Rescue of Photoreceptors From the Damaging Effects of Constant Light by Midkine, a Retinoic Acid-Responsive Gene Product

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Purpose. To evaluate the protective effects of midkine (MK), the product of a retinoic acid-responsive gene, on constant light-induced retinal degeneration in albino Sprague-Dawley rats.

Methods. Midkine, basic fibroblast growth factor (bFGF), MK plus heparin, or buffer controls were injected intravitreally 2 days before constant light exposure. After 7 days of continuous light exposure, the eyes were perfused with fixative, bisected along the vertical meridian, embedded in paraffin, and sectioned. The degree of retinal light damage was assessed for paraffin-embedded sections by cytologic analysis, by measuring the thickness of the outer nuclear layer (ONL), and by counting the number of macrophages.

Results. After 1 week of constant light exposure, uninjected controls and those injected with phosphate-buffered saline (PBS) lost most of the photoreceptor inner and outer segments, and the thickness of the ONL was decreased. Eyes that were injected with MK or bFGF demonstrated a significant rescue in the photoreceptor layer with a two- to threefold increase in the ONL thickness. The number of macrophages in eyes injected with MK was significantly suppressed compared with controls. Those injected with bFGF had a 1.5-fold increase in number compared with controls.


Retinoic acid, a retinoid and metabolite of vitamin A, is required for growth, morphogenesis, development, and differentiation.1-3 In the retina, retinoic acid and retinoic acid-binding protein are located in amacrine and Müller cells.4-6 Retinoic acid does not support the visual function, but it may play an unknown role in the retina.

Midkine (MK) is a heparin-binding growth factor that is expressed temporally during the early stages of retinoic acid-induced differentiation of embryonal carcinoma cells and during the midgestation period of mouse embryogenesis.7-9 This protein enhances the survival and neurite outgrowth of cultured embryonic neurons and promotes mitosis in some fibroblast cell lines.10-12 In the mouse eye, MK is expressed in the retina and its surrounding region and in the cornea on the 11th to 13th embryonic days, but not in later embryos.8

In retinal degeneration induced by constant light in the albino Sprague-Dawley rat, the heparin-binding growth factors, basic fibroblast growth factor (bFGF), and acidic fibroblast growth factor, as well as brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), induced strong survival-promoting activity, rescuing photoreceptors from constant light damage.13 The light damage system in the rat is a relatively simple, fast, and efficient means of...
evaluating the rescue effects of various agents in vivo. We therefore examine the protective role of MK in the light-damaged retina of the rat.

MATERIALS AND METHODS

Animals

Sprague-Dawley albino rats were obtained at 2 to 3 months of age (Kyudo, Kumamoto, Japan) and maintained in a 12-hour light/12-hour dark cycle (with an in-cage illuminance of less than 15 foot-candles) for at least 7 days before use. All procedures followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Kagoshima University Faculty of Medicine for Animal Research.

Factors

Midkine, bFGF, and heparin were injected into the vitreous cavity of rat. Midkine was purified from the culture medium of L-cells transfected with an MK expression vector, as described in detail elsewhere, and was concentrated to 1 \( \mu g/\mu l \). Human recombinant bFGF (1 \( \mu g/\mu l \); R & D Systems, Minneapolis, MN) and heparin (8.2 units/\( \mu l \); Nakarai Tesque, Kyoto, Japan) were commercially available. The control vehicle was phosphate-buffered saline (PBS).

Injection and Histologic Procedures

Two days before constant light exposure, we anesthetized rats with an intramuscular injection of ketamine (84 mg/kg)–xylazine (6 mg/kg) mixture. We then injected a single 1 \( \mu l \) of solution containing various agents into the vitreous of one eye.

The other eye of each rat was injected with the same volume of PBS as a control. The injections were performed with a 32-gauge beveled needle injected through the sclera, choroid, and retina at a point midway between the ora serrata and the equator of the eye. Two days later, the rats were placed into constant light at an illuminance of 130 fc to 150 fc (1 fc = 10.76 lux) for 1 week. After the constant light exposure, the rats were killed by an overdose of carbon dioxide and maintained in a 12-hour light/12-hour dark cycle (with an in-cage illuminance of less than 15 foot-candles) for at least 7 days before use. All procedures followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Kagoshima University Faculty of Medicine for Animal Research.

RESULTS

Degeneration of photoreceptor cells after 1 week of constant light in uninjected rats and those injected with PBS was most severe in the posterior to equatorial region of the superior hemisphere. The ONL was reduced in thickness from the normal nine to ten rows of retina, the six measurements were made at defined points 100 \( \mu m \) apart using a scale on the screen. In this way, 48 measurements in two hemispheres sampled representative regions of almost the entire section.

We analyzed the rescue effects of each agent assigning a relative score to the control eye described. This method considered not only ONL thickness but also the integrity and organization of the inner and outer segments, as well as the distribution and extent of rescue and degeneration within each eye. For assessing the overall degree of photoreceptor rescue, we compared each section with its contralateral control eye and scored the degree of rescue from 0+ to 4+. Zero indicated no rescue and 4+ was maximal, with at least some regions of the retina appearing almost normal.

For macrophage counts, the number of cells was counted in the photoreceptor, inner plexiform, and ganglion cell layer, which had the appearance of a macrophage in a single section from each rat eye. We omitted cells that were obviously neurons, glia, or those associated with blood vessels.

For each of the experiments, the number of eyes measured is given in parentheses at the bottom of the figures. The measurements of ONL thickness and macrophage counts of treated eyes were compared with those of control eyes, using Student’s \( t \) test.
of photoreceptor nuclei (Fig. 1A) to one to three rows (Fig. 1B). Only a few fragments of photoreceptor inner and outer segments were saved in this most damaged region. The retinal pigment epithelium (RPE) did not show any damage. In other parts of the light-damaged retina, the inner and outer segments of photoreceptors were damaged to a lesser degree, and the ONL was thicker. The peripheral region of the inferior hemisphere had the least damage from the constant light exposure.

Intravitreal injection with MK, MK plus heparin, and bFGF significantly rescued the photoreceptors and ONL (Figs. 1C, 1D). The photoreceptors had inner and outer segments and sometimes appeared even normal, although they were a little shortened and disorganized. The ONL had seven to eight rows of nuclei, although pyknotic nuclei were scattered throughout the layer.

The thickness of the ONL in the uninjected and PBS-injected eyes was approximately 25% of that seen in normal cyclic light (Fig. 2). The thickness of the eye injected with MK showed a considerable rescue in constant light-damaged retina (approximately 75% of the normal thickness; \( P < 0.0001 \)). The rescue activity of bFGF was similar to that of MK and also to that previously demonstrated. The combination of MK and heparin revealed a slightly greater degree of rescue than did MK alone, as did bFGF; the difference between the degree of rescue for bFGF and that of MK alone was, however, not statistically significant. The injection of heparin alone did not show any rescue effects compared with that of PBS.

Figure 3 shows the rescue effect of various agents as determined by the scoring system described in the Methods section. We gave the eye that received the factor a relative score to that injected with PBS, as previously reported. The overall results were similar to those of the ONL thickness. Midkine and bFGF revealed similar survival effects on constant light-induced retinal degeneration, and MK plus heparin had the highest score (Fig. 3). The eyes injected with heparin had a low score.

Injection of bFGF into the RCS (Royal College of Surgeons) rat with inherited retinal dystrophy and the albino Sprague-Dawley rat with light-induced retinal degeneration increased the number of retinal macrophages. We counted the number of macrophages in the retina...
FIGURE 2. Measurements (mean ± standard deviation) of the ONL thickness in eyes exposed to cyclic light (Cyc L), exposed to constant light for 7 days without any injection (CL), and with various agents injected 2 days before light exposure. The agents are midkine (MK), MK plus heparin (MK + Hep), bFGF, and heparin. Controls for each agent (PBS) injected 2 days before light exposure. The number of rats injected (with an equal number of control eyes) was nine for MK, six for MK plus heparin, five for bFGF, and five for heparin. Bar = the mean value; error bar = standard deviation. Outer nuclear layer thickness of eyes injected with MK, MK plus heparin, and bFGF shows significant differences (shaded bars) when compared with their control eye (solid black bars). *P < 0.001, **P < 0.0001. Heparin does not show any significant difference from the control.

FIGURE 3. The scores for the degree of photoreceptor rescue (mean ± standard deviation) by various agents. The scores of photoreceptors rescued by various agents were similar to those obtained by measuring the outer nuclear layer thickness. The rats scored were the same as those used in the experiments described in Figure 2. The abbreviations are also the same as those described in the legend to Figure 2.

**DISCUSSION**

These results indicate that MK, the product of a retinoic responsive gene, promotes the survival of the eye subjected to constant light-induced retinal degeneration in the albino Sprague-Dawley rat. The degree of rescue from light damage by MK was almost the same as that by bFGF. Midkine is the first member of a new family of heparin-binding growth factors that have recently been discovered; it is structurally unrelated to FGF. Midkine is expressed temporally during the mid-gestation period of mouse embryogenesis and in the eye of 11- to 13-day mouse embryos. It is expressed intensely in the retina, its surrounding region, and the cornea. This protein has been shown to promote neurite outgrowth and survival of embryonic neurons in culture. Retinoic acid, a derivative of vitamin A, is a key regulator of epithelial cells, as well as of neuronal cell growth and differentiation and development of several cell types. Furthermore, retinoic acid modulates the expression of a number of proteins at the gene transcription level. Many of these genes code for constituents of the cytoskeleton or extracellular matrix. Although retinoic acid has no known function in the visual system, the intraretinal synthesis of retinoic acid by amacrine and Müller cells and the presence of the retinoic acid-binding protein in retinal neurons and Müller cells suggest that it plays an important role in the maintenance and organization of the retina. One possible role is to induce MK during retinal damage. Further experiments should be performed along these lines.

Several growth factors and neurotrophic agents promote survival in the central and peripheral nervous systems. In the retina, bFGF injection into either the subretinal space or the vitreous cavity delays inherited photoreceptor degeneration in the RCS rat. Also, a number of survival-promoting factors injected intravitreally can rescue photoreceptors from constant light damage in the albino rat. In the
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MK and BDNF are independent of macrophage involvement. Retinoic acid prevents the outgrowth of RPE cells in vitro and shows antiproliferative effects on proliferative vitreoretinopathy in an animal model. Because MK is the product of a retinoic acid-responsive gene, the injection of MK may be effective and harmless for the treatment of human retinal diseases.

Key Words
light damage, retinal degeneration, photoreceptor rescue, midkine, rat

FIGURE 4. The counts of macrophages (mean ± standard deviation) in various retinas. Macrophages were counted in the same rats used in Figure 2. The counts of macrophages in the constant light-damaged retina with or without injection of PBS increased significantly compared with those in normal retinas (solid black bars). *P < 0.0001. MK reduced the macrophage incidence (P < 0.01). MK plus heparin and heparin only suppressed the number of macrophages to 30% of the uninjected and PBS-injected constant light-damaged retina (P < 0.0001). bFGF intravitreal injection significantly increased the number of macrophages compared with un.injected eyes and those injected with PBS (P < 0.0001). *P < 0.01; **P < 0.001; ***P < 0.0001.

inner retina, bFGF, BDNF, and CNTF have been demonstrated to promote survival in pressure-induced retinal ischemia of rats. The mechanisms by which those growth factors and neurotrophic agents afford protection from light damage and ischemic injury remain unclear. Light damage is thought to result from the generation of oxygen free radicals and the peroxidation of lipids. It remains to be elucidated how MK ameliorates light damage of the retina.

The injection of bFGF into the vitreous of rats with either inherited or light-induced retinal degeneration increases the incidence of retinal macrophages; this is one of several potentially harmful side effects. It is of interest in the present study that the injection of MK into the vitreous reduced the number of macrophages from that usually seen in light damage. Brain-derived neurotrophic factor also decreases the number of macrophages to 30% of the uninjected and PBS-injected constant light-damaged retina (P < 0.0001).

References

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