Hiroshima J. Med. Sci.
Vol. 55, No. 4, 109~116, December, 2006
HIJM 55–17

Pigment Epithelium-Derived Factor Promotes Neurite Outgrowth of Retinal Cells

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ABSTRACT

The ability of pigment epithelium-derived factor (PEDF) to promote neurite outgrowth of retinal cells through mitogen-activated protein kinase (MAPK) pathways was examined. Neurite outgrowth effects of PEDF were determined by quantifying the neurite length extending from cultured chick embryo retinal explants, and neurite outgrowth ratio of R28 cells (a neural cell line derived from the neonatal rat retina). MAPK activity levels were determined by inhibition assays. The contribution of signaling pathway was quantified with a specific inhibitor for MAPK: PD98059. PEDF (50 ng/ml) promoted chick retinal neurite elongation and increased the extent of R28 cell neurite outgrowth. PD98059 decreased neurite elongation of chicken retinal explants and the extent of R28 cell neurite outgrowth. PEDF possibly promotes neurite outgrowth for retinal cells by activating MAPK pathways. These data suggest that PEDF provides a useful support for retinal cells through the MAPK pathway and leads to the progress of therapy for many retinal diseases.

Key words: *Pigment epithelium-derived factor (PEDF), Neurite outgrowth, MAPK signaling, Retina*

The chick embryo is a useful model for studying retinal cell fate determination and neurogenesis because of its accessibility and the availability of molecular tools[24,35)]. The neural retina contains a population of proliferating progenitor cells that differentiate into 7 types of cells, including retinal ganglion cells (RGCs)[11,22)]. During chicken retinal development, RGCs are generated from embryonic day 2 (E2) to embryonic day 9 (E9)[32)]. Recent studies have identified the molecular events that control the differentiation and specification of RGCs[3,15,20,21)], but the mechanism of RGC development is still not clear.

Pigment epithelium-derived factor (PEDF), a 50-kDa protein, was initially identified in medium conditioned by cultured fetal human retinal pigment epithelium (RPE) cells[44)]. It is structurally a member of the serpin superfamily[6,40)], which is expressed widely in the developing and adult nervous system including the retina[7)]. In the ocular compartment, its expression appears to decline with age in the aqueous humor[29)], lens epithelial cells[36)], and retina[7)].

Previous studies have demonstrated that PEDF has two major actions, both as a neurotrophic factor and antiangiogenic factor[43)]. PEDF exerts neurotrophic effects on retinoblastoma cells[5,40)]. In addition, it promotes neuronal survival of cerebellar granule neurons[28,41)] and both survival and differentiation of developing motor neurons[18)]. PEDF, however, has no effect on the proliferation rate and neurite outgrowth on cerebellar granule cells[41)]. A recent study revealed that NFκB activation is required to produce neuroprotective effects of PEDF on cerebellar granule neurons[46)]. In neuronal differentiation, PEDF has so far been shown to affect only two types of neurons: retinoblastoma cells[44)] and chick spinal cord neurons[18)]; however, the neuronal differentiation effect of PEDF on retinal cells remains unclear.

ERKs are a subfamily of the mitogen-activated protein kinase (MAPK) family of serine/threonine kinases[9)]. In neuronal cells, activation of ERK in the PC12, a pheochromocytoma cell line, is suffi-

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Grant: Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS): No. 15790991.

cient to induce some aspects of neuronal differentiation[33]. Previous studies of the ERK cascade in neuronal cells using transformed cell lines have led to conclusions about the importance of the ERK pathway in neurite growth[14,30]. Other studies have shown the mechanistic role of ERK MAP kinases in the regulation of neurite elongation; the duration of ERK activation determines the switch from proliferation to differentiation[17,23,25]. Neurite elongation of PC12 cells and other neurons, including chick retinal neurons, was inhibited by MAPK inhibitor PD98059[8,30,31].

In this report, we examine the neuronal differentiation effects of PEDF on retinal precursor cells by culturing chicken embryo retinal explants and R28 cells with PEDF. Furthermore, we examine the signal transduction pathways that mediate the cell differentiation-promoting actions of PEDF. To our knowledge, this is the first time that the neuronal differentiation activity of PEDF on retinal cells and its transduction signaling during differentiation have been investigated.

MATERIALS AND METHODS

Embryos and cells

Fertilized White Leghorn eggs were purchased from local suppliers and incubated at 38°C in a humidified incubator. Chick embryos were staged according to Hamburger and Hamilton (HH)[16]. R28 cells (retinal precursor cell line derived from neonatal rat retina)[38] were kindly supplied by Dr. M. Nakamura (Kobe University, Japan).

Retinal tissue culture and cell culture

Chick embryos were decapitated and the retina was isolated from retinal pigment epithelium in PBS for tissue culture. Freshly prepared embryonic day 6 (E6) chick retinal small explants were placed in collagen gel (Cellmatrix Type 1-A, Nitta gelatin, Osaka, Japan) and cultured for 48 h in Dulbecco's Modified Eagle's Medium (DMEM) /F12 culture medium supplemented with N2 supplement, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all reagents in the culture medium were from Gibco, Invitrogen Corp., Carlsbad, CA, USA) in an incubator at 37°C in an atmosphere of 95% air and 5% CO_2.

R28 cells were cultured as described previously[4, 26,27,34,37,42]. Briefly, they were grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with Minimal Essential Medium (MEM) nonessential amino acids, MEM vitamins, gentamicin, and 10% newborn calf serum (Gibco, Invitrogen Corp., Carlsbad, CA, USA). In the neuronal differentiation assay, R28 cells were transferred to a laminin-coated dish in medium containing 1% serum.

Immunohistochemistry

For immunohistochemical staining of the developing neural retina, eyes from E7 and E11 chick embryos were fixed with 4% formaldehyde. Cryostat sections were permeabilized with 0.1% Triton X-100, blocked with Protein Block Serum-Free (DAKO Corp., Carpinteria, CA, USA), and then incubated with the primary antibody, mouse anti-PEDF antibody (CHEMICON International, Inc., Temecula, CA, USA). Next, the sections were incubated with the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA). Sections were counterstained with DAPI. Immunolabeled sections were examined using an Axioskop 2 plus microscope (Carl Zeiss, Jena, Germany) and images were captured using an Axio Cam CCD camera and Axio Vision 3.0 (Carl Zeiss, Jena, Germany).

For immunohistochemical staining of R28 cells, cells cultured on the chamber slide were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 2% BSA, and then incubated overnight at 4°C with mouse anti-PEDF antibody (CHEMICON International, Inc., Temecula, CA, USA) as a primary antibody, and then with Alexa Fluor 546 goat anti-mouse IgG (H+L) (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA). As a negative control, this immunostaining was absorbed by rPEDF. Immunostained cells were examined using an Axioskop 2 plus microscope, and images were captured using an Axio Cam CCD camera and Axio Vision 3.0.

Western analysis

Isolated retinas from chick embryos were lysed with lysis buffer (8 M urea, 0.5% dithiothreitol), and total lysates were clarified by centrifugation and subjected to SDS-page. Proteins were transferred to a nitrocellulose membrane, Hybond C (GE Healthcare UK Ltd, Buckinghamshire, England), blocked with 5% skim milk containing Tris-buffered saline (TBS) -T buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20), and incubated with mouse anti-PEDF antibody (CHEMICON International, Inc., Temecula, CA, USA). Secondary antibodies and enhanced chemiluminescence (ECL) detection were performed as described previously[19].

Neuronal differentiation assay

Cultured retinas were examined using a 10× objective on an ECLIPSE TS100 microscope (Nikon, Tokyo, Japan), photographs of each retina were taken in 4 directions with CAMEDIA C4040 ZOOM (OLYMPUS, Tokyo, Japan), and digital images were analyzed with Scion Image software (http://www.scioncorp.com/). As previously described[10], lengths of the 5 longest neurites per explant were measured perpendicular to the explant tissue. Neurite length measurements

were statistically analyzed by Scheffé post-hoc test (Stat View Version 5.0; SAS Institute Inc.). PD98059 (Promega, Madison, WI, USA), a MAPK/ERK kinase 1 inhibitor, was added directly to the collagen gel culture medium for 1 h prior to treatment with PEDF (CHEMICON International, Inc., Temecula, CA, USA) or brain-derived neurotrophic factor, BDNF (Sigma, St. Louis, MO, USA). Chick retinal explants were treated by PEDF or BDNF and specific signaling pathway inhibitors for 2 days. Several assays were performed independently.

R28 cells were incubated with PEDF in medium containing 1% new born calf serum for 48 h, and the proportion of neurites extending to cultured R28 cells was examined using an ECLIPSE TS100 microscope (Nikon, Tokyo, Japan). Photographs of 15 random fields per well were taken with a CAMEDIA C4040 ZOOM (OLYMPUS, Tokyo, Japan). Processes with lengths longer than 2 diameters of the cell body were counted as extended neurites and these cells were counted as differentiated cells. For inhibition of the PEDF effect on R28 cells, PD98059 was added for 30 min prior to treatment with PEDF and incubated for 2 days. For each datum point, the mean value was calculated from 5 random-field observations of 3 replicate experiments, and a minimum of 100 cells per field were counted. The ratio of differentiated cells to all cells was statistically analyzed by Scheffé post-hoc test.

RESULTS

Expression of PEDF in the chicken embryo retina

To confirm the PEDF expression of the chicken embryo retina, we performed immunohistochemistry using mouse anti-PEDF antibody. In the retina, PEDF protein localized to the ganglion cell layer at E7 and E11 (Fig. 1). The relative intensity of PEDF immunofluorescence in the ganglion cell layer decreased with the increasing stage of the chick embryo. At E11, PEDF immunofluorescence in the ganglion cell layer was relatively weak. Also, PEDF staining was weaker in the posterior retina than in the peripheral retina. No PEDF staining was seen in retinal pigment epithelium cells (RPE), photoreceptors, or Müller cells at any time point. PEDF immunofluorescence was detected in the ciliary body, and PEDF immunofluorescence in the epithelium of the ciliary body was stronger than that of the peripheral retina.

No immunofluorescence was detectable in the inner retina or ciliary body without the primary antibody as a negative control (data not shown).

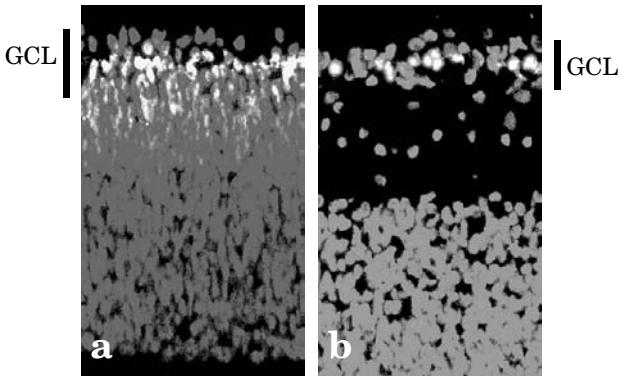

Fig. 1. Expression of PEDF in the chicken embryo retina on E7 and E11.
Retinal sections were stained with anti-PEDF antibody and DAPI. Expression of PEDF is found in GCL of chicken embryo retina. On the section of E7 retina, more retinal ganglion cells were stained with anti-PEDF antibody compared with E11 retina (a: E7, b: E11, GCL: ganglion cell layer)

Change of PEDF protein levels in the developing chicken embryo retina

PEDF protein levels in the developing chick retina were quantified by Western blot analysis. Protein levels during the formation of retinal ganglion cells were examined on E5, E7, and E11 (Fig. 2). No change in retinal PEDF protein levels occurred in chick development between E5 and E7. A large decrease in retinal PEDF protein levels occurred between E7 and E11.

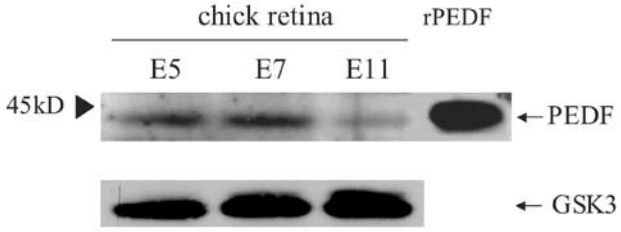

Fig. 2. PEDF protein levels of chicken embryo retina during prenatal development. Protein levels in the retina were examined on E5, E7, and E11, respectively. Endogenous PEDF was expressed at the same molecular size of recombinant PEDF (rPEDF). No change in retinal PEDF protein levels occurred in chick development between E5 and E7. A large decrease in retinal PEDF protein levels occurred from E7 to E11. GSK3 was used to standardize the total proteins.

Effect of PEDF on neurite elongation

For evaluation of the neuronal differentiation induced by PEDF, we assayed PEDF for its ability to enhance retinal cell differentiation (Fig. 3)

Neurite elongation with PEDF was observed within a day of culture, and the length and density of outgrowth increased over the following day. Neurites grew out from explants derived from E6, 7, and 8 chick retinas, but not from E10 retinas (data not shown), suggesting that neurite elongation with PEDF may be gradually regulated during embryonic retinal development. The most

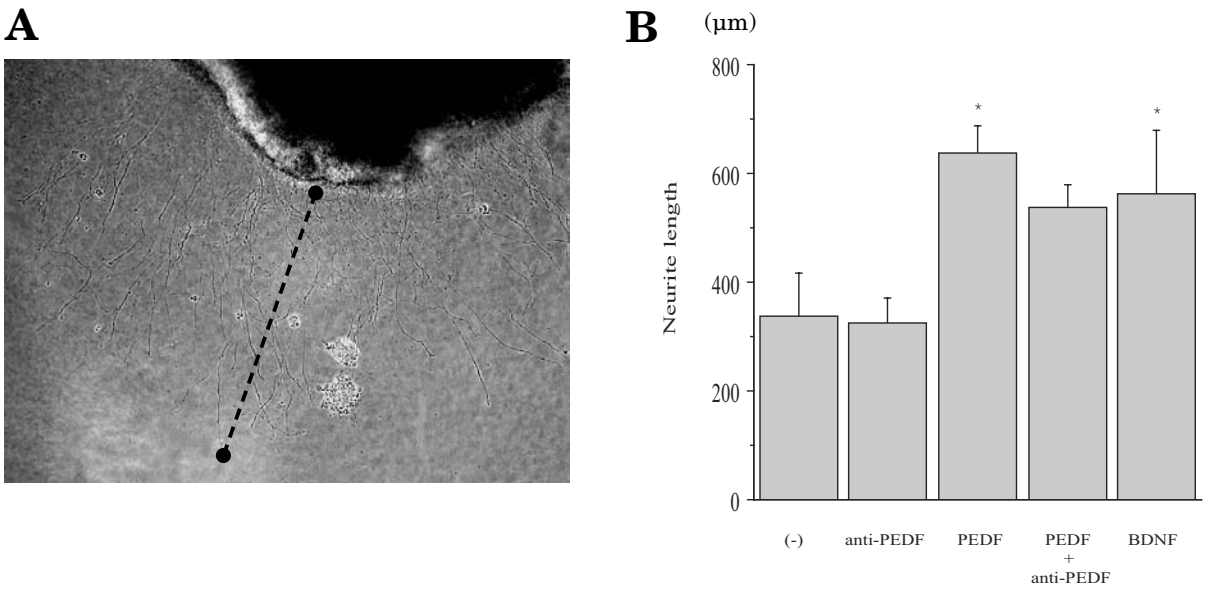

Fig. 3. Neurite elongation effect of PEDF.
E6 chick retinal explants were cultured with PEDF and BDNF in three-dimensional collagen gel culture for 48 h. To quantify neurite outgrowth, the lengths of the five longest neurites per explant were measured perpendicular to the explant tissue as indicated in Fig. 3A (dotted line). PEDF (50 ng/ml) and BDNF (20 ng/ml) promoted neurite elongation up to 189.3% and 166.5%, respectively, compared with the negative control (without growth factor), as shown in Fig. 3B. The neurite elongation effect of PEDF was almost the same as or higher than that of BDNF. An asterisk indicates significant difference from the control with $p<0.005$. (Scheffé post-hoc test, graph shows the average of 4~8 independent experiments.)

pronounced elongation occurred from E6 retinas; consequently, we used the neural retina at this developmental stage exclusively for the experiments below. PEDF enhanced neurite elongation from E6 retinas as shown in Fig. 3A. At a concentration of 50 ng/ml, PEDF increased neurite elongation up to 189.3% (Fig. 3B). The longest neurite was more than 800 μm. At a concentration of 10 ng/ml, PEDF did not increase neurite elongation remarkably. At a concentration of 100 ng/ml, PEDF increased neurite elongation only up to 105.5% (almost the same level) compared with the concentration of 50 ng/ml, and this effect was almost the same at a concentration of 500 ng/ml compared with 50 and 100 ng/ml (data not shown). We therefore deduced that this elongation effect reached a plateau at a concentration of 50 ng/ml.

Compared to brain-derived neurotrophic factor (BDNF)-induced neurite elongation, PEDF has sufficient ability to induce neurite elongation of the chicken embryo retina, suggesting that PEDF may be useful as a unique signaling mechanism to promote neurite elongation.

Promotion of neurite outgrowth of R28 cells by PEDF

To test whether R28 cells have endogenous PEDF, we performed immunohistochemical assessment using anti-PEDF antibody. PEDF expression was found mainly in the cytoplasm of the cell body (Fig. 4A), suggesting that R28 cells have endogenous PEDF.

Furthermore, to investigate the role of PEDF as a neurotrophic factor in retinal physiology, we analyzed the extent of R28 cell differentiation cultivated with PEDF. Such analysis revealed a clear difference between R28 cells cultured with and without PEDF (Fig. 4B). PEDF-dependent neurite outgrowth on R28 cells was observed in a dose-dependent manner. At a concentration of 50 ng/ml, PEDF promoted neurite outgrowth up to 163.3%.

Effect of ERK inhibitor on the differentiation of retinal cells

To analyze the signaling pathway leading to neuronal differentiation, neurite assays were performed in the presence or absence of PD98059. In this experiment, we used PD98059 as a MAPK inhibitor, which has an inhibitory effect on the phosphorylation of ERK by MEKK[2,13,30]. PD98059 partially blocked PEDF-mediated changes in axonal length in tissues pretreated with PD98059 when compared to untreated controls (Fig. 5A). PD98059 of 20 2M markedly inhibited neurite elongation of retinal tissues, and this effect occurred in a dose-dependent manner, ranging from 20 to 100 aM.

We also found that PEDF promoted neurite outgrowth in R28 cells, and PD98059 caused a significant inhibition of PEDF-mediated neurite outgrowth (Fig. 5B). PD98059 decreased PEDF-mediated retinal precursor cell differentiation, suggesting that the MAPK pathway is involved in PEDF signaling in this system.

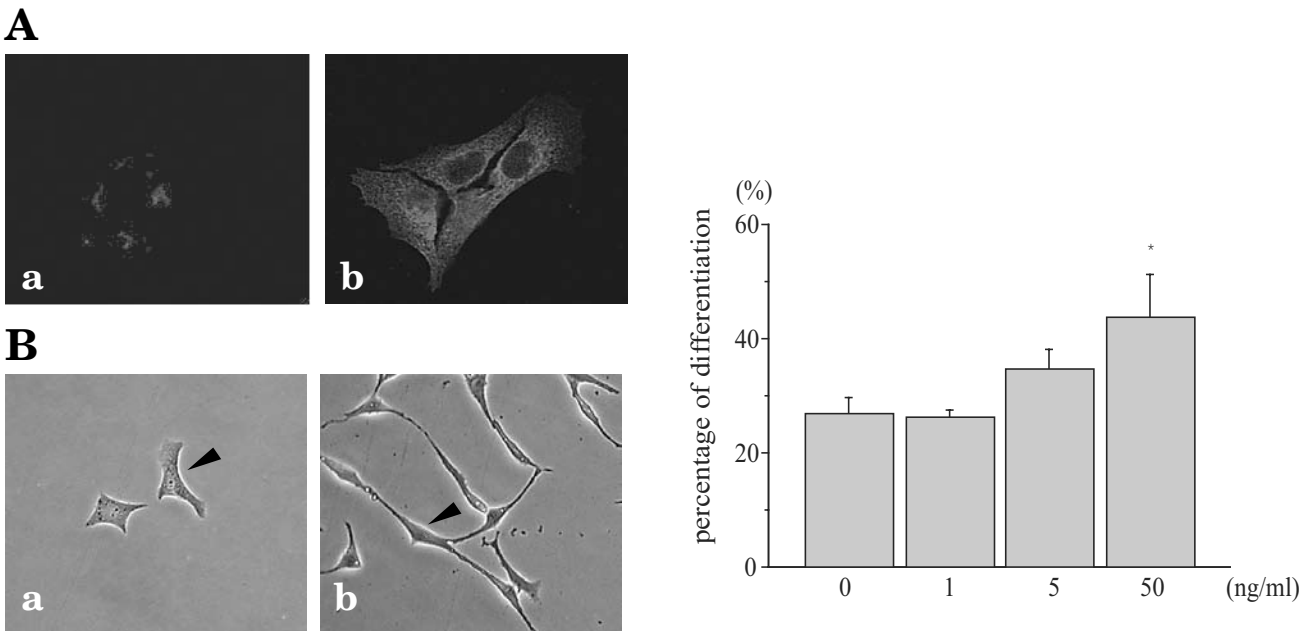

Fig. 4. Neurite outgrowth effect of PEDF in R28 cells.
A: Endogenous PEDF expression of R28 cells was visualized by immunohistochemistry using anti-PEDF antibody. a: negative control, b: with anti-PEDF. Expression of PEDF is found mainly in the cytoplasm of R28 cells. B: Quantification of the effect of PEDF on neurite outgrowth of R28 cells. Phase contrast micrographs of R28 cells cultured with differentiation medium containing 1% serum for 48 h. a: control (without PEDF), b: with PEDF (50 ng/ml). Arrowheads indicate a cell without neurite outgrowth and a cell with neurite outgrowth of more than twice the length of the cell body, respectively. PEDF promoted neurite outgrowth. The graph shows the extent of differentiation. Cells with neurites more than twice the length of the cell body were counted as differentiated cells, and the ratio of differentiated cells to all cells was analyzed. PEDF (50 ng/ml) promoted neurite outgrowth up to 163.3% compared with the negative control (without PEDF). An asterisk indicates a significant difference from the control with $p<0.05$. (Scheffé post-hoc test, graph shows 5 average rates of 3 independent experiments.)

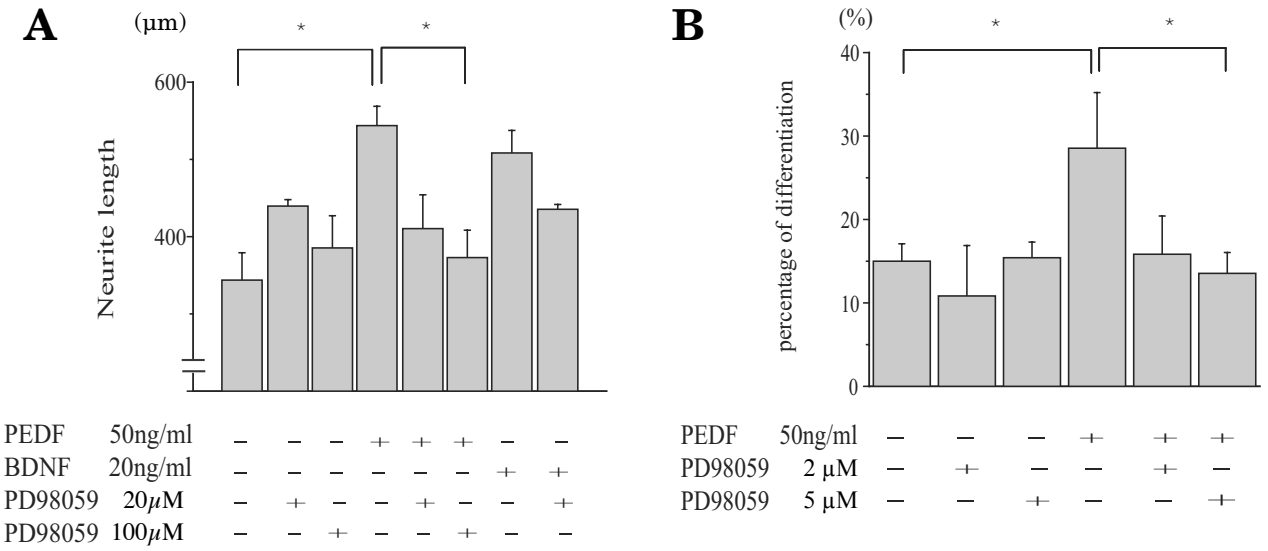

Fig. 5. Effect of MAPK activation on retinal cell differentiation.
Activation of ERK induced by PEDF was inhibited by PD98059. A: Quantitative analysis of neurite elongation of E6 chick retinal explants is shown. After pre-incubation with PD98059 (20, 100 μM), retinas were cultured for 48 h with PEDF and BDNF. PD98059 (100 μM) inhibited neurite elongation down to 68.7%. An asterisk indicates a significant difference with $p<0.0001$. (Scheffé post-hoc test, graph shows the average of 4~6 independent experiments.) B: Quantitative analysis of the extent of R28 cell differentiation is shown. After pre-incubation with PD98059 (2, 5 μM), R28 cells were cultured for 48 h with PEDF. PD98059 (5 μM) inhibited the extent of differentiation down to 47.7%. An asterisk indicates a significant difference with $p<0.0001$ (Scheffé post-hoc test, graph shows the average of 3 independent experiments.)

DISCUSSION

PEDF expression in the developing mouse eye exists initially in the ciliary body and choroids, and relative protein levels increase in the ganglion cell layer[7]. Another report has shown that PEDF is expressed in the ciliary epithelium (most prominently in the unpigmented layer of cells), in the outer retina (in retinal pigment epithelium cells and in the extracellular matrix that surrounds the outer segment layer of retinal photoreceptors), in the cornea (in epithelial cells at the corneal margin), and in the inner retina (retinal ganglion cells)[43]. In our study, PEDF was expressed in the ciliary body and retinal ganglion cell layer (not in the cornea) in the chick embryo eye on E7 and E11, and the degree of immunostaining of the retinal ganglion cell layer was stronger on E7 than on E11, suggesting that PEDF may be involved in retinal ganglion cell development. This deduction is supported by the fact that retinal ganglion cells are formed from E2 to E9 in a phased manner in the chicken embryo retina[1,12,39].

Changes in PEDF expression level during chick retinal development are shown in Fig. 2. Previous studies also showed that PEDF relative protein levels of mouse retina increased in the ganglion cell layer near term and remained high through the first 2 postnatal weeks, and then the levels decreased but persisted through adulthood[7]. As retinal ganglion cell layers are already formed near term (in the previously mentioned study, E18.5 retina was immunostained), PEDF protein levels during the formation of retinal ganglion cells were not examined, so we performed Western blotting to identify protein expression levels during retinal ganglion cell development. PEDF protein levels of the whole lysate retina did not change from E5 to E7, when essentially all RGCs had been generated, but remarkably decreased from E7 to E11, when developmental ganglion cell death had not yet begun. This result also suggested that PEDF is involved in the development of chick embryo retina. From these results, we hypothesized that a high expression level of PEDF induces retinal ganglion cell differentiation, and PEDF expression level decreases after the ganglion cell layer is formed, because this layer is formed by E7 or E8 and completely formed at E11.

To explore our hypothesis that PEDF is involved in retinal ganglion cell differentiation, we examined the effect of PEDF on cultured chicken embryo retinal tissue. When we cultured E6 retina with PEDF for 48 h, the neurite elongation of the retina was observed at the same level compared to the cultured retina with BDNF (Fig. 3B), and neurite elongation was inhibited by a specific antibody, anti-PEDF, suggesting that this effect was caused directly by PEDF. We showed that PEDF has neuronal differentiation activity in the chicken embryo retina, which is compatible with the hypothesis that PEDF may be involved in retinal cell differentiation. In the previous study, PEDF had no effect on the proliferation rate and neurite outgrowth on cerebellar granule cells[41]. In this study, however, we proved that PEDF has a neurite outgrowth effect on the development stage of the retina.

Next, we examined PEDF activity on R28 cells. A previous study reported that insulin-like growth factor-1 (IGF-1) and its analogs were able to inhibit neuroretinal R28 cell death[37]. Another study reported that insulin promotes R28 cell survival[45]. No other report, however, has explored the PEDF effect on R28 cells. The result of our study indicated that PEDF has differentiation activity on these neuroretinal R28 cells (Fig. 4B). From these experiments, using two different types of samples, chicken embryo retina and R28 cells, we concluded that PEDF has sufficient ability to promote the neuronal differentiation effect of retinal cells.

Furthermore, we investigated the molecular mechanism by which PEDF promoted the differentiation of cultured retina (Fig. 3B) and R28 cells (Fig. 4B). We examined the signal transduction pathways that mediate the cell differentiation-promoting actions of PEDF. Our results showed that a specific inhibitor of MAPK, PD98059, inhibited neurite elongation of the developing retina (Fig. 5A) and also inhibited neurite outgrowth of retinal precursor cells (Fig. 5B). These results suggested that PEDF promotes retinal cell differentiation through its unknown receptor and the activation of ERK. We could not confirm that this effect acts through the receptor, but this is suggested by the fact that ERK is activated when PC12 cells and other neuronal cells differentiate, and this differentiation effect is inhibited by PD98059. However, another signaling pathway of cell differentiation induced by PEDF may exist, requiring further experiments.

In this report, we showed that PEDF acts as a differentiation factor for immature retinal cells, and one of the transduction signaling mechanisms during differentiation was also identified. These findings increase our understanding of PEDF biology and may lead to progress in new therapies useful for treating many retinal diseases that cause blindness.

ACKNOWLEDGMENTS

We thank Dr M. Nakamura for R28 cells. This study was financially assisted in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS): No. 15790991.

(Received August 31, 2006)
(Accepted November 7, 2006)

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