Photoreceptor Protection by Cardiotrophin-1 in Transgenic Rats with the Rhodopsin Mutation S334ter

Ying Song,1 Lian Zhao,1 Weng Tao,2 Alan M. Laties,1 Zbijun Luo,3 and Rong Wen1

Purpose. This work examines the effects of cardiotrophin (CT)-1 on photoreceptor survival in transgenic rats that carry the rhodopsin mutation S334ter.

Methods. Recombinant CT-1 was injected intravitreally into eyes of heterozygous animals. Photoreceptor survival was analyzed by histology. Phosphorylation of signal transducer and activator of transcription I (STAT1), STAT3, extracellular signal-regulated kinase (ERK), or Akt was assessed by immunoblot analysis. Localization of phosphorylated STAT3 was determined by immunocytochemistry.

Results. Heterozygous S334ter rats experience rapid photoreceptor degeneration. By postnatal day (PD)20, the outer nuclear layer (ONL) retained only 1 to 2 rows of nuclei compared with 10 to 12 rows in wild-type animals. Repeated administration of CT-1 resulted in significant survival of photoreceptors. At PD20, a CT-1-treated eye (2 μg/2 μL every 3 days, starting at PD9) had six to seven rows of nuclei, and the vehicle-treated eyes had only one to two rows. At PD30, eyes treated every 3 days still had five to six rows of nuclei, in contrast to no rows to one row in vehicle-treated eyes. Eyes treated every 4 days retained three to four rows, whereas eyes treated every 5 days had two to three rows. There was a significant increase in phosphorylated STAT1 and -3 in the retina after CT-1 injection. The increase in phosphorylated STAT3 was colocalized with glutamine synthetase, a Müller cell marker, by immunocytochemistry.

Conclusions. These results indicate that CT-1 promotes photoreceptor survival and that Müller cells probably mediate this effect. They also suggest that sustained delivery of the protein is essential for long-term rescue of photoreceptors. (Invest Ophthalmol Vis Sci. 2003;44:4069–4075) DOI:10.1167/iovs.02-1130

Retinitis pigmentosa is a group of inherited retinal degenerative disorders. In the early stage of the disease, patients typically experience night blindness and a decline in peripheral vision due to loss of rod photoreceptors. As the degeneration progresses, central vision is also affected, leading eventually to total blindness. Mutations in any one of many different genes can be responsible for photoreceptor degeneration, including rhodopsin, peripherin, the β subunit of the cGMP phosphodiesterase, and the rod outer segment protein ROM1.1,2

In animal models of photoreceptor degeneration, photoreceptors are partially protected by neurotrophic factors. This was first demonstrated in the Royal College of Surgeon (RCS) rat in which a mutation in the Merth gene renders the retinal pigment epithelial (RPE) cells incapable of proper phagocytosis,3,4 resulting in accumulation of the shed outer segments of photoreceptors in the subretinal space and eventually to degeneration of the photoreceptors. Subretinal injection of basic fibroblast growth factor (bFGF) was found to rescue photoreceptors temporarily in the RCS rats.5 Subsequent studies showed that ciliary neurotrophic factor (CNTF), a member of the interleukin (IL)-6 family of cytokines, protects photoreceptors in a broad range of animal models in several species, including rat,6 mouse,7 and dog.8

CT-1, also a cytokine of the IL-6 family, was originally identified as a factor that induces hypertrophy of cardiac myocytes.9 Further characterization revealed that it also promotes cardiac myocyte survival10,11 and supports the long-term survival of spinal motor neurons.11 Studies have shown that the biological effects of CT-1 are initiated by the binding of CT-1 to a receptor complex containing gp130 and leukemia inhibitory factor receptor (LIFR)-β,12 and are mediated through specific signaling pathways. CT-1 promotes survival of myocardial cells through the extracellular signal-regulated kinase (Erk)-mitogen-activated protein (MAP) kinase and the phosphatidylinositol 3 (PI3) kinase/Akt signaling pathways,10,13 whereas it induces cardiac myocyte hypertrophy by activating a signal transducer and activator of transcription (STAT)-3-dependent pathway.14

The present work examines the potential of CT-1 to protect photoreceptors in a line of transgenic rats that carry the rhodopsin mutation S334ter. These animals experience rapid photoreceptor degeneration soon after birth.15 Repeated intravitreal injection of CT-1 leads to greatly enhanced survival of photoreceptor cells in these animals. Results also show that CT-1 activates the STAT3 signaling pathway in retinal Müller cells. These findings suggest that long-term protection of photoreceptors could be achieved by sustained delivery of neurotrophic factors such as CT-1. They also indicate that CT-1 promotes photoreceptor survival indirectly through the agency of Müller cells.

Materials and Methods

Expression and Purification of Recombinant CT-1 Protein

The open reading frame of human CT-1 cDNA was PCR cloned into an expression vector (pQE30; Qiagen, Valencia, CA), fused to a 6xHis tag at the amino terminus, to generate plasmid pQE-CT1. Recombinant human CT-1 protein was expressed in Escherichia coli (XL-blue; Stratagene, La Jolla, CA) and purified by immobilized-metal affinity chroma-
microsyringes (Hamilton, Reno, NV). The right eye of an animal was repeated every 3, 4, or 5 days. Eyes were collected at PD20 or PD30.

The numbers of transgenic animals used were as follows: 2D); nine for multiple injections at 3-day intervals, end point PD30 (Fig. 2A). Severe degeneration was observed in PBS-treated eyes of transgenic animals. Shown in Figure 2B is a section of the superior retina of the right eye of an S334ter-3 rat that received a single injection of CT-1 (2 μL PBS at PD9). It had only one visible outer segments. In the left eye of this animal, which was treated with a single intravitreal injection of CT-1 (2 μg from each sample was electrophoresed on 10% polyacrylamide gel (NuPage; Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Blots were stained briefly with ponceau S for visual inspection of transfer efficiency. Immunoblot analysis was performed, and signals were visualized using chemiluminescent substrates (SuperSignal; Pierce) and recorded on autoradiograph (Hyperfilm; Amersham). All experiments were repeated three times to verify the consistency of the results.

RESULTS

Protection of Photoreceptors by CT-1

Heterozygous transgenic rats that carry the rhodopsin mutation S334ter (the S334ter-3 rats) were used to evaluate the potential of CT-1 for photoreceptor protection. These animals experience rapid photoreceptor degeneration shortly after birth. Degeneration is evident as early as PD8, and by PD20 more than 90% photoreceptors are lost. PD20 was therefore chosen as the end point for our routine screening for potential photoreceptor protective agents. Figure 2 shows representative superior retinal sections from control and S334ter-3 animals collected at PD20. In wild-type Sprague-Dawley rats, the outer nuclear layer (ONL) had 11 to 12 rows of photoreceptor nuclei and the inner and outer segments were well developed (Fig. 2A). Severe degeneration was observed in PBS-treated eyes of transgenic animals. Shown in Figure 2B is a section of the superior retina of the right eye of an S334ter-3 rat that received a single injection of 2 μL PBS at PD9. It had only one row of nuclei in the ONL and very short inner segments with no visible outer segments. In the left eye of this animal, which was treated with a single intravitreal injection of CT-1 (2 μg in 2 μL PBS) at PD9, the retina retained two to three rows of nuclei in the ONL (Fig. 2C). Repeated injection of the same dose at an interval of 3 days resulted in significant protection of photoreceptors. Figure 2D shows a superior retina from an eye that was treated with CT-1 every 3 days starting at PD9. The ONL still had six to seven rows of nuclei at PD20, and the inner segments were better preserved. Control retina treated with...
PBS (2 μL) every 3 days (data not shown) were similar in appearance to the section in Figure 2B. The significant preservation of photoreceptors in CT-1–treated eyes at PD20 encouraged us to extend our experiments to PD30. Figure 3 shows representative tissue sections of superior retinas collected at PD30. The retinas of wild-type control rats at PD30 were similar to those in PD20 animals. The ONL generally had 10 to 12 rows of nuclei (Fig. 3A). The retina treated with a single dose of CT-1 (2 μg in 2 μL PBS at PD9) from the left eye of the same animal (C), the ONL had two to three rows of nuclei. The ONL of a retina from a rat that received repeated injections every 3 days (2 μg CT-1 in 2 μL PBS per injection, starting at PD9) had six to seven rows of nuclei. The IS were better preserved, although shorter than those in normal control eyes. Some dislocated cells were found in the subretinal space next to the RPE (D). Sections were stained with toluidine blue. OPL, outer plexiform layer. Scale bar, 20 μm.

CT-1–Induced Phosphorylation of Signaling Proteins

Effects of CT-1 are mediated through specific signaling pathways: CT-1 promotes survival of myocardial cells through the MAP kinase and the PI3 kinase/Akt signaling pathways, whereas it induces cardiac myocyte hypertrophy through the STAT3 pathway.10,13,14 To explore the signaling pathways through which CT-1 protects photoreceptors, we examined the phosphorylation state of proteins in several signaling pathways. In these experiments, S334ter-3 rats at PD9 were treated with PBS (2 μL) in the right eye and CT-1 (2 μg CT-1 in 2 μL PBS) in the left eye. Retinas were collected at various time points after injection, and the amount of phosphorylated STAT1, STAT3, Erks, and Akt was examined by immunoblot analysis. As shown in Figure 4A, CT-1 induced a dramatic increase in STAT3 phosphorylation. The increase was detected as early as 30 minutes after injection and lasted 24 hours. A smaller increase of shorter duration in STAT3 phosphorylation was observed in PBS-injected retinas (Fig. 4B). Injection of CT-1 also induced STAT1 phosphorylation of duration similar to that of STAT3 phosphorylation (Fig. 4C). There was, however, no impressive preservation of the photoreceptor cell body, no clear outer segment development was observed, even at PD30.

Figure 2. Protection of photoreceptors by CT-1 (PD20). Plastic-embedded sections of retina from a normal rat (A), or retinas from transgenic rats treated with PBS (B), a single injection of CT-1 (C), or multiple injections of CT-1 (D) were examined at PD20 by light microscopy. The normal retina had well-developed outer and inner segments (OS, IS). The ONL had 11 to 12 rows of nuclei (A). In the PBS-treated retina (2 μL, single injection at PD9) from the right eye of an S334ter-3 rat, the ONL had only one row of nuclei, and the inner segments became very short stumps (B). In the retina treated with a single dose of CT-1 (2 μg in 2 μL PBS at PD9) from the left eye of the same animal (C), the ONL had two to three rows of nuclei. The ONL of a retina from a rat that received repeated injections every 3 days (2 μg CT-1 in 2 μL PBS per injection, starting at PD9) had six to seven rows of nuclei. The IS were better preserved, although shorter than those in normal control eyes. Some dislocated cells were found in the subretinal space next to the RPE (D). Sections were stained with toluidine blue. OPL, outer plexiform layer. Scale bar, 20 μm.
detectable increase in STAT1 phosphorylation in PBS-treated eyes (data not shown).

A parallel increase was demonstrated in Erk1/2 phosphorylation in CT-1–treated retinas (Fig. 5A). The increase reached its maximum within 1 hour, and by 12 hours it had already declined to a level close to control. PBS injection induced a small increase in Erk1/2 phosphorylation of a shorter duration (Fig. 5B). CT-1 treatment did not alter Akt phosphorylation in the retina (Fig. 5C), nor did PBS injection (data not shown).

Localization of Phospho-STAT3

To localize the CT-1–induced increase in STAT3 phosphorylation, normal Sprague-Dawley rats were treated in the left eyes with CT-1 (2 μg in 2 μL PBS), and the untreated right eyes were the control. Eyes were collected 1 hour after treatment, and frozen sections (10 μm) were prepared for immunocytochemical analysis using phospho-STAT3–specific antibodies. In the control retina, immunostaining for phospho-STAT3 is detected mainly in the nuclei of retinal ganglion cells. Slight staining is also visible in some cells in the inner nuclear layer (INL; Fig. 6A). A dramatic increase in phospho-STAT3 immunostaining in the CT-1–treated retina (left eye of the same animal, 1 hour after intravitreal injection of CT-1) was seen in a specific band of cells in the INL (Fig. 6B). No significant alteration of the intensity of signals in the ganglion cells was observed, however. The location and morphology of the cells that responded to CT-1 treatment suggested that they were Müller cells. To confirm the identity of these cells, we performed double-labeling experiments using antibodies against phospho-STAT3 and glutamine synthetase (GS), a Müller-cell–specific marker. The immunostaining of phospho-STAT3 (Figs. 6C, 6E, green) and GS (Figs. 6D, 6E, red) are colocalized in cells (Fig. 6E, yellow) in which an increase in STAT3 phosphorylation was observed after CT-1 treatment. Thus, in our experiments, CT-1 induced STAT3 activation specifically in Müller cells.
DISCUSSION

Although retinal degenerative disorders have similar clinical manifestations—namely, the degeneration of rod photoreceptors, which eventually leads to cone degeneration and blindness—the underlying genetic abnormalities can be very different.1,2 Given the genetic heterogeneity of retinitis pigmentosa, one practical treatment strategy would be to promote photoreceptor survival through a common mechanism, rather than to attempt to target a large number of specific mutations. Evidence is accumulating in this regard that activation of a gp130-dependent mechanism in Müller cells by neurotrophic factors, such as members of the IL-6 family of cytokines, represents a viable approach to treatment without identifying the specific gene mutation. Photoreceptor rescue by neurotrophic factors first came to prominence when it was demonstrated in the RCS rat that injection of bFGF protects photoreceptors.5 Subsequently, a screen of a panel of neurotrophic factors in a second model, the light-damage model of photoreceptor degeneration in the albino rat, revealed the photoreceptor protective properties of CNTF.6 Still later, studies showed that whereas some factors, such as bFGF, rescue photoreceptors in only one or a few animal models, CNTF is protective over a broad range of models and species.6–8 The breadth of protection afforded by CNTF leads immediately to the hypothesis that a common mechanism exists in mammalian retina through which CNTF or CNTF-like molecules can promote photoreceptor survival. That mechanism probably involves a gp130-dependent signaling pathway. A recent report that cardiortrophin-like cytokine (CLC) protects photoreceptors is consistent with this hypothesis (Wen R, et al. IOVS 2001;42:ARVO Abstract 3380), as is the present work, which provides clear evidence that CT-1 also protects photoreceptors. Because all three are members of the IL-6 family of cytokines, it is very probable that they work by the same mechanism.

CT-1, originally identified in a screening conducted to find factors that induce a hypertrophic response in neonatal cardiac muscle cells,9 is a polypeptide of approximately 200 amino acid (aa) residues (human CT-1: 201 aa, mouse CT-1: 203 aa) with 80% amino acid identity between mouse and human. Similar to CNTF, the N-terminal of CT-1 does not have a conventional hydrophobic secretion signal sequence9,17 although CT-1 is secreted nonetheless.11 Being a member of the IL-6 family of cytokines, CT-1 has 24% amino acid identity to LIF and 19% identity to CNTF. Analysis of the secondary structure predicted for CT-1 also indicates similarity with other members of the IL-6 family.9,18 CT-1, originally identified in a screening conducted to find factors that induce a hypertrophic response in neonatal cardiac muscle cells,9 is a polypeptide of approximately 200 amino acid (aa) residues (human CT-1: 201 aa, mouse CT-1: 203 aa) with 80% amino acid identity between mouse and human. Similar to CNTF, the N-terminal of CT-1 does not have a conventional hydrophobic secretion signal sequence9,17 although CT-1 is secreted nonetheless.11 Being a member of the IL-6 family of cytokines, CT-1 has 24% amino acid identity to LIF and 19% identity to CNTF. Analysis of the secondary structure predicted for CT-1 also indicates similarity with other members of the IL-6 family.

FIGURE 4. CT-1 induced phosphorylation of STAT3 and -1. Immunoblot analyses were performed to detect the phosphorylation of STAT3 and STAT1 at 0 (control), 0.5, 1, 6, 12, 24, and 48 hour after intravitreal injection of CT-1 (2 μg in 2 μL PBS) in P09 S334ter-3 rats. (A) CT-1 induced a significant STAT3 phosphorylation (pSTAT3), which was detected as early as 30 minutes and lasted 24 hours. It also induced a slight increase in total STAT3 between 6 to 24 hours after injection (STAT3). (B) An increase in STAT3 phosphorylation was detected after PBS injection with a lesser amplitude and shorter duration than CT-1 treatment (pSTAT3). There was no alteration in total STAT3 protein after PBS treatment (STAT3). (C) CT-1 injection induced STAT1 phosphorylation occurred within 30 minutes after injection and lasted 24 hours (pSTAT1) with a small, late increase in total STAT1 protein (STAT1).

B. PBS (2μl)

C. CT-1 (2μg/2μl)

pSTAT3

STAT3

pSTAT1

STAT1

FIGURE 5. CT-1 induced phosphorylation of Erk1/2. Immunoblot analyses were performed to detect the phosphorylation of Erk1/2 at 0 (control), 0.5, 1, 6, 12, 24, and 48 hours after intravitreal injection of CT-1 (2 μg in 2 μL PBS) in P09 S334ter-3 rats. (A) CT-1 induced a significant increase in Erk1/2 phosphorylation (ppERKs), which was detected within 30 minutes and lasted 12 hours, with a peak occurring at 1 hour after injection. No changes in the total Erk1/2 protein were observed (ERKs). (B) PBS injection also induced an increase in Erk1/2 phosphorylation, but it was of less amplitude and shorter duration than CT-1 treatment (pERKs). There were no changes in total Erk1/2 protein after PBS injection (ERKs). (C) CT-1 injection induced Erk1/2 phosphorylation occurred within 30 minutes after injection and lasted 24 hours (pSTAT1) with a small, late increase in total STAT1 protein (STAT1).
of the family. More important, CT-1 binds to a receptor complex containing LIFRβ and gp130, commonly shared by other members of the family.12 Functionally, CT-1 not only induces a hypertrophic response in cardiac myocytes,9,17 but also has cytoprotective effect on them.10 Expression of CT-1 mRNA is found to be high in heart and skeleton muscles, suggesting that CT-1 is also a candidate motor neuron survival factor.9,17 Indeed, CT-1 supports long-term survival of spinal motor neurons.11

CT-1 activates several signal transduction pathways. The cytoprotective effect of CT-1 on myocardial cells is clearly mediated through the Erk-dependent and the PI3 kinase/Akt pathways,10,13,14 whereas the STAT3-dependent pathway is responsible for the hypertrophic response of cardiac muscle cells.9,17 In the present work, intravitreal injection of CT-1 activated STAT1, STAT3, and Erks, entirely consistent with previous findings by Peterson et al.18 that a CNTF mutein axokine also stimulates phosphorylation of STAT1, STAT3, and Erks. The localization of CT-1-induced phosphorylated STAT3 in Müller cells in the present work is consistent with the finding that axokine-induced STAT3 and Erk phosphorylation occurs in Müller cells.18 These findings are consistent with the hypothesis that CT-1 acts through signaling pathways similar to those of CNTF in the retina and that Müller cells are the major target cells.

An important finding of the present work concerns dose and regimen. Long-term protection of photoreceptors can be achieved by repeated injection of CT-1. For the potential clinical use of any neurotrophic factor, such as CNTF or CT-1, a crucial issue is whether the factor in question offers long-term protection, and if so, how best to achieve it. In the present work, we used an animal model with rapid photoreceptor degeneration. Photoreceptor death in these animals was evident as early as PD8 and by PD20, only 1 to 2 rows of photoreceptor nuclei remain in the ONL from the original 11 to 12 rows. The peak of degeneration occurs between PD10 and PD12 during which approximately 50% of photoreceptors disappeared.13 Considering the rapidity and severity of the degeneration, it is surprising to see that nearly 50% of photoreceptors remained at PD30 (the arbitrary end point of these experiments) with repeated CT-1 injections at 3-day intervals. Of specific note, relatively few photoreceptors died between PD20 and PD30 with repeated CT-1 injections (every 3 days), indicating that such treatment effectively prevented the degenerative process. It is also worth noting that less of a protective effect was observed when the intervals were extended to 4 or 5 days and that the protective effect was roughly inversely proportional to the length of the interval (Fig. 3). Two important points can be deduced from these observations: that the protein available to the responsive cells rapidly diminishes after a bolus injection and that the memory of the responsive cells is short. It is therefore reasonable to presume that more frequent treatment would result in enhanced protection. More to the point, maximum efficacy is likely to require sustained delivery.

In summary, the present work demonstrates the photoreceptor protective properties of CT-1 and highlights the role of a gp130-dependent mechanism in Müller cells in photoreceptor protection. It also points out the potential importance of sustained delivery of neurotrophic factors such as CT-1 for long-term rescue of photoreceptors.

Acknowledgments

The authors thank Yun Liu and Xinyu Zhao for excellent technical assistance.

References