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Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light

(light damage/retinal degeneration/photoreceptor rescue/neurotrophic factor/basic fibroblast growth factor)

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ABSTRACT **Recent demonstrations of survival-promoting activity by neurotrophic agents in diverse neuronal systems have raised the possibility of pharmacological therapy for inherited and degenerative disorders of the central nervous system. We have shown previously that, in the retina, basic fibroblast growth factor delays photoreceptor degeneration in Royal College of Surgeons rats with inherited retinal dystrophy and that the growth factor reduces or prevents the rapid photoreceptor degeneration produced by constant light in the rat. This light-damage model now provides an efficient way to assess quantitatively the survival-promoting activity *in vivo* of a number of growth factors and other molecules. We report here that photoreceptors can be significantly protected from the damaging effects of light by intravitreal injection of eight different growth factors, cytokines, and neurotrophins that typically act through several distinct receptor families. In addition to basic fibroblast growth factor, those factors providing a high degree of photoreceptor rescue include brain-derived neurotrophic factor, ciliary neurotrophic factor, interleukin 1β, and acidic fibroblast growth factor; those with less activity include neurotrophin 3, insulin-like growth factor II, and tumor necrosis factor α; those showing little or no protective effect are nerve growth factor, epidermal growth factor, platelet-derived growth factor, insulin, insulin-like growth factor I, heparin, and laminin. Although we used at least one relatively high concentration of each agent (the highest available), it is still possible that other concentrations or factor combinations might be more protective. Injecting heparin along with acidic fibroblast growth factor or basic fibroblast growth factor further enhanced the degree of photoreceptor survival and also suppressed the increased incidence of macrophages produced by either factor, especially basic fibroblast growth factor. These results now provide the impetus for determining the normal function in the retina, mechanism(s) of rescue, and therapeutic potential in human eye diseases for each agent.**

Pharmacological treatment is presently unavailable for most inherited and degenerative disorders of the central nervous system (CNS), particularly for the prevalent yet relatively poorly characterized groups of retinal degenerations known collectively as retinitis pigmentosa and macular degeneration. In the past several years, a number of neurotrophic factors have shown survival-promoting activity in a wide range of neuronal systems both *in vitro* and *in vivo* (1–16). We recently explored the survival-promoting activity of basic fibroblast growth factor (bFGF) on degenerating photoreceptor cells in the Royal College of Surgeons (RCS) rat with inherited retinal dystrophy (17). The intravitreally and subretinally injected bFGF dramatically delayed photoreceptor degeneration in this inherited disorder (17). Injection of bFGF was not without its side effects, however, since it resulted in an increased incidence of retinal macrophages (17) and cataracts (ref. 18; E. G. Faktorovich, R.H.S., and M.M.L., unpublished data) in RCS rats (although an increased incidence of cataracts is not seen following bFGF injection in light damage experiments on albino F344 or Sprague–Dawley rats; M.T.M., D.Y., R.H.S., and M.M.L., unpublished data). Moreover, bFGF is a powerful mitogen and angiogenic factor, and it has been shown to increase the proliferation of some retinal pigment epithelium (RPE) and Müller glial cells in the retina (19). Thus, we cautioned against the therapeutic use of bFGF until more could be learned of its potentially harmful side effects (17).

We subsequently demonstrated that intravitreally injected bFGF also reduces or prevents photoreceptor degeneration produced by constant light in the rat (20, 21). With its rapid photoreceptor degeneration, the light-damage model seemed an efficient way to assess quantitatively the survival-promoting activity of other potential neurotrophic agents, some of which, hopefully, would not have the potentially harmful side effects of bFGF. Therefore, we have now examined the protective role in the light-damage model of many of those growth factors, neurotrophins, and cytokines that either are present in the eye, stimulate biological activity in the retina or RPE, or have demonstrated survival-promoting or survival-potentiating activity in other parts of the nervous system (1–4, 6–16, 22–27). We report here that eight different agents that typically act through several different receptor families can rescue photoreceptors from the damaging effects of constant light, and most do so without obvious side effects. The findings also illustrate that the light-damage model is perhaps the most efficient bioassay thus far described for testing quantitatively the survival-promoting activity of neurotrophic agents in an *in vivo* model.

MATERIALS AND METHODS

Animals, Injection, and Histological Procedures. We injected the various agents individually into the vitreous of the superior hemisphere of one eye (17) of anesthetized, 2- to 3-month-old, male Sprague–Dawley rats 2 days before they were placed into constant fluorescent light at an illuminance

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Abbreviations: aFGF, acidic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNS, central nervous system; CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IL-1β, interleukin 1β; NGF, nerve growth factor; NT-3, neurotrophin 3; ONL, outer nuclear layer; PDGF, platelet-derived growth factor; RCS, Royal College of Surgeons; RPE, retinal pigment epithelium; TNF-α, tumor necrosis factor α.

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of ≈115-200 footcandles (1 footcandle = 10.76 lux) for 1 week (28). For buffer control experiments, the other eye of each rat was injected with the same volume of phosphate-buffered saline (PBS) or other appropriate buffer. The volume of injection was 1 μl in all cases. After 1 week of light exposure, the rats were killed by overdose of carbon dioxide followed by vascular perfusion of mixed aldehydes, the eyes were embedded in epoxy resin, and 1-μm-thick histological sections were taken along the vertical meridian to allow comparison of all regions of the eye in the superior and inferior hemisphere, as described elsewhere (21). Retinas from uninjected rats kept in cyclic light and in constant light for 1 week were also examined with each group of experiments. All procedures involving the rats adhered to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and the guidelines of the University of California, San Francisco, Committee on Animal Research.

Factors Injected. All of the agents were injected at a concentration of 1 μg/μl, unless otherwise indicated. The agents injected were the following: human recombinant brain-derived neurotrophic factor (BDNF) (Amgen/Regeneron Partnership, Tarrytown, NY), rat recombinant and human recombinant ciliary neurotrophic factor (CNTF) (0.5 μg/μl; Regeneron Pharmaceuticals), human recombinant neurotrophin 3 (NT-3) (Amgen/Regeneron Partnership), acidic fibroblast growth factor (aFGF) (two sources: bovine brain, R & D Systems, Minneapolis; human recombinant, Upstate Biotechnology, Lake Placid, NY), human recombinant bFGF (a gift of D. Gospodarowicz, University of California, San Francisco); heparin (8.2 units/μl; Kabi Pharmacia Hepar, Franklin, OH), nerve growth factor (NGF) (two sources: a gift of W. Mobley, University of California, San Francisco; Bioproducts for Science, Indianapolis, IN), human recombinant epidermal growth factor (EGF) (Upstate Biotechnology); platelet-derived growth factor (PDGF) (two sources: 0.05 μg/μl, Boehringer Mannheim; 0.25 μg/μl, Upstate Biotechnology), insulin (bovine pancreas, Sigma), human recombinant insulin-like growth factor I (IGF-I) (two sources: 0.1 μg/μl, Boehringer Mannheim; 1 μg/μl, Upstate Biotechnology), human recombinant insulin-like growth factor II (IGF-II) (two sources: 0.1 μg/μl, Boehringer Mannheim; 1 μg/μl, Upstate Biotechnology), laminin (Boehringer Mannheim), human recombinant interleukin 1β (IL-1β) (0.5 μg/μl, Upstate Biotechnology), and human recombinant tumor necrosis factor α (TNF-α) (two sources: 0.1 and 0.5 μg/μl, Upstate Biotechnology; 0.5 μg/μl, R & D Systems). The number of controls and animals injected with each agent is given in Fig. 2. Where 10 or more rats were tested with a given factor, the experiments were done at least twice with 5 or more rats each time.

Quantification of Photoreceptor Rescue and Macrophage Incidence. We used two methods to quantify photoreceptor rescue. With the first, a measurement of mean outer nuclear layer (ONL) thickness for each retina provided an index of photoreceptor cell number (21, 28). Fifty-four measurements were made in the two hemispheres of each section in order to sample representative regions of almost the entire retinal section, so that a single mean ONL thickness was obtained for each eye as described (28). In pilot experiments, we found that the two eyes of a given rat showed statistically indistinguishable degrees of light damage to the retina in control (uninjected and PBS-injected) animals. There was, however, a moderate degree of interanimal variability, even within the same litter and light-exposure cage. Therefore, in almost all cases we compared the experimentally injected eyes with control eyes in the same rats. As found in RCS rats (17), when intravitreal bleeding occurred, an irregular and often extensive photoreceptor rescue was observed; this occurred several times in the present study, and those animals were omitted from further analysis.

For the second method of quantifying rescue, we assigned a relative score to the eye receiving the factor by comparing it to the opposite control eye. 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 the eye. For assessing the overall degree of photoreceptor rescue, four of us compared each experimental section with its contralateral control eye and scored the degree of rescue 0–4+. Zero indicated no rescue and 4+ was maximal, with at least some regions of the retina appearing almost normal. The score for degree of rescue was relative to the degree of degeneration of the contralateral eye, because those rats that showed less damage to the control eye inevitably showed greater rescue in the experimental eye for a given agent. Those that showed greater damage to the control eye inevitably had less rescue for a given agent.

For macrophage counts, all cells in the photoreceptor, inner plexiform, and ganglion cell layers that had the appear-

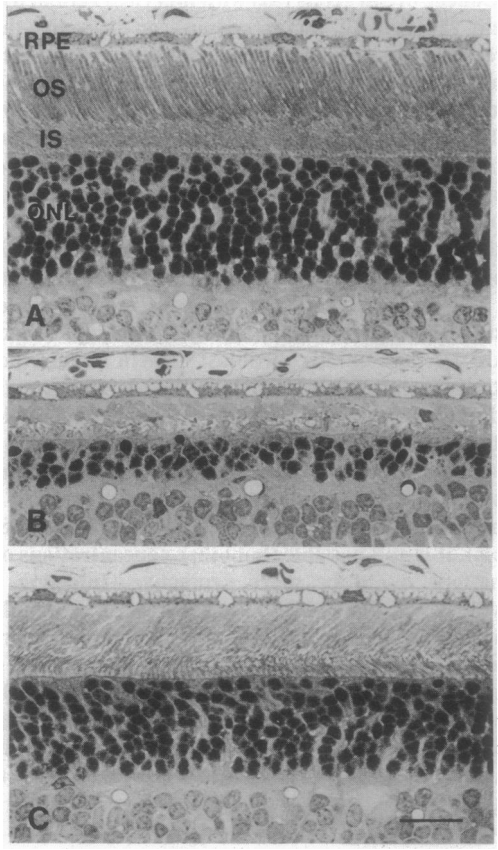

FIG. 1. Plastic-embedded sections of Sprague–Dawley rat retinas. (*A*) Normal retina of rat reared in cyclic light. The photoreceptor outer segments (OS) are apposed to the RPE; distinct photoreceptor inner segments (IS) are seen; and the ONL consists of 9–10 rows of photoreceptor cell nuclei. (*B*) Retina of rat exposed to 1 week of constant light beginning 2 days after intravitreal PBS injection. In this superior posterior region of the retina, the ONL is reduced to 2–3 rows of nuclei; the inner segments are missing or are reduced to very short stumps; and the few remaining outer segments are abnormally rounded and are shorter and larger in diameter than normal (compare with *A*). (*C*) Retina from the same rat as *B*, but in which BDNF was injected intravitreally 2 days before the 1-week light exposure. The same region of the retina as in *B* is shown, and the retina appears almost normal (compare with *A*; same labels of retinal laminae apply), except for the loss of some photoreceptor nuclei, somewhat shorter inner and outer segments, and somewhat disorganized outer segments. (Toluidine blue; bar = 20 μm.)

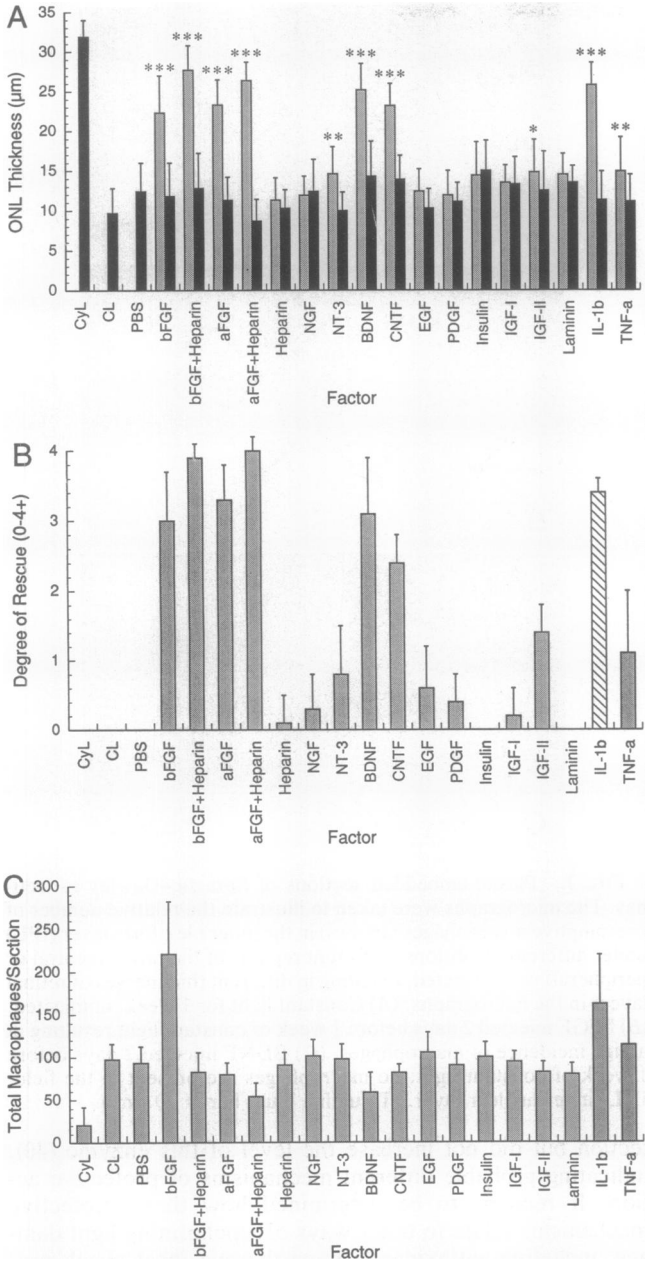

FIG. 2. Measurements of ONL thickness (*A*), degree of photoreceptor rescue (*B*), and number of presumptive macrophages (*C*) in the retinas of rats exposed to 1 week of constant light or maintained in cyclic light (CyL). Two days before the onset of light exposure, each rat received an intravitreal injection of one of the listed factors in one eye and a buffer control (usually PBS) injection in the other eye. Uninjected controls were also exposed to light (CL). In each case, the bars represent the mean value, and the error bars are the standard deviation. (*A*) ONL thickness of eyes injected with bFGF, aFGF, bFGF or aFGF with heparin, BDNF, CNTF, and IL-1β showed the significantly greatest difference in numbers of photoreceptor nuclei surviving (shaded bars) compared to their control eyes (solid black bars). NT-3, IGF-II, and TNF-α produced less photoreceptor rescue, but still significantly greater numbers of photoreceptor nuclei survived than in control eyes (*, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.0001$). The remaining agents failed to show significant differences from controls. The number of rats injected (with an equal number of control eyes) was 17 for bFGF, 13 for bFGF plus heparin, 14 for aFGF, 11 for aFGF plus heparin, 14 for heparin, 12 for NGF, 6 for NT-3, 16 for BDNF, 11 for CNTF, 6 for EGF, 14 for PDGF, 6 for insulin, 10 for IGF-I, 5 for IGF-II, 5 for laminin, 8 for IL-1β, and 10 for TNF-α. Although we did not compare agents in the same animal, the bFGF plus heparin value is significantly greater than that for bFGF alone ($P < 0.0025$), as is aFGF plus heparin over aFGF alone ($P < 0.05$). We excluded results from additional rats injected with different lots of three of the agents in order to demonstrate the maximal rescue effect with each agent. These were NT-3 (11 rats with two earlier lots of NT-3 that showed less potency; however, the mean of the 17 eyes still was significantly greater than controls; $P < 0.015$) and EGF and IGF-II (6 and 4 additional eyes, respectively, with lots from different vendors, which showed no rescue effect). (*B*) The scores for the degree of rescue are based on the same number of animals (i.e., the same histological slides) given in *A*. The results closely parallel the ONL measurements shown in *A*. The bar representing IL-1β is hatched to represent the presence of retinal folds, rosettes, and inflammatory cells, in addition to the significant rescue of photoreceptors (see text). (*C*) The counts of macrophages are also based on the same number of animals given above in *A*. Only bFGF ($P = 0.0001$) and IL-1β ($P < 0.0025$) produced significant increases in the incidence of macrophages over control values. Most of the control values ranged from 85 to 100 per section and are omitted for clarity. The striking reduction in macrophage incidence with bFGF plus heparin compared to bFGF alone is highly significant ($P = 0.0001$), as is the reduction using aFGF plus heparin ($P < 0.0015$). The low number seen after BDNF injection is significantly less than control values ($P = 0.0001$).

ance of macrophages (21) were tabulated in a single section from each rat. We omitted any cells that were obvious neurons or glia or that were directly associated with blood vessels. Statistical comparisons of the macrophage counts, as well as the ONL measurements and scores for degree of rescue, were made using the Student *t* test between the eyes that received the agents and the opposite eyes from the same rats that received buffer control injections.

RESULTS

After 1 week in constant light, uninjected rats or those injected with PBS showed a loss of most photoreceptor cells in the most sensitive (superior posterior) region of the retina (21). The ONL was reduced from the normal 9–10 rows of photoreceptor nuclei (Fig. 1*A*) to 1–3 rows (Fig. 1*B*). In this region, virtually no photoreceptor inner segments survived, and only a few fragments of outer segments remained. The remainder of the central retina was somewhat less damaged, and even more photoreceptors survived in the far peripheral retina, where the ONL consisted of 3–6 rows of nuclei in some regions. Overall, light-damaged retinas of control eyes showed only very small inner segments, if any at all, and almost no outer segments of normal caliber, length, or organization, with no obvious damage to the RPE. When the various agents rescued photoreceptors, not only were more cell nuclei present, but also the surviving photoreceptors had greater integrity, with inner and outer segments present, sometimes appearing almost normal in structure and organization (Fig. 1*C*). With all factors that rescued photoreceptors, both rods and cones were saved.

Those agents producing the greatest rescue, as determined by ONL thickness (Fig. 2*A*), were bFGF, aFGF, bFGF or aFGF with heparin, BDNF, CNTF, and IL-1β. In these retinas, the ONL was at least 70% the normal thickness and was about twice (in some cases more) as thick as in the contralateral light-damaged control eye that received only buffer injection (Fig. 2*A*). Combining heparin with bFGF or aFGF gave a substantially greater rescue than either growth factor alone (Fig. 2*A*). NT-3, IGF-II, and TNF-α produced less rescue, but ONL thicknesses were statistically greater than controls (Fig. 2*A*). None of the other factors rescued photoreceptors by the criterion of ONL thickness. We should emphasize that, in most cases, only one, relatively high concentration of each agent was used, so it is possible that those that failed to rescue might do so at a higher concentration (in some cases not available), at a lower concentration, or in combination with other factors.

Overall, the results of scoring the degree of photoreceptor rescue (Fig. 2*B*) closely paralleled those with ONL thickness (Fig. 2*A*). Retinas with agents producing the most rescue (BDNF, CNTF, and bFGF and aFGF with or without heparin) showed extensive regions that were almost devoid of damage after 1 week of constant light exposure. Retinas with agents that gave very low scores (heparin, NGF, EGF, PDGF, and IGF-I) showed mostly no rescue, with only one or two eyes in which photoreceptor integrity appeared slightly greater than in the control eye for each factor; thus, we assume these factors had little or no rescue activity.

Of the agents producing the highest scores, only two gave complications, IL-1β and bFGF. While IL-1β consistently produced one of the thickest ONL measurements and inner and outer segments were almost normal in some regions, these retinas also showed variable numbers of photoreceptor rosettes, retinal folds, and other focal disruptions of the ONL. Also, inner and outer segments were significantly shorter than normal in some regions, and there were signs of inflammation, as noted in other studies where IL-1β was given intraocularly (29).

In the case of bFGF, we previously found an increased macrophage incidence in retinas of RCS rats treated with this growth factor, particularly in the inner plexiform layer (17). Although blood-derived macrophages are known to invade the retina during light damage (Figs. 2*C* and 3*A*) (30), the bFGF-exposed retinas showed more than twice the number of presumptive macrophages compared to uninjected or PBS-injected eyes (Figs. 2*C* and 3*B*). Significantly, the addition of heparin to bFGF suppressed the increase in number of macrophages (Fig. 2*C*), and the same held for heparin combined with aFGF (Fig. 2*C*). Surprisingly, BDNF reduced the incidence of macrophages from that seen in light-damaged retinas (Figs. 2*C* and 3*C*).

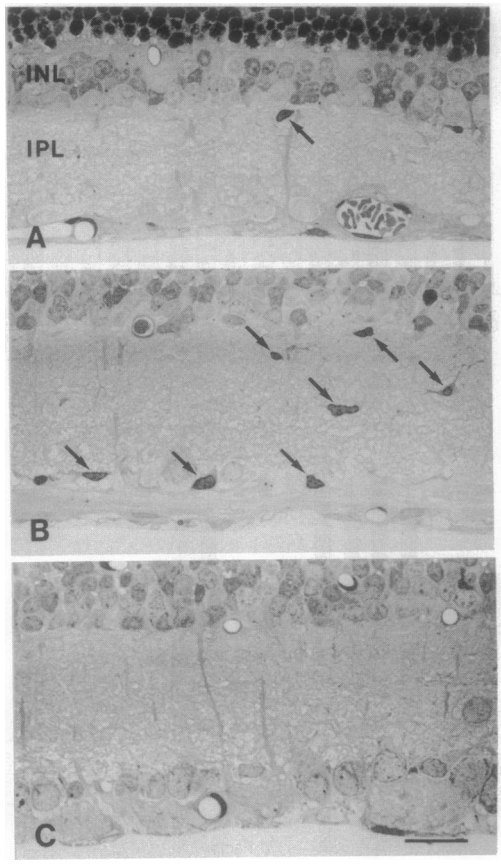

FIG. 3. Plastic-embedded sections of Sprague–Dawley rat retinas. The micrographs were taken to illustrate the relative number of presumptive macrophages (arrows) in the inner plexiform layer (IPL) under different conditions. Different regions of the retina (central or peripheral) were selected, resulting in different thicknesses of retinal layers in the micrographs. (*A*) Constant light for 1 week, uninjected. (*B*) bFGF injected 2 days before 1 week of constant light resulting in a high incidence of macrophages. (*C*) BDNF injected 2 days before 1 week of constant light; no macrophages are present in the field. INL, inner nuclear layer. (Toluidine blue; bar = 20 μm.)

DISCUSSION

We have shown that eight different agents, which use several different receptor families (31–34), can rescue photoreceptors from cell death and reduce injury from the damaging effects of constant light. Of these agents, bFGF was shown previously to rescue photoreceptors *in vivo* (17, 20, 21); aFGF (35) and bFGF (16) prolong photoreceptor survival *in vitro*; and BDNF (2, 7) and CNTF (36) have been found to promote retinal ganglion cell survival *in vitro*. However, none of the other agents, to our knowledge, have previously been shown to have survival-promoting activity in the retina. In other regions of the nervous system, BDNF, NT-3, CNTF, bFGF, and aFGF have been shown to rescue neurons *in vitro* and *in vivo* (1–4, 6–11, 13–15), but as far as we are aware, IL-1β, TNF-α, and IGF-II have not yet been shown to do this. Our finding of rescue by this diverse group of agents raises obvious questions about the mechanism of cell injury and death in light damage, the cellular location and molecular mechanisms of the protective and rescue effects, and which mechanisms (if any) are shared by the different agents.

Light damage is thought to result from the generation of oxygen free radicals and the ensuing peroxidation of lipids (37), and when viewed in the microscope, outer segments usually are the first part of the photoreceptor cell to exhibit damage (38, 39). Preservation, by the injected factors, of some intact outer segments in most of the rescued retinas indicates that these agents actually protect the cells from damage at an early stage in the injury event. Although the nature of the protective effect against injury and its relation to protection from cell death remain to be worked out for each factor, it has been shown recently that BDNF appears to protect against oxidative stress in an *in vitro* system by increasing the activity of glutathione reductase (40). In the same system, however, bFGF (and NGF) also offered protection but did not increase the level of this enzyme (40), indicating probable different mechanisms of protective action. It remains to be determined how these protective mechanisms relate to other ways of ameliorating light damage, including antioxidants, hyperthermia (heat shock proteins), calcium channel blockers, age, prior lighting history, and as yet undefined genetic factors (see refs. 28, 41, and 42 for further references).

The actions of the growth factors, neurotrophins, and cytokines that show rescue potential are complex, since many of them inhibit or induce the expression of others, or of themselves, in paracrine, autocrine, and inhibitory feedback loops in various biological processes. Moreover, in other CNS systems, even the general issue of direct action of survival-promoting agents on neurons versus glial-mediated action of the factors is unresolved. In the retina, we must also take into account the special nature of interactions thought necessary for normal photoreceptor function and viability, interactions between photoreceptors, RPE, the intervening interphotoreceptor matrix, and the Müller glial cells (43). These cells and the interphotoreceptor matrix either contain, synthesize, or respond to many growth factors and cytokines. For instance, some of the agents examined here cause biological responses in RPE cells (24, 26); a few of them are known to be synthesized or secreted by RPE cells (16, 25, 27, 44, 45); and receptors for some have been demonstrated on RPE (16, 27) or photoreceptor (46, 47) cells. The interpho-

toreceptor matrix has also been shown to contain bFGF (48), IGF-I (27), and an IGF binding protein (27), as well as an undefined (non-bFGF) survival-promoting factor (49). Müller glial cells have both high- and low-affinity binding sites for aFGF and bFGF (16, 50), and IL-1 is expressed in Müller cells (51).

In light damage, as in most neuronal degenerations, there is the additional complication of invading macrophages. More than 30 growth factors and cytokines have been detected in macrophages (52), including at least three that rescue photoreceptors (bFGF, IL-1β, and TNF-α), and these cells are known to respond to some growth factors and cytokines by releasing various factors (52). Most of the rescuing agents did not increase the macrophage numbers, and BDNF actually reduced the number of invading cells from that usually seen in light damage, indicating that the increased incidence of macrophages is not responsible for photoreceptor rescue. Nevertheless, the macrophages typically present following light damage (30) cannot be excluded from participating in the rescue process.

Light damage in the rat retina provides a relatively simple, fast, and efficient system for the *in vivo* assessment of the photoreceptor survival-promoting activity of various agents. The importance of such an *in vivo* model vs. an *in vitro* model is underscored by the failure of CNTF to rescue ciliary neurons from programmed cell death during development *in vivo*, whereas CNTF clearly rescues these neurons *in vitro* (15). The utility of the light-damage model as an *in vivo* bioassay system is highlighted by the present study—an *in vivo* assay of the survival-promoting activity of a wide range of growth factors, cytokines, and neurotrophins on a single CNS tissue. Although our findings pertain to only one class of neurons in the vertebrate retina, photoreceptor cells, they may have significance for neurons in other regions of the CNS.

The ultimate therapeutic potential of each agent for use in any inherited or acquired human retinal disease remains to be determined. The rationale for such use should come from further study of each agent's mechanism of action in the normal retina, as well as in the diseased retina. Most importantly, it will be necessary to evaluate the ability of each agent to rescue photoreceptors or other retinal neurons in animal models that are directly relevant to human retinal disease. Our present findings now provide the impetus for undertaking such studies.

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