Protective Effect of Adult T-Cell Leukemia-Derived Factor on Retinal Ischemia-Reperfusion Injury in the Rat

Hiroto Shibuki, Naomichi Katai, Sachiko Kuroiwa, Toru Kurokawa, Junji Yodoi, and Nagahisa Yoshimura

PURPOSE. To evaluate the protective effects of recombinant adult T-cell leukemia-derived factor (ADF)-human thioredoxin against ischemia-reperfusion injury in the rat retina.

METHODS. Retinal ischemia was induced in rats by increasing the intraocular pressure to 110 mm Hg for 60 minutes. Various doses of recombinant human ADF (rhADF) or vehicle were administered intravenously before ischemia induction and immediately after reperfusion. The degree of retinal damage was assessed by electroretinogram (ERG) recording, by measuring the inner retinal thickness, and by counting the number of TdT-dUTP terminal nick-end labeling (TUNEL)-positive cells in the inner nuclear layer.

RESULTS. The amplitudes of the ERG b-wave and oscillatory potentials were increased significantly by treatment before ischemia and after reperfusion with 0.5 mg or 5 mg rhADF and by treatment after reperfusion with 1 mg rhADF, compared with those of vehicle-treated control rats (P < 0.01). On day 28 after reperfusion, the thickness of the inner retina of control rats and of rats treated before ischemia and after reperfusion with 0.5 mg rhADF were 46.1 ± 6.4 μm and 78.5 ± 8.9 μm, respectively (P < 0.01). The number of TUNEL-positive cells on days 1 and 2 after reperfusion was decreased significantly by treatments with 0.5 mg rhADF compared with the number of TUNEL-positive cells in control rats (P < 0.01).

CONCLUSIONS. Electrophysiologic and histologic studies showed that ischemia for 60 minutes produces severe damage in vehicle-treated control rat retina, particularly in the inner retinal layer. Intravenous injection of rhADF protects the rat retina from ischemia-reperfusion injury. (Invest Ophthalmol Vis Sci. 1998;39:1470-1477)

Formation of reactive oxygen species such as superoxide and hydrogen peroxide is thought to play an important role in the pathogenesis of ischemia-reperfusion injury. Previous studies have demonstrated that antioxidant agents that scavenge reactive oxygen species, including superoxide dismutase, catalase, and mannitol, reduce ischemia-reperfusion injury of the retina.3-7 Adult T-cell leukemia-derived factor (ADF), originally identified as an inducer of an interleukin-2 receptor [IL2R/p55(Tac)], is a polypeptide consisting of 104 amino acids with a molecular weight of approximately 12,000. Cloning of cDNA for ADF from the cDNA library of a human T-cell leukemia virus-1-positive human T-cell line revealed its primary structure and, as a result, it became evident that ADF is a human homologue of thioredoxin (TRX). ADF-TRX is ubiquitously distributed among many species, and human and rat molecules share 89% homology in the amino acid sequences.12-16 ADF-TRX is a redox protein that has a characteristic center of activity: Trp-Cys-Gly-Pro-Cys-Lys. These two cysteine residues play a role in the redox reaction. The reducing activity of ADF-TRX catalyzes proton transfer between the thiol radical of cysteine-containing proteins. In many cells, ADF-TRX is induced by cell division and differentiation and by a variety of stresses, including exposure to x-ray and UV light, virus infection, and ischemia.17-24

In normal rat retina, ADF-TRX is expressed weakly in the internal limiting membrane, ganglion cell layer, and inner plexiform layer. Under ischemic conditions, however, expression is upregulated in glial cells and more specifically, in retinal pigment epithelial cells. Furthermore, ADF-TRX is expressed in glial cells in the gerbil brain after transient ischemia. The upregulation of ADF-TRX after ischemic injury is thought to play a role in neuroprotection against reactive oxygen species. Recombinant human ADF (ADF)-TRX has a protective effect against reactive oxygen species that are formed by xanthine oxidase and other pathways. This protective effect was described in an in vivo model of ischemia-reperfusion injury of the lung and the heart and in an in vitro model of oxidative stress of the central nervous system.26-30

Using a rat in vivo model of retinal ischemia-reperfusion, we examined the possible protective effect of rhADF against such injury. Retinal damage and repair were evaluated by
TABLE 1. Animal Groups Used in the Experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>rhADF Treatment before Ischemia/after Reperfusion</th>
<th>ERG Recording</th>
<th>TUNEL Staining</th>
<th>Retinal Thickness Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 9)</td>
<td>0.1 mg/0.1 mg</td>
<td>Days -1, 1, 2, 4, 7, 14, and 28 (n = 9 for each time point)</td>
<td>Days 1, 2, 4, 7, 14, and 28 (n = 8 for each time point)</td>
<td>Day 28 (n = 7)</td>
</tr>
<tr>
<td>B (n = 98)</td>
<td>0.5 mg/0.5 mg</td>
<td>Days -1, 1, 2, 4, 7, 14, and 28 (n = 8 for each time point)</td>
<td>Days 1, 2, 4, 7, 14, and 28 (n = 7 for each time point)</td>
<td>Day 28 (n = 6)</td>
</tr>
<tr>
<td>C (n = 7)</td>
<td>5 mg/5 mg</td>
<td>Days -1, 1, 2, 4, 7, 14, and 28 (n = 7 for each time point)</td>
<td>Days 1, 2, 4, 7, 14, and 28 (n = 7 for each time point)</td>
<td>Day 28 (n = 6)</td>
</tr>
<tr>
<td>D (n = 7)</td>
<td>Vehicle/1 mg</td>
<td>Days -1, 1, 2, 4, 7, 14, and 28 (n = 7 for each time point)</td>
<td>Days 1, 2, 4, 7, 14, and 28 (n = 7 for each time point)</td>
<td>Day 28 (n = 6)</td>
</tr>
<tr>
<td>E (n = 101)</td>
<td>Vehicle/vehicle</td>
<td>Days -1, 1, 2, 4, 7, 14, and 28 (n = 11 for each time point)</td>
<td>Days 1, 2, 4, 7, 14, and 28 (n = 8 for each time point)</td>
<td>Day 28 (n = 7 for each time point)</td>
</tr>
<tr>
<td>F (n = 7)</td>
<td>Normal rats: no ischemia, no treatment</td>
<td></td>
<td></td>
<td>12-week-old rats (n = 7)</td>
</tr>
</tbody>
</table>

ERG, electroretinogram; rhADF, recombinant human adult T-cell leukemia-derived factor; TUNEL, TdT-dUTP terminal nick-end labeling.


electroretinogram (ERG), by measurement of the inner retinal thickness (the thickness between the inner limiting membrane and the boundary of the outer nuclear layer and the outer plexiform layer), and by counting the number of cells in the inner nuclear layer (INL) labeled by the TdT-dUTP terminal nick-end labeling (TUNEL) method.

MATERIALS AND METHODS

Animals

Two hundred twenty-nine adult male Sprague-Dawley rats weighing 250 g to 300 g were used in this study. All experiments involving rats adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinal ischemia was induced in the left eye and a sham procedure was performed on the right eye. Animals were divided into six groups. Group A animals (n = 9) were treated by intravenous injection into the tail vein of 0.1 mg rhADF in 0.4 ml phosphate-buffered saline (PBS) 1 minute before ischemia and by another 0.1 mg rhADF immediately after reperfusion. Group B rats (n = 98) were treated in the same way, but were administered 0.5 mg instead of 0.1 mg rhADF. Group C animals (n = 7) were treated by intravenous injection into the tail vein of 0.1 mg rhADF in 0.4 ml phosphate-buffered saline (PBS) 1 minute before ischemia and by another 0.1 mg rhADF immediately after reperfusion. Group D rats (n = 7) received 1 mg rhADF immediately after reperfusion, but 0.4 ml PBS rather than rhADF was administered before ischemia. Group E (control group; n = 101) rats were treated with 0.4 ml PBS before ischemia and immediately after reperfusion. Group F rats served as normal control animals and were not subjected to retinal ischemia–reperfusion (n = 7). Detailed information of the measurements performed in the various groups is summarized in Table 1.

Recombinant Adult T-Cell Leukemia-Derived Factor

A plasmid that carries the ADFTRX gene was transformed in Escherichia coli. After incubation with E. coli, rhADF was extracted from the bacteria without bacterial endotoxin (Oriental Yeast, Nagahama, Japan). Recombinant human ADF was dissolved in PBS at a concentration of 5 mg/ml. The purity of the rhADF is greater than 99%, determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the content of bacterial endotoxin in rhADF is less than 4 pg/mg, assessed by the quantitative chromogenic lipopolysaccharide method.12,31

Ischemia Model

Rats were anesthetized with intraperitoneal injections of 60 mg/kg pentobarbital, and the pupils were dilated with topical phenylephrine hydrochloride and tropicamide. The anterior chamber of the left eye was cannulated with a 27-gauge infusion needle connected to a bag containing normal saline. Intraocular pressure was increased to 110 mm Hg for 60 minutes by elevating the saline bag. Retinal ischemia was confirmed by whitening of the iris and fundus. In preliminary experiments, the actual intraocular pressure of 110 mm Hg was confirmed, using a recorder (RT-3208N; NEC San-ei, Tokyo, Japan), by cannulation of the anterior chamber with another 27-gauge infusion needle connected to a pressure transducer (AMPL-1829; NEC San-ei). The temperature of the rats was measured by a rectal sensor (TD-320; Shibaura Electronics, Urawa, Japan) and maintained at 37°C with a heating blanket during the ischemic insult. Control right eyes (sham procedure) underwent similar procedures but without elevation of the saline bag, so that normal ocular tension was maintained. Rats in which severe cataract and corneal opacity developed were excluded from the study.
Electroretinograms

The rats were anesthetized by intramuscular injections of 70 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, and the pupils were dilated with phenylephrine hydrochloride and tropicamide. Rats were dark adapted for at least 60 minutes before ERGs were recorded. The temperature of the rats was measured by a rectal sensor and was maintained at 37°C with the heating blanket during anesthesia. Electroretinograms were recorded 1 day before ischemia and on days 1, 2, 4, 7, 14, and 28 after reperfusion. A recording electrode of the contact lens type made of platinum (Kyoto Contact Lens, Kyoto, Japan) was placed on the cornea, and a reference electrode wire and a ground electrode wire made of stainless steel (Nihon Kōhden, Tokyo, Japan) were connected to the nose and an ear, respectively. A photostimulator lamp (SLS-3100; Nihon Kōhden) was placed in front of the eye; light intensity was approximately $3.5 \times 10^6 \text{ cd/m}^2$ using a neutral-density filter (Eastman Kodak, Rochester, NY). The responses were amplified with a time constant of 0.3 seconds and with low- and high-frequency-cut filters of 0.1 Hz and 1000 Hz (MEB-5304; Nihon Kōhden). When oscillatory potentials (OPs) were recorded, the frequency band-pass filter was reset at 100 to 500 Hz. The amplitudes and the latency of the ERG a-wave, b-wave, and OPs were measured three times and the results averaged.

Histologic Study

On days 1, 2, 4, 7, 14, and 28 after reperfusion, rats were killed with an overdose of sodium pentobarbital. The eyes were immediately enucleated and fixed in 2.5% glutaraldehyde in phosphate buffer for morphometric measurements and in 4% paraformaldehyde in phosphate buffer for the TUNEL studies. Specimens fixed in glutaraldehyde were osmicated, dehydrated, and embedded in epoxy resin. One-micrometer sections, cut along the vertical meridian of the eye and passing through the optic nerve head, were stained with toluidine blue. The ischemic changes were evaluated by measuring the inner retinal thickness at 10 points for each eye. Measurements were made at 100-μm intervals and extended 800 μm to 1200 μm above and below the optic nerve head, according to a previously described method.32 Data are represented as mean value from seven retinas with 10 measurements made on each retina. Specimens fixed in paraformaldehyde were dehydrated and embedded in paraffin, and 4-μm sections were obtained. The sections were stained by the TUNEL method, using 3,3’-diaminobenzidine as the substrate.33 The number of TUNEL-positive cells was counted in 20 areas of approximately 6000 μm$^2$ in the INL of each section. Data are represented as findings per square millimeter. The measurement of the inner retinal thickness and count of TUNEL-positive cells were digitized by a computer-controlled display on a computer screen, using a scanning laser confocal microscope with an area measurement function (LSM410; Zeiss, Oberkochen, Germany).

Statistical Analysis

Data regarding amplitude and latency of ERG a-wave, b-wave, and OPs were analyzed by repeated measures of analysis of variance (ANOVA) and by Scheffé’s post hoc test. Data from the measurement of the inner retinal thickness and the count of TUNEL-positive cells were analyzed by one-way ANOVA, two-way ANOVA, and Scheffé’s and Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Electroretinograms

As shown in Figure 1, an ischemic insult of 60 minutes decreased the amplitudes of ERG a-wave, b-wave, and OPs of group E animals (vehicle treatment). On day 28, all components of the ERG showed some, but not full, recovery. The difference between the experimental groups in the latencies of ERG a-wave, b-wave, or OPs was not statistically significant (data not shown). Furthermore, in all treatment groups, amplitude of the ERG a-wave showed approximately 90% recovery on day 28, with no statistically significant difference between the treatment groups.

Repeated measures of ANOVA, however, showed different patterns of response in the amplitudes of ERG b-wave and OPs. The amplitudes of b-wave and OPs from animals in group B (treatment with 0.5 mg rhADF before ischemia and after reperfusion), group C (treatment with 0.5 mg rhADF before ischemia and after reperfusion), and group D (treatment with 1 mg rhADF after reperfusion) showed statistically significant changes compared with those seen in groups A (treatment with 0.1 mg rhADF before ischemia and after reperfusion; $P < 0.01$) and group E (vehicle only) rats ($P < 0.01$). No statistically
**Table 2.** ERG Amplitude Changes (in Microvolts) after Intravenous Injection of rhADF or Vehicle

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Before Ischemia</th>
<th>Days after Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>a wave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>194.8 ± 32.51</td>
<td>172.0 ± 21.64</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>185.6 ± 28.94</td>
<td>166.9 ± 22.37</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>176.0 ± 35.41</td>
<td>159.3 ± 27.03</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>177.2 ± 23.71</td>
<td>140.2 ± 22.83</td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>169.1 ± 27.12</td>
<td>141.3 ± 12.88</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Group A: treatment with 0.1 mg rhADF before ischemia and after reperfusion; group B: treatment with 0.5 mg rhADF before ischemia and after reperfusion; group C: treatment with 5 mg rhADF before ischemia and after reperfusion; group D: treatment with 1 mg rhADF after reperfusion; group E: treatment with vehicle.

* P < 0.05, compared with group E.
† P < 0.05, compared with group A.
‡ P < 0.01, compared with group E.
§ P < 0.01, compared with group A.

**Histologic Findings**

Typical histologic changes after ischemia-reperfusion injury are shown in Figure 2. In vehicle-treated control rat retina, there were many cells with pyknotic nuclei in the INL (Fig. 2E). With rhADF treatment before ischemia and after reperfusion, fewer cells showed pyknotic nuclei (Fig. 2B). On day 28, the inner retinal layer showed well-maintained structure compared with that of group E (vehicle control) animals (Figs. 2C, 2F).

Cells in the ganglion cell layer (GCL) were also attenuated after ischemia-reperfusion injury, but with rhADF treatment, some cells remained in the GCL (Fig. 2C). In the retina of vehicle-treated rats, little GCL remained on day 28 (Fig. 2F). No appreciable histologic changes were observed in the right eyes (sham procedure) of animals that received rhADF, compared with normal rat retina (Figs. 2A, 2D).
eyes, no TUNEL-positive cells were observed on days 4, 7, 14, and 28 after reperfusion (Fig. 4), and no TUNEL-positive cells were observed in control eyes (sham procedure; graphic data not shown). Analyses by two-way ANOVA and Bonferroni post hoc test showed a statistically significant decrease in the number of TUNEL-positive cells in group B (2251 ± 351 cells/mm² on day 1 and 807 ± 83 cells/mm² on day 2; \( P < 0.01 \)), compared with those of group E (3589 ± 406 cells/mm² on day 1 and 1531 ± 138 cells/mm² on day 2; Fig. 5).

**DISCUSSION**

In this experiment, intravenously administered rhADF reduced retinal ischemia–reperfusion injury in the rat, especially that which developed in the INL, when evaluated by ERG recordings (Fig. 1, Table 2), measurement of inner retinal thickness (Figs. 2, 3), and determination of number of TUNEL-positive cells in the INL (Figs. 4, 5). We found no statistically significant difference between group B and group C in the ERG recording and inner retinal thickness (Table 2 and Fig. 3B). Accordingly, detailed examination was performed, using the lower dose of rhADF.

In this ischemia–reperfusion model, the GCL is also damaged. However, because cells in the GCL are easily damaged and there were few TUNEL-positive cells in the GCL after day 1 (data not shown), it was not practical to analyze cell death in this layer. In the INL, TUNEL-positive cells were most prominent on day 1, decreased in number on day 2, and were...
Rescue of Retinal Ischemia by Recombinant Human ADF

Figure 3. (A) Measurement of thickness of the inner retinal layer (IRL) after ischemia-reperfusion injury, with and without treatment. Hatched column, normal control retina without ischemia-reperfusion (n = 7); open columns, vehicle-treated control retina (n = 7 for each time point); filled columns, recombinant human adult T-cell leukemia-derived factor (rhADF) treatment (0.5 mg before ischemia and 0.5 mg immediately after reperfusion; n = 7 for each time point). The inner retinal thickness in the sham-treated rat right eye was determined on day 28 (n = 7). Results are mean ± SD. Asterisks indicate a statistically significant difference between rhADF- and vehicle-treated eyes (***P < 0.01; Bonferroni post hoc test).

(B) Measurement of inner retinal thickness on day 28 after reperfusion. Results are mean ± SD (n = 6–7). Asterisks indicate a statistically significant difference (*P < 0.05; **P < 0.01; Scheffe’s post hoc test).

ADF-TRX is a protein with a molecular weight of approximately 12,000. Under normal physiologic conditions, proteins of this high molecular weight cannot cross the blood-retina barrier. However, ischemia-reperfusion injury damages the barrier, and rhADF exerts protective effects on neuronal cells. It is also possible that this protein scavenges reactive oxygen species that are formed in the vascular bed during reperfusion, although the plasma concentration of rhADF was not determined in our experiment.

It took a long time before the protective effects of rhADF became evident. Electrophysiologic and histologic evidence of retinal rescue appeared 4 days after reperfusion (Table 2, Fig. 3A). In previous studies using antioxidants, electrophysiologically appreciable retinal rescue after ischemia-reperfusion was shown in the early phase of reperfusion. Our results may indicate that mechanisms other than scavenging of reactive oxygen species play a role in the effect of rhADF, because it is difficult to believe that formation of peroxynitrite and hydrogen peroxide takes so much time. Delayed recovery of ERG amplitudes may also be explained by the period of recovery from retinal damage, for example amelioration of retinal edema, phagocytosis of apoptotic bodies by macrophages, and repair by glial cells. Also, electrophysiologic function is related to the concentration of glutamate and phospholipase C in the retina after reperfusion. The retina during this period is an abnormal milieu, and the protective effects of rhADF take time to become apparent. Other possible mechanisms include decreased protein phosphorylation by ADF, upregulation of transcription factors, and increased cellular glucocorticoid responsiveness. Another possibility is that metabolites of rhADF play a role in neuroprotection. Studies in which oxidized rhADF is used may answer the question.

Our finding that treatment after reperfusion with 1 mg rhADF is as effective as is the combination of treatment before ischemia and after reperfusion with 0.5 mg rhADF is encouraging when the possible practical applications are considered. Clinically, only treatment after ischemia is feasible. The electrophysiologic and histologic studies show no apparent side effects in sham-treated eyes, which encourages clinical use of rhADF. More detailed studies on possible side effects of this protein are needed, however, before clinical applications can be seriously considered.

Electron microscopic observations of cellular morphologic structure and investigation of DNA ladder formation were not performed in this study. However, results of previous studies in our laboratory have revealed that retinal ischemia-reperfusion injury induces apoptosis in retinal cells of the inner nuclear layer, judged by TUNEL staining, electron microscopic observation, and DNA ladder formation. Recombinant human ADF may protect against retinal ischemia-reperfusion injury by reducing apoptotic neuronal cell death. Detailed observation of the cell types that suffered apoptosis and of the no longer apparent on day 4 (Fig. 4). Retinal ischemia was induced by increasing intraocular pressure in the present study, but the time course of appearance of TUNEL-positive cells in vehicle-treated control retinas was similar to that of a ligation model of ischemia used previously in our laboratory. Dying cells seemed to be removed quickly from the retina. Although the number of TUNEL-positive cells was different between rhADF-treated rats and vehicle-treated control rats, the morphologic appearance of TUNEL-positive cells was similar.

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FIGURE 4. In situ labeling of retina by the TdT-dUTP terminal nick-end labeling (TUNEL) method. (A, B, C) Rat retina treated with 0.5 mg recombinant human adult T-cell leukemia-derived factor (rhADF) before ischemia and after reperfusion was labeled on days 1, 2, and 4, respectively, after the ischemic insult. (D, E, F) Vehicle-treated retina 1, 2, and 4 days, respectively, after ischemia. Scale bar, 50 μm.

FIGURE 5. Time course of TdT-dUTP terminal nick-end labeling (TUNEL)-positive cells after 60 minutes of ischemia-reperfusion injury. Data are presented as mean ± SD (n = 8 for each time point). Asterisks indicate a statistically significant difference between eyes treated before ischemia and after reperfusion with 0.5 mg recombinant human adult T-cell leukemia-derived factor (rhADF) and vehicle-treated eyes (**P < 0.01; Bonferroni post hoc test).

cells rescued by rhADF treatment should increase our understanding of the mechanism of action of rhADF, as well as deepen our understanding of retinal ischemia-reperfusion injury.

References
Rescue of Retinal Ischemia by Recombinant Human ADF


