Necrostatin-1 Protects Photoreceptors from Cell Death and Improves Functional Outcome after Experimental Retinal Detachment

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Necroptosis is a recently discovered programmed necrosis.1–3 Evidence demonstrated the importance of necroptosis in neuronal cell death. Necrostatin-1 is a specific inhibitor of necroptosis. In this study, we investigated the role of necrostatin-1 on photoreceptor survival and functional protection after experimental retinal detachment (RD) in rats. Necrostatin-1/inactive analogue of necrostatin-1 was introduced into the subretinal space at RD induction and 6 hours afterward, respectively. We found that necrostatin-1 attenuated retinal histopathological damage and reduced plasma membrane breakdown (a morphological hallmark of necroptosis) in outer retinal layers. Transmission electron microscopy showed that necrostatin-1 directly protected neurons by inhibiting necroptotic, not apoptotic, cell death. Treatment with necrostatin-1 inhibited the induction of receptor-interacting protein kinase phosphorylation after RD (a biomarker of necroptosis). Finally, electroretinographic recording proved that necrostatin-1 contributed to objective functional improvement after RD. These findings indicate that necrostatin-1 is a promising therapeutic agent that protects photoreceptors from necroptosis and improves functional outcome.

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Necroptosis is a recently discovered programmed necrosis.1–3 Evidence demonstrated the importance of necroptosis in physiological, pathophysiological, and embryonic development.4–6 Although necroptosis shares some morphological features of necrosis, it is different from necrosis by chromatin condensation, severe vacuolation, early loss of plasma membrane integrity, and many autophagosomes.1,2,7,8 Receptor-interacting protein kinase (RIP1), a death domain–containing kinase, is specifically involved in regulating necroptosis.2 Necrostatin-1 is a specific inhibitor of necroptosis, which inhibits RIP1.9–11 Neuronal cell death, caused by ischemic injury, contains a substantial nonapoptotic component,12,13 and necrostatin-1 has reduced infarction size in a mouse middle cerebral artery occlusion stroke model.2 These data suggest that ischemic injury generates conditions that are nonoptimal for apoptosis but suitable for necroptosis, and necroptosis may be a prominent mode of ischemic cell death in vivo.2

Our previous studies have shown that retinal detachment (RD), defined as the separation of the neurosensory retina from subjacent retina pigment epithelium, is a common cause of visual impairment.14,15 To our knowledge, retinal blood flow significantly decreases in retinal-detached eyes. This may contribute to the limited recovery of visual acuity.16,17 Therefore, decreased blood flow velocity in RD generates optimal conditions for necroptosis. Trichonas et al18 discovered that receptor-interacting protein kinase–mediated necroptosis is a significant mode of photoreceptor cell loss after experimental RD. However, it remains unknown whether necrostatin-1 inhibits necroptosis, protects neurons directly, and improves functional outcome after RD. Thus, we hypothesize that necroptosis contributes to neuronal damage,
and necrostatin-1, a specific inhibitor of necroptosis, protects photoreceptors from necroptosis and improve functional outcome.\textsuperscript{19}

In this study, we tested the hypotheses of whether necrostatin-1 attenuates retinal histopathological damage and whether it reduces plasma membrane breakdown (a morphological hallmark of necroptosis) in outer retinal layers. Transmission electron microscopy (TEM) was performed to investigate if necrostatin-1 directly protects neurons by inhibiting necroptotic or apoptotic cell death. Then, to determine whether inhibition of necroptosis leads to objective functional improvement after RD, we studied the electrophysiological effects of necrostatin-1. Finally, we addressed whether necrostatin-1 can reduce RIP1 phosphorylation, a biomarker of necroptosis. The results suggest that necrostatin-1, the specific necroptosis inhibitor, is a promising therapeutic agent against neuronal necroptosis, improving functional outcome in RD.

**Materials and Methods**

**Animals and Induction of RD**

All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines established by the University Committee on Use and Care of Animals of Shanghai JiaoTong University (Shanghai, China). Male Sprague-Dawley rats, weighing 260 to 280 g, were provided by the Laboratory Animal Center of the institution. Experimental RD was induced as previously described.\textsuperscript{15,20} Briefly, the rats were anesthetized with an i.p. injection of 10% chloral hydrate and their pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceutical, Suzhou, China); a 30-gauge needle was inserted into the subretinal space via an external trans-scleral approach. The 1% sodium hyaluronate (Bausch & Lomb Freda, Jinan, China) was gently injected into the subretinal space to enlarge the RD (50 μL each).\textsuperscript{21} The RD was confirmed by surgical microscope on every animal and was generated only in the right eye of each animal, with the left eye serving as the control.

**Administration of Necrostatins and Related Compounds**

At RD or 6 hours afterward, 5 μL of 400 μmol/L necrostatin-1, an inactive analogue of necrostatin-1 (necrostatin-1i), or vehicle [dimethyl sulfoxide (DMSO)] was administered into the subretinal space of the experimental eyes. Necrostatin-1 and necrostatin-1i are available from Merck (Darmstadt, Germany). The time point was selected based on previous studies.\textsuperscript{22,23} In separate experiments, the pan-caspase inhibitor, a 300 μmol/L solution of Z-VAD-FMK (Enzo Life Sciences, Farmingdale, NY), was administered at RD induction (5 μL each). The dose and volume of compounds were selected based on previous studies.\textsuperscript{2,22,24}

**Assessment of Histopathological Damage**

As previously described,\textsuperscript{23,25} the right (treatment) and left (control) globes were enucleated 1 week after RD and were embedded in paraffin. Encuclated globes were then divided into sections in the vertical meridian and the inferior portion of the eye wall. Sections (5-μm thick) were stained with H&E. The retinal histoarchitecture was evaluated by light microscopy. The ratio of outer nuclear layer (ONL) thickness/total retinal thickness was determined by Image-Pro Plus software (Media Cybernetics, Rockville, MD) and standardized by in the attached retina. Because retinal thickness varies with distance from the optic nerve, the thickness of the inner nuclear layer was used as a control to ensure that ONL measurements were taken at the same distance from the optic nerve head.\textsuperscript{26} Five sections were randomly selected in each eye. Then, the outer nuclear layer (ONL) thickness ratio was measured at 10 points in each section by masked observers. The data are expressed as normalized ONL thickness ratio: [(ONL/neuroretina thickness in detached retina)/(ONL/neuroretina thickness in attached retina)].\textsuperscript{18,23,27}

**Administration of PI and Detection of PI-Positive Cells**

The effects of necrostatin-1 on plasmalemma integrity loss in ONL regions were assessed using in vivo propidium iodide (PI) labeling, a technique to demonstrate cells with a disrupted cell membrane.\textsuperscript{24} The PI (Sigma, St Louis, MO) was diluted in double-distilled H₂O by 2:1. The PI (5 μL at 50 μg/mL), was injected into the subretinal space, 3 days after RD, 2 hours before sacrifice. Then, the eyes were enucleated and cryosections (10 μm thick) were cut and air dried. For detection of PI-labeled cells, retinal sections were fixed in 100% ethanol for 10 minutes at room temperature. DAPI was used to counterstain the nuclei. The center of the detached retina was photographed with a fluorescence microscope, using excitation/emission filters 568/585 for PI. The number of PI-positive cells in the ONL was analyzed by Image-Pro Plus software.\textsuperscript{25}

**TEM Photomicrographs in the ONL**

As previously described,\textsuperscript{15} 3 days after RD, TEM was performed. Approximately 200 photoreceptors per sample were photographed and subjected to quantification of cell death modes in a masked manner. Photoreceptors showing cellular shrinkage and nuclear condensation were defined as apoptotic cells, whereas photoreceptors associated with cellular and organelle swelling and discontinuities in plasma and nuclear membrane were defined as necrotic cells. Electrondense granular materials were simply labeled as end-stage cell death/unclassified, because these materials occur subsequent to both apoptotic and necrotic cell death.\textsuperscript{28,29}
Immunoprecipitation and Western Blot Analysis

As presented in our previous works,14,30 the neural retina was collected on 3 days after RD. Equal amounts of retinal lysates (200 µL) were incubated with 1 µL (1 mg/mL) anti-RIP1 antibody (Cell Signaling Technology, Boston, MA) and 40 µL of protein A/G agarose beads (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer’s instructions, at 4°C overnight. Beads were washed five times with Tris-buffered saline solution, and the immunopellets were then subjected to Western blot analysis. Samples were run on 8% SDS-PAGE. After electrophoretic separation, the proteins were transferred to nitrocellulose membranes (Whatman, Maidstone, UK). After blocking with 5% bovine serum albumin, the membrane was reacted with RIP1 (1:1000; Cell Signaling Technology) or phosphoserine (1:100; Enzo) antibody. Membranes were then washed three times and incubated with horseradish peroxidase–labeled secondary antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature. Bands were visualized by electrochemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), according to the manufacturer’s instructions, and were exposed to X-ray film. The density of the signal was quantified using Bandscan43 (Glyko, Inc., Novato, CA).

Assessment of Neuronal Function with ERGs

Flash electroretinograms (ERGs) with overnight dark adaptation were recorded 7 days before treatment and 7 days after treatment. The rats were anesthetized with an i.p. injection of 10% chloral hydrate, and their pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops. The body temperature was maintained with a heating pad setting to 37°C during the procedure. ERGs were recorded with a corneal gold wire electrode, a reference electrode at the head, and a ground electrode in the tail. All procedures were performed in dim red light, and the rats were kept warm during and after the procedure. The responses to a light flash (3.0 candela·seconds/m²) from a photic stimulator were amplified, and the preamplifier bandwidth was set at 0.2 to 300 Hz. The amplitude of the a-wave was measured from baseline to the maximum a-wave trough, and the amplitude of the b-wave was measured from the maximum a-wave trough to the maximum b-wave peak.31–33

Statistical Analysis

Data are expressed as mean ± SD. Assuming that the data meet normal distribution and that variances are equal, the one-way analysis of variance, followed by validation using Student-Newman-Keuls tests, was used to analyze the statistical differences in retinal thickness, ERG data, and cell number among groups. The analysis was performed by computer software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL). For all comparisons, $P < 0.05$ was considered statistically significant.

Results

Effects of Necrostatin-1 Supplementation on Structure and Morphological Characteristics of the Detached Retina and ONL Thickness

To verify if necrostatin-1 would reduce injury after RD, we examined the effects of necrostatin-1 on neuronal damage. The time point was selected because disorders of the outer retinal layer are observed in the model of RD at 7 days after RD.23,25 There were no significant histopathological changes in the inner retina at 7 days after RD, but evident changes were detected in the outer neural retina. Both inner and outer segments of photoreceptor cells were disordered and shortened. Both pretreatment and post-treatment of necrostatin-1 led to significant preservation in histoarchitecture of the outer retina, compared with vehicle-treated controls or inactive necrostatin-1–treated animals (Figure 1, A and B). The ratio of ONL thickness was reduced after RD (0.68 ± 0.04) but was thicker in the necrostatin-1 pretreatment groups (0.87 ± 0.06) ($n = 6$ per group, $P < 0.05$) (Figure 1C). Pretreatment with an inactive analogue of necrostatin-1, which previously lacked necroptosis-inhibitory activity, abolished this protective effect (0.70 ± 0.04) (Figure 1C). When given 6 hours after RD induction, necrostatin-1 also led to significant preservation in histoarchitecture of the outer retina and ONL thickness ($n = 6$ per group, $P < 0.05$) (Figure 1D).

Data are expressed as mean ± SD. Assuming that the data meet normal distribution and that variances are equal, the one-way analysis of variance, followed by validation using Student-Newman-Keuls tests, was used to analyze the statistical differences in retinal thickness, ERG data, and cell number among groups. The analysis was performed by computer software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL). For all comparisons, $P < 0.05$ was considered statistically significant.

Figure 1. Both pretreatment and post-treatment of necrostatin-1 (Nec-1) leads to significant preservation in the histoarchitecture of the outer retina, compared with vehicle-treated controls or inactive necrostatin-1 (Nec-1i)–treated eyes. A and B: Representative photomicrographs of the different treatment groups [pretreatment (A) and post-treatment (B)]. C and D: Percentage thickness ratio of outer nuclear layer (ONL)/total retinal thickness in detached retina compared with normal attached retina [pretreatment (C) and post-treatment (D)] ($n = 6$ per group). *$P < 0.05$. Scale bar = 50 μm. GCL, ganglion cell layer; INL, inner nuclear layer.
Necrostatin-1 Reduces Acute Plasmalemma Permeability in Injured Cells after RD

Because plasma membrane permeability is an early hallmark of necrotic cell death, we assessed the effects of necrostatin-1 on loss of plasmalemma integrity in ONL regions, using in vivo PI labeling.24 The PI-labeled cells in ONL were measured using image analysis software; the numbers in pretreatment of the necrostatin-1, necrostatin-1i, and DMSO groups were 428.42 ± 33.03, 803.40 ± 87.78, and 800.20 ± 48.39 mm², respectively. Compared with vehicle-treated retinas, pretreatment with necrostatin-1 led to a significant reduction in PI-labeled cells at 3 days after RD, suggesting reduction of necrotic cellular injury (n = 6 per group, P < 0.01) (Figure 2, A and C). Pretreatment with necrostatin-1i abolished this protective effect of suppressing the number of PI-positive cells in the ONL (Figure 2, A and C). The administration of necrostatin-1, 6 hours after the induction of RD, also led to a significant reduction in PI-labeled cells at 3 days after RD (n = 6 per group, P < 0.01) (Figure 2, B and C).

Necrostatin-1 Inhibits Necrotic, But Not Apoptotic, Cell Death after RD

Because PI-positive cells may also occur late in apoptosis,34 and necroptosis shares some morphological features of necrosis, we performed TEM to investigate whether necrostatin-1 directly protected neurons by inhibiting necrotic cell death (Figure 3, A and B). PI staining and an EM examination were performed at 3 days after RD induction, necrostatin-1 reduced the percentage of necrotic cells after RD (14.60% ± 2.03%) compared with the necrostatin-1i (22.01% ± 1.08%) or vehicle-treated (22.62% ± 0.90%) groups (Figure 3E) (n = 6 per group, P < 0.01). On the other hand, pretreatment with necrostatin-1 did not affect the percentage of apoptotic cells (Figure 3F). At 6 hours after RD induction, necrostatin-1 also reduced the percentage of necrotic cells at 3 days after RD (n = 6 per group, P < 0.01) (Figure 3, E and F).

Necrostatin-1 Prevents RD-Induced Photoreceptor Necroptosis by Inhibiting RIP1 Phosphorylation

Necrostatin-1 is a specific inhibitor of necroptosis, which inhibits the RIP1 phosphorylation that may be a key early signaling event in necroptosis.9 To further explore the protective mechanism of action of necrostatin-1 in the RD model, we tested whether necrostatin-1 inhibited RIP1 phosphorylation. At 3 days after RD induction, Western blot densitometry analysis demonstrated that RIP1 phosphory-
lation was elevated in the DMSO-treated retina compared with the untreated retina. Necrostatin-1 pretreatment substantially inhibited this increase of RIP1 phosphorylation, whereas necrostatin-1i had no effect on this induction (Figure 4, A and B). The administration of necrostatin-1, 6 hours after the induction of RD, also inhibited the increase of RIP1 phosphorylation at 3 days after RD (Figure 4, C and D). The data showed that necrostatin-1 prevented RD-induced photoreceptor necroptosis by inhibiting RIP1 phosphorylation.

**Effect of Necrostatin-1 on ERG Responses under Scotopic Conditions**

Although the previously described data suggested that necrostatin-1 reduced photoreceptor cell death after RD, a crucial question was whether inhibition of necroptosis was functionally significant. We addressed this question by assessing the effects of necrostatin-1 using electroretinography. The ERGs were obtained 7 days before and 7 days after RD (Figure 5A). Compared with 7 days before RD, necrostatin-1 pretreatment presented an approximate 65% recovery of dark-adapted ERG amplitudes in the a-wave, which was notably different from both the DMSO (28.44 ± 0.05%) and necrostatin-1i (30.75 ± 0.04%) treatment groups (Figure 5C) (n = 6 per group, P < 0.01). The b-wave amplitude showed similar recovery behavior (Figure 5D). However, necrostatin-1i had no protective effect. These results showed that necrostatin-1 administration increased the functional recovery of the a- and b-waves after RD. We then addressed the question of whether necrostatin-1 was neuroprotective when administered after RD. Most important, the administration of necrostatin-1, at 6 hours after RD, also led to a significant functional recovery of the a- and b-waves after RD.
b-waves, at 7 days after RD (n = 6 per group, P < 0.05) (Figure 5, B–D).

Discussion

Several previous reports have identified the activation of cell death pathways as a mechanism of photoreceptor loss in RD, yet little is known about inhibition of the necroptosis pathway that promotes photoreceptor survival. In this study, we provide strong evidence that necroptosis is an important mechanism of photoreceptor cell death after RD. Necrostatin-1, a specific inhibitor of necroptosis by inhibiting RIP1 phosphorylation, attenuated retinal degeneration, preserved retinal thickness, rescued neurons in the outer retinal layers, and reduced functional impairment after RD. These results, together with prior work, provide evidence that necrostatin-1 is a promising therapeutic agent that protects photoreceptors from necroptosis and improves functional outcome.

Necroptosis is a pathway of regulated necrotic cell death triggered by death receptor ligands, and ischemic injury generates conditions that are nonoptimal for apoptosis while suitable for necroptosis. Several lines of evidence suggest that necroptosis may be an important mode of cell death in retinal injury. For example, Arimura et al showed that the vitreous level of high-mobility group box 1, which is released from necrotic, but not apoptotic, cells, is increased in human eyes with RD. Therefore, we hypothesize that necroptosis, as an essential pathway for cell loss, also contributes to neuronal damage in RD. The H&E staining results supported this hypothesis because necrostatin-1, a specific inhibitor of necroptosis, reduced post-RD lesion size in the retina.

Necroptosis is a regulated cell death that has the morphological features of necrosis (early membrane and organelle swelling, followed by cell lysis). Han et al have demonstrated a necrotic death with the following characteristics: morphological characteristics of necrotic cell death and loss of plasma membrane integrity. Another study showed that retinal ganglion cells were not rescued from death by cotreatment with apoptosis inhibitors and, instead, followed an alternative pathway of PI-positive cell death. Our data showed that, compared with vehicle-treated retinas, both pretreatment and post-treatment of necrostatin-1 led to a significant reduction in PI-labeled cells at 3 days after RD, suggesting reduction of necroptotic cellular injury. On the other hand, treatment with an inactive analogue of necrostatin-1 abolished this protective effect.

We know that necrostatin-1 specifically inhibits necroptosis but does not affect apoptosis. However, PI staining merely refers to plasma membrane integrity and is not specific for necroptosis, because PI-positive cells may also occur late in apoptosis. One study observed that nuclei stained with PI in the corneas could occur late in apoptosis or necrosis. Further analysis with TEM, however, indicated that many cells had cell morphological changes more consistent with necrosis. Therefore, we next assessed the effect of necrostatin-1 by TEM. Our data indicated that, at 3 days after RD, necrostatin-1 pretreatment and post-treatment reduced the percentage of necrotic cells after RD, but did not affect the percentage of apoptotic cells.

Nevertheless, necroptosis is different from necrosis in that it displays the following features: chromatin condensation, severe vacuolation, early loss of plasma membrane integrity, and many autophagosomes. Martinet et al discovered treatment of macrophages with Z-VAD-fmk induced necroptosis cell death and was characterized by extensive vacuolization. Han et al andDegterev et al demonstrated that necroptosis cell death exhibited the following features: chromatin condensation, severe vacuolation, and many autophagosomes that can be inhibited by necrostatin-1. In this study, we showed, for the first time to our knowledge, that photoreceptors exhibited necroptotic morphological characteristics after RD, which can be inhibited by necrostatin-1.

To our knowledge, the phosphorylation of RIP1 may be a key early signaling event in necroptosis and necrostatin-1 can inhibit this phosphorylation. To further explore the protective mechanism of action of necrostatin-1 in the RD model, we tested whether necrostatin-1 inhibited RIP1 phosphorylation, a key step involved in necroptosis. Consistent with the previous findings, at 3 days after RD, our results demonstrated that RIP1 phosphorylation was elevated in the DMSO-treated retina compared with untreated retina. Necrostatin-1 pretreatment and post-treatment substantially inhibited this increase of RIP1 phosphorylation, whereas necrostatin-1i had no effect on this induction.

Although the previously described data suggest that necrostatin-1 reduces neuronal cell death after RD, a critical question is whether inhibition of necroptosis is functionally significant. Dysfunction of the outer retinal layer is involved in the ERG a- and b-wave abnormalities observed in this model at 7 days after RD. Such functional data may be a more predictive sensitive measure of clinical outcome than histological characteristics. We showed, for the first time to our knowledge, that necrostatin-1 administration increased the functional recovery of the a- and b-waves after RD. Functional protection in this study was even seen when the drug was administered at 6 hours after RD, a time window that is clinically relevant.

In summary, by using necrostatin-1, a stringent special inhibitor of necroptosis, the current study provides proof of principle that necrostatin-1 is a promising therapeutic agent that protects photoreceptors from necroptosis and improves functional outcome; necroptosis may be a prominent mode of cell death in RD. Therefore, necrostatin-1, in combination with other cell death inhibitors, may provide us with a more effective regimen to fight against cell death in RD.
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