Localization of mRNAs for Insulin-like Growth Factor-I (IGF-I), IGF-I Receptor, and IGF Binding Proteins in Rat Eye

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Purpose. To localize mRNAs for insulin-like growth factor (IGF)-I, IGF-I receptor (IGF-IR), and IGF binding protein (BP)-I to IGFBP-6 in the rat eye.

Methods. cDNA sequences for IGF-I, IGF-IR, and IGFBP-1 to IGFBP-6 were used to synthesize 35S-CTP labeled antisense and sense probes for in situ hybridization on 5-μm sections of the rat eye, including the retina, choroid, sclera, ciliary body, and cornea.

Results. IGF-I mRNA was demonstrated over ganglion cells of the retina and endothelial cells of the choroid and ciliary processes. IGF-IR mRNA showed more extensive distribution, localizing to the retinal ganglion cell layer, inner nuclear layer, and outer limiting membrane and also the outer nonpigmented epithelium of the ciliary processes and cornea, conjunctiva, and lens. IGFBP-2 mRNA localized to outer nonpigmented epithelia of the ciliary processes and the germinal layer of corneal epithelium as well as iris, conjunctiva, and sclera. Messenger RNAs for IGFBP-3 to IGFBP-6 localized to choroidal endothelial cells and chromatophores and also to the inner pigmented epithelium of the ciliary processes. Messenger RNAs for IGFBP-5 and IGFBP-6 were seen in the inner and outer nuclear layers of the neural retina. IGFBP-1 mRNA was not detected within the rat eye.

Conclusions. Using in situ hybridization, we have demonstrated mRNAs for IGF-I, IGF-IR, and IGFBP-2 to IGFBP-6 in specific histologic layers of the retina, choroid, ciliary body, and cornea in the rat. The characterization of the IGF system in vivo suggests specific roles in the normal eye and provides a basis for studying the IGF system in eye pathology. Invest Ophthalmol Vis Sci. 1996;37:1459–1468.

The insulin-like growth factors (IGF)-I and -II are peptides that play an important role in cell proliferation and cell differentiation, and they have been identified in many tissues throughout the body.¹ Actions of IGF-I, the predominant IGF in adult life, are partially growth hormone dependent and are mediated by binding to the IGF-1 receptor. In addition, six IGF-binding proteins (IGFBP-1 to IGFBP-6) modulate IGF-I action, either by binding circulating IGF or by enhancing IGF binding to receptors.²⁻⁴

The importance of IGF-I within the eye is highlighted by the alterations in the IGF system occurring under pathologic conditions, such as the increased amounts of vitreal IGF-I present in proliferative diabetic retinopathy and other neovascular eye disease.⁵ Further evidence suggesting the IGF system in proliferative diabetic retinopathy has been that serum IGF-I levels are elevated⁶ and that hypophysectomy, by ablationing growth hormone, has been successfully used for severe proliferative diabetic retinopathy.⁷

In the eye, identification of elements of the IGF system has focused primarily on IGF-I and its receptor, whereas there is incomplete data regarding the IGFBPs. IGF-I, IGF-II, IGFBP-2, and IGFBP-3 are present in the vitreous; however, sites of synthesis have not been established.⁸ In vitro data suggest that endothe-
Figure 1. Localization of insulin-like growth factor (IGF)-I mRNA in paired bright (A) and dark (B) fields of retina hybridized with IGF-I antisense probe showing an intense signal on ganglion cells (arrows). Magnification ×330. Bar = 30 μm. V = vitreous; AH = aqueous humor.

(C) Dark field of retina hybridized with IGF-I sense probe. Magnification ×330. Paired bright (D) and dark (E) fields of ciliary processes showing IGF-I mRNA on the endothelium of blood vessels (arrows). Bar = 25 μm.

Hyal cells, pericytes, and retinal pigment epithelial (RPE) cells secrete IGF-I and IGFBPs, and have IGF-I receptors, although the in vivo significance of these findings is unclear. IGF-I mRNA has been demonstrated in rat retina by in situ hybridization, and distribution of IGF-I receptor mRNA has been shown in mouse retina. Documentation of cellular mRNA expression for the IGFBPs is limited to IGFBP-2 mRNA, which has been demonstrated in the retina. We have undertaken a systematic study of components of the IGF system in the adult rat eye to determine the sites of expression of IGF-I, IGF-I-R, and IGFBPs mRNA throughout the eye.

Materials and Methods

Animals and Tissue Fixation

Adult male Sprague–Dawley rats (N = 3), each 12 to 14 weeks of age and each weighing 200 to 250 g, were used in this study. Animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were prepared for vascular fixation as previously described. Briefly, animals were anesthetized with Nembutal (60 mg/kg body weight; Boehringer Ingelheim, Artarmon, Australia) by intraperitoneal injection. The abdominal aorta was cannulated with an 18-gauge needle filled with Hank’s balanced salt solution (CSL, Melbourne, Australia). Perfusion was begun at 200 mm Hg with 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4, and the inferior vena cava was severed adjacent to the renal vein. Tissue fixation was achieved after 5 minutes (100 to 200 ml of fixative). Whole eyes were then excised and placed in 4% paraformaldehyde for 24 hours at room temperature. Tissues were processed through graded ethanol, cleared in two changes of Histolene (Riverstone, Australia) and infiltrated with paraffin wax. Five-micron sections were cut and placed on aminoalkylsilane-treated slides and baked overnight at 37°C.

Riboprobe Transcription

Complementary DNA for rat IGFBP-1 to IGFBP-6 were kindly provided by Dr. S. Shimasaki (Whittier Institute, La Jolla, CA). IGF-I and IGF-I-receptor cDNA probes were kindly provided by Dr. C. T. Roberts, Jr. (Oregon Health Sci University, Portland, OR.). Antisense and sense RNA probes were synthesized in 20-μl reactions containing 100 μCi[35S] deoxy-cytidine triphosphate (New England Nuclear, Dupont, Boston, MA) 1000 to 1500 Ci/mmol; 40 nmol/L Tris-HCl, pH 7.5; 6 mmol/L MgCl2; 2 mmol/L spermidine; 10 mmol/L NaCl; 10 mmol/L dithiothreitol; 660 μmol/L of adenosine triphosphate, guanosine triphosphate, and uridine triphosphate; 20 U RNasin; 100 ng linearized DNA template; and 20 U T3, T7, or Sp6 polymerase.
FIGURE 2. Localization of insulin-like growth factor (IGF-I) receptor (IGF-IR) mRNA in rat eye. V = vitreous; AH = aqueous humor; C = lens capsule; S = lens stroma. Paired bright (A) and dark (B) fields of retina hybridized with IGF-IR antisense probe showing intense signal on ganglion cells (arrows), inner nuclear layer (INL), and outer limiting membrane (asterisk). Magnification X400. Bar = 25 μm. Paired bright (C) and dark (D) fields of ciliary processes showing IGF-IR mRNA in nonpigmented ciliary epithelium (arrows). Magnification X165. Bar = 61 μm. (E) Bright field of lens showing IGF-IR mRNA expression localized to lens epithelium (arrows). Magnification X530. Bar = 19 μm. (F) Bright field of retina hybridized with IGF-IR sense probe. Bar = 22 μm.

(Fromega, Madison, WI). For RNA longer than 200 bp, an excess of cold deoxy-cytidine triphosphate was added (170 pmol). The reaction was then incubated at 37°C for 90 minutes, after which the DNA template was digested with 1 U deoxyribonuclease for 15 minutes at 37°C. Complementary RNA was precipitated with yeast RNA as a carrier. Precipitated IGF-I, IGF-I-R, and IGFBP RNA probes were adjusted to an average length of 150 bases by alkaline hydrolysis. The average specific activity generated by the riboprobes was approximately $2 \times 10^9$ cpm/μg RNA.

In Situ Hybridization
Tissue sections were prepared for in situ hybridization as previously described. Briefly, sections were de-waxed in Histolene, hydrated by passage through graded ethanol and ultrapure water (Liquipure Continental Water System, Melbourne, Australia), equilibrated in P buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA, pH 8) and treated with Pronase E (Sigma Chemical, St. Louis, MO), 125 μg/ml in P buffer for 10 minutes at 37°C. Sections were then washed in 0.1 M sodium phosphate buffer (pH 7.2), fixed in 4% paraformaldehyde-phosphate-buffered saline, at room temperature for 10 minutes, rinsed again in 0.1 M sodium phosphate buffer (pH 7.2), and ultrapure water, dehydrated in 70% ethanol, air dried, and hybridized. Antisense and sense riboprobes ($5 \times 10^5$ cpm/25 μl hybridization buffer) were added to hybridization buffer (300 mM NaCl; 10 mM Tris-HCl, pH 7.5).

FIGURE 3. Localization of insulin-like growth factor binding protein (IGFBP)-2 mRNA in rat eye. Paired bright (A) and dark (B) fields of ciliary processes hybridized with IGFBP-2 antisense probe showing specific localization to ciliary epithelium (arrows). Magnification ×165. Bar = 61 μm. (C) At a higher magnification, signal is seen in the cytoplasm of nonpigmented ciliary epithelium (arrows). Magnification ×396. Bar = 25 μm. AH = aqueous humor. (D) A section of ciliary processes hybridized with IGFBP-2 sense probe. Magnification ×462. Bar = 22 μm. (E,F) Bright field of cornea showing intense IGFBP-2 expression in basal cells of the anterior stratified epithelium (arrows), fibroblasts in corneal stroma (double arrows), and posterior epithelium (asterisk). Magnification ×330. Bar = 30 μm. AC = anterior chamber.

pH 7.5; 10 mM Na2HPO4, pH 6.8; 5 mM EDTA, pH 8; 1 x Denhardt’s solution; 0.8 mg/ml yeast RNA; 50% formamide; and 10% dextran sulfate), heated to 85°C, and 25 μl of hybridization buffer added to the sections. Coverslips were placed on the sections, and the slides were put in humidified (50% formamide) chambers at 59.8°C for 14 to 16 hours. Slides were washed in 2 x standard sodium citrate (2 x SSC: 0.3 M NaCl, 0.3 M Na2CaH2O7·2H2O) containing 50% formamide at 55°C to remove coverslips, then washed in 2 x SSC containing 50% formamide at 55°C for 1 hour. Sections were rinsed three times in RNase buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA pH 8; 0.5 M NaCl) at 37°C, treated with 150 μg/ml RNase A in RNase buffer for 1 hour at 37°C, and then washed in 2 x SSC at 55°C for 45 minutes. Finally, sections were dehydrated through graded ethanol, air dried, and exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, New York) for 3 days at room temperature. Slides were dipped in Amersham LM-1 emulsion (Amersham, Buckinghamshire, UK) and stored with desiccant at 4°C for 10 to 21 days, developed in D-19 (Kodak) at room temperature for 4 minutes, washed in 1% acetic acid, and counterstained with hematoxylin and eosin. Sections were photographed using an Olympus (Tokyo, Japan) photomicroscope and using Agfa-Copex (Leverkusen, Germany) black-and-white film 50 ASA.

RESULTS

In situ hybridization studies for each component of the IGF system showed specific hybridization with antisense probes indicating distinct and discrete areas of mRNA expression with varying abundance. In sections of adult rat eye hybridized with sense probes, there
IGF-I, IGF-I Receptor, and IGFBP mRNAs in Rat Eye

FIGURE 4. Localization of insulin-like growth factor binding protein (IGFBP)-3 mRNA in rat eye. V = vitreous; C = choroid; B = choroidal blood vessel; AH = aqueous humor. Paired bright (A) and dark (B) fields of retina hybridized with IGFBP-3 antisense probe showing intense hybridization in chromatophores and fibroblasts of the choroid (arrows). Magnification $\times$330. Bar = 30 $\mu$m. (C) Dark field of retina hybridized with IGFBP-3 sense probe. Magnification $\times$330. Paired bright (D) and dark (E) fields of ciliary processes showing localization of IGFBP-3 mRNA to chromatophores (arrows). Bar = 25 $\mu$m.

was minimal nonspecific hybridization. The following description will address IGF-I, IGF-I receptor (IGF-IR), and each IGFBP separately. The specific hybridization findings are shown in Figures 1 to 7, and findings for the sense probes are given in a number of the figures. Table 1 summarizes the findings anatomically.

IGF-I mRNA
IGF-I mRNA localized to the ganglion cell layer of the neural retina (Figs. 1A, 1B). No specific localization was seen in eye sections hybridized with the sense probe (Fig. 1C). Endothelial cells in ciliary processes showed more intense hybridization to IGF-I mRNA than the choroid (Figs. 1D, 1E). IGF-I mRNA was not detected within the retinal capillary networks, the cornea, or the lens.

IGF-I-Receptor mRNA
IGF-I receptor mRNA was distributed widely throughout the neural retina. Specific hybridization to IGF-IR mRNA was found in the ganglion cell layer, the inner nuclear layer, and the outer limiting membrane (Figs. 2A, 2B). In the ciliary body, IGF-IR hybridization was located along the nonpigmented ciliary epithelium (Figs. 2C, 2D). IGF-IR mRNA also was detected in lens (Fig. 2E), the anterior epithelium of the cornea (not shown), and the conjunctival epithelium (not shown). No specific hybridization was seen when the IGF-IR sense probe was hybridized to sections of eye (Fig. 2F).

IGFBP-1 mRNA
No specific hybridization with the IGFBP-1 antisense probe was seen in any of the compartments of the eye.

IGFBP-2 mRNA
There was prominent hybridization to IGFBP-2 mRNA along the outer nonpigmented epithelium of the ciliary processes (Figs. 3A, 3B, 3C). Specific localization was not detected in sections hybridized with the IGFBP-2 sense probe (Fig. 3D). Within the cornea, IGFBP-2 mRNA showed intense hybridization in the basal layer of the anterior epithelium, fibroblasts within the corneal stroma, and the posterior epithelium adjacent to the aqueous humor (Figs. 3E, 3F). Specific hybridization for IGFBP-2 mRNA also was seen in fibroblasts within the iris and sclera and the conjunctival columnar epithelium (not shown). There was no specific hybridization for IGFBP-2 mRNA within any layers of the neural retina or the choroid.
IGFBP-3 mRNA
IGFBP-3 mRNA was detected in fibroblasts and chromatophores within the choroid (Figs. 4A, 4B) and occasionally was detected in endothelial cells. In sections hybridized with the IGFBP-3 sense probe, no specific hybridization was detected (Fig. 4C). IGFBP-3 mRNA localized to the chromatophores and, to a lesser extent, to endothelial cells within the ciliary processes (Figs. 4D, 4E). IGFBP-3 mRNA was not identified within the cornea or the neural layers of the retina.

IGFBP-4 mRNA
Intense hybridization for the IGFBP-4 mRNA antisense probe was detected within the choroid and was localized to endothelial cells, fibroblasts, and chromatophores (Figs. 5A, 5B, 5C). IGFBP-4 mRNA localized to the endothelial cells in ciliary processes (Figs. 5E, 5F) but was not identified in retinal capillaries, the cornea, or the lens.

IGFBP-5 mRNA
IGFBP-5 mRNA localized to the retinal capillary network, ganglion cells, and the internal aspect of the inner nuclear layer (corresponding to the location of amacrine cells) (Figs. 6A, 6B). Within the choroid, IGFBP-5 mRNA was prominent in endothelial cells, chromatophores, and fibroblasts (Figs. 6A, 6B). IGFBP-5 mRNA was localized to endothelial cells within the ciliary processes (Figs. 6C, 6D). IGFBP-5 mRNA was not detected within the cornea or lens.

IGFBP-6 mRNA
IGFBP-6 mRNA localized to ganglion cells, the inner nuclear layer, and the outer limiting membrane (Figs. 7A, 7B). IGFBP-6 mRNA also localized to the endothelial cells.
IGF-I, IGF-I Receptor, and IGFBP mRNAs in Rat Eye

**FIGURE 6.** Localization of IGFBP-5 mRNA in rat eye. V = vitreous; AH = aqueous humor. Paired bright (A) and dark (B) fields of retina hybridized with insulin-like growth factor binding protein (IGFBP)-5 antisense probe showing hybridization in endothelium of retinal blood vessels (asterisk), ganglion cells (double asterisk), and the inner nuclear layer (INL). In the choroid, endothelium of blood vessels (arrow) and stroma (double arrows) showed intense hybridization for IGFBP-5 mRNA. Magnification ×230. Bar = 44 μm. Paired bright (C) and dark (D) fields of ciliary processes showing localization of IGFBP-5 mRNA to endothelium of blood vessels (arrow). Bar = 30 μm.

**DISCUSSION**

We have completed a comprehensive survey of IGF-I, IGF-IR, and IGFBP mRNAs in the adult rat eye that showed mRNAs were localized to specific histologic layers. The retina showed mRNAs for IGF-I, IGF-IR, IGFBP-5, and IGFBP-6, whereas the adjacent choroid demonstrated mRNAs for IGF-I and IGFBP-3 to IGFBP-6. The ciliary processes showed specific distribution of IGF-I, IGF-IR, and IGFBP-2 to IGFBP-6 mRNAs. Less abundant IGF-I mRNA was present in the iris, cornea, sclera, and conjunctiva, and IGFBP-2 mRNA was present in the lens, cornea, sclera, and conjunctiva. These in situ hybridization studies significantly extend existing knowledge of the IGF system in ocular tissues.

Our findings clearly show IGF-I mRNA localization in retinal ganglion cells, choroid, and ciliary processes. Documentation of IGF-I mRNA in the retina supports the ongoing role of IGF-I in adult life, whereas an earlier study emphasized the importance of IGF-I only in the developing retina. The IGF-I mRNA is likely to be physiologically significant because IGF-I is known to have anabolic effects on neural and glial cells in vitro and to influence postnatal neural tissue survival. Adjacent choroidal IGF-I mRNA suggests that locally produced IGF-I may exert a paracrine effect and may contribute to the IGF-I protein identified by immunohistochemistry in the neural retina by other workers. We did not identify IGF-I mRNA in situ in retinal endothelial cells that contrasts with in vitro studies.

Documentation of IGF-IR mRNA expression over the ganglion cell and bipolar layers is consistent with earlier IGF-I binding studies. IGF-IR localization in these neuronal layers suggests that IGF-I acts extensively throughout the retina. The outer limiting membrane was a further site of IGF-IR mRNA expression. This is important because it has been suggested that the RPE layer secretes IGF-I into this region around the rods and cones. We showed IGFBP-5 mRNA co-localization with IGF-IR mRNA in the ganglion cell layer and bipolar layer of the retina. This suggests that IGFBP-5 may have an IGF-I modulating role in the retina because IGFBP-5 binds to extracellular matrix, thus potentially retaining IGF-I in the tissue. Although IGFBP-5 mRNA also showed co-localization with IGF-IR mRNA, this binding protein is not matrix...
A paracrine method of IGF-I action can be proposed because the sites of IGF-IR and IGF-I synthesis are closely situated, similar to other neural tissues. The distribution and co-localization of IGFBP-5 mRNA with IGF-I mRNA in retinal ganglion neurons is analogous to their distribution in mitral neurons, a similar cell type, in the olfactory bulb, where it is proposed that IGFBPs may be involved in neuronal transport of IGF-I.

IGFBP-2 may have a role in the developing retina, although it appears to have a lesser role in ongoing retinal function because we did not detect IGFBP-2 mRNA in adult rat retina. IGFBP-2 mRNA has been detected in the mouse photoreceptor layer. The reason for the difference between the rat and mouse retina is unclear. Species variation is possible, although components of the IGF system have been shown to be highly conserved across mammalian species. Although RPE cells express IGFBP-2 mRNA in vitro, we found no evidence of IGFBP-2 mRNA in RPE cells, which suggests that they do not express significant levels of IGFBP-2 in vivo. IGFBP-1 appears to have a negligible role in any region of the eye because IGFBP-1 mRNA was absent in our study of adult rats and in the developing retina.

Ciliary processes were a prominent site of synthesis of mRNA for all the IGFBPs, except IGFBP-1. In the ciliary body, the only binding protein previously demonstrated was IGFBP-2. We have shown IGFBP-2 mRNA to be abundant in adult life with specific distribution to the outer nonpigmented epithelium. Furthermore, we demonstrated expression of IGFBP-3 to IGFBP-6 mRNAs in the ciliary body, which has not been shown before. IGFBP-3 to IGFBP-6 mRNAs were expressed in the interior of the ciliary processes in chromatophores and endothelial cells, similar to in vitro retinal endothelial cell data.

The IGFBP-2 mRNA in the outer epithelium of the ciliary processes may be the origin of IGFBP-2 identified in the aqueous. This postulated secretion of IGFBP-2 into the aqueous humor has an interesting parallel with other areas of the central nervous system; IGFBP-2 synthesis has been demonstrated in the choroid plexus, where secretion into cerebrospinal fluid leads to postulated interaction with IGFs at distant sites in the brain. Because there is some communication between the posterior compartment of aqueous humor and the vitreous humor, it is possible that the ciliary processes are the source of IGFBP-2 and IGFBP-3 identified in the vitreous humor. IGFBP-4 to IGFBP-6 have not been demonstrated within the vitreous, and the mRNA expression identified within the ciliary processes suggests local action for these IGF binding proteins.

Within the cornea, the intense specific hybridization for IGFBP-2 mRNA along the internal epithelium may represent an additional source of aqueous IGFBP-2. The other sites of IGFBP-2 mRNA expression in the cornea were the superficial stratified epithelium and fibroblasts of the substantia propria, which may contribute to paracrine IGF action. This proposed IGF action in the cornea is reinforced by the identification of IGF-IR mRNA expression, which suggests that IGFBP-2 may influence targeting of IGFs to the receptors.

This work demonstrates that IGF-I mRNA is expressed predominantly in neuronally rich areas of the retina in vivo, analogous to our earlier findings in the olfactory bulb. This contrasts with much work on the IGF system in the retina, which focused previously on such vascular and connective tissues as endothelial cells and pericytes. Insulin-like growth factor action is likely to be complex and specific for each component of the eye with the potential effect of IGF influenced
TABLE 1. Distribution of mRNAs for IGF-1, IGF-1 Receptor, and IGFBP-1 to IGFBP-6 in Normal Rat Eye

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Inner nuclear layer contains nuclei of bipolar, horizontal, and amacrine neurones and Müller cells.
Outer limiting membrane represents end feet processes of Müller cells wrapped around photoreceptors.

by regional expression of the IGFBPs. The distribution of IGF-IR mRNA indicates neural tissue sites of action, and the local expression of IGFBP-5 and IGFBP-6 mRNAs suggests potential targeting of IGF action to these tissues. The ciliary processes are an important site of expression of mRNAs for IGFBP-2 to IGFBP-6, and these ciliary processes potentially represent the source of IGFBP-2 and IGFBP-3 in the aqueous and vitreous humors. Further studies are in progress to explore a possible role in the adult eye for IGF-II, particularly in view of the abundance in many regions of the eye of IGFBP-2, IGFBP-5, and IGFBP-6 mRNA, which are relatively IGF-II selective binding proteins.

Our documentation of the sites of mRNA expression using in situ hybridization in the eye significantly expands existing knowledge and is unique particularly in relation to the IGF binding proteins 1 to 6. The localization of mRNA for IGF-I, IGF-IR, and the IGFBPs to specific histologic layers of the retina, choroid, ciliary body, and cornea suggests that insulin-like growth factors have extensive sites of action within the eye. This systematic study of the components of the IGF system in the normal eye provides the basis for studies in such diseases as hypoxic eye disease, proliferative vitreoretinopathy, and diabetic retinopathy.

Key Words
ciliary epithelium, in situ hybridization, insulin-like growth factors, retinal ganglion cells, retinal vasculature

References

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