Microglia-Mediated IGF-I Neuroprotection in the rd10 Mouse Model of Retinitis Pigmentosa

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PURPOSE. To characterize the effect of IGF-I in the rd10 mouse model of retinitis pigmentosa at the cellular level, focusing on the role of microglia in the neurodegenerative process.

METHODS. Both organotypic retinal explants and intravitreal injections were used to assess the effect of IGF-I on photoreceptor cell death in the Pde6b

RESULTS. Both ex vivo and in vivo IGF-I treatment reduced the number of TUNEL-positive nuclei in rd10 mouse retinas. In addition, IGF-I treatment in explants increased the number of microglial cells in the ONL. Depletion of microglia in explants with liposomes containing clodronate diminished the neuroprotective effect of IGF-I but also moderately reduced photoreceptor cell death in rd10 retinas cultured in the absence of IGF-I.

CONCLUSIONS. IGF-I is able to attenuate photoreceptor cell death both ex vivo and in vivo in the rd10 mouse retina. Microglia is required for the neuroprotective effect of IGF-I in the dystrophic retina. In addition, microglial cells play a detrimental role, seemingly led to neuroprotection by IGF-I. (Invest Ophthalmol Vis Sci. 2011;52:9124–9130) DOI:10.1167/iovs.11-7736

Retinitis pigmentosa (RP) comprises a large group of inherited retinal dystrophies that are clinically similar but genetically heterogeneous (http://www.sph.uth.tmc.edu/Retnet/disease.htm). RP evolves with a loss of visual function that, in most models studied, parallels photoreceptor cell death. Consequently, moderate success in attenuating vision loss in animal models of RP has been achieved using molecules that promote cell survival, some of which have progressed to the clinical development stage (clinicaltrials.gov/ct2/results?term=retinitis+pigmentosa).

Proinsulin, insulin, and insulin-like growth factors (IGFs) are well characterized attenuators of cell death in the developing and adult nervous system. Proinsulin delays photoreceptors death and prolongs visual function in the rd10 mouse model of RP, whereas insulin extends cone survival in the rd1 mouse. Recently, we demonstrated that IGF-I also attenuates photoreceptor apoptosis ex vivo in both genetic and experimentally induced RP models. To fully explore the therapeutic potential of growth factors of this family in RP, it is crucial to understand the molecular and cellular mechanisms underlying these observed effects.

Among other effects, IGF-I promotes the proliferation of macrophages and microglial cells. Microglia constitute a particular macrophage/monocyte lineage in the central nervous system (CNS) and thus perform the usual immune surveillance function. As in other parts of the CNS, microglial cells in the retina continuously monitor the environment and respond to alterations with morphologic, migratory, and proliferative adaptations that include the autocrine/paracrine secretion of growth factors and proinflammatory cytokines. Extensive studies in the injured brain have revealed that exacerbation of damage is a common consequence of the microglial response. However, a graded response ranging from the induction of neuroprotection to the exacerbation of injury has also been described, depending on multiple factors, such as the nature and severity of the damage and the local environmental features. Studies in the retina have linked microglial activation to increased neuronal injury in experimental models of RP, glaucoma, light-induced photoreceptor degeneration, diabetic retinopathy, and age-related macular degeneration. Further studies are required to determine whether microglia play a beneficial role in retinal dystrophies and, more important, to identify strategies to prevent their detrimental effects.

The rd10 mouse is the most commonly studied mouse model of RP, though it is not without disadvantages. The onset of RP in this model occurs early, and disease progression is aggressive. Consequently, electroretinographic recordings of visual function are of very low intensity, even when partial rescue is reported. Of particular relevance to studies of cell death regulation, a partial overlap is observed between developmental cell death in the inner nuclear layer (INL) and pathologic cell death in the outer nuclear layer (ONL). To circumvent these problems, we performed our studies in the rd10 mouse, which carries a missense mutation in the Pde6b gene (as opposed to the nonsense mutation in the same gene of the rd1 mouse), resulting in a less aggressive phenotype, and used this model to further characterize the effects of IGF-I on retinal dystrophies. IGF-I induced a decrease in photoreceptor apoptosis in retinal explants, in parallel to an increase in microglial cell number. The IGF-I neuroprotective effect was corroborated by intravitreal injection. Microglia depletion with clodronate-encapsulated liposomes reduced the neuroprotective ef-
ffect of IGF-I on photoreceptor cells. Interestingly, retinas treated only with clodronate-encapsulated liposomes exhibited a moderate reduction of photoreceptor cell death, revealing a complex and possibly dual role of microglia in this model of retinal degeneration.

**MATERIALS AND METHODS**

**Animals**

Experiments were carried out in accordance with the European Union guidelines and the ARVO Statements for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the Institutional Bioethics Committee. Control wild-type (WT) C57BL/6j mice were obtained from local facilities. The rd10 mouse model of retinal degeneration, homozygous for the Pde6brd10 allele and on a C57BL/6j background, was kindly provided by Bo Chang (The Jackson Laboratory, Bar Harbor, ME). Mice were reared in local facilities under a 12-hour light/12-hour dark cycle at 20°C. All assays were performed with postnatal day 23 (P23) or P24 WT and rd10 mouse retinas.

**Ex Vivo Retinal Explants**

P23 mice were euthanized, and their eyes were enucleated. Neuroretinas were dissected from the anterior segment, vitreous body, sclera, and pigmented epithelium and were mounted photoreceptor-side up on nitrocellulose inserts (Millipore; Billerica, MA). Explants were cultured for 24 hours in 1.2 mL R16 medium (formulation kindly provided by Per A. Ekstrom, Wallenberg Retina Centre, Lund University, Lund, Sweden) with no additional serum (basal), and were treated, where indicated, with 20 nM IGF-I (Sigma; St. Louis, MO) or with liposomes. Liposomes were a kind gift from The Foundation Clodronate Liposomes (www.clodronateliposomes.org). Clodronate was a kind gift from Roche Diagnostics GmbH (Mannheim, Germany). Clodronate- and PBS-encapsulated liposomes were prepared as previously described34 and applied, where indicated, to the retinal explant cultures for the entire 24-hour period. Retinas were then washed in PBS, fixed in 10% (vol/vol) neutral buffered formalin solution (Sigma) for 24 hours at 4°C, and infiltrated with sucrose 25% (wt/vol) in PBS. They were then cryosectioned at a thickness of 12 μm, and the sections were mounted on positively charged slides (Menzel-Gläser, Braunschweig, Germany). Cryosections were also prepared from freshly dissected retinas at P24. Alternatively, cultured retinas were directly processed for ELISA (Cell Death; Roche Diagnostics).

**Intravitreal Injection**

P23 mice were anesthetized with an intraperitoneal injection of ketamine chlorhydrate (89.44 mg/kg) and xylazine chlorhydrate (3.89 mg/kg) solution in saline. Pupils were dilated by applying a topical drop of 1% tropicamide, and intravitreal injection was performed our study in the rd10 mouse model, in which the peak of photoreceptor cell death occurs around P25, well after the developmental cell death process has ended.33 Organotypic culture, IGF-I significantly reduced photoreceptor cell death (Fig. 1). The effect of IGF-I on photoreceptor cell death was quantified either by TUNEL in retinal sections (Fig. 1E) or by ELISA of free nucleosomes in retinal extracts (Supplementary Fig. S1A, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11-7736/DCSupplemental). As clearly seen, IGF-I prevented photoreceptor cell death in rd10 retinal explants.

**Cell Death Assays**

DNA fragmentation characteristic of apoptosis was visualized by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Promega, Madison, WI) in retinal cryosections, as previously described.12 After labeling, the retinas were mounted in mounting medium (Fluoromount-G; Southern Biotechnology, Birmingham, AL), counterstained with and DAPI (4′, 6-diamino-2-phenylindole; Sigma), and observed with a laser confocal microscope (TCS SP2; Leica, Microsystems, Wetzlar, Germany).

Free nucleosomes originating at late stages of apoptosis were quantified by Cell Death ELISA, according to the manufacturer’s instructions. Measurements were performed at 405 nm with an automatic microplate analyzer (Multiskan Bichromatic; Labsystems, Helsinki, Finland). All samples were run in duplicate in each assay. Background measurements were performed and subtracted from the sample results. The relative levels of cell death in each sample were normalized to their respective controls.

**Immunofluorescence**

Retinal cryosections, from either freshly dissected or cultured retinas, were washed in TBS containing 0.1% (wt/vol) BSA and 0.1% (vol/vol) Triton X-100 (this same buffer was used for all subsequent washes) and then were blocked and permeated for 2 hours in TBS containing 3% (wt/vol) BSA and 1% (vol/vol) Triton X-100. The sections were incubated overnight in a humid chamber at 4°C with rat anti-CD11b or goat anti-β1a (1:250; Abcam, Cambridge, UK) in blocking solution. The sections were then washed and incubated for 90 minutes with anti-rat or anti-goat immunoglobulin antibodies conjugated to Alexa-488 (1:500; Molecular Probes, Eugene, OR), washed, and mounted with medium (Fluoromount G) containing DAPI. Staining was observed with a laser confocal microscope.

**Microscopy and Cell Quantification**

Images were acquired by confocal microscopy, and stained cells or nuclei were scored by two masked observers. Stained microglial cells or TUNEL-positive nuclei were counted in the indicated retinal layer of an entire section of the retina, using a 40× objective. At least three retinas and four nonadjacent sections per retina were scored for each experimental point.

**Statistical Analysis**

All data were analyzed using a statistical program (Statgraphics Plus 5.1; Statpoint Technologies Inc., Warrenton, VA). Each experimental design was composed of replicate samples and was performed independently at least three times. Data were analyzed by one-way ANOVA followed by Bonferroni t-test. Differences were considered significant at P ≤ 0.05.

**RESULTS**

**Attenuation of Photoreceptor Cell Death by IGF-I in the rd10 Mouse**

Given the antiapoptotic effect of IGF-I in rd1 mouse retinas,12 we sought to identify the underlying cellular mechanisms. However, in the rd1 model, both pathologic and developmental cell death overlap,28 hindering discrimination between the individual contributions of each process to the cellular response to death, which may include modulation of microglia and reactive gliosis. We thus performed our study in the rd10 mouse model, in which the peak of photoreceptor cell death occurs around P25, well after the developmental cell death process has ended.33 Organotypic rd10 retinas were cultured for 24 hours in the absence or the presence of IGF-I (Fig. 1). The effect of IGF-I on photoreceptor cell death was quantified either by TUNEL in retinal sections (Fig. 1E) or by ELISA of free nucleosomes in retinal extracts (Supplementary Fig. S1A, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.11-7736/DCSupplemental). As clearly seen, IGF-I prevented photoreceptor cell death in rd10 retinal explants.

We corroborated this finding in vivo by intravitreal injection of IGF-I (Fig. 2). Despite the fact that the unformulated peptide may be rapidly eliminated from the vitreous and that the small volume of the murine vitreous does not ensure a homogenous distribution of the factor, as opposed to the treatment in culture, IGF-I significantly reduced photoreceptor cell death (Fig. 2C).
Microglial Cell Number and Distribution in the rd10 Retina In Vivo and Ex Vivo

Among other effects at the molecular and cellular levels and data not shown, IGF-I treatment induced a microglial response (Fig. 3). Although microglial cells in the rd1 retina have been partially characterized, no equivalent data for the rd10 retina is available. We thus investigated the presence of microglial cells in the dystrophic rd10 retina in vivo by immunolabeling with Cd11b and Iba1 antibodies (Fig. 3). Cd11b preferentially labeled ramified microglia (Fig. 3A), whereas Iba1 preferentially labeled amoeboid microglia (Fig. 3B). At P24 in vivo, both WT and rd10 retinas exhibited a similar number and distribution of microglial cells (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental).

Next, we studied the response of microglial cells to cell culture conditions for 24 hours and to IGF-I treatment (Figs. 3C–E). Culturing the rd10 retinas, which in itself constitutes a stressor, resulted in an overall, twofold increase in microglial cell numbers. In striking contrast to the in vivo situation (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental), a high proportion of microglia—approximately 50% of total microglial cells—were observed in the ONL (Fig. 3E). Conversely, WT retinas showed no increase in microglial cell number in culture (Supplementary Fig. S3A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental, and data not shown), suggesting that the preexisting damage in the rd10 retina somehow primes the microglial response in culture. IGF-I treatment further enhanced the microglial presence in the ONL in cultured rd10 retinas and increased the number of microglial cells in the GCL as well (Fig. 3E), and IGF-I also exerted an antiapoptotic effect on RGC death (Figs. 1C, 1D, and data not shown). No increase in microglial cell number in WT retinas was observed in response to IGF-I (Supplementary Fig. S3B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental, and data not shown). Thus, in cultured rd10 retinas, the prevention of apoptosis by
IGF-I correlated with a selective increase in the number of microglial cells in the ONL.

Abolishment of IGF-I Antiapoptotic Effect by Microglia Depletion

The previous finding points to a link between the observed microglial response and the neuroprotective effect of IGF-I. To investigate this correlation, we exploited the phagocytic capability of microglial cells. Liposomes filled with clodronate were used to eliminate retinal microglia. As expected, the presence of microglial cells in the ONL induced by IGF-I treatment of cultured rd10 retinas was clearly reduced by clodronate liposomes compared with control PBS liposomes (Fig. 4 and data not shown). No effect of either lipid type was observed in WT retinas (Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental, and data not shown), which responded to neither the culture conditions nor IGF-I treatment. Of importance, the antiapoptotic effect of IGF-I on rd10 retinal explants was strongly reduced by the presence of clodronate liposomes (Fig. 5), demonstrating the need for microglial cells for IGF-I-mediated neuroprotection. Again, no effect was observed in cultured WT retinas (Supplementary Fig. S4, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental, and data not shown). The effect of microglial depletion on photoreceptor cell death was quantified either by TUNEL in retinal sections (Fig. 5E) or by ELISA of free nucleosomes in retinal extracts (Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental). Little change in free nucleosome values was observed between conditions (see also Supplementary Fig. S1A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7736/-/DCSupplemental), likely because of cell death occurring in other retinal layers and the presence of nucleosome residues in neighboring engulfing cells. In contrast, TUNEL staining and scoring of TUNEL-positive nuclei in the ONL yielded clear differences that revealed an additional effect of clodronate liposomes in the absence of IGF-I treatment (Fig. 5E). In this situation, microglial depletion reduced the amount of TUNEL-positive photoreceptors.

Taken together, our observations in rd10 retinal explants indicate that the neuroprotective effect of IGF-I is mediated to a large extent by the microglial response. Conversely, resident microglia appear to play a proapoptotic role, evident in the absence of IGF-I. The fine-tuning of this dual role, which has
We have analyzed the neuroprotective role of IGF-I in retinal degeneration using organotypic rd10 retinal explants and intravitreal injections as a model system. Previously we had investigated the biochemical basis of the neuroprotective effect of IGF-I in rd11 retinas and in a model of photoreceptor cell death induced by Ca^{2+} overload.\textsuperscript{1,2} Here we demonstrate the effectiveness of IGF-I in a new model and the necessity of a microglial response, involving increased cell number and selective localization in the ONL, for the antiapoptotic effect of IGF-I.

Retinitis pigmentosa normally begins with primary apoptosis of the cell type, usually rod photoreceptors, in which the mutated gene exerts its function. This primary degeneration spreads to other cell types in the retina, causing secondary apoptosis of cone photoreceptors and retinal pigment epithelial cells, microglial recruitment, reactive gliosis of Mülller glial cells, and vascular disorganization and regression.\textsuperscript{2} This complex cellular scenario should be considered as a whole when designing therapeutic strategies. Retinal organotypic cultures provide a physiologically relevant environment in which to study retinal cells, maintaining cell-cell contacts and microenvironmental conditions. These kinds of cultures have been successfully used to characterize physiological cell death processes related to retinal development and pathologic cell death processes related to retinal degeneration.\textsuperscript{1,2,38–41} The combination of IGF-I treatment and retinal organotypic cultures revealed an unexpected effect of IGF-I and a complex, dual role of microglia in the progression of degeneration.

The rd10 retina in vivo showed a moderate microglia presence, similar to that found in age-matched WT retinas. However, a masked microglial response in the dystrophic retina was revealed solely by culturing. This potentially stressing process doubled the number of microglial cells in rd10 but not in WT retinas. Because retinas had been isolated from all surrounding tissues before culturing, these additional microglial cells could originate from a preexisting, hidden population. We speculate that these microglia might have originated from bone marrow-derived phagocytic cells that have been described to enter the damaged retina, where they exert a protective role.\textsuperscript{42,43}

Derived phagocytic cells that have been described to enter the retina in vivo showed a moderate microglia presence, similar to that found in age-matched WT retinas. However, a masked microglial response in the dystrophic retina was revealed solely by culturing. This potentially stressing process doubled the number of microglial cells in rd10 but not in WT retinas. Because retinas had been isolated from all surrounding tissues before culturing, these additional microglial cells could originate from a preexisting, hidden population. We speculate that these microglia might have originated from bone marrow-derived phagocytic cells that have been described to enter the damaged retina, where they exert a protective role.\textsuperscript{42,43}

IGF-I treatment reduced photoreceptor cell death and, in parallel, doubled again the number of microglial cells, specifically in those layers in which IGF-I acts, namely ONL and GCL. IGF-I may induce microglial proliferation because of its action as a potent mitogen for a wide variety of cell types, including peripheral macrophages, an aspect that remains to be studied.\textsuperscript{13–44} Microglial cell depletion with clodronate-encapsulated liposomes in IGF-I-treated retinas strongly attenuated the antiapoptotic effect of IGF-I, demonstrating a potential direct or indirect role of microglia in neuroprotection. Microglia-mediated neuroprotection has been described in other parts of the nervous system, in response to other pathologic insults.\textsuperscript{20–21} Conversely, in the retina, the best described effect of microglia was neurotoxic.\textsuperscript{22–29} After microglial cell depletion, the neuroprotective effect of IGF-I was considerably diminished, a somewhat surprising observation because of the multiple biochemical and cellular effects displayed by IGF-I.\textsuperscript{6–9} We favor the existence of an amplifying cellular network involving microglial cell recruitment with possible additional effects on Mülller glial cells.\textsuperscript{45} Indeed, glial response to retinal damage includes reactive Mülller cell gliosis, which was observed in the rd10 retinas (data not shown), microglia-Mülller cell crosstalk, and secretion of neurotrophic factors by Mülller cells.\textsuperscript{46–48} Certainly, the effects of a single factor, even one as potent as IGF-I, should be considerably less than the effects produced by a putative IGF-microglia-Mülller amplifying axis or loop. Further, Mülller cells seem to be able to proliferate and transdif-

**FIGURE 5.** Effect of microglia depletion in rd10 mouse retinal explants. P23 retinal explants from rd10 mice were cultured for 24 hours in basal medium (A, C) or in the presence of IGF-I (20 nM; B, D) in conjunction with control PBS-encapsulated liposomes (A, B) or clodronate-encapsulated liposomes (C, D). Explants were then processed for cryosectioning. Cell death was visualized by TUNEL (A–D, green), and the structure of the retina was counterstained with DAPI (A–D, blue). Retinal layers are labeled ONL, INL, and GCL. (E) Cell death was quantified by scoring TUNEL-positive nuclei in the ONL as shown in (A–D). Data represent the mean ± SEM of the normalized number of labeled nuclei located in the ONL per entire retinal section out of four sections from three independently cultured retinas. *P ≤ 0.01; **P ≤ 0.005. Scale bar, 75 μm (A–D).

been described in other parts of the nervous system but not in the retina, merits further studies oriented to attenuate retinal dystrophies. Further, more investigation is required to identify additional retinal cell types involved in the neuroprotective response to IGF-I and related molecules.
ferentiate into photoreceptors in damaged retinas. A scenario not covered by our short-term treatments but of interest for possible future therapies.

The rd10 organotypic retinas treated with clodronate-encapsulated liposomes but no IGF-I exhibited reduced levels of apoptotic photoreceptors compared with retinas treated with control liposomes. Thus the retina, as in the rest of the nervous system, appears to exhibit dual, graded microglial behavior, the effects of which can range from neuroprotective to deleterious. Interestingly, this dual function may be mediated by distinct subpopulations (i.e., endogenous resident versus bone marrow-derived microglia), a possibility that merits further investigation. Our results reveal a complex signaling network mediating the neuroprotective effects of IGF-I in the dystrophic retina. These findings provide an important basis for further detailed characterization of this pathway, with a view to developing valid therapeutic approaches for the treatment of patients with RP.

Acknowledgments

The authors thank Carolina Isegas and José María Ruiz for performing intravitreal injections; Julio Navascues, Amanda Sierra, Teresa Suárez, Patricia Vázquez, and Patricia Boya for critical reading of the manuscript; Flora de Pablo for continuous encouragement and ideas; and Maite Seisdedos, Ana Robles, and the staff of the animal house for technical support.

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