Insulin-like Growth Factor I (IGF-I)-induced Chronic Gliosis and Retinal Stress Lead to Neurodegeneration in a Mouse Model of Retinopathy*

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Background: In the retina, insulin-like growth factor I (IGF-I) is neuroprotective and essential for vasculature homeostasis.

Results: Transgenic mice overexpressing Igf-I in the retina present chronic gliosis and retinal stress leading to neurodegeneration.

Conclusion: IGF-I induces deleterious processes in the retina that, chronically, may overcome its neurotrophic properties.

Significance: Increased intraocular IGF-I may contribute to the pathogenesis of conditions such as ischemic or diabetic retinopathies.

Insulin-like growth factor I (IGF-I) exerts multiple effects on different retinal cell types in both physiological and pathological conditions. Despite the growth factor’s extensively described neuroprotective actions, transgenic mice with increased intracellular levels of IGF-I showed progressive impairment of electroretinographic amplitudes up to complete loss of response, with loss of photoreceptors and bipolar, ganglion, and amacrine neurons. Neurodegeneration was preceded by the overexpression of genes related to retinal stress, acute-phase response, and gliosis, suggesting that IGF-I altered normal retinal homeostasis. Indeed, gliosis and microgliosis were present from an early age in transgenic mice, before other alterations occurred, and were accompanied by signs of oxidative stress and impaired glutamate recycling. Older mice also showed overproduction of pro-inflammatory cytokines. Our results suggest that, when chronically increased, intraocular IGF-I is responsible for the induction of deleterious cellular processes that can lead to neurodegeneration, and they highlight the importance that this growth factor may have in the pathogenesis of conditions such as ischemic or diabetic retinopathy.

IGF-I is a growth factor with mitogenic, differentiating, anti-apoptotic, and metabolic functions (1) that also plays a key role in pathological retinal neovascularization (2). IGF-I is increased in the vitreous of patients with proliferative ocular diseases, such as diabetic retinopathy (3), and pharmacological inhibition or genetic deletion of the Igf-I receptor in the retinal vasculature of rodents prevent neovascularization under hypoxic conditions (4, 5). Similarly, in humans, mutations in genes coding for the growth hormone receptor, Igf-I, or Igf-I receptor are associated with reduced retinal neovascularization (6). IGF-I is a potent inducer of the expression of the pro-angiogenic factor VEGF, through hypoxia-inducible factor 1α stabilization (7). In addition to the effects on VEGF transcription, IGF-I may act cooperatively with VEGF to stabilize newly formed vessels (4).

Besides its actions on retinal vasculature, IGF-I is a pro-survival molecule that can act on neurons (8). Humans carriers of homozygous mutations associated with reduced expression of the Igf-I gene present mental retardation and deafness, associated with severe prenatal growth retardation, postnatal growth failure, and microcephaly, confirming the role of IGF-I as a neurotrophic factor (9, 10). Because of these properties, IGF-I has been used to counteract neurodegeneration in experimental animal models of brain injury (11) and retinal neurodegeneration (12). Moreover, IGF-I-deficient mice develop slow retinal neurodysfunction with loss of ERG responses (13). This evidence demonstrates that IGF-I plays an important role in neuronal development and survival.

Transgenic mice overexpressing Igf-I in photoreceptors (TgIgf-I) have increased intraocular VEGF levels and develop many of the retinal vascular alterations characteristic of human diabetic eye disease, despite not being hyperglycemic (14, 15). Nonproliferative vascular alterations are found in young animals (2–3 months), and the phenotype progresses to overt preretinal neovascularization in adult mice (7–8 months) (14).
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IGF-I, which plays a pivotal role in the development of proliferative retinopathies (16), is expressed by glial cells in transgenic retinas, the cell type that also overproduces this factor in diabetic patients (17). Gliosis is observed in Igf-I-overexpressing retinas at nonproliferative stages, before neovascularization occurs, suggesting that IGF-I overexpression is likely causing other alterations on transgenic retinas that contribute to the phenotype.

Given the apparently antagonistic effects of IGF-I on the retina, with deleterious effects on retinal vasculature (6) but positive effects on neuronal survival (12), we evaluated the consequences of the chronic elevation of intraocular IGF-I on retinal functionality in TgIGF-I mice. We observed a progressive decline in ERG amplitudes in transgenic animals, leading to complete loss of response in old animals. This study demonstrates that the chronic overproduction of IGF-I in the retina induces a series of deleterious cellular processes, independent of neovascularization, that eventually surpass IGF-I pro-survival properties leading to neurodegeneration.

**EXPERIMENTAL PROCEDURES**

**Animals**—CD1 heterozygous mice overexpressing Igf-I in the retina were used (14). Animal care and experimental procedures were approved by the Ethics Committee in Animal and Human Experimentation of Universitat Autònoma de Barcelona.

**Electroretinography**—ERG recordings were performed in deeply anesthetized and dark-adapted mice. Recordings were taken between a mouse electrode fixed on a corneal lens over the mouse eye (Burian-Allen electrode, Hansen Ophthalmic Development Lab), a reference electrode located in the mouth, and a ground electrode located in the tail. After pupil dilatation, flash-induced ERG responses were recorded from the right eye in response to light stimuli produced with a Ganzfeld stimulator. Light stimuli intensities ranged from $-4$ to $2 \text{ log cd} \cdot \text{m}^{-2}$ and, for each intensity, 4–64 consecutive light presentations were averaged. Each range of flashes allowed recording of particular responses as follows: from $-4$ to $-1.52$ for rod-mediated responses; from $-1.52$ to 0.48 for mixed rod and cone responses; 0.48 and at a recording frequency range of 100–1000 Hz for oscillatory potentials; from $-0.52$ to 2 $\text{ log cd} \cdot \text{m}^{-2}$ on a rod-saturating background of 30 $\text{cd/m}^2$ for cone-mediated responses; and at 1.48 $\text{ log cd} \cdot \text{m}^{-2}$ on a rod-saturating background for flicker responses (20 Hz). The ERG signals were amplified, band filtered between 0.3 and 1000 Hz (Grass CP511 AC amplifier), and digitized at 10 kHz with a Power Lab data acquisition board (ADI Instruments). ERG measurements were taken by an observer blinded to the experimental conditions of the animals.

**Histological Analysis**—Formalin-fixed, paraffin-embedded eye sections were incubated with tomato lectin *Lycopersicon esculentum* (Sigma), anti-rhodopsin (Abcam, Cambridge, UK), anti-calretinin (Invitrogen), anti-PKCα (Sigma), anti-Brn3a (Santa Cruz Biotechnology, Santa Cruz, CA), GFAP (Dako Cytomation, Glostrup, Denmark), and anti-cleaved caspase3 (Cell Signaling, Danvers, MA). Nuclei were counterstained with DAPI (Sigma) Images were obtained with a laser-scanning confocal microscope (TCs SP2; Leica Microsystems GmbH, Wetlar, Germany) or a Nikon Eclipse 90i microscope (Nikon Instruments Inc., Tokyo, Japan).

**Morphometric Analysis**—Retinal layers (INL and ONL) length was measured with NIS Elements software (Nikon) in six central and six peripheral images ($\times20$) from retinal sections showing the optic nerve. Three measurements were performed in each image for each layer, ensuring perpendicular distances between layer limits. The length of the posterior segment of rod photoreceptors was determined by the same method in central sections. For quantification of specific neuronal populations, total positive cells were counted per 20 images of central retinal sections with optic nerve.

**Microarray Samples and Analysis**—The protocol for RNA extraction recommended by Affymetrix was followed (Expression Analysis Technical Manual). Microarray analysis was performed by Progenika (Bilbao, Spain). The final gene list contained only those probe sets with a $p < 0.05$. For the interpretation, cross-checking, and visualization of the data, Fatigo Term Enrichment (release date Nov. 20, 2010), Database for Annotation, Visualization, and Integrated Discovery (DAVID, david.abcc.ncifcrf.gov), and Kyoto Encyclopedia of Genes and Genomes (KEGG database), and GeneCodis were used. Array data have been submitted to GEO database (accession number GSE46246).

Reduction of RNAs were purified using RNeasy mini kit (Qiagen Sciences, Germantown, MD). cDNA was synthesized with transcript first strand cDNA synthesis kit (Roche Applied Science). Quantitative real time PCR was performed using LightCycler® 480 SYBR Green I Master (Roche Applied Science) with specific primers.

**Protein Analysis**—For Western blot, retinas were homogenized in lysis buffer and 50–100 $\mu$g of retinal extract were separated by 12% SDS-PAGE. Immunoblot was performed with anti-acetyl-NF-κB (Cell Signaling), anti-ERK1/2 (Cell Signaling), anti-phospho-ERK1/2 (Cell Signaling), anti-GFAP (DAKO Cytomation), anti-MCP-1 (Abcam), or anti-tubulin (Abcam). Detection was performed using Immobilon Western reagent (Millipore). The pixel intensity of the bands obtained was determined with GeneSnap software for Gene Genius Bio Imaging System (Syngene, Synoptics Ltd.), and the ratio protein content/tubulin content was calculated for each sample to allow loading-independent comparison.

**Retinal Explants**—Wild-type C57 mice were sacrificed, and eyes were enucleated. Retinas were dissected and incubated in DMEM (reference E15-810, PAA) complemented with 10% FBS (PAA, ref. A15–151) and antibiotic/antimycotic (PAA, ref. P11–002) at 37 °C and 5% CO₂. Medium was refreshed 24 h later. After 48 h, recombinant IGF-I (Sigma) was added to DMEM without FBS. Between 2 and 5 h prior to IGF-I addition, 5 $\mu$L of 200 $\mu$m wortmannin (Sigma) or DMSO (Sigma) were added to the culture media. After 48 h of stimulation, retinas were collected and frozen at $-80 ^\circ$C for total RNA isolation.

**Glutamine Synthetase Activity and Oxidized Glutathione**—Protein extracts were obtained as for Western blot analysis. 40 $\mu$L of a 1:10 dilution of the extracts was added to the reaction mixture containing 50 $\mu$m sodium glutamate (Sigma), 20 $\mu$m
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**RESULTS**

**Neuronal Dysfunction in Transgenic Retinas Overexpressing IGF-I**—Retinal neurophysiology was assessed in wild-type (WT) and TgIGF-I mice through ERG studies. Both rod and cone-driven responses were evaluated by performing measurements in scotopic and photopic conditions, respectively. At 3 months of age, transgenic mice did not show any difference in comparison with WT littermates in the amplitudes of the responses obtained with different light intensities (Fig. 1, A–F). At 6 months of age, however, responses were heterogeneous among different animals (Fig. 1, A–F). The abnormal responses in TgIGF-I mice became statistically significant at 7.5 months of age (Fig. 1, A–F). By 9 months of age, the average response in transgenic mice was reduced to about 20% of that recorded in WT animals (Fig. 1, A–F). Clearly, retinal dysfunction was progressive, from normal responses at 3 months of age to almost complete loss of response by 9 months.

To evaluate if this progressive retinal neurodegeneration was the consequence of the development of neovascularization, young (1.5-month-old) TgIGF-I mice were treated intravitreally with adenoassociated viral vectors carrying the gene of pigment epithelium-derived factor (PEDF), a well known antiangiogenic factor (18). This treatment leads to long-term production of PEDF, normalization of intraocular levels of VEGF, and prevention of neovascularization and retinal detachment in this animal model (19). However, the evaluation of ERG responses at 7.5 month of age, i.e. 6 months after gene transfer, did not reveal any recovery in PEDF-treated animals (Fig. 1G), suggesting that vascular alterations were not the main mechanism underlying neurodegeneration in transgenic retinas.

**Decreased Thickness of Retinal Layers in Transgenic Mice**—The thickness of retinal layers is a general indicator of the loss of neuronal populations. ONL and INL thicknesses were quantified in both the peripheral and central areas of retinal sections containing the optic nerve (Fig. 2A). In agreement with functional observations, at 3 months, the thickness of retinal layer was preserved in TgIGF-I retinas and only a slight, nonstatistically significant decrease was observed in 6-month-old animals, mainly in the ONL (data not shown). At 7.5 months of age, retinas of TgIGF-I mice were clearly thinner, with a 40% reduction in the width of both ONL and INL (Fig. 2A). These results suggest that a chronic increase in intraocular IGF-I levels resulted in neurodegeneration of photoreceptors and other neuronal cells.

**Statistical Analysis**—Values are expressed as the mean ± S.E. Differences between groups were compared by unpaired Student's t test. Differences were considered statistically significant at p values less than 0.05.

**FIGURE 1.** Progressive alteration of electroretinographic responses in transgenic mice with increased intraocular IGF-I. A–F, evolution over time of ERG responses in TgIGF-I mice (pink lines) compared with healthy littermates (black lines). With age, TgIGF-I showed a progressive decline in the recorded amplitudes in response to all stimuli tested. A, scotopic threshold response, representing highly sensitive responses of rod photoreceptors; B, scotopic b-wave, representing rod responses; C, mixed scotopic, reflecting stimulation of both rod and cones under scotopic conditions; D, oscillatory potentials, which measure INL neuronal activity; E, photopic b-wave, depicting cone responses; F, flicker, repetitive photopic stimulations that analyze cone recovery. There were statistically significant reductions in the responses in animals aged more than 7.5 months. G, ERG responses were recorded 6 months after a single intravitreal administration of AAV2-hPEDF (left eye) and AAV2-null (right eye) vectors to TgIGF-I mice aged 1.5 months. Age-matched WT littermates were used as controls. Scotopic b-wave (B<sub>scot</sub>), mixed a and b-waves, oscillatory potentials, and photopic b-wave (B<sub>phot</sub>) were analyzed. Despite the counteraction of retinal neovascularization, the overexpression of PEDF was not able to ameliorate the electroretinographic responses of treated eyes, which presented reduced amplitudes in all tests performed, similar to those of null-injected, untreated eyes. Values are expressed as the mean ± S.E. of 5–9 animals/group. *p < 0.05; **p < 0.01; ***p < 0.001.
Loss of Specific Neuronal Populations in TgIGF-I Retinas—To identify which type of neurons was degenerating in transgenic retinas, specific markers were used to detect and quantify different neuronal populations in 7.5-month-old animals. The length of rod photoreceptor outer segment was markedly reduced in transgenic retinas, as determined by morphometric analysis on rhodopsin-immunostained sections (Fig. 2B). Nevertheless, TgIGF-I retinas had normal cone arrestin content (data not shown), indicating that cone photoreceptors were not altered. Collectively, these observations suggested that the reduced ONL thickness observed (Fig. 2A) was likely due to the loss of rods, as rods and cones are the only cell types found in this nuclear layer.

Bipolar and amacrine neurons specifically express the α1-isoform of protein kinase C (PKCα) and calretinin, respectively. Retinal sections from 7.5-month-old TgIGF-I animals had a
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To identify the changes in gene expression that could account for the neuronal dysfunction induced by increased intraocular IGF-I, we performed microarray analysis on total retinal RNA from 4-month-old animals. At this age, transgenic retinas do not yet show neurodegeneration but only evidence of nonproliferative vascular alterations and gliosis (14). After data normalization and filtering, we detected 37 genes differentially expressed in transgenic retinas, of which 25 were up-regulated and 12 were down-regulated more than 1.5-fold (Table 1). Most of the up-regulated genes were classified in three main categories as follows: retinal stress, gliosis, and angiogenesis, whereas down-regulated genes were mostly associated with angiogenesis and CNS development. Several of the up-regulated genes in the category of retinal stress corresponded to acute-phase pro-

### TABLE 1

Microarray expression analysis in young transgenic mice

Classification of up- and down-regulated genes in retinas of 4-month-old IGF-I transgenic mice according to their biological function reported in the literature. Up-regulated genes (≥1.5-fold change) were classified in three main categories as follows: retinal stress, gliosis, and angiogenesis. Down-regulated genes (<1.5-fold change) were classified in two main categories, angiogenesis and CNS formation.

| Gene description       | Gene symbol | Fold change | p value | RefSeq   |
|------------------------|-------------|-------------|---------|----------|
| **Stress (retinal stress)** |             |             |         |          |
| Nuclear protein 1      | Nupr1       | 2.03        | 0.0003  | NM_019738|
| Lipocalin 2             | Lcn2        | 2.00        | 0.0013  | NM_008491|
| Oncostatin M receptor  | Osnr        | 1.59        | 0.0039  | NM_011019|
| Interferon-induced transmembrane protein 3 | Ifitm3 | 1.58 | 0.0050 | NM_025378|
| Complement component 4b | C4b       | 1.76        | 0.0084  | NM_009780|
| Complement component 1, q subcomponent, α-polypeptide | C1qa | 1.55 | 0.0095 | NM_007572|
| Inhibitor of DNA binding 2 | Id2       | 1.70        | 0.0214  | NM_010496|
| Histidine decarboxylase | Hdc        | 1.50        | 0.0260  | NM_008230|
| Endothelin 2            | Edn2        | 2.00        | 0.0497  | NM_007902|

| **Gliosis** |             |             |         |          |
| Glial fibrillary acidic protein | Gfap       | 3.10        | 0.0014  | NM_010277|
| S100 protein, β polypeptide | S100b      | 2.02        | 0.0060  | NM_009115|
| Doublecortin-like kinase 1 | Dclk1      | 1.54        | 0.0111  | NM_019978|
| Gap junction protein, α1 | Gja1       | 2.06        | 0.0173  | NM_010288|
| Aquaporin 4             | Aqp4        | 1.52        | 0.0199  | NM_009700|

| **Angiogenesis** |             |             |         |          |
| Carboxic anhydrase 3 | Car3       | 1.59        | 0.0005  | NM_007606|
| EGF-containing fibulin-like extracellular matrix protein 1 | Efemp1 | 1.52 | 0.0056 | NM_146015|
| Rac GT-Pase-activating protein 1 | Racgap1 | 1.53 | 0.0068 | NM_012025|
| α2-Macroglobulin | A2m         | 1.73        | 0.0090  | NM_175628|
| Procollagen C-endopeptidase enhancer protein | Pcolce | 1.60 | 0.0215 | NM_008788|
| Polycystic kidney disease 1 homolog | Pkd1 | 1.58 | 0.0272 | NM_013630|
| Progesterone receptor membrane component 2 | Prm2c | 1.58 | 0.0348 | NM_027558|
| Rho guanine nucleotide exchange factor | Rgeo6 | 3.45 | 0.0426 | NM_012026|

| **CNS formation** |             |             |         |          |
| Nardilysin, N-arginine dibasic convertase, NRD convertase 1 | Nrd1 | 1.54 | 0.0002 | NM_146150|
| Sema domain, immunoglobulin domain (lg), short basic domain | Sema3f | 1.52 | 0.0354 | NM_011349|
| Cut-like homeobox 2 | Cux2 | 1.54 | 0.0426 | NM_007804|

| **Miscellaneous** |             |             |         |          |
| Keratin 18 | Krt18       | 1.65        | 0.0027  | NM_010664|
| CDK2 (cyclin-dependent kinase 2)-associated protein 1 | Cd2tap1 | 1.89 | 0.0027 | NM_013812|
| β2-Microglobulin | B2m | 1.52 | 0.0086 | NM_009735|
| Actin, α2, smooth muscle, aorta | Acta2 | 1.51 | 0.0107 | NM_007392|
| Hemoglobin α, adult chain 1 | Hba-a1 | 1.66 | 0.0206 | NM_008218|
| Hemoglobin α, adult chain 2 | Hba-a2 | 1.66 | 0.0206 | NM_007902|
| RIKEN cDNA 1200015N20 gene | 1200015N20Rik | 1.67 | 0.0325 | NM_024244|
| Chromodomain helicase DNA-binding protein 7 | Chd7 | 1.55 | 0.0343 | NM_001081417|
| RIKEN cDNA 5830417I10 gene | 5830417I10Rik | 2.23 | 0.0345 | NM_027389|
| Mitochondrial ubiquitin ligase activator of NFκB 1 | Mul1 | 2.32 | 0.0491 | NM_026689|

**FIGURE 3. Detection of apoptotic cells in the retinas of transgenic mice with increased intraocular IGF-I.** Cells positive for the apoptosis marker cleaved caspase 3 (green, arrows) were found in the ganglion cell layer of transgenic retinas at 6 months of age. Cleaved caspase 3-positive cells could not be detected in retinal sections from age-matched wild-type animals. Nuclei were stained with DAPI (blue). Scale bar, 7.6 μm (left panel), and 11.43 μm (right panel).
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Proteins, such as α2-macroglobulin, lipocalin2, and components of the complement system. Microarray results were confirmed by quantitative PCR, and the expression of up-regulated genes was studied at different ages to identify the onset of each response (Fig. 4A). For most genes, expression was increased already by 1.5 months of age, and this rise was maintained or even exacerbated through the animal’s life (Fig. 4A). Gliosis-related genes, including Gfap, S100B, Edn2, and C4b were already up-regulated in 3-week-old transgenic retinas when analyzed by quantitative PCR. Values are expressed as mean ± S.E. of four animals/group. *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, quantitative RT-PCR analysis of the expression of the transcription factor CEBP-δ in WT and transgenic retinas at the indicated ages. Statistically significant increases in the expression of CEBP-δ were observed in transgenic retinas at 3 and 7.5 months of age. Values are expressed as the mean ± S.E. of 4–5 animals/group. *, p < 0.05.

By performing functional analysis using the GeneCodis web tool (21), we found in the promoter of genes whose expression was altered in the microarray using the GeneCodis software. The table shows the percentage of altered genes whose promoters contained target sequences for the indicated transcription factors. E, retinal content of the acetylated, nuclear form of NF-κB (a-NF-κB) at 7.5 months of age. tub, tubulin. Transgenic mice presented higher levels of acetyl-NF-κB when compared with age-matched WT retinas, indicating activation of this pathway. Values are expressed as mean ± S.E. of 2–6 animals/group. *, p < 0.05.
reports describing the presence of gliosis in the retinas of TgIGF-I mice (14, 15). To determine whether there is a temporal progression of glial alterations, GFAP immunohistochemistry was performed at different ages. We observed increased GFAP staining in both astrocytes and Müller cells in TgIGF-I animals as young as 1.5 months of age, and this GFAP up-regulation was maintained and even intensified throughout the animal’s life (Fig. 5A). Quantification by Western blot confirmed a marked increase in GFAP retinal content from an early age (Fig. 5B). Another cellular response characteristic of activated Müller cells is activation of the ERK1/2 pathway (23). In agreement with the presence of gliosis in TgIGF-I mice, a modest but statistically significant increase in the phosphorylated form of ERK was observed in 3-month-old animals, with very evident activation of ERK signaling in old animals (Fig. 5C).

Microglial cells are resident macrophages of the retina and become activated upon retinal injury (24). IGF-I induces prolif-
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**FIGURE 6. Increased gliosis in wild-type retinas exposed to recombinant IGF-I.** A, GFAP; B, S100b expression levels in WT retinas incubated with increasing concentrations of IGF-I in the presence or absence of wortmannin, an inhibitor of IGF-I downstream signaling. The expression of both markers of gliosis was increased in the presence of IGF-I, and this effect was abrogated by the addition of wortmannin. Values are expressed as the mean ± S.E. of 3–4 retinas/group. *, p < 0.05.

eration and activation of microglial cells in retina and brain (25, 26). We previously reported that old TgIGF-I mice had increased numbers of bone marrow-derived microglia (15). When analyzed by staining with tomato lectin, we found that the number of microglial cells was higher in TgIGF-I retinas at all ages, confirming increased microglial infiltration starting early in the animal’s life (Fig. 5D). Moreover, few of the tomato lectin-positive cells showed a rounded shape, a characteristic morphology of activated microglia (Fig. 5E, insets).

**Gliosis Is a Direct Consequence of IGF-I Signaling**—Further confirmation of the direct effects of IGF-I on retinal glial cells was obtained by culturing retinas isolated from WT mice with different concentrations of recombinant IGF-I protein. After 48 h of exposure to 100 ng/ml IGF-I, there was a nonstatistically significant increase in retinal expression of Gfap over levels of untreated retinas, which became clearly significant (about 4-fold) when incubated with 200 ng/ml, suggesting a direct activation of glial cells by IGF-I (Fig. 6A). This effect was abrogated in the presence of wortmannin, a specific inhibitor of PI3K, a downstream effector of IGF-I signaling pathway (Fig. 6A). A similar pattern of IGF-I-mediated up-regulation was observed when the expression of S100b was analyzed (Fig. 6B). These data clearly demonstrate the link between IGF-I signaling and gliosis.

**Increased Oxidative Stress in IGF-I-overexpressing Retinas**—Oxidative stress, which can potentially cause neurodegeneration, is especially likely to occur in the retina due to the tissue’s constant exposure to radiation and its high oxygen consumption. Glutathione acts as a scavenger of free radicals and reactive oxygen species (ROS) (27). In the retina, glutathione is produced mostly by Müller cells and astrocytes, which rapidly release it to provide neurons with antioxidant equivalents in situations of oxidative stress (27). The levels of oxidized glutathione were increased from an early age in TgIGF-I (Fig. 7A). Moreover, the expression of Nrf2, a transcription factor that activates a battery of antioxidant and cytoprotective genes (28), was also increased in transgenic retinas (Fig. 7B). NADPH oxidase is one of the main enzymes involved in the production of ROS in microglia, astrocytes, and neurons (29). The expression of NADPH oxidase regulatory subunits, p67Phox and p22Phox, was up-regulated in transgenic retinas (Fig. 7, C and D). Depending on the cell type, NADPH oxidase can be formed by different catalytic subunits such as Nox1, Nox2, and Nox4 (29). All three subunits were strikingly up-regulated in transgenic retinas at 7.5 months of age (Fig. 7, E–G). In addition, retinal iNOS expression was also markedly increased at this age (Fig. 7H), suggesting that there might be overproduction of nitric oxide (NO) in transgenic retinas. Altogether, these results suggest a raise in oxidative stress in TgIGF-I retinas.

**Impaired Glutamate Recycling in TgIGF-I**—Persistent gliosis can impair the recycling by Müller cells of neurotransmitters, such as glutamate, whose accumulation in the retinal milieu causes neurotoxicity (30). Glutamine synthetase (GS), which converts glutamate into glutamine (30), showed reduced activity in transgenic retinas (Fig. 8A), suggesting impairment in glutamate detoxification.

**Increased Production of Pro-inflammatory Cytokines in Transgenic Retinas**—Activated Müller and microglial cells have direct neurotoxic effects through the release of pro-inflammatory cytokines such as TNF-α and MCP-1, which can cause the death of ganglion neurons (31) and photoreceptors (32). Retinal Tnf-α expression was not altered in younger transgenic mice, but increased substantially (about 7-fold) by 7.5 months of age (Fig. 8B). Similarly, the measurement of MCP-1 content at 7.5 months of age showed much higher levels (9-fold increase) in transgenic retinas (Fig. 8C).

**DISCUSSION**

IGF-I is a pleiotropic growth factor involved in multiple biological and pathological processes. In addition to the diabetes-like vascular alterations previously reported for transgenic mice with increased intraocular IGF-I (14, 15), this study has unraveled a complex phenotype in transgenic retinas involving neuronal and glial cells. TgIGF-I mice showed a progressive decline in electroretinographic responses and a decrease in specific neuronal populations that resulted in significantly impaired neuronal functionality. Markers of retinal stress, gliosis, and microgliosis were detected at early ages, before major vascular alterations occur in this model. Transgenic mice also showed signs of oxidative stress and alterations in glutamate metabo-
lism, likely secondary to gliosis. Alterations showed a progressive pattern, worsening as animals aged. These changes in normal retinal metabolism may underlie the neuronal dysfunction observed in transgenic retinas, which would be further exacerbated by the increased production of pro-inflammatory cytokines such as TNF-α and MCP-1.

In TgIGF-I mice, ERG recordings showed a pattern compatible with progressive retinal neurodegeneration, from normal responses at 3 months of age to alterations of all recorded parameters in both scotopic and photopic conditions by 6–7 months and flat ERG in old animals. Histological analysis showed loss of retinal neurons (rod photoreceptors, bipolar, ganglion, and amacrine cells) in aged mice, consistent with the ERGs. Overt retinal dysfunction occurred at an age, 7.5 months, at which ≈75% of TgIGF-I mice present retinal detachment (14, 19). However, when neovascularization and retinal detachment are prevented by long term overexpression of the anti-angiogenic factor Pedf in the retina (19), ERG responses do not improve. This observation suggests that additional causes contribute to the dysfunction observed in transgenic retinas.

The analysis of the gene expression profile before onset of neuronal dysfunction in Tg-IGF-I retinas revealed that the genes whose expression was altered fell into three main categories: retinal stress, which included genes involved in the acute-phase response (APR), gliosis, and angiogenesis. Nearly every major disease of the retina, including retinitis pigmentosa and diabetic retinopathy, is associated with reactive gliosis involving Müller cells (33). This physiological response contributes to tissue repair and neuroprotection but, if persistent, may also contribute to tissue damage (33). Likewise, the acute-phase response is part of the body’s early defense mechanisms after infection, tissue injury, stress, or neoplasia (34). However, chronic APR can lead to disturbances in normal physiology (35). Increased production of acute-phase proteins has been reported for animal models of ocular pathologies, such as glaucoma, experimental diabetes, and photoreceptor degeneration

FIGURE 7. Increased oxidative stress in mice with increased intraocular IGF-I. A, analysis of markers of oxidative stress in WT and TgIGF-I animals at different ages. A, levels of oxidized glutathione were higher in transgenic retinas at all ages studied. Values are expressed as the mean ± S.E. of five animals/group. *, p < 0.05. Retinal expression of the p67Phox (C) and p22Phox (D) regulatory subunits of the NADPH oxidase enzyme, assessed by quantitative RT-PCR. A marked up-regulation of both subunits was observed in transgenic mice at 3 and 7.5 months of age. E–G, retinal expression of the catalytic subunit of NADPH oxidase, which are expressed in different retinal cell types. E, Nox1; F, Nox2, and G, Nox4 were all markedly up-regulated in transgenic retinas at 7.5 months of age. nd, not detected. Values are expressed as mean ± S.E. of 4–5 animals/group. *, p < 0.05. H, retinal expression by quantitative RT-PCR of the enzyme iNOS. There was a marked increase in iNOS expression in transgenic retinas at 7.5 months of age. Values are expressed as mean ± S.E. of 4–5 animals/group. *, p < 0.05.

FIGURE 8. Impaired retinal glial functionality in mice with increased intraocular IGF-I. A, retinal GS activity was assayed in WT and TgIGF-I mice by spectrophotometric monitoring of γ-glutamyl hydroxamate. GS activity was significantly reduced in transgenic animals from an early age. Values are expressed as mean ± S.E. of 8–13 animals/group. *, p < 0.05. B, follow up of retinal TNF-α expression by quantitative PCR. TNF-α was noticeably increased in TgIGF-I at 7.5 months of age. Values are expressed as mean ± S.E. of 4–5 animals/group. *, p < 0.05. C, retinal MCP-1 content in 7.5 month-old WT and TgIGF-I mice analyzed by Western blot. MCP-1 levels were significantly higher in transgenic retinas. Values are expressed as mean ± S.E. of four animals/group. **, p < 0.01. tub, tubulin.
(36–38). In our animal model, the increase in APR gene expression may have resulted from the up-regulation, at an early age, of the transcription factor CEBP-δ, one of the main transcriptional regulators of acute-phase genes (20). IGF-I is also a well-known activator of the transcription factor NF-κB (39), through several signaling pathways, including the Akt pathway, which is activated in transgenic retinas (15). Promoter analysis suggested that 6% of the genes altered in the microarray had target sequences for NF-κB. Activation of NF-κB contributes to the early activation of APR genes, maintained in later stages by the interaction between CEBP-β and CEBP-δ (20).

In our transgenic mice, high intraocular IGF-I led to increased expression of acute-phase proteins and markers of gliosis early in the course of the animal’s life (3 weeks), before neuronal or vascular phenotypes appear (14, 15), suggesting that these alterations were a direct consequence of IGF-I and not secondary responses. The observation of up-regulated expression of Gfap and S100b in wild-type retinas exposed to recombinant IGF-1 confirmed this hypothesis. Noticeably, increased levels of S100b, produced by reactive astrocytes, have been associated with a wide range of pathological conditions of the CNS, including Alzheimer, epilepsy, and diabetic retinopathy (40–42). S100b, through its binding to the receptor for the CNS, including Alzheimer, epilepsy, and diabetic retinopathy, has been associated with a wide range of pathological conditions of increased levels of S100b, produced by reactive astrocytes, have been postulated to be responsible for this overexpression in reactive astrocytes in the ischemia/reperfusion model (47). Moreover, the up-regulation of iNOS in transgenic eyes suggests increased production of NO, probably by activated Müller and/or microglial cells, contributing to the formation of reactive species. Finally, the up-regulation of Nrf2, a master regulator of endogenous antioxidant protection involved in the transcriptional control of detoxifying enzymes, may be considered a compensatory mechanism against oxidative stress in IGF-I-overexpressing retinas. Indeed, the up-regulation of Nrf2 has been reported after prolonged activation of NF-κB (48).

Müller cells maintain the homeostasis of the retinal milieu, controlling pH, the concentrations of ions, and the recycling of neurotransmitters, such as glutamate (33). However, all these essential functions are impaired when gliosis is established. Glutamate accumulation causes neurotoxicity (30). The retinal activity of the enzyme GS, which detoxifies glutamate to glutamine, is decreased in experimental models of retinal injury (49). Similarly, this activity was significantly reduced in transgenic retinas from an early age, suggesting impaired glutamate detoxification. In addition, in situations of retinal injury and gliosis, activated Müller and microglial cells can produce and secrete pro-inflammatory cytokines, which may contribute to the dysfunction of the blood-retinal barrier and also cause neuronal death (31, 50). Among cytokines, TNF-α and MCP-1 directly contribute to neuronal loss (31, 32). In agreement with the extensive gliosis and microgliosis observed in IGF-I mice, the levels of TNF-α and MCP-1 were considerably increased at 7.5 months of age, when severe loss of vision occurs, according to ERG measurements and the marked reduction of retinal neurons. The increased production of cytokines may act synergis-
tically with the impairment of glial supportive functions to cause neuronal loss in TgIGF-I retinas.

In our model, glial alterations are observed early in the animals' life, whereas neuronal loss and functional impairment do not become evident until mice are 6 months or older. This probably results from the fact that IGF-I is a pro-survival molecule for neurons (8). Despite the profound alterations in retinal homeostasis caused by glial dysfunction and oxidative stress, it is likely that IGF-I overexpression delayed neuronal death in this environment, preventing neuronal functional alterations in young TgIGF-I retinas. As animals aged, the pro-survival effects of IGF-I in the retina were progressively overcome leading to neurodegeneration (Fig. 9).

In conclusion, high intraocular levels of IGF-I trigger a series of cellular processes that bring about retinal stress, impair homeostatic functions of the glia, and lead to neuronal dysfunction and death. This work highlights the importance that increased intraocular levels of IGF-I may have in the progression of diseases such as ischemic or diabetic retinopathy.

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REFERENCES

1. Laviola, L., Natalicchio, A., and Giorgino, F. (2007) The IGF-I signaling pathway. Curr. Pharm. Des. 13, 663–669
2. Shaw, L. C., and Grant, M. B. (2004) Insulin-like growth factor-I and insulin-like growth factor binding proteins: their possible roles in both maintaining normal retinal vascular function and in promoting retinal pathology. Rev. Endocr. Metab. Disord. 5, 199–207
3. Inokuchi, N., Ikeda, T., Imamura, Y., Sotozono, C., Kinoshita, S., Uchihori, Y., and Nakamura, K. (2001) Vitreous levels of insulin-like growth factor-I in patients with proliferative diabetic retinopathy. Curr. Eye Res. 23, 368–371
4. Smith, L. E., Shen, W., Perruzzi, C., Soker, S., Kinose, F., Xu, X., Robinson, G., Driver, S., Bischoff, J., Zhang, B., Schaeffer, J. M., and Senger, D. R. (1999) Regulation of vascular endothelial growth factor-dependent retinal neovascularization by insulin-like growth factor-I receptor. Nat. Med. 5, 1390–1395
5. Kondo, T., Vicent, D., Suzuma, K., Yanagisawa, M., King, G. L., Holzenberger, M., and Kahn, C. R. (2003) Knockout of insulin and IGF-1 receptors on vascular endothelial cells protects against retinal neovascularization. J. Clin. Invest. 111, 1835–1842
6. Hellström, A., Carlsson, B., Niklasson, A., Segnestam, K., Boguszewski, M., de Lacerda, L., Savage, M., Svensson, E., Smith, L., Weinerberger, D., Albertsson Wikland, K., and Laron, Z. (2002) IGF-I is critical for normal vascularization of the human retina. J. Clin. Endocrinol. Metab 87, 3413–3416
7. Treins, C., Giorgetti-Peraldo, S., Murdaca, J., Monthouèil-Kartmann, M. N., and Van Obbergen, E. (2005) Regulation of hypoxia-inducible factor (HIF)-1 activity and expression of HIF hydroxylases in response to insulin-like growth factor-I. Mol. Endocrinol. 19, 1304–1317
8. D’Ercole, A. J., Ye, P., Calikoglu, A. S., and Gutierrez-Ospina, G. (1996) The role of the insulin-like growth factors in the central nervous system. Mol. Neurobiol. 13, 227–255
9. Bonapace, G., Concolino, D., Formicola, S., and Strisciuglio, P. (2003) A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J. Med. Genet. 40, 913–917
10. Woods, K. A., Camacho-Hübner, C., Savage, M. O., and Clark, A. J. (1996) Intraperitoneal growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N. Engl. J. Med. 335, 1363–1367
11. Saatman, K. E., Contreras, P. C., Smith, D. H., Raghupathi, R., McDermott, K. L., Fernandez, S. C., Sanderson, K. L., Voddi, M., and McIntosh, T. K. (1997) Insulin-like growth factor-1 (IGF-I) improves both neurological motor and cognitive outcome following experimental brain injury. Exp. Neurol. 147, 418–427
12. Kermer, P., Klöcker, N., Labes, M., and Bähr, M. (2000) Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 in vivo. J. Neurosci. 20, 2–8
13. Rodriguez-de la Rosa, L., Fernandez-Sanchez, L., Germain, F., Murillo-Cuesta, S., Varela-Nieto, I., de la Villa, P., and Cuenca, N. (2012) Age-related functional and structural retinal modifications in the Igf1−/− null mouse. Neurobiol. Dis. 46, 476–485
14. Ruberto, J., Ayuso, E., Navarro, M., Carretero, A., Nacher, V., Haurigot, V., George, M., Llombart, C., Casellas, A., Costa, C., Bosch, A., and Bosch, F. (2004) Increased ocular levels of IGF-I in transgenic mice lead to diabetes-like eye disease. J. Clin. Invest. 113, 1149–1157
15. Haurigot, V., Villacampa, P., Ribera, A., Llombart, C., Bosch, A., Nacher, V., Ramos, D., Ayuso, E., Segovia, J. C., Buener, J. A., Ruberte, J., and Bosch, F. (2009) Increased intraocular insulin-like growth factor-I triggers blood-retinal barrier breakdown. J. Biol. Chem. 284, 22961–22969
16. Aiello, L. P., Avery, R. L., Artrig, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thieme, H., Iwamoto, M. A., and Park, J. E. (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N. Engl. J. Med. 331, 1480–1487
17. Amin, R. H., Frank, R. N., Kennedy, A., Elloitt, D., Puklin, J. E., and Abrams, G. W. (1997) Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with nonproliferative diabetic retinopathy. Invest. Ophthal. Vis. Sci. 38, 36–47
18. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W., and Bouck, N. P. (1999) Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 285, 245–248
19. Haurigot, V., Villacampa, P., Ribera, A., Bosch, A., Ramos, D., Ruberte, J., and Bosch, F. (2012) Long-term retinal PEDF overexpression prevents neovascularization in a murine adult model of retinopathy. PLoS One 7, e41511
20. Puli, V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J. Biol. Chem. 273, 29279–29282
21. Nogales-Cadenas, R., Carmona-Saez, P., Vazquez, M., Vicente, C., Yang, X., Tirado, F., Carazo, J. M., and Pascual-Montano, A. (2009) GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. Nucleic Acids Res. 37, W317–W322
22. Moshage, H. (1997) Cytokines and the hepatic acute phase response. J. Pathol. 181, 257–266
23. Takeda, M., Takamiya, A., Yoshida, A., and Kiyama, H. (2002) Extracellular signal-regulated kinase activation predominantly in Müller cells of retina with endothoxin-induced uveitis. Invest. Ophthal. Vis. Sci. 43, 907–911
24. Karlstetter, M., Ebert, S., and Langmann, T. (2010) Microglia in the healthy and degenerating retina: insights from novel mouse models. Immunobiology 215, 685–691
25. Zelinka, C. P., Scott, M. A., Volkov, L., and Fischer, A. J. (2012) The reactivity, distribution, and abundance of non-astrocytic inner retinal glial (NIRG) cells are regulated by microglia, acute damage, and IGF1. PLoS One 7, e44477
26. O’Donnell, S. L., Frederick, T. J., Krady, J. K., Vannucci, S. J., and Wood, T. L. (2002) IGF-I and microglia/macrophage proliferation in the ischemic mouse brain. Glia 39, 85–97
27. Schütte, M., and Werner, P. (1998) Redistribution of glutathione in the ischemic rat retina. Neurosci. Lett. 246, 53–56
28. Wakabayashi, N., Slocum, S. L., Skoko, J. I., Shin, S., and Kessler, T. W. (2010) When NRFP2 talks, who’s listening? Antioxid. Redox Signal. 13, 1649–1663
29. Bedard, K., and Krause, K. H. (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 245–313
30. Bringmann, A., Pannicke, T., Biedermann, B., Francke, M., Iandiev, I., Grosche, I., Wiedemann, P., Albrecht, J., and Reichenbach, A. (2009) Role of retinal glial cells in neurotransmitter uptake and metabolism. Neuro...
31. Lebrun-Julien, F., Duplan, L., Pernet, V., Osswald, I., Sapieha, P., Bourgeois, P., Dickson, K., Bowie, D., Barker, P. A., and Di Polo, A. (2009) Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism. *J. Neurosci.* **29**, 5536–5545

32. Nakazawa, T., Hisatomi, T., Nakazawa, C., Noda, K., Maruyama, K., She, H., Matsubara, A., Miyahara, S., Nakao, S., Yin, Y., Benowitz, L., Hafezi-Moghadam, A., and Miller, J. W. (2007) Monocyte chemoattractant protein 1 mediates retinal detachment-induced photoreceptor apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2425–2430

33. Bringmann, A., Pannicke, T., Grosche, J., Francke, M., Wiedemann, P., Skatchkov, S. N., Osborne, N. N., and Reichenbach, A. (2006) Müller cells in the healthy and diseased retina. *Prog. Retin. Eye Res.* **25**, 397–424

34. Gabay, C., and Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.* **340**, 448–454

35. Venteclef, N., Jakobsson, T., Steffensen, K. R., and Treuter, E. (2011) Metabolic nuclear receptor signaling and the inflammatory acute phase response. *Trends Endocrinol. Metab.* **22**, 333–343

36. Samardzija, M., Wariwoda, H., Imsand, C., Huber, P., Heynen, S. R., Gubler, A., and Grimm, C. (2012) Activation of survival pathways in the degenerating retina of rd10 mice. *Exp. Eye Res.* **99**, 17–26

37. Gerhardinger, C., Costa, M. B., Coulombe, M. C., Toth, I., Hoehn, T., and Grosu, P. (2005) Expression of acute-phase response proteins in retinal Müller cells in diabetes. *Invest. Ophthalmol. Vis. Sci.* **46**, 349–357

38. Steele, M. R., Inman, D. M., Calkins, D. J., Horner, P. J., and Vetter, M. L. (2006) Microarray analysis of retinal gene expression in the DBA/2J model of glaucoma. The Penn State Retina Research Group. *Exp. Eye Res.* **70**, 723–730

39. Nair, S., Doh, S. T., Chan, J. Y., Kong, A. N., and Cai, L. (2008) Regulatory potential for concerted modulation of Nrf2- and Nf-κB-mediated gene expression in inflammation and carcinogenesis. *Br. J. Cancer* **99**, 2070–2082

40. Krady, J. K., Basu, A., Allen, C. M., Xu, Y., LaNoue, K. F., Gardner, T. W., and Levison, S. W. (2005) Minocycline reduces glutamate oxidation and glutamine synthesis in the retina. The Penn State Retina Research Group. *Exp. Eye Res.* **70**, 723–730

41. Van Eldik, L. J., and Wainwright, M. S. (2003) The Janus face of glial-derived S100B: beneficial and detrimental functions in the brain. *Restor. Neurol. Neurosci.* **21**, 97–108

42. Zong, H., Ward, M., Madden, A., Yong, P. H., Limb, G. A., Curtis, T. M., and Stitt, A. W. (2010) Hyperglycaemia-induced pro-inflammatory responses by retinal Müller glia are regulated by the receptor for advanced glycation end-products (RAGE). *Diabetologia* **53**, 2656–2666

43. Bianchi, R., Giambanco, I., and Donato, R. (2010) S100B/RAGE-dependent activation of microglia via NF-κB and AP-1 Co-regulation of COX-2 expression by S100B, IL-1β, and TNF-α. *Neurobiol. Aging* **31**, 665–677

44. Zong, H., Ward, M., and Stitt, A. W. (2011) AGEs, RAGE, and diabetic retinopathy. *Curr. Diab. Rep.* **11**, 244–252

45. Barakat, D. J., Dvoriantchikova, G., Ivanov, D., and Shestopalov, V. I. (2012) Astroglial NF-κB mediates oxidative stress by regulation of NADPH oxidase in a model of retinal ischemia reperfusion injury. *J. Neurochem.* **120**, 586–597

46. Nair, S., Doh, S. T., Chan, J. Y., Kong, A. N., and Cai, L. (2008) Regulatory potential for concerted modulation of Nrf2- and Nf-κB-mediated gene expression in inflammation and carcinogenesis. *Br. J. Cancer* **99**, 2070–2082

47. Lieth, E., LaNoue, K. F., Antonetti, D. A., and Ratz, M. (2000) Diabetes reduces glutamate oxidation and glutamine synthesis in the retina. The Penn State Retina Research Group. *Exp. Eye Res.* **70**, 723–730

48. Krady, J. K., Basu, A., Allen, C. M., Xu, Y., LaNoue, K. F., Gardner, T. W., and Levison, S. W. (2005) Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. *Diabetes* **54**, 1559–1565