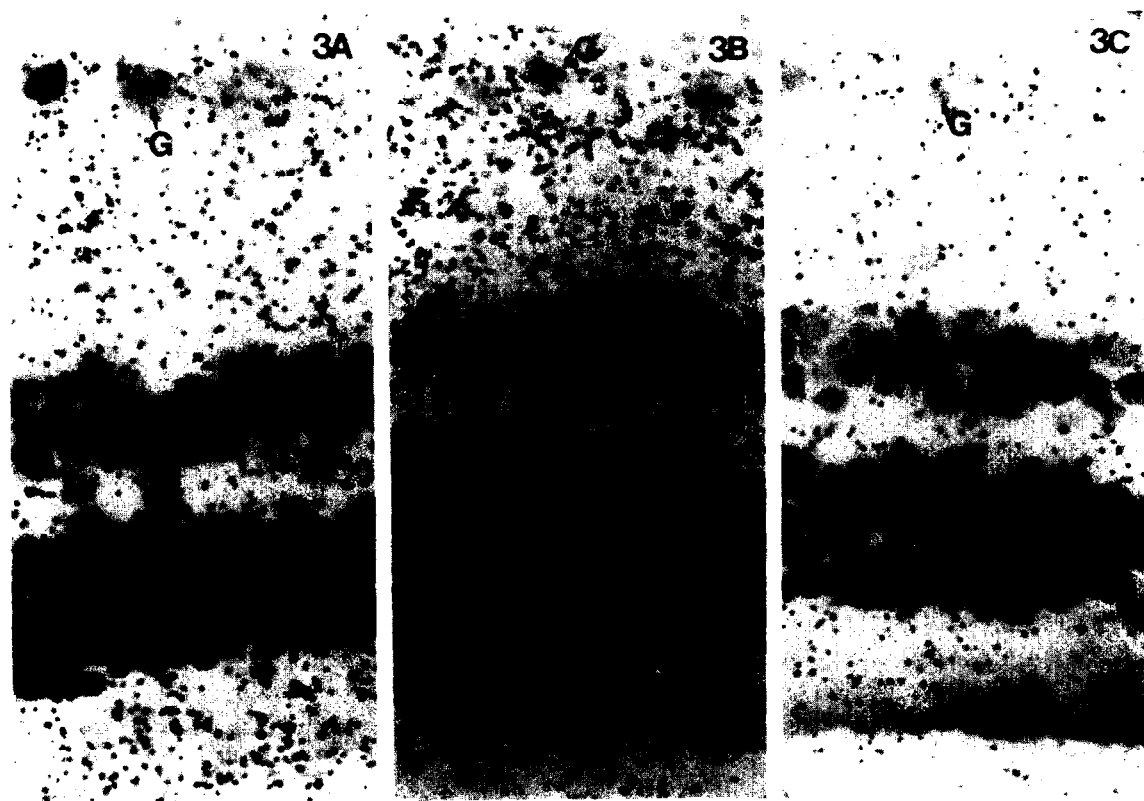

Fig. 3. Autoradiographs of retinas following receptor binding with [^{125}I]-IGF-I in (A) non-diabetic BB-rat and (B) diabetic rat. Incubation with unlabelled IGF-I together with [^{125}I]-IGF-I served as controls (C). Note increased grain density in 'B' compared to 'A'.

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Discussion

In the present study we have demonstrated the presence of IGF-I in the RPE, neuronal, and glial components of the normal retina as well as an increase in IGF-I immunoreactivity of these cell constituents in the retina of diabetic BB/W-rats. Surprisingly, endothelial cells of retinal capillary walls exhibited a decreased IGF-I immunoreactivity in diabetic rats. Composite retinal IGF-I was nonsignificantly increased in diabetic BB/W-rats.

This distribution of IGF-I immunoreactivity in the retina is similar to that of insulin and the localization of IGF-I receptors is consistent with recent data describing their presence in the RPE and in the neural retina [11,19,20]. Retinal IGF-I receptors have characteristics similar to those of insulin receptors and are more concentrated in the non-photoreceptor region of the retina [21]. In situ hybridization revealed a diffuse distribution of signals suggesting IGF-I synthesis by Muller cells, although we cannot exclude the possibility that IGF-I might be synthesized by other retinal cell components as well. Aldose reductase and insulin mRNA has been demonstrated in Muller cells [19,22]. Since both aldose reductase mRNA and protein content are increased in diabetic rat retinas [23] and IGF-I synthesis appears to be unchanged, these findings suggest, that IGF-I is not affected by an increased activity of the polyol pathway in the diabetic retina and that different mechanisms are probably involved in the regulation of these two genes.

IGF-I levels are elevated in serum and the vitreous from diabetic patients with proliferative retinopathy [5]. Some investigators have demonstrated a transient elevation of serum IGF-I during the early phases of neovascularization or during intensive insulin therapy with continuous subcutaneous insulin infusion [6,24]. Increased IGF-I in diabetic vitreous could possibly reflect increased IGF-I synthesized by retina. However, in the present study of diabetic BB/W-rats displaying the preretinopathic stage of retinal changes, increased IGF-I immunoreactivity, in the absence of increased IGF-I mRNA suggests increased uptake of IGF-I, possibly facilitated by a defect in the blood-retinal barrier, rather than an increase in locally synthesized IGF-I. This hypothesis is supported by the demonstration of increased [^{125}I]-IGF-I binding in the retina of diabetic BB/W-rats. In contrast, retinal microvessels demonstrated decreased immunoreactivity in diabetic BB/W-rats. How these findings, suggesting a downregulation of IGF-I receptor activity on diabetic retinal endothelial cells, can be reconciled with enhanced in vitro DNA synthesis by the same cells in response to IGF-I [12] remains to be investigated.

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