Lens Epithelium-Derived Growth Factor Promotes Photoreceptor Survival in Light-Damaged and RCS Rats

Shigeki Machida,1 Prem Chaudhry,1 Toshimichi Shinohara,2 Dhirendra P. Singh,2 Venkat N. Reddy,1 Leo T. Oblyack, Jr,2 Paul A. Sieving,1 and Ronald A. Bush1

PURPOSE. To investigate possible protective effects of lens epithelium-derived growth factor (LEDGF) against photoreceptor death in light-damaged, Royal College of Surgeons (RCS) and P23H rhodopsin transgenic rats.

METHODS. Twelve-week-old Sprague-Dawley (SD), 6-week-old RCS, and 10-day-old P23H (line 1, heterozygote) rats received an intravitreal injection of LEDGF fused with glutathione-S-transferase (GST-LEDGF). Fellow eyes received vehicle and served as control specimens. Two days after the injections, the SD rats were exposed to light of 2000 lux for 48 hours. Corneal Ganzfeld ERGs were recorded 10 days after light damage, at 10 weeks of age in RCS rats, and at 4 weeks of age in P23H rats. The eyes were then processed for histologic analysis. Heat shock protein (hsp) content in the sensory retina was analyzed quantitatively by protein immunoblot.

RESULTS. In light-damaged rats, the ERG indicated retinal protection in GST-LEDGF–injected eyes, with b-wave and STR thresholds being 1.14 ± 0.50 (mean ± SD) and 0.60 ± 0.26 log candela (cd)/m² lower, respectively, than in vehicle-injected eyes (P < 0.01). The GST-LEDGF-treated eyes had maximum b-wave amplitudes that were significantly larger (P < 0.0005), had more than twice as many remaining photoreceptors, and had better organized outer segments than the control eyes. In RCS rats, the treated eyes had 2.76 ± 0.73 and 0.83 ± 0.09 log cd/m² lower thresholds for the b-wave and STR, respectively (P < 0.005), and had significantly larger maximum b-wave amplitude (P < 0.0005). GST-LEDGF–treated eyes of RCS rats also had more photoreceptors remaining (P < 0.005) and a thinner debris layer than control eyes. In P23H rats, GST-LEDGF treatment did not protect either retinal function or structure. The retinas from GST-LEDGF–treated eyes of SD and RCS rats had higher levels of hsp25 and α-crystallin than vehicle-injected eyes.

CONCLUSIONS. GST-LEDGF protects photoreceptor structure and function in both light-damaged and RCS rats. The increased expression of hsp25 and α-crystallin may play a role in this protection. The absence of rescue in P23H raises the possibility that some forms of inherited retinal degeneration may not be amenable to treatment by intraocular injection of LEDGF. (Invest Ophthalmol Vis Sci. 2001;42:1087–1095)

In a previous study, a clone encoding a novel adhesion, growth, and survival factor was isolated from a cDNA library of human lens epithelial cells (LECs) and was named lens epithelium-derived growth factor (LEDGF).1,2 LEDGF belongs to a family of homologous proteins including hepatoma-derived growth factor (HDGF)3 and HDGF-related protein-1 and -2.4 LEDGF was found in culture medium of LECs and cos7 cells, suggesting that it is a secreted protein.1 LEDGF enhanced adhesion, stimulated growth and prolonged survival of mouse and human lens epithelial cells, mouse keratinocytes, human fibroblasts, chick photoreceptor cells, and retinal pigment epithelial (RPE) cells cultured in a serum-free environment.1,2,5,6 Without exogenous LEDGF, most of these cells died after 2 to 7 days in culture, although LEDGF was synthesized endogenously.1,2,5,6

Immunohistologic analysis, showing widespread distribution of LEDGF in the retina1 and enhancement of survival of cultured chick photoreceptor cells under serum deprivation and thermal stress by LEDGF treatment,2 suggests that LEDGF may play a role in the survival of photoreceptor cells in vivo. One of the reasons that LEDGF enhanced the resistance to oxidative and thermal stresses and prolonged survival in the above-described cell types may be the increase in the synthesis of heat shock proteins (hsp).1 Hsps are generally assumed to play protective roles in a wide range of cell types. They enable cells to survive and recover from a variety of stresses. A previous study demonstrated that hsp expression correlates well with the protective effect against retinal light damage.5 All this evidence suggests that exogenous application of LEDGF may enhance photoreceptor survival in retinal degeneration caused by stress.

In the present study, we investigated whether LEDGF would protect photoreceptor cells against light damage in normal rats and against inherited photoreceptor degenerations in the Royal College of Surgeons (RCS) rat and a transgenic rat model of human retinitis pigmentosa carrying the rhodopsin mutation Pro23His (P23H).3 We found that in LEDGF-treated eyes there was significant preservation of rods and cones compared with the vehicle-injected eyes in the first two animal models, but LEDGF was not effective in delaying photoreceptor cell degeneration in P23H rats. These results indicate that LEDGF played a significant role in protecting photoreceptor cells against environmental stress in normal rats and against hereditary degeneration in RCS rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats 5 to 8 weeks of age, purchased from Harlan (Indianapolis, IN), and RCS (p−) and P23H rhodopsin transgenic (line 1, heterozygote) rats,7 which were born in our laboratory, were used in this study. All were housed in 12-hour-on, 12-hour-off cyclic fluorescent white light (5 lux for P23H rats and 50 lux for SD and RCS rats at cage level). They were fed a high-fat breeding chow (Formula PB; PMI Feed, Richmond, IN) ad libitum. These studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
Preparation and Injection of LEDGF

A fusion protein between glutathione-S-transferase (GST) and LEDGF was expressed in a prokaryotic expression system and was purified with GST column chromatography, as described previously.1 The GST-LEDGF was dissolved in phosphate-buffered saline (PBS) with 100 U/ml of heparin (Sigma, St. Louis, MO).

SD, RCS, and P23H rats received intravitreal injections at 12 weeks, 6 weeks, and 10 days of age, respectively. The ERG rapidly deteriorates after 5 weeks of age in RCS rats10 and photoreceptor loss progresses rapidly from 10 days in P23H line 1 rats.11 (Matthew LaVail, personal communication, May 1997). Three SD rats were not treated and were kept as nondamaged control animals. Three untreated RCS rats were kept as baseline control animals.

All injection procedures were performed using a microscope under fluorescent light. Intravitreal injection of 1 µl of GST-LEDGF (1000 ng/µl) was administered into the right eye of each treated animal using a syringe (model 80001; Hamilton, Reno, NV) with a 30-gauge needle inserted approximately 1 mm behind the corneal limbus. The estimated concentration of GST-LEDGF at the retina was approximately 25 ng/µl, assuming full mixing in a 38-µl vitreous volume. The left eyes received 1 µl heparin (100 U/ml) as a control. To test the effect of GST alone, the right eyes of SD and RCS rats were treated with 220 ng of GST (equivalent to 1000 ng GST-LEDGF), and the left eyes received 1 µl heparin (100 U/ml) using the same injection procedure and treatment parameters as with GST-LEDGF. Shortly after the injections, we observed the eyes for signs of hemorrhage or cataract using a direct ophthalmoscope. If either occurred, the animals were not used. The injections were performed between 10:00 A.M. and 12:00 P.M., after which all animals were returned to the animal colony room under the standard cyclic light condition for 2 days before light exposure.

Light Exposure

Bright light exposure was started at 7:00 A.M. after 12 hours of darkness and consisted of diffuse cool white fluorescent light of 2000 lux for 48 hours. Each animal was housed in a separate well-ventilated transparent plastic cage so that one animal could not hide behind another. During light exposure, the temperature was kept at 25.0°C ± 1.0°C. After light exposure, the animals were returned to the colony room where they were kept for 9 days under the standard cyclic light.

Recording ERGs

ERG recordings were obtained 10 days after cessation of light exposure in the SD rats and in the RCS and P23H rats at 10 weeks and 4 weeks of age, respectively. Animals were kept in total darkness for 12 hours before recording and were prepared under dim red light. They were anesthetized intramuscularly with a loading dose of xylazine (13 mg/kg) and ketamine (86 mg/kg) and then maintained with subcutaneous infusion of the same mixture by pump (Razel, Stamford, CT). The pupils were dilated with 0.1% atropine and 0.1% phenylephrine HCl. The animals were held steady with a bite bar and nose clamp. A heating pad maintained body temperature at 37°C.

Scotopic and photopic ERGs were recorded in all animals from both eyes simultaneously using a chlorided silver wire loop on the center of the cornea, with 1% tetracaine topical anesthesia and a drop of methyl cellulose to maintain corneal hydration. A chlorided silver reference electrode was positioned at the temporal sclera approximately 1 mm behind the corneal limbus. An alligator clip attached to the left ear served as a ground electrode. Responses were amplified at 10,000 gain from 0.1 Hz to 1000 Hz, filtered to remove 60-Hz noise, and digitized at a rate of 10 kHz. Twenty to 40 responses were computer averaged with flush intervals from 3 seconds to 60 seconds for scotopic recordings and 1 second for photopic recordings. ERGs were recorded using a Ganzfeld bowl and a white (2800 K) 50-milli-second stimulus with a maximum intensity of 2.2 log candela (cd)/m², which was attenuated with neutral-density filters. Photopic ERGs were recorded in the presence of a white rod-suppressing background of 54 cd/m². All animals were light adapted for 10 minutes before the photopic ERG recording was begun. Recording intensities began below ERG threshold and progressed upward in 0.5- or 0.4-log-unit steps.

Histology

One day after ERG recording, the rats were killed with an overdose of sodium pentobarbital. The eyes of each animal were removed for histology and kept overnight at 4°C in Karnovsky’s fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in 0.1 M cacodylate buffer. Eyes were trimmed and postfixed in 1% osmium for 1 hour. Epon-embedded tissue was cut into 1-µm sections and stained with toluidine blue for light microscopy. All sections for light microscopy were cut along the vertical meridian of the eye passing through the optic nerve.

We assessed the thickness of the outer nuclear layer (ONL) in number of nuclei and width in micrometers. The total number of cones was counted in 100-µm lengths of the retina. The combined rod outer segment (ROS) and inner segment (RIS) length, designated as ROS+RIS, was also measured in light-damaged and P23H rats. Because intact ROS were generally not distinguishable from ROS debris in RCS rats,12 ROS and the debris layer were measured separately. The number of pyknotic nuclei in 100-µm lengths of the retina was normalized by ONL cell counts in the same area to obtain a rate of pyknosis in RCS rats. All measurements were made every 400 µm in each retinal half, beginning 200 µm from the optic nerve head, to obtain the average of each morphologic parameter in every section. The total number of cells in the inner plexiform layer (IPL) and the subretinal space, which had the appearance of macrophages, was counted in a section from each eye.13,14

Immunoblot Analysis

Thirty hours after intravitreal injection of GST-LEDGF and vehicle into the right and left eyes, respectively, the sensory retinas were taken from 6- to 10-week-old SD rats without light damage and 6-week-old RCS rats for immunoblot analysis. To detect hsp, the soluble fractions were prepared by homogenizing sensory retinas in Eppendorf tubes containing lysis buffer (50 mM Tris-HCl, [pH 7.2], 2 mM EDTA, 100 mM sodium fluoride, 25 mM b-glycerophosphate, 50 mM sodium chloride, 1% Nonidet P-40, 5 mM benzamidine, and a cocktail of protease inhibitors) and rocking the tubes at 4°C for 1 hour. The homogenates were centrifuged (15,000g, 20 minutes, 4°C) and supernatants thus obtained were used for Western blot analysis. Proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Bio-Rad, Hercules, CA). Immunoblot analysis was performed using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. Primary antibodies were used at a dilution of 1:4000, rabbit-anti-hsp25 from Stressgen (Victoria, British Columbia, Canada) and rabbit-anti B-crystallin from Chemicon (Temecula, CA). Followed by incubation with anti-rabbit immunoglobulin horseradish peroxidase at a dilution of 1:20,000. The blots were exposed to hyperfilm ECL and scanned, and the relative band density of hsp25 and B-crystallin from Chemicon was also measured in light-damaged and P23H rats. Because the scotopic a-wave was a more sensitive communication, May 1997). Three SD rats were not treated and were kept as nondamaged control animals. Three untreated RCS rats were kept as baseline control animals.

Data Analysis

Amplitude of the scotopic and photopic b-waves and the scotopic threshold response (STR) were plotted against the stimulus intensity to determine the threshold of each ERG component for light-damaged and RCS rats. Because the scotopic a-wave was a more sensitive measure than the b-wave and it correlated well with photoreceptor loss and ROS shortening in P23H rats,15 the a-wave maximum amplitudes of the scotopic a-wave (Vbmax), and b-wave (Vbmax), and photopic b-wave (photopic Vbmax) were defined as the...
amplitudes elicited by maximum intensity (2.2 log cd/m²). The statistical significance of the difference between right and left eyes was determined with the Student’s two-tailed t-test.

RESULTS

Histology

GST-LEDGF conveyed protection for both light-damaged and RCS rats (Fig. 1). In the light-damaged rat (Fig. 1A), the inferior retina of the vehicle-injected eye showed only a one- to two-nuclei-thick ONL with severely truncated ROS. The GST-LEDGF-treated eye had four to five rows of ONL cells with better preserved ROS structure than in the vehicle-injected eye. In the RCS rat (Fig. 1B), the vehicle-injected eye had an ONL that consisted of less than one complete row of nuclei with pyknosis. Whereas two to three rows of nuclei remained in the GST-LEDGF-treated eye, note that the GST-LEDGF treated eye has a thinner debris zone than the vehicle-injected eye.

many ONL cells remained in the GST-LEDGF-treated eyes than in the vehicle-injected eyes. In RCS rats (Fig. 3 and Table 1), rows of ONL cells and ONL thickness, RIS length, and the number of cones were significantly better maintained in the GST-LEDGF-treated eyes than in the vehicle-treated eyes ($P < 0.005$), although progressive thinning of the ONL was noted even in GST-LEDGF-treated eyes compared with baseline data at the age of 6 weeks. Pyknotic cell rates were significantly reduced in GST-LEDGF–treated eyes compared with baseline or vehicle-injected eyes ($P < 0.005$).

FIGURE 1. Photographs of the GST-LEDGF–treated and vehicle-injected eyes from a light-damaged and RCS rat. (A) In a light-damaged rat, the GST-LEDGF–treated eye exhibited a two- to three-times thicker ONL with better preserved ROS than the vehicle-injected eye. (B) In an RCS rat, the vehicle-injected eye had an ONL that consisted of less than one complete row of nuclei with pyknosis, whereas two to three rows of nuclei remained in the GST-LEDGF–treated eye. Note that the GST-LEDGF treated eye has a thinner debris zone than the vehicle-injected eye.

FIGURE 2. Morphologic comparison between GST-LEDGF–treated and vehicle-injected eyes from light-damaged rats. ONL cell counts and width, cone counts, and ROS+RIS length showed significantly better preservation in GST-LEDGF–treated eyes than in vehicle-injected eyes. Error bars, SE.

FIGURE 3. Morphologic comparison between GST-LEDGF–treated and vehicle-injected eyes from RCS rats. ONL cell counts and width, cone counts, and RIS lengths showed significantly better preservation in GST-LEDGF–treated eyes than in vehicle-injected eyes. Pyknotic rate and debris+ROS thickness were significantly lower in GST-LEDGF–treated eyes than in vehicle-injected eyes. Error bars, SE.
It was also found that the debris+ROS zone was significantly thinner in the GST-LEDGF-treated eyes than that at baseline or in vehicle-injected eyes (P < 0.005, Fig. 3 and Table 1). The counts of surviving ONL cells from GST-LEDGF–treated and vehicle-injected eyes were plotted against debris+ROS to see whether there was a correlation between these two factors (not shown). Although a good correlation was not obtained (R = 0.27 and 0.49 for the lower and upper hemispheres, respectively), the slope deviated significantly from zero (P < 0.01 and 0.0001 for the lower and upper hemispheres, respectively). This indicates that thinning of the debris zone in the GST-LEDGF-treated eyes may in some way be related to photoreceptor survival.

In P23H rats, there was no difference between GST-LEDGF–treated and vehicle-injected eyes in either ONL cells (GST-LEDGF–treated: 6.22 ± 0.70; vehicle-injected: 6.53 ± 0.55; P = 0.423, n = 3), ONL thickness (GST-LEDGF–treated: 37.5 ± 0.99 µm; vehicle-injected: 38.23 ± 1.78 µm; P = 0.409, n = 3), or ROS+RIS length (GST-LEDGF–treated: 22.8 ± 1.75 µm; vehicle-injected: 23.27 ± 0.06 µm; P = 0.628, n = 3).

Electroretinogram

Light-Damaged Rats. ERG study of light-damaged rats showed functional rescue in GST-LEDGF–treated eyes. The ERG intensity series from the GST-LEDGF–treated eye (Fig. 4A) showed the scotopic b-wave emerging from the negative-going STR at −2.5 cd/m², whereas in the vehicle-injected eye, the apparent b-wave was detectable at −1.5 cd/m², giving an approximate 1.0-log-cd/m² threshold difference (P < 0.01). The b-wave amplitude of the GST-LEDGF–treated eye reached a maximum amplitude approximately seven times larger than that of the vehicle-injected eye (P < 0.0005). The photopic b-wave amplitude reached the 5-µV criterion at 1.2 and 1.5 log cd/m² in GST-LEDGF–treated and vehicle-injected eyes, respectively, a 0.5-log-unit threshold difference (P < 0.01). At the maximum intensity, the photopic b-wave amplitude seen in the GST-LEDGF–treated eye was three times larger than in the vehicle-injected eye (P < 0.005). The scotopic and photopic b-wave intensity–response functions of the vehicle-injected eyes were shifted down and toward the right compared with the GST-LEDGF–treated eyes, indicating that GST-LEDGF conveyed protection for both b-wave sensitivity and maximum amplitude (Figs. 4B, 4C).

As summarized in Table 2, the GST-LEDGF–treated eyes reached the 10-µV criterion at a 1.14-log-cd/m² lower intensity (P < 0.01) and had 0.97 log µV greater Vb,max (P < 0.0005) than the vehicle-injected eyes. In comparison to the b-wave threshold difference, the shift in the STR threshold was smaller (0.6 log cd/m²), consistent with our previous results in which the STR threshold showed a much slower elevation with reduction of ONL cells than the scotopic b-wave threshold in light-damaged rats. The photopic b-wave threshold and photopic Vb,max were significantly better preserved in the GST-LEDGF–treated eyes than in the vehicle-injected eyes (Fig. 4C and Table 2, P < 0.01 for the photopic b-wave threshold, P < 0.005 for the photopic Vb,max).

RCS Rats. In the GST-LEDGF–treated eye shown in Figure 5A, the scotopic b-wave appeared at −1.5 log cd/m² and grew with stimulus intensity. In the vehicle-injected eye, a negative response dominated the ERG over the whole intensity range, as previously reported, and the scotopic b-wave did not appear until nearly maximum stimulus intensity. There was a 3-log-unit threshold difference between GST-LEDGF–treated and vehicle-injected eyes (P < 0.005). The photopic Vb,max in GST-LEDGF–treated eyes was also approximately four times larger than the vehicle-injected eye (P < 0.005).

The averaged scotopic and photopic b-wave amplitude from RCS rats was plotted against stimulus intensity (Figs. 5B, 5C). In GST-LEDGF–treated eyes, the intensity–response function curve for the scotopic b-wave was shifted to the right by only 0.5 log units compared with the baseline control, whereas the curve from vehicle-injected eyes was shifted to the right by 2.76 log units (P < 0.005, Fig. 5B and Table 2). As shown in Table 2, all ERG parameters were better preserved in the GST-LEDGF–treated eyes than those in the vehicle-injected eyes (P < 0.005 for the thresholds of scotopic b-wave, STR, and photopic b-wave threshold;
Table 1. Morphological Comparison between GST-LEDGF–Treated and Vehicle-Injected Eyes

<table>
<thead>
<tr>
<th></th>
<th>ONL Thickness (µm)</th>
<th>ONL Cell Counts</th>
<th>ROS+RIS Length (µm)</th>
<th>Cone Counts (100-µm width)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nondamaged (n = 3)</td>
<td>34.77 ± 0.97</td>
<td>9.47 ± 0.12</td>
<td>38.13 ± 1.24</td>
<td>3.07 ± 0.38</td>
</tr>
<tr>
<td>Light-damaged (n = 5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEDGF-treated eyes</td>
<td>25.64 ± 3.07</td>
<td>4.50 ± 0.57</td>
<td>22.26 ± 2.30</td>
<td>2.24 ± 0.47</td>
</tr>
<tr>
<td>Vehicle-injected eyes</td>
<td>11.66 ± 1.18</td>
<td>1.94 ± 0.20</td>
<td>8.87 ± 4.24</td>
<td>1.41 ± 0.40</td>
</tr>
<tr>
<td>Difference</td>
<td>13.98 ± 3.05</td>
<td>2.59 ± 0.44</td>
<td>13.30 ± 3.78</td>
<td>0.81 ± 0.50</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0005</td>
<td>&lt;0.0005</td>
<td>&lt;0.0005</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

|                                |                   |                 |                    |                             |
| RCS rats                       |                   |                 |                    |                             |
| 6 weeks of age (baseline, n = 3)| 19.77 ± 1.61      | 3.80 ± 0.35     | 8.35 ± 0.81        | 17.23 ± 3.14                |
| 10 weeks of age (n = 5)        | 10.60 ± 0.89      | 1.80 ± 0.09     | 4.30 ± 1.23        | 4.00 ± 1.01                 |
| GST-LEDGF                      |                   |                 |                    |                             |
| Vehicle                        | 3.69 ± 0.86       | 0.57 ± 0.09     | 0.74 ± 0.43        | 23.98 ± 7.29                |
| Difference                      | 6.91 ± 1.60       | 1.25 ± 0.11     | 3.56 ± 0.97        | 25.58 ± 7.16                |
| P                              | <0.0005           | <0.0005         | <0.0005            | <0.0005                     |

Tabulated values are expressed as mean ± SD.

P < 0.005 for the photopic Vmax, and P < 0.0005 for Vbmax.

P23H Rhodopsin Transgenic Rats. No difference was found between GST-LEDGF–treated and vehicle-injected eyes in either Vmax (GST-LEDGF–treated: 2.11 ± 0.08; vehicle-injected: 2.09 ± 0.06 log µV, P = 0.46), or in the a-wave threshold (GST-LEDGF–treated: 0.22 ± 0.09; vehicle-injected: 0.24 ± 0.13 log cd/m², P = 0.76). Thus, using these specific treatment parameters, GST-LEDGF does not appear to have a protective effect against loss of photoreceptor function in these P23H rats.

Immunoblot Analysis

Expression of hsp25 and αB-crystallin in the sensory retina of SD rats without light damage and RCS rats in response to exogenous application of GST-LEDGF is shown in Figure 6. Mean densities on immunoblots of vehicle-injected eyes relative to those of GST-LEDGF–treated eyes, with standard errors for five animals are shown in the graphs. Hsp25 and αB-crystallin were elevated approximately fivefold in GST-LEDGF–treated eyes compared with vehicle-injected eyes in normal SD rats. In RCS rats, a similar pattern of upregulation was seen in GST-LEDGF–treated eyes with approximately a threefold increase in hsp25 and αB-crystallin.

Figure 5. (A) Scotopic and photopic electoretinogram (ERG) waveform series from an LEDGF-treated eye and a vehicle-injected eye of an RCS rat. Both eyes were recorded simultaneously in one animal. Average scotopic (B) and photopic (C) b-wave amplitudes versus stimulus intensity from 6- and 10-week-old RCS rats on a log–log scale. Error bars, SE.
crease, suggesting that these small hsps may play a role in enhanced photoreceptor survival in GST-LEDGF–treated eyes in vivo. It was noted that elevation of the small hsp level was more prominent in normal SD rats than in RCS rats, probably due to a smaller number of surviving photoreceptor cells in RCS rats than in normal rats (Table 1). Intraocular injection of GST did not alter the expression level of these small hsps in normal SD rats (n = 3).

Ocular Side Effects

Cataract formation is a major complication in RCS rats treated with basic fibroblast growth factor (bFGF). 17 In the present study, ophthalmoscopically detectable cataracts did not develop after treatment in light-damaged or RCS rats. Although invasion of macrophages into the sensory retina is evident after an intravitreal injection of bFGF and IL-1β,13,17,18 significant differences were not found in macrophage counts in the IPL between the GST-LEDGF–treated and vehicle-injected eyes. In light-damaged rats, GST-LEDGF–treated eyes contained 40.0 ± 9.4 macrophages/section, and vehicle-injected eyes contained 39.6 ± 4.7 macrophages/section (P = 0.90). In RCS rats, GST-LEDGF–treated eyes contained 30.6 ± 7.6 macrophages/section, and vehicle-injected eyes contained 36.2 ± 12.2 macrophages/section (P = 0.45). Slightly higher numbers of macrophages could be observed in debris+ROS+RIS in vehicle-injected eyes of RCS rats (GST-LEDGF–treated: 51.8 ± 13.3 macrophages/section; vehicle-injected: 70.4 ± 30.1 macrophages/section, P < 0.05).

GST Effect

Photoreceptor rescue by the fusion protein GST-LEDGF raises the question of whether GST itself plays a role in this protection. To address this question, the protective effect of GST alone was evaluated in light-damaged and RCS rats. In light-damaged (n = 5) and RCS (n = 5) rats, eyes treated with GST alone did not have significantly lower thresholds (P > 0.50) or

<table>
<thead>
<tr>
<th></th>
<th>Scotopic ERG</th>
<th>Photopic ERG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>b-Wave Threshold* (log cd/m²)</td>
<td>Vb&lt;sub&gt;max&lt;/sub&gt;† (log µV)</td>
</tr>
<tr>
<td>SD rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nondamaged (n = 3)</td>
<td>-3.50 ± 0.05</td>
<td>3.09 ± 0.04</td>
</tr>
<tr>
<td>Light damage (n = 5)</td>
<td>-2.52 ± 0.17</td>
<td>2.61 ± 0.11</td>
</tr>
<tr>
<td>GST-LEDGF</td>
<td>-1.30 ± 0.41</td>
<td>1.64 ± 0.18</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.14 ± 0.50</td>
<td>0.97 ± 0.19</td>
</tr>
<tr>
<td>Difference</td>
<td>&lt;0.01</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>RCS rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 weeks of age (baseline, n = 3)</td>
<td>-1.91 ± 0.09</td>
<td>2.58 ± 0.01</td>
</tr>
<tr>
<td>10 weeks of age (n = 5)</td>
<td>-1.39 ± 0.21</td>
<td>2.25 ± 0.07</td>
</tr>
<tr>
<td>GST-LEDGF</td>
<td>1.24 ± 0.91</td>
<td>1.42 ± 0.09</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2.76 ± 0.71</td>
<td>0.83 ± 0.09</td>
</tr>
<tr>
<td>Difference</td>
<td>&lt;0.005</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

* Criterion amplitude: 10 µV.
† Stimulus intensity: 2.2 log cd/m².
‡ Criterion amplitude: 5 µV.
significant higher \( V_{b,\text{max}} \) \((P > 0.1, \text{LD}; 0.3 \text{ RCS})\) than heparin-injected eyes. These results indicate that GST itself did not have a major impact on protection seen in GST-LEDGF–treated eyes.

**DISCUSSION**

**Possible Mechanism of LEDGF**

This study demonstrates that a single intravitreal injection of GST-LEDGF results in preservation of rod and cone structure and function in light damage and RCS degeneration. This result indicates that exogenous application of GST-LEDGF promotes photoreceptor survival in the face of environmental stress and in a genetic form of retinal degeneration. Because exogenous GST alone produced no significant protection against photoreceptor degeneration in light-damaged and RCS rats, it is unlikely that it contributed to the protection provided by the fusion protein. The possibility that cellular protection by the fusion protein may be much greater than LEDGF alone has also been considered. It was demonstrated in vitro that LEDGF, cleaved from GST-LEDGF by proteolytic enzyme, had an effect similar to that of GST-LEDGF in promoting cell survival and growth in serum-free media, suggesting that the protective effect provided by GST-LEDGF mainly represents a biologic response induced by LEDGF itself.

One mechanism by which LEDGF protected photoreceptors from these degenerations may be through upregulated synthesis of hsps. We have previously shown that LEDGF stimulates synthesis of hsp27 and \( \alpha \)-crystallin in cultured LECs, and hsp90 in cultured retinal neural cells. The present study has demonstrated that exogenous GST-LEDGF applied to the intact or pathologic eye increased the synthesis of hsp25 and \( \alpha \)-crystallin in the sensory retina and extends previous observations to this in vivo preparation.

Hsps are assumed to enhance cell survival against a range of stress factors. After mild thermal stress, the retina showed resistance against light damage, and the protective effect was well correlated with expression of hsps. Sensory neurons expressing hsp27 survived better under deprivation of nerve growth factor (NGF) and after axotomy than those without hsp27 expression. Hsps function as molecular chaperons to repair unfolding proteins impaired by various stresses. Hsps can also inhibit signal transduction in apoptosis, which is believed to be the final common pathway of various retinal degenerations.

An alternative explanation is that the thinner debris zone found in GST-LEDGF–treated eyes, also observed in RCS rats protected by light exposure, may enhance the movement of metabolites to photoreceptors and thereby affect their survival. There are at least three possible mechanisms that could yield a thinner debris zone in GST-LEDGF–treated eyes. First, LEDGF may induce phagocytic activity in mutant RPE, as suggested by a report that bFGF increases ROS phagocytosis by cultured mutant RPE cells obtained from RCS rats. Second, LEDGF may cause downregulation of ROS synthesis by surviving photoreceptors. This would reduce recovery from light damage involving the synthesis of new ROS or result in a smaller amplitude ERG than expected for the number of ONL cells remaining. However, GST-LEDGF–treated eyes of light-damaged rats had ERG amplitudes and thresholds equivalent to those expected from our previous parametric study of ERG responses in light damage. Third, an increased number of macrophages in the subretinal space could reduce debris. This seems unlikely, because we counted fewer macrophages in the debris zone in GST-LEDGF–treated eyes than in vehicle-injected eyes.

**ERG Change in RCS Degeneration**

A high degree of photoreceptor cell protection was evident from the enhanced preservation of the b-wave threshold and amplitude in GST-LEDGF–treated eyes of RCS rats. However, other studies have shown that the b-wave does not have a simple relationship to loss of cells or visual function in this model. Perlman demonstrated that the scotopic b-wave sensitivity of RCS rats declines more rapidly than could be explained by loss of photon-absorbing capacity of photoreceptors. In addition, visual function in RCS rats, measured by a psychophysical method and pupillary reflex, is better preserved than indicated by the scotopic b-wave sensitivity. This indicates that the b-wave measurement may not be the best parameter to track retinal sensitivity or cellular loss in RCS degeneration. We have noted the presence of a large negative component from the inner retina in RCS rats with severe damage (Fig. 5) that reduces the b-wave. We have also found that intracocular application of NMA (N-methyl-DL-aspartic acid), which blocks synaptic transmission between the bipolar and third-order neuron cells, causes the scotopic b-wave to become much larger in 10-week-old RCS rats (Machida, unpublished observation), suggesting that the negative-going STR (driven by the third-order neurons) can obscure the b-wave in the lower stimulus range. This characteristic interaction between positive and negative responses would increase the b-wave threshold and may result in a larger apparent b-wave threshold difference than is suggested by the difference in cell survival between GST-LEDGF–treated and vehicle-injected eyes in RCS rats.

**Cone Preservation by LEDGF**

Structure and function of cones are better preserved in GST-LEDGF–treated eyes than in the vehicle-injected eyes of both light-damaged and RCS rats. Preservation of cones is of practical importance for daily human visual performance. Cones can be directly impaired by light damage and indirectly affected in RPE dysfunction or secondary to rod death. In rhodopsin knockout mice and P23H rats, which have genetic mutations in rods but not in cones, there is a substantial cone function loss that begins after approximately 50% of rods are lost. Similarly, cone loss in rats, light-damaged with 1000 to 2000 lux, is not significant until more than 50% of the rods are lost, suggesting that cone loss in this model is also an indirect result of rod cell death. Because ONL cell counts were below 50% of normal values, even in the GST-LEDGF–treated eyes of light-damaged and RCS rats, cone rescue could have resulted indirectly from the preservation of rods. Although there may have been a direct effect of treatment on cones, further experiments comparing rod and cone survival are needed.

**Intraocular Dynamics and Effective LEDGF Concentration**

This study did not analyze an optimal concentration of LEDGF. Previous work showed that LEDGF protected survival of LECs and photoreceptor cells against serum starvation at 0.1 to 1 ng/ml. Considering that LEDGF is washed out by ocular circulation and that the permeability of a 60-kDa protein through the multiple layers of retinal cells may be very low, it is not surprising that the effective in vitro concentration is \( 4 \times 10^{-4} \) to \( 4 \times 10^{-7} \) of the ocular LEDGF concentration used in our study. Both studies indicate that LEDGF works at very low concentrations. Previous studies using intravitreal growth fac-
tor injection used a concentration similar to that used in the present study.14,18

The protective effect of GST-LEDGF was observed, even if it was administered 14 days before light damage (unpublished observation), implying that it stays in or acts on the eye for long periods. This prolonged intraocular effect of GST-LEDGF is consistent with the protective effect against chronic degeneration in RCS rats provided by a single intravitreal injection of GST-LEDGF and with earlier results showing long-term rescue by a single intraocular injection of bFGF in the RCS rat.18

Interpretation of Negative Results in P23H Rats

Intravitreal injections of various survival-promoting factors have failed to protect the retina from degeneration in two different transgenic mice with the P23H mutation.37–39 Thus, the negative result of GST-LEDGF treatment of P23H rat is consistent with results using other growth factors. As was pointed out in previous studies, this could be due to the short biological lifetime of survival factors in the eye or the existence of a critical period during which photoreceptor degeneration can be reversed by growth factors. The former does not fully explain our findings, because GST-LEDGF had a rescue effect against light damage for at least 14 days after injection (Machida, unpublished observation). Therefore, our results imply a more fundamental reason for the failure of LEDGF to rescue photoreceptors from degeneration in P23H rats, perhaps relating to the mechanism of degeneration itself.

An exact mechanism of photoreceptor death in P23H mutation remains to be investigated. However, it is evident that the opsin gene mutation itself plays a critical role in cell death. We found that hsp25 was also upregulated by GST-LEDGF application in P23H rats (data not shown), raising the possibility that upregulated hsp25 is ineffective or not effective enough to rescue photoreceptors in this model.

Comparison with Other Growth Factors

Multiple cytokines, growth factors, and neurotrophins are effective against retinal light damage in rats.15,14,40 Among these agents, acidic (a)FGF, bFGF, ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), interleukin (IL)-1β, and midkine provide a high degree of photoreceptor rescue against light-damage in rats with the ONL remaining in treated eyes being twice as thick as in control eyes. In the present study, GST-LEDGF–treated eyes had ONL thickness more than twice that of light-damaged vehicle-injected eyes, indicating that LEDGF provides a similar degree of rescue to other agents.

Although bFGF exhibits protection in light-damaged and RCS rats,14,18 it causes an increased incidence of retinal macrophages and cataracts as unfavorable side effects.15,17,18 In the present study, GST-LEDGF–treated eyes showed neither phalloidins nor significant invasion of macrophage into the sensory retina, indicating that LEDGF has less potential for cataractogenesis and macrophage attraction than bFGF. Further study is required using various doses or long-term observation to determine ocular complications of this treatment.

Conclusions

In summary, a single intravitreal injection of GST-LEDGF provided structural and functional preservation of rods and cones in light-damaged and RCS rats. Our current results open the possibility that LEDGF can be used as a therapeutic agent for photoreceptor degeneration induced by some environmental factors and genetic mutations. The absence of rescue effect in P23H rats suggests that some forms of inherited retinal degeneration are not ameliorated by this treatment.

Acknowledgments

The authors thank Brad Nelson for excellent technical assistance in histology; Austra Liepa for maintaining the animal colonies; Hiroonri Matsui for helpful discussion of this study and preparing the solution of LEDGF; Rong Wen for technical suggestions about intravitreal injection; and Matthew LaVail for providing mating pairs of P23H transgenic rats.

References