Neuroprotective Effects of FGF2 and Minocycline in Two Animal Models of Inherited Retinal Degeneration

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Purpose. The purpose of this study was to study the effect of minocycline and several neurotrophic factors, alone or in combination, on photoreceptor survival and macro/microglial reactivity in two rat models of retinal degeneration.

Methods. P23H-1 (rhodopsin mutation), Royal College of Surgeon (RCS, pigment epithelium malfunction), and age-matched control rats (Sprague-Dawley and Pievald Viro Glaxo, respectively) were divided into three groups that received at P10 for P23H-1 rats or P33 for RCS rats: (1) one intravitreal injection (IVI) of one of the following neurotrophic factors: ciliary neurotrophic factor (CNTF), pigment epithelium-derived factor (PEDF), or basic fibroblast growth factor (FGF2); (2) daily intraperitoneal administration of minocycline; or (3) a combination of IVI of FGF2 and intraperitoneal minocycline. All animals were processed 12 days after treatment initiation. Retinal microglial cells and cone photoreceptors were immunodetected and analyzed qualitatively in cross sections. The numbers of microglial cells in the different retinal layers and number of nuclei rows in the outer nuclear layer (ONL) were quantified.

Results. IVI of CNTF, PEDF, or FGF2 improved the morphology of the photoreceptors outer segment, but only FGF2 rescued a significant number of photoreceptors. None of the trophic factors had qualitative or quantitative effects on microglial cells. Minocycline treatment reduced activation and migration of microglia and produced a significant rescue of photoreceptors. Combined treatment with minocycline and FGF2 had higher neuroprotective effects than each of the treatments alone.

Conclusions. In two animal models of photoreceptor degeneration with different etiologies, minocycline reduces microglial activation and migration, and FGF2 and minocycline increase photoreceptor survival. The combination of FGF2 and minocycline show greater neuroprotective effects than their isolated effects.

Keywords: royal college of surgeons, P23H-1, microglia, minocycline, FGF2
could further increase photoreceptor survival and therefore be used for the treatment of retinal degenerations.

In this study, we analyze in the P23H line 1 (P23H-1) and RCS rat strains the effects of (1) different neurotrophic factors, (2) minocycline, or (3) a combination of the neurotrophic factor that gave best results (FGF2) and minocycline on photoreceptor survival and microglial cell activation during the period of early photoreceptor degeneration.

**Materials and Methods**

**Animals and Experimental Groups**

Homozygous albino female P23H-1, homozygous pigmented RCS rats, and their healthy controls albino Sprague-Dawley (SD) and pigmented Piebald Virol Glaxo (PVG) rats were used in this work. Transgenic homozygous P23H-1 animals were obtained from M. LaVail (University of California at San...
Francisco School of Medicine; http://www.ucsfeye.net/mla
vailRDratmodels.shtml; Steinberg RH, et al. IOVS 1996;37:AR-
VO Abstract 3190), and bred at the University of Murcia; RCS,
SD, and PVG rats were obtained from the breeding colony of
the University of Murcia, Murcia, Spain. Rats were housed in
temperature- and light-controlled rooms with a 12-hour light/
dark cycle (light from 8 AM to 8 PM) and had food and water ad
libitum. Animal manipulations were carried out following the
Spanish and European Union regulations for the use of animals
in research (Council Directive 86/609/EEC), the indication of
the University of Murcia ethical committee, and the ARVO
statement for the use of animals in ophthalmic and vision
research.

P23H and RCS rats were divided into different experimental
groups (Fig. 1). In the first experimental group, the animals
received an intravitreal injection (IVI) of one of the following
neurotrophic factors: CNTF (B), PEDF (C), FGF2 (D), minocycline (E), or the combination of
neurotrophic factors, FGF2 (F) showing S (red) and L/M (green) opsin immunoreactivity and DAPI counterstaining (blue). (G, H) Graphs showing mean numbers ± SD of nuclei rows in the ONL of dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of
neurotrophic factors and FGF2. Retinas treated with FGF2, minocycline, or its combination showed a higher number of nuclei rows in the ONL. Scale bar: 100 μm. One-way ANOVA: F(6,41) = 222.843; P < 0.001.

IVIs
IVIs were performed only in the left eye following previously
described methods that are standard in our laboratory.59
Briefly, the injections were made through the supratemporal
sclera using a Hamilton microsyringe (26G; Hamilton 701 N,
Esslab, Benfleet, UK). Once the needle was visualized retroreflectively, the liquid was injected and the needle was removed.

The following quantities of neurotrophic factors were injected: (1) 0.4 μg CNTF (R&D Systems, Vitro S.A. Madrid, Spain), 2 μg PEDF (Preprotech, London, UK), both diluted in PBS, or 1 μg FGF2 (Preprotech) diluted in Tris-Cl 2 mM, pH 7.6. Matched groups of P23H-1 and RCS rats were treated with the corresponding vehicle solutions. These doses were used following previously published works.28,32,36–38 The injection volume was always 5 μL.

Intraperitoneal Minocycline
Minocycline (45 mg/kg) or vehicle (saline, NaCl 0.9%) intraperitoneal injections were administered twice the first day and daily for the remaining days. This dose was used following previously published results.33–35 For the group of animals that received the combined treatment, the first dose of minocycline was given 1 day before the IVI.

Tissue Processing
Rats were first sedated by an intraperitoneal injection of diluted sodium pentobarbital (1:1 in saline; Dolethal Vetoquinol, S.A., Lure, France) and then killed with a lethal dose of sodium pentobarbital. Then, rats were perfused transcardially through the ascending aorta, first with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Finally, all the retinas were prepared for cryostat cross sections following previously described methods.27,40,41

Immunohistofluorescence
Immunohistofluorescence staining was performed on retinal whole mounts following standard procedures in our laboratory.27 Briefly, cross sections were washed in PBS containing 0.1% Triton X-100 (Tx; Sigma-Aldrich) and incubated overnight at 4°C with a mixture of the primary antibodies (see next paragraph) diluted in blocking buffer (PBS containing 2% Tx and 2% normal donkey serum). After washing with PBS-0.1%Tx, the sections were incubated for 1 hour at room temperature with a mixture of the secondary antibodies (see next paragraph) diluted in PBS-2%Tx. Sections were washed again with PBS-0.1%Tx and, after a rinse in PBS, mounted with a mounting media containing DAPI (4′,6-diamidino-2-phenylindole; Vectashield Mounting Medium con DAPI; Vector Atom, Alicante, Spain) to counterstain all retinal nuclei.

Antibodies

Primary Antibodies. Microglial cells were detected with rabbit anti-Iba1 antibody (1:1000, 019-19741; Wako Chemicals, Neuss, Germany). L- and S-cone outer segments were detected with rabbit anti-red/green opsin (1:1200, AB5405; Chemicon-Millipore Ibérica, Madrid, Spain) and goat anti-blue opsin (1:1000, N-20; OPN1SW; Santa Cruz Biotechnology, Heidelberg, Germany), respectively. Cones outer and inner segments
were immunodetected with rabbit anti-cone arrestin (1:1000, AB15282; Chemicon-Millipore Iberica).

Secondary Antibodies. For secondary detection, two antibodies were used: Donkey anti-goat Alexa Fluor 594 and donkey anti-rabbit Alexa Fluor 488, both purchased from Molecular Probes (Invitrogen, ThermoFisher, Madrid, Spain) and diluted at 1:500.

Image Analysis

Image analysis was performed following previously described methods.\textsuperscript{27} Briefly, retinal cross sections spanning the optic disk were selected (three cross sections/retina and antibody combination; \( n = 6 \) retinas/group). Samples were examined and photographed under a fluorescence microscope (Axioskop 2 Plus; Zeiss Mikroskopie, Jena, Germany) equipped with various filters and a digital high-resolution camera (ProgRes C10; Jenoptik, Jena, Germany).

Eight images (530 \( \times \) 390 \( \mu \)m) were taken per retinal section: four from the dorsal (superior) retina and four from the ventral (inferior) retina. Images were acquired at equidistant distances from the optic disk to the retinal periphery (25\%, 50\%, 75\%, and 95\%, considering 100\% the length of each hemi-retina). Images were further processed with Adobe Photoshop CS 6 (Adobe Systems, Inc., San Jose, CA, USA) when needed.

Some photographs of the cross sections were also taken with confocal microscope Leica SP8 (Leica Microsystems, Wetzlar, Germany).

Nuclei Rows and Microglial Cell Quantification

The number of nuclei rows in the outer nuclear layer (ONL) and the number of microglial cells in each retinal layer were manually quantified in each image\textsuperscript{27} (\( n = 3 \) retinal sections from six different retinas per time point and experimental group).

Statistics

The number of nuclei rows in the ONL and the number of microglial cells were compared among retinal regions and vehicle, CNTF, PEDF, FGF2, minocycline, and combination of minocycline and FGF2 by one-way ANOVA (GraphPad Software, San Diego, CA, USA). Tukey’s post-hoc test was performed for multiple comparisons.
groups. Statistical analysis was carried out using SigmaStat 3.11 for Windows (Systat Software, Inc., Richmond, CA, USA). The Student’s t-test was used to compare between two different groups (i.e., different strains following the same treatments) and 1-way ANOVA (followed by Tukey’s post hoc test) was used to compare several groups (i.e., different treatments on the same strain). Differences were considered significant when $P < 0.05$.

RESULTS

Untreated P23H-1 and RCS Retinas

P23H-1 and RCS animals were processed at P22 and P45, respectively (Fig. 1). At this age, there were no significant differences in photoreceptor loss between the untreated or vehicle-treated animals. Photoreceptor degeneration had started in both groups; cones showed shortened outer segments and the mean number of nuclei rows in the ONL were significantly reduced to four to six or two to four rows in the P23H-1 (Figs. 2, 3) or RCS (Figs. 4, 5) compared with 12 to 14 rows in controls (SD, PVG rats; data not shown). In addition, we could observe activated retinal microglial cells, with ameboid morphology and migration of these cells from their normal location to the photoreceptors layers: ONL and outer segment layer (Figs. 6, 7), in accordance with previously published data.27

Efficacy of CNTF, PEDF, or FGF2

CNTF, PEDF, or FGF2 was administered intravitreally at P10 (P23H) or P33 (RCS), and the retinas were examined 12 days later. These three factors improved the morphology of the photoreceptors outer segment compared with the vehicle-treated retinas (Figs. 2A–2D, 4A–4D). The outer segment of the photoreceptors appeared straightened, longer, and more strongly labeled with arrestin in the trophic factor-treated retinas, both in P23H-1 rats and RCS rats (Figs. 3, 5). However, despite the observed morphologic rescue, only FGF2 produced a significant increase in the mean number of nuclei rows in the ONL (Figs. 2G, 2H, 4G, 4H, 6E) in the dystrophic animals, and this effect was more pronounced in the dorsal retina in the P23H strain (Fig. 2G). When expressed as percentages of increased survival in relation to vehicle-treated animals, FGF2 increased $+21\%$ or $+23\%$ the number of nuclei rows in P23H-1 or RCS rats (Fig. 8), respectively.

None of the trophic factors had an effect on the numbers of microglial cells or on their migration, as their numbers in the different retinal layers were similar to those observed in vehicle-treated P23H and RCS rats (Figs. 6H, 7H).

Efficacy of Minocycline

In both dystrophic strains, intraperitoneal treatment with minocycline induced an important reduction of the microglial cell activation and migration (Figs. 6F, 6H, 7F, 7H). Indeed, compared with vehicle- or trophic factor-treated retinas, the
Microglial cell expression and quantification in P23H-1 rats. Photomicrographs of representative retinal cross sections taken from SD rats (A) and P23H-1 rats treated with vehicle (B) CNTF, (C) PEDF, (D) FGF2, (E) minocycline, (F) or combination of minocycline and FGF2 (G) showing microglial cells (green) and DAPI counterstaining (blue). (H) Graph showing mean number ± SD of microglial cells counted in each retinal layer in control naive Sprague-Dawley (SD) rats and in dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of both. In the animals treated with minocycline (alone or in combination with FGF2), the mean number of microglial cells observed in the outer retinal layers was significantly lower than that observed in the animals treated with vehicle, CNTF, PEDF, or FGF2. However, for the retinal ganglion.
cell layer, the mean number of microglial cells observed was higher in the animals treated with minocycline (alone or in combination with FGF2). One-way ANOVA: for the retinal ganglion cell layer, \( F(4,29) = 29.698, P < 0.001 \); for the ONL, \( F(4,29) = 10.809, P < 0.001 \); for the outer segment layer, \( F(4,29) = 1834.715, P < 0.001 \).
mean numbers of microglial cells in the minocycline group were significantly higher in the GCL and significantly lower in the ONL and outer segment layer (Figs. 6H, 7H), indicating less microglial cell migration from the inner to the outer retina.

In addition to its effect on microglial dynamics, minocycline treatment protected photoreceptors, with a significant rescue of the nuclei rows of +22% in P23H-1 and +17% in RCS rats compared with vehicle-treated animals (Fig. 8). This rescue was similar to that observed when the animals were treated with FGF2 (see above).

**Efficacy of FGF2 and Minocycline Combined Treatment**

Finally, we analyzed the animals treated with FGF2 and minocycline to assess whether their combined neuroprotective effect was additive. As expected, the effect of the combination of treatments on the photoreceptors outer segment morphology and microglial modulation was similar to the corresponding single treatments, FGF2 and minocycline, respectively (compare Figs. 3D, 5D, 6D, 6E, 6F, 7E, 7F with Figs. 3E, 5E, 6G, 7G, respectively). However, both treatments together induced a significantly higher photoreceptor neuroprotection than each treatment alone; for instance, the increase of nuclei rows was +30% in P23H-1 and +15% in RCS rats compared with FGF2 alone, and +29% in P23H-1 and +12% in RCS rats compared with minocycline alone (Fig. 8). Compared with vehicle-treated animals, this increase reached +51% in P23H-1 rats and +38% in RCS rats, an increased neuroprotection that was greater than that obtained by each treatment alone (Fig. 8).

**Rescue Effects in P23H-1 and RCS Rats**

As stated in the previous paragraphs, the different neurotrophic factors, minocycline or the combination of FGF2 and minocycline, increased photoreceptor survival both in P23H-1 rats and RCS rats. However, the combination of FGF2 and minocycline produced significantly more photoreceptor survival in P23H-1 rats than in RCS rats (Fig. 8).

**DISCUSSION**

In this work, we studied the effect on photoreceptor survival and microglial behavior of different neurotrophic factors and minocycline either alone or in combination during the early period of photoreceptor degeneration in two different animal models of inherited retinal degeneration.

Because the loss of photoreceptors is irrecoverable, strategies to rescue them depend on the number of remaining photoreceptors, and therefore, patients should be treated in the early stages of these progressive diseases. Early therapies may delay the progression of photoreceptor loss and in doing so prevent the retinal remodeling that occurs following photoreceptor degeneration. This is of great impor-

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**FIGURE 7.** Microglial cell expression and quantification in RCS rats. Photomicrographs of representative retinal cross sections taken from PVG rats (A) and RCS rats treated with vehicle (B), CNTF (C), PEDF (D), FGF2 (E), minocycline (F), or combination of minocycline and FGF2 (G) showing microglial cells (green) and DAPI counterstaining (blue). (H) Graph showing mean number ± SD of microglial cells counted in each retinal layer in control naive Sprague-Dawley (SD) rats and in dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of both. In the animals treated with minocycline (alone or in combination with FGF2), the mean number of microglial cells observed in the outer retinal layers was significantly lower than that observed in the animals treated with vehicle. CNTF, PEDF, or FGF2. However, for the retinal ganglion cell layer, the mean number of microglial cells observed was higher for the animals treated with minocycline (alone or in combination with FGF2). One-way ANOVA: for the retinal ganglion cell layer, F(4,29) = 103.856, P < 0.001; for the ONL, F(4,29) = 518.717, P < 0.001; for the outer segment layer, F(4,29) = 885.111, P < 0.001.

**FIGURE 8.** Percentage of effectiveness of each treatment studied. Graph showing in percentage the increase of nuclei in ONL and, consequently, photoreceptor survival as a measure to evaluate the effectiveness of each treatment. Combination of minocycline and FGF2 produces an additive neuroprotective effect of +51% in P23H-1 and +38% RCS rats. Student’s t-test, P < 0.05.
rance, because most therapeutic strategies for RP (i.e., retinal prosthesis or cell transplantation) are based on the assumption that the retina remains viable after photoreceptor degeneration.

Trophic factors are small proteins with essential functions such as promoting proliferation, growth, regeneration, maturation, or neuronal survival. Several studies have shown their role in the prevention of photoreceptor degeneration. Our results showed that IVI of neurotrophic factors (CNTF, PEDF or FGF2) produced a morphologic rescue of the photoreceptors outer segment, both in the P23H-1 and in the RCS rats, indicating that neurotrophic factors have a neuroprotective effect that is independent of the etiology of the degeneration (rhodopsin mutation or ERP malfunction). However, only FGF2 treatment produced a significant rescue in the mean number of nuclei rows in the ONL. This is in accordance with previous studies showing that treatment with CNTF or PEDF rescues photoreceptor morphology but does not improve the electroretinographic responses. On the other hand, several authors have shown that FGF2 not only rescues photoreceptor morphology, but also increases photoreceptor survival, reducing apoptosis, and ameliorates the ERG responses in accordance with our results.

Microglial activation is an early event of retinal degenerations, and thus modulating these cells could be a potential treatment to provide photoreceptor neuroprotection. Conversely, some studies have suggested that microglia is required to promote photoreceptor survival. Indeed, different roles (neuroprotective and neurodestructive) have been proposed for resident, activated, and migrating microglia. The neuroprotective effect of minocycline against photoreceptor apoptosis in retinal degenerations is explained by mainly two different mechanisms: (1) a direct antiapoptotic effect on photoreceptors and/or (2) an effect through its anti-inflammatory properties.

Minocycline (a microbial inhibitor) is widely used to study neuroprotection in central nervous system diseases. Minocycline is a broad-spectrum tetracycline antibiotic, and previous studies have demonstrated that minocycline has antimicrobial, anti-inflammatory, antiapoptotic, and neuroprotective properties. The neuroprotective effect of minocycline has been proposed for resident, activated, and migrating microglia.

In this work, we show that the combined treatment with FGF2 and minocycline elicited a photoreceptor rescue (morphology and number of nuclei in the ONL) that was higher than that observed with the individual treatments. Because previous studies have shown that increasing the dose of the substances does not increase survival, our results suggest that the observed improvement is due to the combined effect of the two treatments acting through different mechanisms. Both treatments have an effect on the apoptotic pathway, but only minocycline has a direct effect in microglial cells.

In summary, our study provides strong support to the concepts that, in retinal degenerations, (1) CNTF, PEDF and FGF2 rescue the photoreceptor outer segment morphology and FGF2 increases photoreceptor survival; (2) minocycline reduces microglial activation and migration and promotes photoreceptor survival; and finally, (3) the combination of FGF2 and minocycline treatments has an additive effect. Thus, here we propose that anti-inflammatory agents (i.e., minocycline) as an adjuvant therapy in combination with neurotrophic factors could be the best choice as potential treatments in the earlier stages of retinal degenerations.

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