Protective Effect against Ischemia and Light Damage of Iris Pigment Epithelial Cells Transfected with the BDNF Gene

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Purpose. Brain-derived neurotrophic factor (BDNF) has been reported to protect retinal neurons against ischemia and light-induced damage. In the current study, the BDNF gene was transfected into iris pigment epithelial (IPE) cells of Long-Evans rats, and the neuroprotective ability of the IPE cells transfected with the BDNF gene against *N*-methyl-D-aspartate (NMDA)–induced neuroretinal cell death and against phototoxic damage was examined.

Methods. The level of BDNF mRNA and protein expressed in the transfected cells was determined by reverse transcription–polymerase chain reaction (RT-PCR) and by sandwich enzyme-linked immunosorbent assay (ELISA). The neuroprotective effects were determined by culturing BDNF gene–transfected IPE cells or nontransfected cells with neuroretinal cells in the presence of NMDA. The neuroprotective activity was also evaluated for the damage induced by constant exposure to light on the photoreceptors by transplanting BDNF gene–transfected IPE cells into the subretinal region of the superior half of the eye.

Results. BDNF gene–transfected IPE cells expressed higher levels of BDNF mRNA and protein than did nontransfected IPE cells. A significant increase in the protection against NMDA was observed in the neuroretinal cells cultured with BDNF-transfected IPE cells than in those cultured with nontransfected IPE cells ($P = 0.0029$) or with nontreated cells ($P = 0.0010$). The effect was partially attenuated by the addition of anti-BDNF antibody. Significant photoreceptor cell protection against injury from constant light was also observed by the subretinal transplantation of BDNF-transfected IPE cells when compared with those receiving transplants of nontransfected cells or vehicle injection.

Conclusions. BDNF-transfected IPE cells demonstrated a neuronal rescue effect in both in vitro and in vivo experiments. IPE cells may be a potential source for autologous cell transplantation for some retinal diseases, and the transfection of the genes of neurotrophic factors into the transplanted cell may be a useful tool for delivering these factors to the retina. (*Invest Ophthalmol Vis Sci.* 2002;43:3744–3753)

The development and maintenance of the nervous system in vertebrates depend, in part, on several neurotrophic factors: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF),[1] and neurotrophin-3, -4, -5, and -6.[2] The similarities of the sequences of these neurotrophic factors have made them easier to clone, and the corresponding mature proteins include approximately 120 residues with approximately 50% of their residues identical.[3]

BDNF has been reported to be the most abundant neurotrophin in the adult brain.[4] It supports the survival of embryonic neurons,[5] basal forebrain cholinergic neurons,[6] and embryonic mesencephalic dopaminergic neurons.[7] Increased amounts of NGF and BDNF have been reported in dentate gyrus granule cells after cerebral ischemia and insulin-induced hypoglycemic coma.[8] BDNF mRNA has also been reported to be lower in the hippocampi of individuals with Alzheimer's disease, and BDNF may be a useful agent in the treatment of this disease.[9]

BDNF can promote survival and prevent death of retinal ganglion cells after axotomy of the optic nerve[10–12] in vivo or in cell cultures.[13–15] In addition, it can promote the sprouting of the dopaminergic fibers that express the receptors for BDNF in the inner plexiform layer.[16] BDNF also supports the survival of rat bipolar cells by stimulating secondary factors, such as basic fibroblast growth factor (bFGF), through the 75-kDa low-affinity neurotrophin receptor s(p75NTR) present on retinal glial cells.[17] Injection of adenovirus vectors containing the BDNF gene into the vitreous cavity results in the selective expression of the gene in Müller cells and the rescue of the neurons of axotomized retinal ganglion cells.[12]

We recently reported the transplantation of autologous iris pigment epithelial (IPE) cells into the subretinal space of patients with age-related macular degeneration (AMD), after the removal of a subretinal neovascular membrane.[18] There were no signs of rejection and/or proliferation of the cultured autologous IPE cells.[18] Although more than 80% of patients showed improved visual acuity after the transplantation, the best visual acuity in these patients was less than 0.3.[19] These results suggest that the transplantation of autologous IPE cells may have a limited effect on final visual acuity. When cultured autologous IPE cells were transplanted into the subretinal space of monkeys, the transplanted cells survived for at least 6 months.[20] The number of the photoreceptor cells on the transplanted IPE cells appeared to be almost the same or somewhat fewer than those in the regions that did not receive the transplant. Some of the IPE cells also appeared to embrace the photoreceptor outer segments. These transplanted autologous IPE cells should be a means of supplying neurotrophic factors to the neighboring cells if the IPE cells are transfected with the genes of neurotrophic factors that are known to have neuron survival-promoting activity.[21–24]

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To determine whether this was possible, we used a lipofection technique to transfect a rat BDNF cDNA directed by a mammalian promoter into rat IPE cells (BDNF-IPE). We then examined the expression of BDNF in the transfected cells and investigated whether cocultures of BDNF-IPE cells protects neuroretinal cells against *N*-methyl-D-aspartate (NMDA)–induced neurotoxicity. We also determined whether the subretinal transplantation of the transfected cells protects photoreceptor cells against light-induced damage.

Materials and Methods

Preparation of Cultured Rat IPE Cells

IPE cell cultures were established with a slight modification of the method used for RPE cells.[25] In brief, eyes of Long-Evans rat were enucleated, and the anterior segment was separated from the lens and vitreous at the posterior surface of the iris. The iris was removed from the anterior segment and incubated in a solution containing 0.25% trypsin-0.2% EDTA (1:250; Gibco BRL, Grand Island, NY) for 20 minutes at 37°C. Each iris was then placed in growth medium containing Ham's F-12 (Gibco BRL) supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 U penicillin, 0.1 mg streptomycin, and 0.25 μg amphotericin B per milliliter of medium). The IPE cells were gently brushed and separated from the stroma with a Pasteur pipette. The isolated cells were collected into a conical centrifuge tube, centrifuged at 1000*g*, and washed with growth medium. With a Pasteur pipette, the IPE cells were dissociated mechanically into a single cell suspension and placed in growth medium. The medium was changed every 3 days. The cells were passaged in CTC solution (collagenase 6 U/mL, 0.1% trypsin, 2% chicken serum, and 4 mM EDTA).[26]

Cells showing confluent conditions were used in all experiments. These cells were first examined with anti-pancytokeratin (a monoclonal antibody mixture; Sigma, St. Louis, MO) to confirm that the cells were epithelial in origin. The number of cells was determined with a Burke-Turk hemocytometer. Phase-contrast photographs of each culture were obtained (IMT-2 camera; Olympus, Tokyo, Japan).

Preparation of Rat Primary Neuroretinal Cells

Primary retinal neuronal cells were established from mechanically separated retinas of eyes of fetal Wistar rats (gestation, 17–20 days).[27] The tissues were filtered through a nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ) after mincing with a razor blade, and plated as single-cell suspensions on plastic dishes (1.0×10^4 cells/well, poly-L-lysine coated; Becton Dickinson Labware) in growth medium containing Eagle's minimum essential medium (Sigma) supplemented with 10% FBS, antibiotics, and 2 mM glutamine. The cultures were incubated at 37°C, and the medium was changed every 2 days. After 4 days in culture, the cell division of nonneuronal cells was stopped by the addition of 10^{-5} M cytosine arabinoside. These cultures were then continued for 8 to 10 days under the same conditions. Retinal neuronal cultures of isolated cells and cell clusters were used for the experiments.

Construction of Vector DNA and BDNF cDNA and Transfection of Plasmid DNA

BDNF cDNA, which was generously supplied by Atsushi Takeda, (Department of Neurology, Tohoku University)[28] was inserted into the plasmid vector pGEM3Sα, which had G418 for an anti-geneticin antibody and a strong mammalian promoter (SRa) just upstream of the multiple cloning site, by a method reported previously.[29] The constructed plasmid was transfected into rat IPE cells with a lipofection technique (Gibco BRL). Briefly, IPE cells at passage 4 were incubated at 37°C in a CO_2 incubator until the cells reached 50% to 80% confluence in 35-mm tissue culture plates. The two solutions, A (1–2 μg of DNA in 100 μL serum-free Ham's F-12) and B (2–25 μL regent [Lipofectoamine; Gibco BRL] in 100 μL serum-free Ham's F-12), were mixed gently and incubated at room temperature (RT) for 45 minutes. After the IPE cells were incubated with the complexes for 5 hours at 37°C, those that had been transfected by BDNF cDNA (BDNF-IPE) were selected and cultured with geneticin (400 ng/mL; Gibco BRL).

After the transfection into the IPE cells, the gene and protein expression of BDNF was examined. For control, we used nontransfected cultured IPE (N-IPE) cells.

Cell Viability Curves of N-IPE Cells

The survival of N-IPE cells was determined by a cell proliferation assay (Cell titer96 AQueous one solution; Promega Co., Madison, WI). N-IPE cells (8×10^3/well) were plated in 96-well plates and incubated at 37°C for 24 hours. Then, 20 μL of a solution composed of the novel tetrazolium compound MTS and an electron-coupling reagent, phenazine ethosulfate (PES), was added to each well. After 1 hour of incubation, cell survival was determined by measuring the reduced absorbance value at 490 nm.

RT-PCR and Quantification of BDNF Gene Expression

mRNA was extracted from BDNF cDNA–transfected IPE cells or from nontransfected cells using oligo dT cellulose (Pharmacia Biotech Inc., Uppsala, Sweden).[25] First, single-strand cDNA was generated by random hexadeoxynucleotides. PCR was performed with a thermocycler (Perkin Elmer, Wellesley, MA) on 50 μL reaction mixture; 20 μM of each primer; 200 mM each of dATP, dCTP, dGTP, and dTTP; 50 mM KCl; 10 mM Tris-Cl (pH 8.3); 1.5 mM $MgCl_2$ and 0.001% gelatin; and 2.5 U *Taq* polymerase. The number of reaction cycles was 25, 28, 30, 33, and 35. The temperatures for PCR were 94°C for 1 minute of denaturation, 60°C for 2 minutes of annealing, and 72°C for 2 minutes of polymerization. Amplified DNA was separated in a 1.5% agarose gel (SeaKem; FMC BioProducts, Rockland, ME) containing 0.05 mg/mL ethidium bromide.

To quantify the changes of expression the BDNF gene in BDNF-IPE cells at different passages, semiquantitative RT-PCR was performed.[30] Briefly, after confirming that the PCR products were increasing exponentially under the described PCR cycles, the density of each PCR product was compared with that of β-actin by computer (Gel Doc software; Bio-Rad, Hercules CA), and the ratio of BDNF to β-actin was calculated.

Primer Preparation

The primer sets used to amplify the rat BDNF gene[31] and β-actin were[25]: for BDNF, 5′-GGAATTCCAGAGTGATGACC-3′ and 5′-GGAATTCCTCCACTATCTTC-3′ yielding a product of 359 bp; and for β-actin, 5′-CTACAATGAGCTGCGTGTGG-3′ and 5′-CGGTGAGGATCTTCATGAGG-3′ yielding a product of 313 bp.

Immunohistochemistry

Cytokeratin immunocytochemistry was performed by preincubating the IPE cells in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 20 minutes at RT.[32] The IPE cells were incubated with anti-cytokeratin overnight at 4°C and then incubated with the peroxidase-labeled second antibody (goat anti-mouse) in Tween (Tw)-PBS and 2% FBS for 30 minutes at RT. After incubation with the second antibody, the color was developed by 3-amino-9-ethylcarbazole (AEC; Dako, Tokyo, Japan). Control slides were made by adding mouse IgG instead of the first antibody to the incubation mixture containing Tw-PBS and 2% FBS.

Quantification of BDNF Expression

The changes of BDNF protein translation were quantified by sandwich enzyme-linked immunosorbent assay (ELISA; Promega Co.).[33] First, 96-well plates were coated with anti-BDNF monoclonal antibody (1 mg/mL). Different amounts of supernatant or cell homogenates of the BDNF-IPE or N-IPE cells were applied on the anti-BDNF monoclonal

antibody–coated plates. The captured BDNF was detected by chicken polyclonal anti-BDNF antibody (pAb; 1 mg/mL). After washing, the amount of specifically bound BDNF pAb was then detected with a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugates were removed by washing, and after incubation with a chromogenic substrate, the color change was measured. The amount of BDNF (0–500 pg/mL) in the test solution was proportional to the color generated in the oxidation-reduction reaction. The protein concentration was assayed with a protein assay kit (Pierce, Rockford, IL). Independent experiments were performed three times.

Evaluation of Neuroprotection

The neurotrophic effects of BDNF on the retinal cultures were quantitatively assessed by combining cultures of BDNF-IPE or N-IPE cells with and rat neuroretinal cells. The trypan blue exclusion method was used to detect live cells.[27] BDNF-IPE and control IPE cells were cultured on permeable membranes (Intercell TP substrate; Kurabou, Osaka, Japan) on 0.45-μm microporous cellulose filters at 37°C for 1 week until just confluent and were then cocultured with rat retinal cells for 3 hours in Eagle's minimum essential medium with 10% FBS before drug application (precombination). The primary rat retinal cell culture was exposed to NMDA (1 mM) for 10 minutes, and further incubation was performed after washing out NMDA.

The effect of BDNF-IPE or N-IPE cells was examined by the combined culture method. BDNF-IPE or N-IPE cells were cocultured with the rat retinal cells for 1 hour after the addition of NMDA. The cells were then stained with 1.5% trypan blue solution at RT for 10 minutes and fixed with isotonic formalin (pH 7.0, 4°C). The fixed cultures were rinsed with Hanks' balanced salt solution (HBSS; BioWhittaker, Walkersville, MD) and examined by phase-contrast (VAREL contrast) microscopy (Carl-Zeiss, Oberkochen, Germany) at 400×. To determine the viability of the cells, more than 200 cells were counted, and the percentage viability was calculated as: number of unstained cells (viable cells)/total number of cells counted (viable cells + nonviable cells). Five experiments were performed and the mean ± SD cell viability determined. Independent experiments were performed three times, and a representative data set is shown.

Animals, Transplantation, and Histologic Procedures

We injected BDNF-IPE and N-IPE cells into the superior subretinal space with a 30-gauge needle (Hamilton, Reno, NV). The needle passed through the sclera between the ora serrata and the equator into one eye of anesthetized, 6- to 8-week-old, male Sprague-Dawley (SD) rats. The number of cells transplanted was $4 \times 10^4/2$ μL. For the control, the same volume of HBSS was injected into the subretinal space. After 24 hours in the usual cyclic light (light–dark, 14:10 hours), the rats were placed in constant light at an illumination of 2000 to 2500 lux for 1 week. After constant exposure to light, the rats were killed by carbon dioxide. The eyes were enucleated, immersion fixed in a 4% paraformaldehyde, and embedded in paraffin wax. Tissue blocks were sectioned at 3 μm along the anterior–posterior axis with the optic nerve head at the center, and the sections were stained with hematoxylin-eosin (H-E). The rats that were not exposed to light and those exposed to constant light for 1 week were examined in the same way. Each experiment was performed on at least five rats in each group.

All procedures were preformed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Quantification of Photoreceptor Rescue

We measured the thickness of the outer nuclear layer (ONL) on photographs of each retina to quantify the surviving photoreceptors. We transferred these photographs to a computer system with a microscope (model DC 100; Leica AG, Heerbrugg, Switzerland) and analyzed them by computer (IPLab software; Scanalytics, Inc. Fairfax VA). The loci in which the thickness of the ONL was measured were 600, 700, 800, 1300, 1400, 1500, 2000, 2100, and 2200 μm along a line from the optic nerve head to the ora serrata through the hemisphere of the region of the transplantation (superior) and the region of nontransplantation (inferior). At each point, the ONL thickness was measured in five continuous sections. Histologic examination on the day after the transplantation showed the transplanted cells at the transplantation site, and only a slight retinal detachment was observed (data not shown). A large retinal detachment was detected in only one eye in histologic sections, and the eye was excluded from further analysis.

Statistical Analysis

Statistical significance was determined by the Bonferroni-Dunnett test. $P < 0.05$ was considered to be statistically significant.

Results

Rat IPE Cell Cultures and Transfection of BDNF cDNA

Phase-contrast photographs of a newly isolated IPE cell culture and a culture at passage 4 of rat IPE cells are shown in Figures 1a and 1b, respectively. The cells at the indicated passage were used for transfection of BDNF cDNA. The IPE cells that were successfully transfected by the BDNF cDNA were selected by geneticin, because the plasmid vector DNA contained the antigeneticin antibody G418. Immunocytochemistry with cytokeratin of passaged BDNF-IPE cells (passage 5) confirmed that the cells selected by geneticin were epithelial in origin (Fig. 1c). The morphology of the cells was comparable to that of N-IPE cells.

Cell viability curves of N-IPE cells with geneticin are shown in Figure 2. With a concentration of 400 ng/mL of geneticin in the medium, N-IPE cells were completely killed within 7 days ($n = 4$). We therefore selected the viable cells at a concentration of 400 ng/mL of geneticin in the medium.

BDNF Expression

As we reported,[30] the degree of gene expression increased exponentially under the PCR procedures used (data not shown). The results of the gene expression of BDNF and β-actin at 30 cycles are shown in Figure 3. The quantities of each PCR product relative to β-actin at the same cycle were compared. The expression of the BDNF gene in the BDNF-IPE cells was higher than that in the N-IPE cells at any passage (passages 1, 3, 5, 7, and 10; Fig. 3). The expression of the BDNF gene in N-IPE cells shown in Figure 3 was at passage 10.

We also examined the amount of BDNF protein in both BDNF-IPE and N-IPE cells. These experiments were performed on cells at approximately passage 5, and we evaluated the BDNF concentration in the homogenates at passage 5 for illustration (Fig. 4, right). The expression of BDNF protein by the BDNF-IPE cells was also higher than that by N-IPE cells at any of the passages examined. Further, the BDNF protein in the supernatant after 72 hours of culture was higher in BDNF-IPE cells than in the N-IPE cells at passage 5 (Fig. 4, left).

Neuroprotective Effect against NMDA-Induced Neurotoxicity

The neuroprotective effect of BDNF-IPE cells against NMDA-induced neurotoxicity was quantitatively assessed by using the methods of combined cultures (Fig. 5a) and trypan blue exclusion. BDNF-IPE and N-IPE cells at passage 5 grew well on 0.45-μm microporous cellulose filters,[34] and the combined cultures with primary rat neuroretinal cells also grew well. The neuroretinal cells just before the experimental procedures are shown in Figure 5b, and the NMDA-induced neurotoxicity in the retinal neurons is shown in Figure 5c. The percentage of

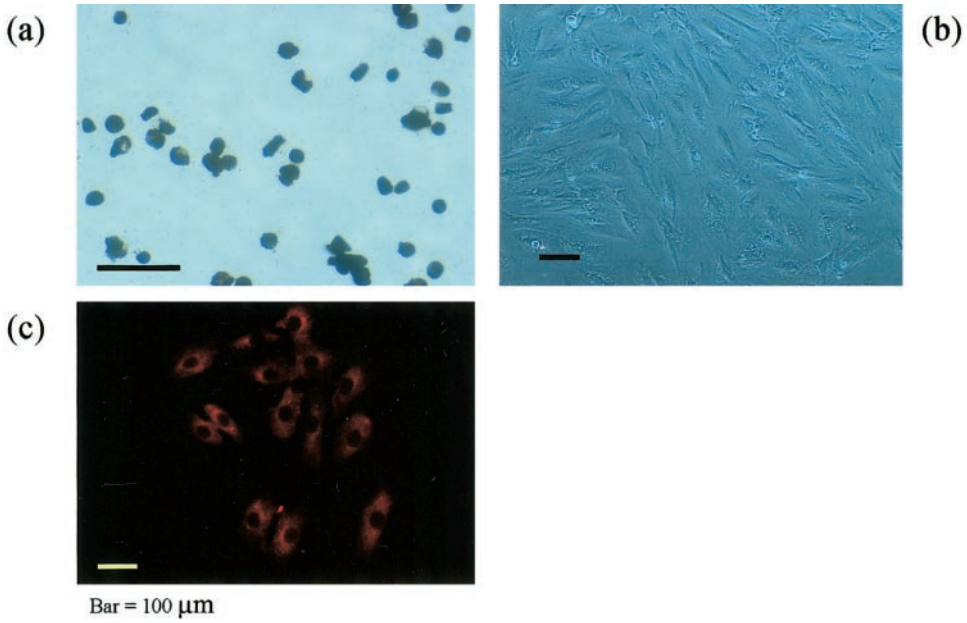

FIGURE 1. Phase-contrast photographs of rat IPE cell cultures. (**a**) Freshly isolated IPE cells and (**b**) cells at passage 4. (**c**) Immunocytochemistry with cytokeratin of BDNF-IPE cells at passage 5. Bar, 100 μm.

viable cells decreased after exposure to NMDA (1 mM) for 10 minutes.

The effect of BDNF-IPE cells in inhibiting NMDA-induced neuroretinal cell death in the combined culture system is shown in Figure 5d. Addition of MK-801 (10 mM), a highly potent and selective noncompetitive NMDA receptor antagonist,[35] increased the number of neurons that survived NMDA-induced cell death.

The percentages of neuroretinal cells treated with MK-801 and NMDA or with NMDA alone that remained viable compared with the controls were 88.6% ± 2.30% and 66.6% ± 9.07% (mean ± SD; $n = 5$), respectively. Of the cells exposed to NMDA in the combination culture of BDNF-IPE and N-IPE cells and those in the control cultures, the percentage viable was 83.0% ± 9.30% and 68.4% ± 7.70%, respectively. Statistical analysis demonstrated a significantly greater cell rescue in the BDNF-IPE cultures than in the NMDA-only ($P = 0.001$) or N-IPE cultures ($P = 0.0029$; Fig. 6).

We also added anti-BDNF antibody (0.1 μg/mL) to the cultures. The effect of BDNF-IPE cells was reduced to 77.6% ± 8.41% by the anti-BDNF antibody, although the effect was not significant when compared with that of BDNF-IPE cells ($P = 0.242$). When both NMDA and MK-801 were applied, the cell viability was 83.0% ± 2.00%, which was close to the control level.

Photoreceptor Rescue

By comparing the thickness of the ONL at 600, 700, 800, 1300, 1400, 1500, 2000, 2100, and 2200 μm from the optic nerve head in the transplant-recipient and nonrecipient sites, we tested whether BDNF-IPE cells at passage 5 or 6 transplanted into the subretinal space would rescue photoreceptor cells from light-induced damage. Light photomicrographs at 700 and 2100 μm from the optic nerve head in the transplantation site in the superior hemisphere of the eye are shown in Figures 7A and 7B, respectively. The ONL was made up of seven to eight rows in the eyes exposed to normal cyclic light (Figs. 7Aa, 7Ba) compared with one to two rows after 1 week of continuous exposure to light without treatment (Figs. 7Ae, 7Be). The ONL

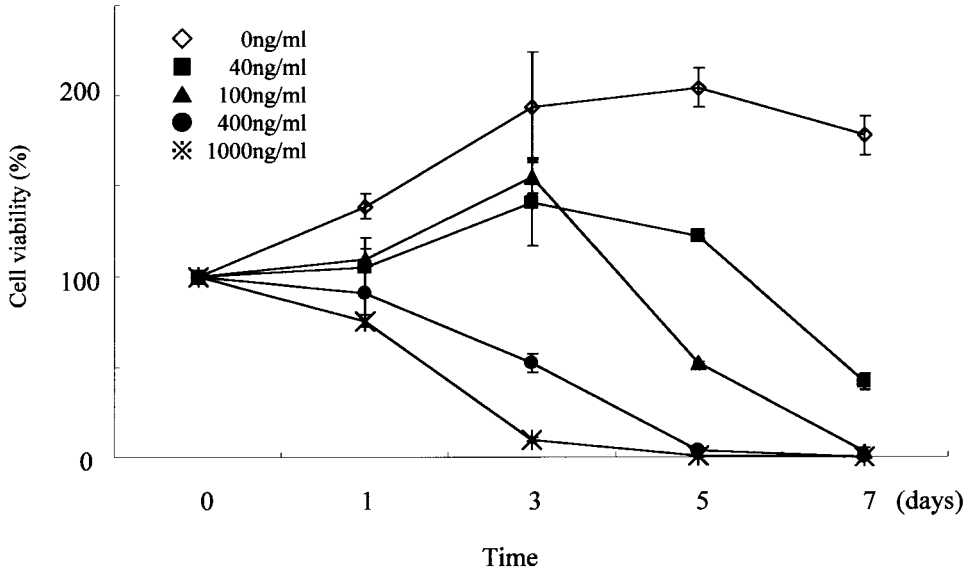

FIGURE 2. Cell viability curves of N-IPE cells with geneticin (mean ± SD, $n = 4$). Geneticin concentration in the medium is shown in the key at *top left*.

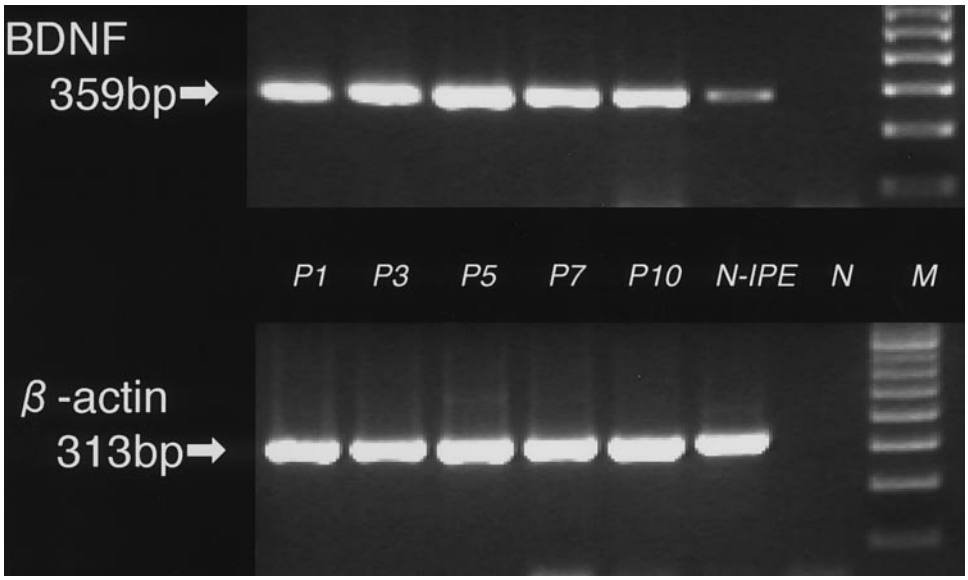

FIGURE 3. Results of RT-PCR of BDNF and β-actin genes of BDNF-IPE and N-IPE cells. P-1, -3, -5, -7, and -10 indicate passage of the cells; N, negative control in which PCR was performed without samples; M, 100-bp marker.

consisted of six to eight rows in eyes injected in the subretinal space with BDNF-IPE cells (Figs. 7Ab, 7Bb), but was reduced to two to three rows with the injection of N-IPE cells (Figs. 7Ac, 7Bc) and to one to three rows in vehicle-injected eyes (Figs. 7Ad, 7Bd). The outer plexiform layer (OPL) was also thinned and was present only in parts of the eyes with photoreceptor loss.

The thickness of the ONL in each group of rats is plotted in Figure 8. The thickness in the BDNF-IPE cell transplant-recipient retinas was thicker at all the measured points, and the differences at the sites of transplantation were significant. The differences were observed not only from the equator to the peripheral area but also toward the central area. Although the thickness of the ONL in the inferior retina of the BDNF-IPE–recipient eyes was thicker than that of the N-IPE– and vehicle-injected eyes, statistical analysis showed that the differences were not significant.

DISCUSSION

It is widely accepted that ischemia produces central neuronal cell death, predominantly through a massive intracellular influx of Ca^{2+} and an excessive efflux of glutamate.[36] This cytotoxic mechanism operates by several different pathways, and the NMDA receptors, which are well-known glutamate receptors, play an important role.[37] Some types of NMDA receptors are

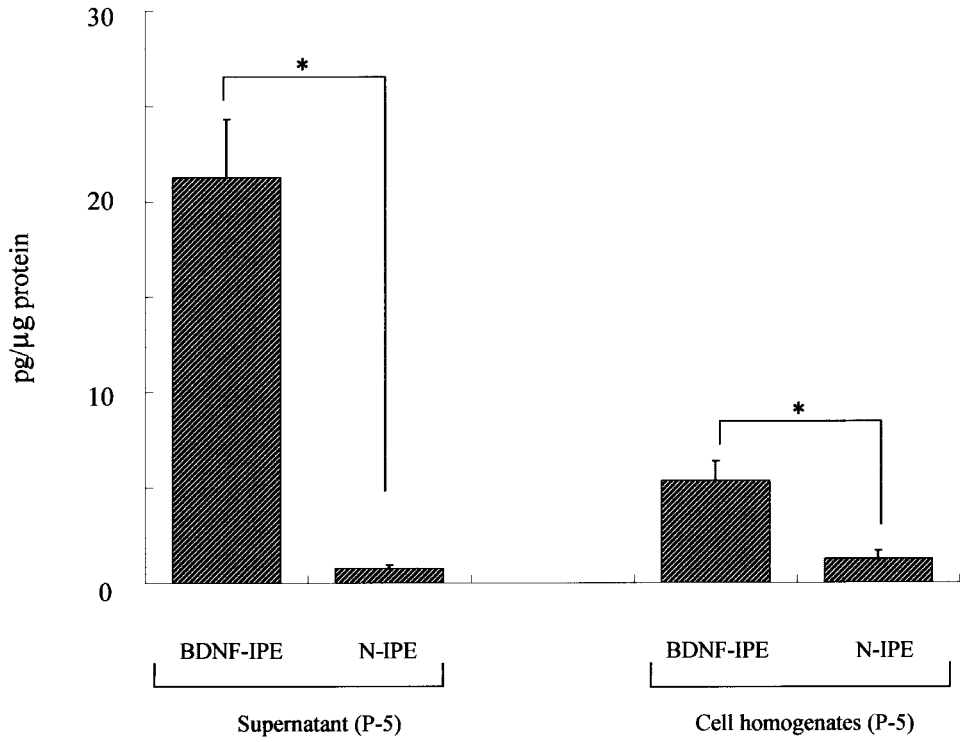

FIGURE 4. Quantity of BDNF protein as determined by sandwich ELISA. The *x*-axis represents the passage of the cell, and the *y*-axis represents the amount of BDNF protein. Statistical analysis was performed with the Bonferroni-Dunnett test. $^*P < 0.0001$ (mean ± SD, $n = 4$).

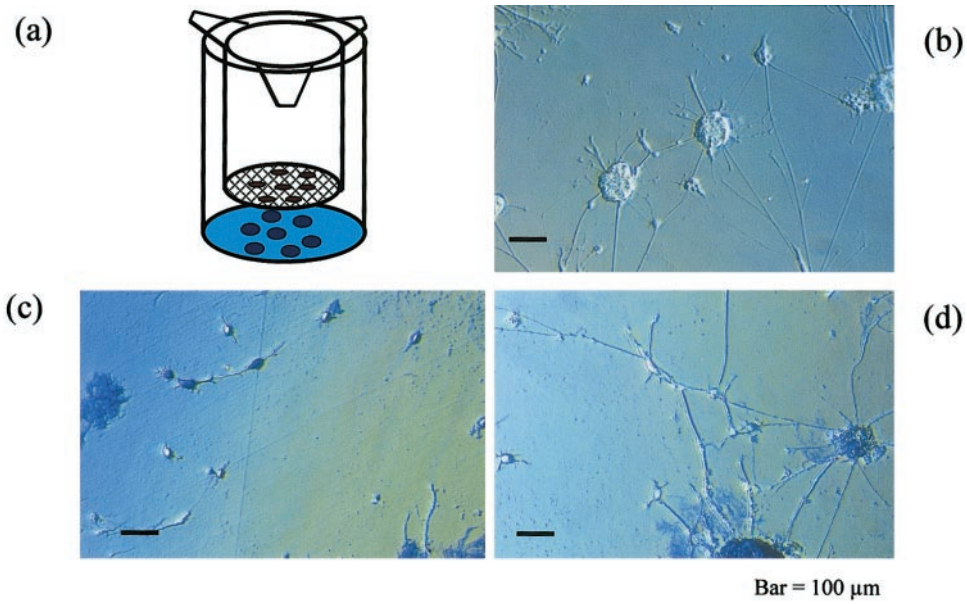

FIGURE 5. (**a**) Method of combination culture of rat retinal cells and BDNF gene-transfected or nontransfected IPE cells. Retinal neurons obtained (**b**) just before treatment by trypan blue exclusion, (**c**) after application of NMDA and treatment with trypan blue, and (**d**) after combination culture with BDNF-IPE cells tested by trypan blue exclusion. (**b**, **c**, **d**) Magnification, ×100.

expressed on rat retinal neurons, whereas all seven types of NMDA receptors are expressed on photoreceptor cells and amacrine cells.[38] Thus, retinal neurons, such as ganglion, horizontal, amacrine, and bipolar cells are sensitive to ischemia, and application of glutamate or NMDA leads to delayed cell death.

Several growth factors or neurotrophic agents are known to promote the survival of neurons in the central and peripheral neuron systems including retinal neurons. However, the role of neurotrophins as survival factors for developing central nervous system (CNS) neurons has not been determined with certainty. BDNF, NGF, and ciliary neurotrophic factor (CNTF), when injected into the vitreous or the superior colliculus, have been reported to have a protective effect against ischemia and light damage in retinal neurons.[23,24,39–41] In this study, we transfected BDNF cDNA into IPE cells and examined whether the cells express sufficient BDNF to protect retinal neurons against NMDA- or light-induced retinal cell death.

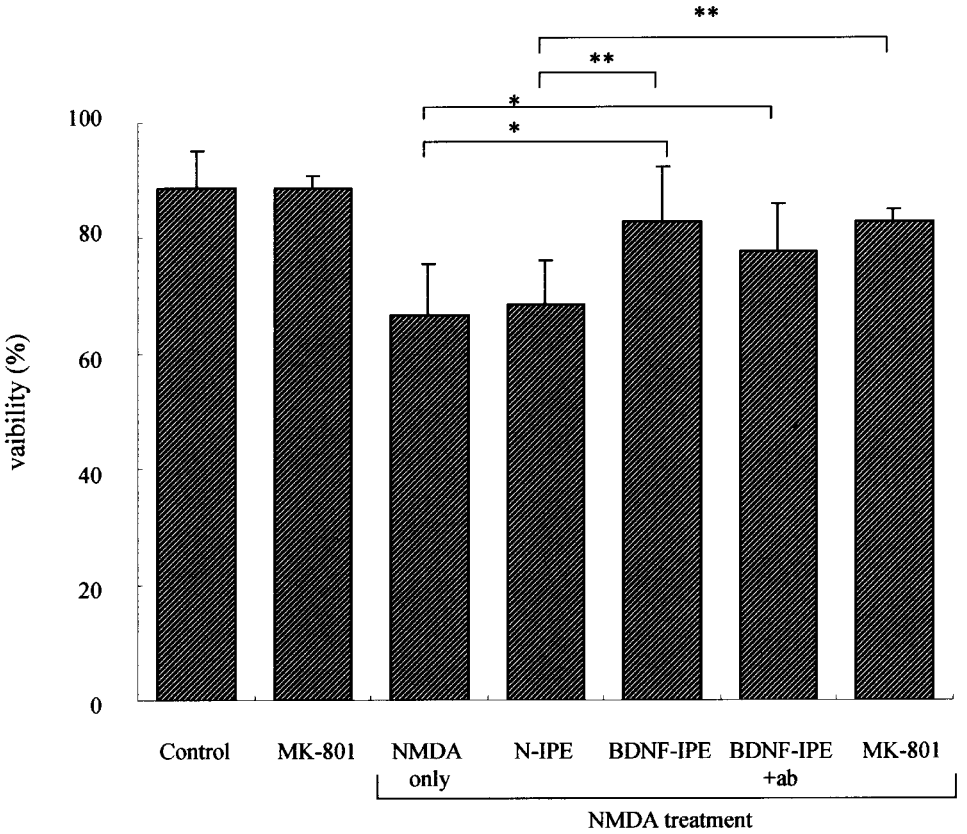

FIGURE 6. Neuroprotective effect of N-IPE cells, BDNF gene-transfected IPE cells, and MK-801 against NMDA-mediated retinal cell death. Control indicates the primary cultured rat retinal cells without treatment; MK-801, a highly potent and selective noncompetitive NMDA receptor antagonist; NMDA, N-IPE cells, a combination culture of rat retinal cells and nontransfected IPE cells; BDNF-IPE cells, a combination culture of the rat retinal cells and BDNF gene-transfected IPE cells; and BDNF-IPE+Ab, a combination culture of the rat retinal cells and BDNF gene-transfected IPE cells with anti-BDNF antibody. Statistical analysis was performed by the Bonferroni-Dunnett test. $P < 0.05$ was considered significant. $^{*}P < 0.01$; $^{**}P < 0.05$ (mean ± SD, $n = 5$). Viability (%) indicates (unstained cells/all cells) × 100.

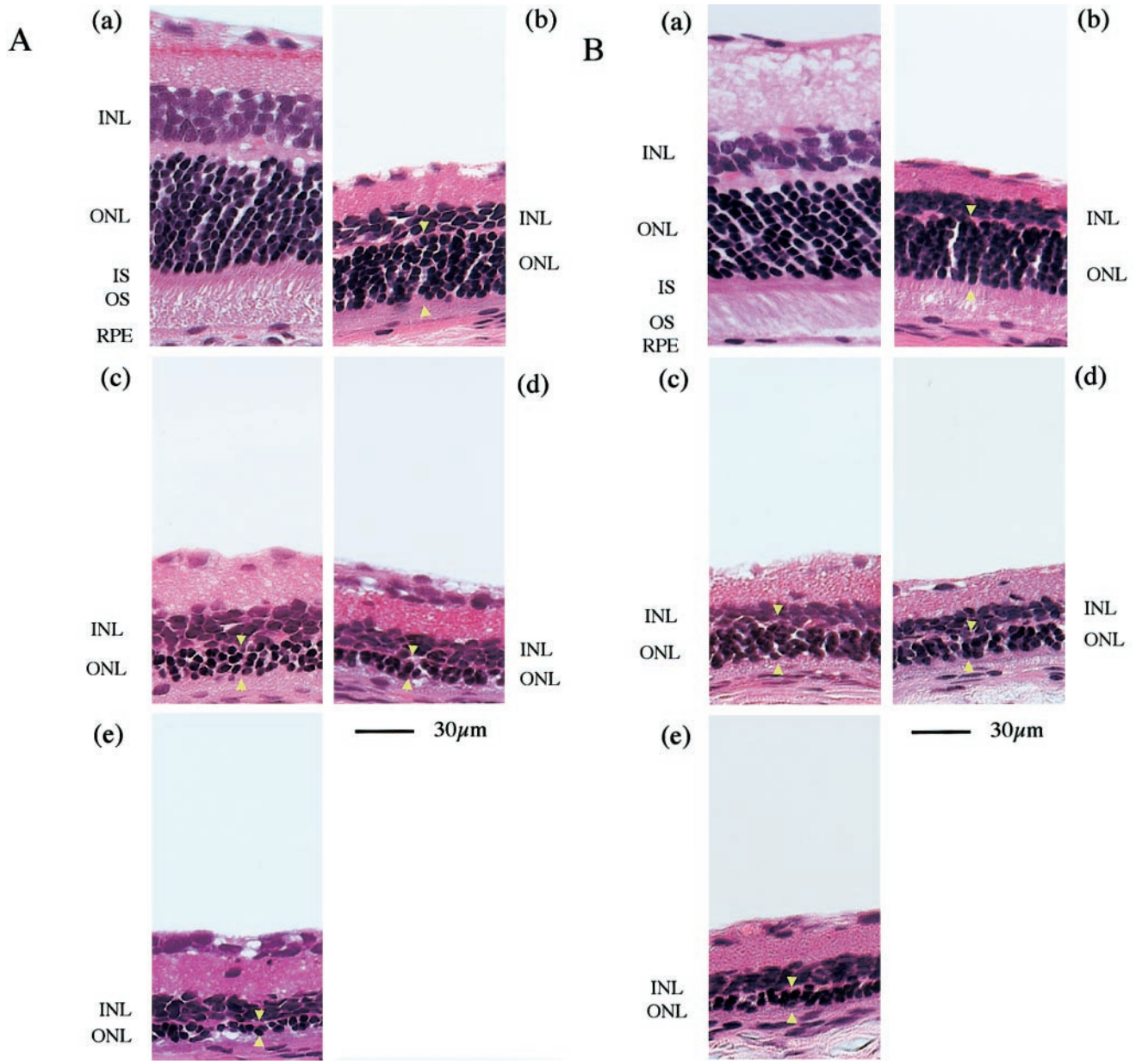

FIGURE 7. Light micrographs of rat retina taken (**A**) 700 and (**B**) 2100 μm from the optic disc in the transplanted superior hemisphere of the eye. (**a**) Normal eye in cyclic light; (**b**) BDNF-IPE transplantation; (**c**) N-IPE transplantation; (**d**) vehicle injection; (**e**) untreated in constant light. INL, inner nuclear layer; IS, photoreceptor inner segments; ONL, outer nuclear layer; OS, photoreceptor outer segments; RPE, retinal pigment epithelium. Bar, 30 μm.

To evaluate the neuroprotective effect of BDNF-IPE cells against NMDA-mediated neuronal death, we cultured BDNF-IPE or N-IPE cells with retinal neurons on a 0.45-μm microporous filter. Heth et al.[34] reported that RPE cultures grown on a 0.45-μm microporous filter or conventional plastic secret an extracellular matrix at the basal surface and ingest isolated rod outer segments. However, RPE cells grown on filters are more cuboidal, form junctional complexes, and show more basal infoldings than RPE cells grown on plastic. N-IPE cells may form more native cell morphology on the filter and may secrete more factors, including neurotrophic factors, than do N-IPE cells grown on plastic. However, no significant protective effect was observed against NMDA-mediated neuroretinal cell death. Conversely, a statistically significant increase in survival of neuroretinal cells was observed when they were cultured with BDNF-IPE cells. It should be noted that BDNF-IPE cells had almost the same neuronal protection as those treated with MK-801, a highly potent and selective noncompetitive NMDA receptor antagonist.

To evaluate the neuroprotective effect of BDNF-IPE cells against light-induced cell death, we measured the thickness of the ONL. When rats were placed in the constant light, a marked decrease of photoreceptor cells was observed.[42] In contrast, the ONL in rats receiving BDNF-IPE cell transplants was significantly better preserved than in those with vehicle injections or no treatment. There was a significant protective effect, even at 700, 800, or 2000 to 2200 μm, in the superior hemisphere, even though light damage is greater in the superior retina.[43] The ONL was thicker in the BDNF-IPE–injected retina than in the N-IPE– or vehicle-injected retinas at the

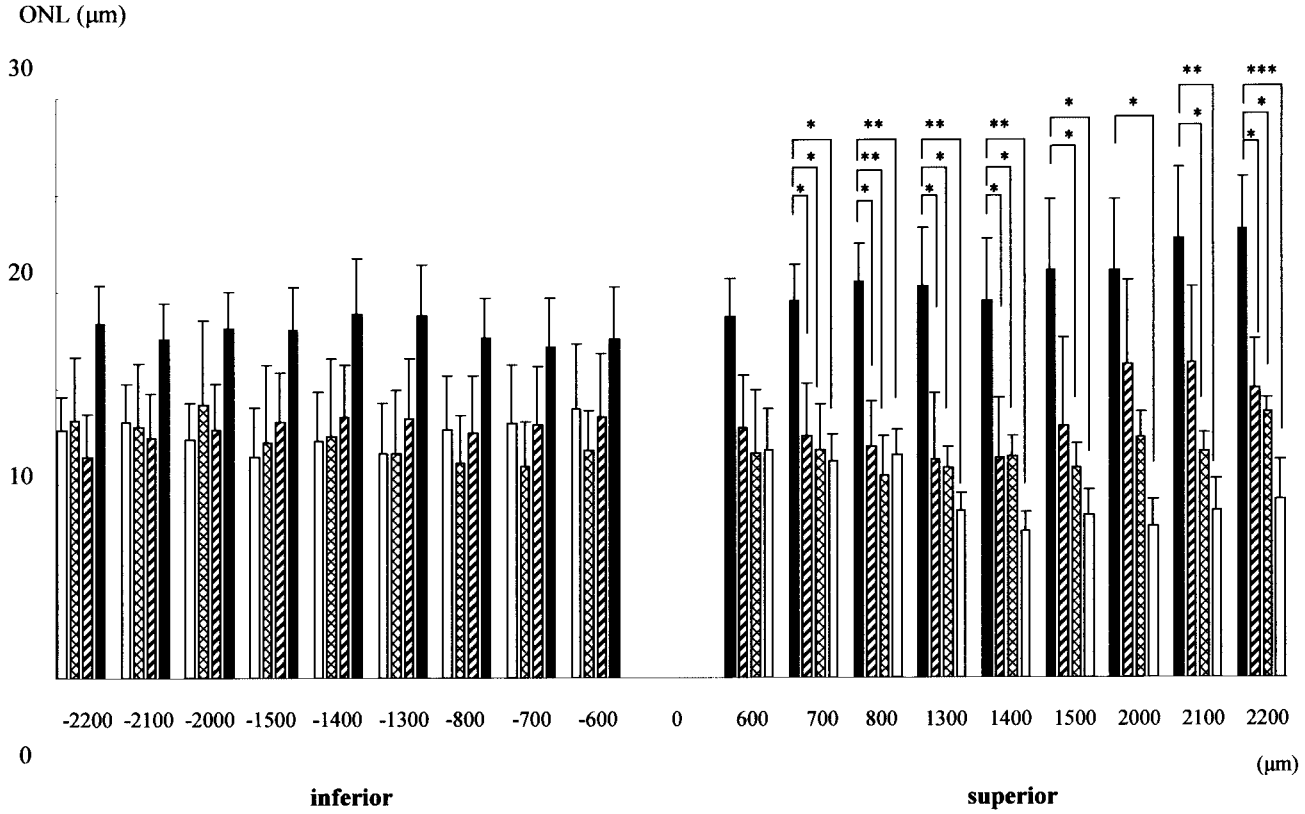

FIGURE 8. The thickness of the ONL is shown. The *y*-axis represents the thickness of the ONL and the *x*-axis indicates the distance from the edge of optic nerve head. (■) Thickness of the ONL in rats treated with BDNF-IPE; (▨) N-IPE; (☒) vehicle only; (□) no treatment. Superior hemisphere was the recipient of transplanted cells; inferior received no transplanted cells. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ (mean ± SE, $n = 5$).

inferior site. These results suggest that the BDNF effect extends not only to the area of transplantation but also to other areas of the retina. These results may be explained by our in vitro experiments, in which we demonstrated that the expression of BDNF was prominent in both the cell homogenates and in the supernatant.

The N-IPE cells also tended to have a more protective effect against light damage than did vehicle injection or no treatment, although it was not significant at any regions examined. Schraermeyer et al.[44] reported that the protective effect against retinal degeneration in Royal College of Surgeons (RCS) rats was less effective with IPE cell transplantation than with RPE cell transplantation. The weaker protective effect of IPE cells in our experiments confirms their results, although the protective mechanism against light damage and retinal degeneration in RCS rats may be different.

Although the protective effects were less, the effect of vehicle injection alone was also somewhat more protective than nontransplantation, as Wen et al.[45] and Cao et al.[46] have reported. As we have reported,[47] even vehicle injection may induce some cytokines at the region of transplantation, and some neurotrophic factors may be expressed in the area of the transplantation and may affect the results.

With the transfection of BDNF into IPE cells, some other neurotrophic factors may be enhanced. We examined for other neurotrophic factors or cytokines, including NT-3 and -4; NGF; IL-2, -4, and -6; and TNF-α in the BDNF-IPE cells by RT-PCR, but found no significant expression in these cells except bFGF (data not shown). The significant expression of bFGF may indicate that it serves as a second neurotrophic factor. Wexler et al.[17] also reported that BDNF promotes the survival of rod bipolar cells by enhancing the expression of bFGF through the TrkB receptor on Müller cells. To date, no evidence has been presented to show autocrine enhancement of the expression of bFGF in IPE cells. The transfection of the BDNF gene may enhance some neurotrophic network in the transfected cells. These findings may provide one reason that anti-BDNF antibody did not completely block the neuroretinal protection against NMDA.

These results, together with the previous reports that neurotrophic factors support the survival of the retinal cells against several types of damage, suggest that autologous IPE cell transplantation may have a protective effect in eyes with different types of retinal diseases when the transplanted cells are manipulated to express some neurotrophic factors.

Cell transplantation is an attractive therapeutic strategy that supplies the appropriate cells to treat some retinal diseases, such as retinitis pigmentosa and AMD. When cultured autologous IPE cells were transplanted into the subretinal space of monkeys, the cells remained there for at least 6 months.[20] The transplanted IPE cells did not show any proliferation or rejection in both monkeys[20] and humans.[18,48]

There have also been many reports on use of gene delivery for therapy for some eye diseases. Recombinant adenoviruses have been the main vector for delivering genes to the appropriate lesions.[49–53] One of the limitations of using the vector is the suppression of the expression of specific proteins.[49–53] Conversely, stable transfection of foreign genes by a noninfectious vector may continue the expression. In this respect, BDNF-IPE cells have a potential for improving neuroprotection of cells in autologous cell transplantation.

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