Quantification and Localization of the IGF/Insulin System Expression in Retinal Blood Vessels and Neurons during Oxygen-Induced Retinopathy in Mice

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PURPOSE. Retinopathy is a result of pathologic angiogenesis influenced by insulinlike growth factor (IGF)-1. The authors examined the local expression of the IGF/insulin family.

METHODS. In retinas with and without oxygen-induced retinopathy, the authors assessed with real-time RT-PCR mRNA expression of the IGF-1 receptor (IGF-1R), insulin receptor (IR), IGF-1, IGF-2, insulin (Ins2), and IGF-binding protein 1 (IGFBP1) to IGFBP6 in total retina from postnatal day (P) 7 to P35 to examine changes over time with the induction of retinopathy and at P17 on laser-captured retinal components to quantitatively localize mRNA expression in the ganglion cell layer, the outer nuclear layer, the inner nuclear layer, normal blood vessels, and neovascular tufts.

RESULTS. IGF-1R and IR are expressed predominantly in photoreceptors and in vessels, with scant expression in the rest of the neural retina. IGF-1R expression is more than 100-fold greater than IR. The major local growth factor (expressed in photoreceptors and in blood vessels) is IGF-2 (approximately 1000-fold greater than IGF-1). IGF-1 (approximately 600 copies/10^6 cyclophilin) is expressed throughout the retina. IGFBP2, IGFBP4, and IGFBP5 expression is unchanged with increasing retinal development and with the induction of retinopathy. In contrast, IGFBP5 expression increased more than 5-fold with hypoxia, found in neovascular tufts.

CONCLUSIONS. IGF-1R, IR, and the ligand IGF-2 are expressed almost exclusively in photoreceptors and blood vessels. IGFBP3 and IGFBP5 expression increases in neovascular tufts compared with normal vessels. IGF-1 is expressed throughout the retina at much lower levels. These results suggest cross-talk between vessels and photoreceptors in the development of retinopathy and retinal vasculature. (Invest Ophthalmol Vis Sci. 2009;50:1831-1837) DOI:10.1167/iovs.08-2905

Many ocular diseases, including diabetic retinopathy and retinopathy of prematurity, are a result of pathologic angiogenesis. At present, we do not understand the contribution of local versus systemic regulation of normal and pathologic vessel growth. Insulinlike growth factor (IGF-1) is an important growth factor involved in retinal angiogenesis.1,2 Low systemic levels of IGF-1 correlate with the degree of retinopathy of prematurity in the clinical setting.3,4 and IGF-1 is implicated in diabetes.5-7 Insulin receptors and IGF-1 receptors in retinal vascular endothelial cells have been implicated in pathologic angiogenesis.8 Although mRNAs for IGF-1, IGF-1 receptor (IGF-1R), and IGF-binding protein 2 (IGFBP2) through IGFBP6 have been described in specific histologic layers of the normal rat retina, choroid, ciliary body, and cornea,9 there has been to date no comprehensive survey of the relative local expression (and, by implication, contribution) of IGF-1 and the IGF/insulin family, in retinal blood vessels and neurons during the induction of retinopathy. This is important because normal retinal signaling through the insulin receptor and the IGF receptor might influence retinal vascular growth. There is also evidence that IGF-2 is expressed in hemangiomatous, proliferative vascular tumors of infancy. The role of IGF-2 in other neovessels has not been established. We sought to find the localization and quantification of insulin, IGF-1R, and the ligands IGF-1, IGF-2, and their binding proteins in the retina to better understand the role of the IGF family in retinopathy.

Diabetes, which is characterized by abnormal insulin levels and function, leads to retinal microvascular disease and diabetic retinopathy. Insulin signaling may play a role in this process, and mice lacking either insulin receptor (IR) or IGF-1 receptor (IGF-1R) in endothelial cells undergo normal vascular development but are protected from hypoxia-induced retinal neovascularization.10 Total loss of IGF-1R in the neural retina and in blood vessels, however, does suppress normal retinal vascular development,1,10 suggesting that IGF-1R in the neural retina influences vascular development. However, localization and quantification of IGF-1R and IR in the neural retina have not been completely established.11 IGF-1 and IGF-2 are potent mitogens that stimulate proliferation but not differentiation in vivo.13 Genetic evidence suggests that in vivo, IGF-1 signals only through the tyrosine kinase IGF-1R, whereas IGF-2 signals through both IGF-1R and IR.14 In contrast, the IGF-2 receptor (IGF-2R) does not signal but acts as an IGF-1 sink.14 The IGFs also have high affinity for a family of six structurally related IGFBPs, IGFBP1 to IGFBP6, which regulate the bioavailability of the IGFs in the circulation. The IGFBPs also modulate the activity of IGFs at the cellular level, either inhibiting or enhancing IGF action.15 There are also independent actions of the IGFBPs. In vivo, with decreasing IGFBP3, there is a dose-dependent increase in oxygen-induced retinal vessel loss and vascular regrowth after vascular destruct-
tion, resulting in less retinal neovascularization.\textsuperscript{14} IGFBP-7 constitutes a low-affinity member of the IGFBP superfamily and primarily functions as a modulator of cell growth in an IGF-independent manner.\textsuperscript{17}

It is not possible to accurately assess local protein levels within retinal neuronal layers and vessels because immunohistochemistry is not quantitative and laser-capture microdissection of retinal layers does not provide sufficient quantities of protein for quantitative analysis. In addition, other methods of protein analysis are problematic because many of the proteins are soluble and diffusible and because the levels in serum are up to 20 to 50 times greater than in tissue,\textsuperscript{18} making accurate assessment of tissue contribution difficult. We have quantitatively evaluated mRNA expression of the IGF-I family with real-time RT-PCR on laser-captured retinal components. With the time course of local production of IGF-1, IR, IGF-1, IGF-2, Ins2, and IGFBP mRNA and their quantified localization in the retina, we can begin to address the question of the contributions and functions of the IGF system during retinopathy.

**MATERIALS AND METHODS**

**Animals**

These studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Children’s Hospital Animal Care and Use Committee. C57BL/6N (Taconic Farms, Germantown, NY) mice were used for the study.

**Model of Ischemia-Induced Proliferative Retinopathy**

Retinopathy was induced in pups (with nursing mothers) by exposure to 75% ± 2% O\textsubscript{2} from postnatal day (P) 7 to P12, followed by exposure to room air until kill.\textsuperscript{19} Age-matched control C57BL/6 mice were housed in room air continuously.

**Isolation of Retinal RNA and cDNA Preparation**

At selected time points (P8, P10, P12, P15, P17, P33), animals were killed by lethal doses of tribromoethanol (Avertin; Sigma, St. Louis, MO), and retinas were dissected immediately, flash frozen, and stored at −80°C. RNA was extracted (RNAsesy Mini RNA extraction kit; Qiagen, Chatsworth, CA) in accordance with the manufacturer’s instructions incorporating a mortar and pestle followed by Qiagen column (QIAshredder) for homogenization. At each time point, total mRNA from 12 pooled retinas (to reduce biological variability) from 12 mice with ischemia-induced proliferative retinopathy or normal age-matched control mice was analyzed. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)-treated H\textsubscript{2}O. To generate cDNA, RNA was first treated with DNase I (Ambion, Austin, TX) to remove contaminating genomic DNA. The DNase-treated RNA (100 ng) was then converted to cDNA using murine leukemia virus reverse transcriptase (Gibco BRL, Life Technologies, Bethesda, MD). cDNA samples were portioned into aliquots and stored at −20°C.

**Laser-Capture Microdissection**

For laser microdissection, eyes with oxygen-induced retinopathy (OIR) or control eyes at P17 were enucleated and embedded in OCT compound, cryosectioned at 10 μm, mounted on RNase-free polyethylene naphthalate (PEN) foil glass slides (11505189; Leica Microsystems, Deerfield, IL), and immediately stored at −80°C. Frozen sections were immersed in 50% ethanol, 75% ethanol, and DEPC H\textsubscript{2}O for 15 seconds each, followed by 30 seconds of staining solution (Arcturus HistoGene; Molecular Devices, Eugene, OR) and a rinse in DEPC H\textsubscript{2}O. After they were air dried for 10 minutes, the sections were laser microdissected for vessels and retinal neuron layers (LMD 6000; Leica Microsystems). Each population was estimated to be greater than 95% homogenous as determined by microscopic visualization of the captured cells. Material was collected directly into lysis buffer (RNasey Micro kit; Qiagen). RNA was subsequently isolated according to the manufacturer’s protocol, treated with DNase, and converted to cDNA.

**Real-Time Quantitative RT-PCR**

Real-time quantitative RT-PCR primer sets targeting murine IGF-1, IGF-2, IGF-1R, IGF-2R, IR, and IGFBP1 to IGFBP7 were designed using the primer bank verified database\textsuperscript{20}; sequences (5′ to 3′) are listed in Table 1. Primers for murine Ins2 were difficult to design, and a verified set was purchased from Qiagen (Quantitect Assays). Reactions including master mix (SYBR Green I; Qiagen) and 1 ng cDNA template were run in triplicate on a PCR system (7300 Real-Time; Applied Biosystems, Foster City, CA), and each target gene was run concurrently with a constitutively expressed control gene for normalization. After amplification, first-derivative melting curve analysis was used to confirm the specificity of the target product. Copy numbers for each target gene were normalized to 1 million copies of cyclophilin A. By comparison to a known dilution of cyclophilin A copies, the data approximate absolute copy numbers. However, some data have been scaled to match parallel experiments based on empirically determined ratios.

For analysis of biological variability, pooling RNA from 12 samples was a more pragmatic approach than processing individual retinas. From our experience, genes that are moderately expressed (>100 copies/10\textsuperscript{5} cyclophilin) vary less than 2-fold between individual retinas within an experimental group. Generally, expression levels below 100 copies/1 million cyclophilin A and fold changes of less than 2 cannot be reliably interpreted, as reflected in the Discussion.

**RESULTS**

**Temporal Expression in Total Retina of IGF-1, IGF-2, Ins2, IGF-1R, IR, IGF-2R mRNA during Oxygen-Induced Retinopathy**

We examined regulation of the IGF-I/insulin system in whole retina of total mRNA from 12 pooled retinas at each time point.
exposed to 75% O2 from P7 to P12, and retinas from selected times were isolated and used for quantitative real-time PCR analysis. Target genes were normalized to 10^6 copies of cyclophilin A. P7 samples (white bars) were collected immediately before OIR and are common to both treatments. (B) Data from P15 replotted for comparison. Error bars represent ±SD.

between P7 and P33 during the induction of OIR and in untreated age-matched controls. Retinas became hypoxic after return to room air at P12 following hyperoxia-induced vessel loss from P7 to P12.

In total retina, IGF-1, IGF-1R, and IGF-2R mRNA expression was essentially unchanged with the development of retinopathy and unchanged with retinal development from P7 to P17. Retinal Ins2 mRNA increased more than 5-fold at P15 and P17 with the induction of retinopathy (Fig. 1A). IGF-2 mRNA decreased with development between P7 and P17, and local IR mRNA production increased modestly (approximately 2-fold) at the peak of neovascularization at P17. However the IGF-1/IGF-1R system predominated. The relative ratio of IR/IGF-1R mRNA production in total retina was approximately 1:10, as was the ratio of local Ins2/IGF-1 mRNA production. In total retina, IGF-2 was not a dominant as IGF-1. The relative amount of IGF-2R was negligible (Fig. 1B).

**Temporal Expression in Total Retina of IGFBP1 to IGFBP7 during Oxygen-Induced Retinopathy**

IGFBP2, IGFB4, and IGFBP5 mRNA expression was essentially unchanged with increasing retinal development between P7 and P33, and was there no change of IGFBP2, IGFB4, and IGFBP5 expression with the induction of retinopathy. In contrast IGFB-1, IGFB3, and IGFB6 all had greater than 5-fold increases in mRNA at P15 with the induction of retinopathy. However, when relative mRNA production was examined at P15, IGFBP1 and IGFBP6 were expressed at very low levels (even after a large induction with retinopathy) compared with the essentially unchanged IGFBP2, IGFB4, and IGFBP5 mRNA expression. In contrast, IGFBP3 mRNA levels were low during development but were upregulated with the onset of hypoxia to a level within 3-fold that of IGFBP2, IGFB4, and IGFBP5 (Fig. 2B).

**Localization and Abundance of the IGF and Insulin Systems in Retinal Cross-Section with OIR and Normal Retina at P17**

To localize and quantify mRNA in retina, we laser captured with microdissection retinal vessels and neovascular vessels extending into the vitreous (tufts) and then captured the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) or photoreceptors in normal retinopathy and OIR at P17. RNA was then quantified to measure absolute abundance in copy numbers per 10 million cyclophilin mRNA copies (Fig. 3).

IGF-1R and IR are expressed predominantly in the photoreceptors in the neural retina, to a lesser extent in normal vessels, and to an even more reduced level in neovascular tufts, with little expression in other parts of the retina (Fig. 4A). However, IGF-1R predominates. The relative abundance of IGF-1R to IR in photoreceptors and vessels was approximately 100:1 (Fig. 4B). In contrast, IGF-1 was expressed in all neural retinal layers and in vessels and tufts (with slightly lower expression in the ONL (photoreceptors; Fig. 4A). IGF-2 mRNA was expressed at a much higher level (up to a 1000-fold difference) than IGF-1 in photoreceptors and in blood vessels. IGF-2 mRNA expression was increased more than 6-fold in neovascular tufts compared with normal vessels. The relative abundance of IGF-1/Ins2 mRNA in photoreceptors in normal retina was approximately 1:10. However, with retinopathy at P17, there was approximately a 5-fold decrease in photorecep-
tor insulin production such that the IGF-1/Ins2 ratio was closer to 1. Nevertheless, the most prominent components of the IGF-1/Ins2 system were IGF-2 and IGF-1R in photoreceptors and vessels.

The localization of the IGFBPs shows large increases in neovascular tufts in IGFBP3, IGFBP5, and IGFBP7 mRNA compared with normal vessels (Fig. 5). The GCL predominantly expresses IGFBP2, IGFBP3, and IGFBP5, as do the ONL and INL (Fig. 5).

**DISCUSSION**

Knowing the retinal distribution and relative expression levels of IGF family components (IGF-1R and Insr, IGF-1, IGF-2, Ins2 and IGFBP1 to IGFBP7) in vessels and specific neuronal layers (the equivalent of quantitative in situ hybridization) as well as changes in mRNA expression in total retina during retinopathy may enhance our understanding of the local IGF system control. Using the technique of quantitative RT-PCR on laser-capture microdissected retinal elements and vessels (normal and neovascular vessels), we found that in the retina the major local IGF system receptor expressed is IGF-1R rather than IR. Surprisingly, the localization of IGF-1R (and IR) showed that these receptors are expressed predominantly in photoreceptors and more modestly in vessels (IGF-1R at approximately 100-fold greater level than IR) with little expression in the rest of the neural retina.

Interestingly, this suggests that IGF-1R in photoreceptors is required for retinal vascular development because of decreased normal retinal vascular development in the IGF-1R null mouse, with its lack of IGF-1R in endothelial cells and in photoreceptors, but normal vascular development in a mouse lacking IGF-1R in vascular endothelial cells alone. Vascular loss occurs when photoreceptors degenerate, and the IGF system in photoreceptors is likely to be one of the growth factor systems required for photoreceptor stability and for...
normal vascular development. Less is known about IR in retinal neurons and vasculature, though IR is also important in retinal neovascularization despite the fact that the expression level is low compared with IGF-1R. Insulin receptor signaling provides a trophic signal for transformed retinal neurons in culture, but the role of IR in retinal neuronal activity in vivo is less understood. In Drosophila, IR guides retinal photoreceptor axons from the retina to the brain during development, and IR influences the size and number of photoreceptors. The lack of IR activation leads to neurodegeneration in brain/neuron-specific IR knockout mice.

We found that the major local retinal IGF-related growth factor expressed in photoreceptors and in blood vessels is IGF-2 rather than IGF-1 (or Ins2). Local production of IGF-2 mRNA at these sites is 100- to 1000-fold greater than IGF-1 or Ins2. IGF-2 signals through IGF-1R and IR, suggesting that IGF-2 may be important for photoreceptor cell survival and for vessel growth. Importantly, IGF-2 is increased 8-fold in neovascular tufts compared with normal vessels. IGF-2 is also highly expressed in a wide variety of neoplasms and hemangiomas, vascular tumors of infancy. Thus, IGF-2 expression may be characteristic of neovessels. We found a very low level of expression of the IGF-2 scavenging receptor, IGF-2R, in photoreceptors and vessels. IGF-2R binds IGF-2 on the cell surface and transports it to the lysosome for degradation, acting as a sink because it does not transduce any signal.

We found that the major IGF-binding proteins expressed in the total retina are IGFBP2, IGFBP3, IGFBP4, and IGFBP5. IGFBP2, IGFBP4, and IGFBP5 are expressed at constant levels with development and with oxygen-induced retinopathy. IGFBP3 is strongly induced by hypoxia and rises to levels comparable to those of IGFBP2, IGFBP4, and IGFBP5. Laser-capture localization shows that neovessels compared with normal vessels strongly express IGFBP3 and IGFBP5, with increases also in IGFBP2 and IGFBP-7 (though to much lower levels) over normal vessels. Ischemia is not a stimulus for the local production of IGFBP3 and IGFBP5 but is a strong stimulus of IGFBP3 in the eye. Although expressed at much lower levels, mRNA of IR, Ins2, IGFBP1, and IGFBP6 also appears to be upregulated with hypoxia.

In cell culture, IGFBP5 controls cell survival, differentiation, and apoptosis independently of IGF-1 or IGF-2. The expression pattern of IGFBP5 in retina in vivo points to a significant role during retinal development. In primary porcine retinal Müller glial cells cultured in vitro, IGFBP5 increases photoreceptor survival. In cell culture, IGFBP2 and IGFBP4 are generally inhibitory, whereas IGFBP5 potentiates IGF-1. IGFBP3, depending on culture conditions, can either inhibit or potentiate the actions of IGF-1. In serum, IGFBP3 is by far the most abundant binding protein, with concentrations in the range of 100 nM, compared with the 2 to 15 nM concentrations of other binding proteins. Thus, serum and local IGFBP3 production appear to play a role in the IGFBP3 effects on retinopathy. IGFBP3 has actions independent of IGF-1. In vivo, IGFBP3, independent of IGF-1, helps to prevent oxygen-induced vessel loss and to promote vascular regrowth after vascular destruction in vivo, resulting in less retinal neovascularization.
A number of studies have examined the serum contribution of the IGF system by comparing levels in vitreous from patients with proliferative retinopathy to vitreous levels obtained from patients with nonproliferative eye disease. IGF-1, IGF-2, IGFBP2, and IGFBP3 were elevated in vitreous 1.5- to 13-fold with proliferative disease. These changes may partially be explained by leakage of serum into the vitreous because IGFs and IGFBPs are 20- to 50-fold higher in serum than in vitreous but may also reflect local production given that vitreous protein content was 1.5- to 5-fold elevated in ischemia patients compared with control subjects.18,38,39

Our results suggest that local IGF-2, IGFBP2, IGFBP3, and IGFBP5 may contribute to vitreous levels because these are produced by blood vessels close to the vitreous and are increased with vasoproliferation.

Measurement of protein levels should enable further elucidation of mechanisms. Interpretation of mRNA results is limited by the purity of laser-captured cells, and mRNA levels may not reflect protein levels. Even low expression levels of some IGF components may have profound effects, and results must be interpreted in that light. However, this is the first study that examines the IGF system in retinopathy, and it improves our understanding of local IGF control with hypoxia and the development of retinopathy. We have shown the importance of local IGF-2 production in blood vessels. We have also shown that IGF-1R is much more abundant than IR and is found almost exclusively in photoreceptors and blood vessels. This system may be part of the cross-talk between vessels and neurons during retinopathy. These studies will help form the basis of future studies of the IGF system in retinopathy.

**Figure 5.** Intraretinal mRNA expression profiles of IGF1 to IGF7 in OIR compared with normoxic controls. (A) Mouse litters were exposed to 75% O2 from P7 to P12. At P17, eyes were collected and cryosectioned, and laser-capture microdissection was used to enrich for GCL, INL, and ONL and for normoxic blood vessels and angiomatous tufts (Vessels/Tufts). RNA was isolated from samples and used for quantitative real-time PCR analysis. Target genes were normalized to 10^6 copies of cyclophilin A. (B) Data replotted for comparison of binding proteins within each retinal layer. Error bars represent ±SD.
References


