Pigment epithelium-derived factor: a novel protein with neurotrophic properties

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Pigment epithelium-derived factor is a protein synthesised by retinal pigment epithelial cells and secreted into the retinal interphotoreceptor matrix. The protein can be purified from culture medium conditioned by retinal pigment epithelial cells or from washes of the interphotoreceptor matrix space. When pigment epithelium-derived factor is added to culture medium of human Y-79 retinoblastoma cells, it induces the cells to elaborate a dense meshwork of neurites and to increase their expression of specific biochemical markers of neuronal differentiation. When pigment epithelium-derived factor is added to culture medium of rat cerebellar granule cells in primary culture, the lifetime of the cells is markedly extended. Thus, this protein is a neurotrophic protein in that it acts as both a differentiating agent and a survival factor to neurons. These properties make pigment epithelium-derived factor an excellent candidate for use in clinical transplantation studies, in the neural retina, and other areas of the central nervous system, as well as in the treatment of neurodegenerative diseases such as retinitis pigmentosa.

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Introduction

In the early stages of development of the eye, cells of the retinal pigment epithelium (RPE) leave the mitotic cycle and gain many differentiated characteristics well before those of the neural retina (NR). Because of this, it has long been thought that the RPE could influence differentiation of NR cells. This is especially true for photoreceptor cells as they abut the RPE and appear to be the last cell type within the NR to achieve terminal differentiation. It is also well-known that RPE cells synthesise many types of growth factors, most of which are secreted into the interphotoreceptor matrix (IPM). Hence, it is reasonable to hypothesise that RPE cells could synthesise and secrete a "neurotrophic factor" that could influence photoreceptor cell development during the embryonic period.

Early evidence that a neurotrophic factor was elaborated by RPE cells was described in 1978 by Israel et al[1] who demonstrated that NR cells co-cultured with more mature RPE cells assumed a more neuronal morphological phenotype. In 1989, a novel activity exhibiting some of the properties expected of a neurotrophic factor was described by Tink et al[2] in medium conditioned from cultured foetal human RPE cells. Using cultured human Y-79 retinoblastoma cells, they demonstrated the elaboration of extensive, neuronal-like processes from the undifferentiated Y-79 cells after exposure to the RPE-conditioned medium. This factor has now been purified and has been shown to be a 50 kDa protein called pigment epithelium-derived factor (PEDF) that is synthesised and secreted in large amounts by RPE cells. In this paper, the properties and biological actions of PEDF are described and the potential clinical uses as applied to the retina and other areas of the CNS are discussed.

Materials and methods

Preparation of IPM wash

After removal of the anterior segments and retinas of adult bovine eyes, the remaining eye cups were gently washed with 1 ml of old 100 mmol/L phosphate-

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buffered saline solution (PBS). Washes were pooled, clarified by low-speed centrifugation and filtered through a 0.2 μm Nalgene (Nalgene Co., Rochester, NY, US) membrane. Prior to use, the soluble IPM wash was diluted with Eagle's minimal essential medium (MEM) [Mediatech, NIH, Md, US].[3]

Preparation of pigment epithelium-derived factor
A combination of the standard biochemical techniques of anion exchange and size exclusion chromatographies were used for the purification of PEDF.[4,5] The starting materials were either conditioned medium from cultured human foetal RPE cells or bovine IPM wash as described above. Recombinant PEDF (rPEDF, Asp^{44}-Pro^{418}) was synthesised and purified.[6]

Retinoblastoma tissue culture
Human Y-79 retinoblastoma cells were obtained (American Type Culture Collection, Rockville, Md, US) and maintained in suspension culture in MEM containing 15% foetal bovine serum.[3] When appropriate, cells were cultured in serum-free medium, and PEDF or IPM wash was added at suitable concentrations. After an eight-day incubation period, the cells were placed in attachment culture in a serum-free medium using tissue culture flasks or glass coverslips coated with poly-D-lysine.[3] Morphological changes were followed on a daily basis using a Nikon Diaphot TMD (Nikon Inc., Rockleigh, NJ, US) inverted microscope. Immunocytochemistry was performed[3] using primary and secondary antibodies (Sigma Chemical Co., St Louis, Mo, US).

Cerebellar granule cell tissue culture
Cerebellar granule cells (CGCs) were prepared from eight-day-old Sprague-Dawley rats using standard methods.[7] Attachment culture was in Eagle's basal medium (Mediatech, NIH, Bethesda, Md, US) with 10% heat-inactivated foetal calf serum or chemically-defined medium lacking serum.[7] Viable neurons were determined using the MTS assay[7] which measures dehydrogenase activity in metabolically active cells.[8] Results of the MTS assay were corroborated by direct cell count under an inverted light microscope.

Results

Pigment epithelium-derived factor identification
As it is very difficult to obtain a pure population of vertebrate photoreceptor neurons and to maintain them in culture, human Y-79 retinoblastoma cells were initially used as a target cell type in assessing the neurotrophic potential of RPE cell-conditioned medium.[2] These cells are an excellent cell type for this purpose as they are undifferentiated retinoblast cells capable of developing along different phenotypic pathways under the influence of differentiating agents.[9] Importantly, retinoblastoma cells appears to have a cone photoreceptor cell lineage,[10] making them a natural target of secreted products of the RPE. Figure 1 demonstrates the synthesis and secretion of PEDF by human foetal RPE cells in culture.

On an SDS-polyacrylamide gel stained with Coomassie blue (Sigma Chemical Co., St. Louis, Mo, US), a comparison can be made between the proteins present in original, unconditioned medium used in the culture which contained 0.9% serum (lane C) and those present in medium conditioned by the RPE cells for one to three days (lane B). Many of the bands, such as the prominent albumin band at 67 kDa, are identical and are present in the original culture medium. However, a relatively prominent new band appears in the

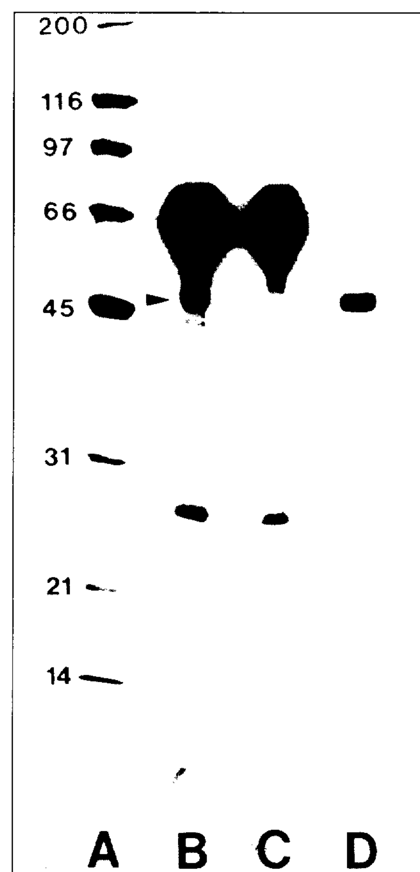

Fig 1. Gel electrophoresis of PEDF protein. A) Protein sizing standards. B) Conditioned medium from human foetal RPE cells. The secreted PEDF doublet is indicated by the arrowhead at 50 kDa. C) Control, unconditioned medium. No band at 50 kDa is apparent. D) Purified PEDF.
Source: Tombran-Tink J, et al. Exp Eye Res 1991.[4]

conditioned medium at about 50 kDa, indicating secretion of at least one new protein by the cells. This band was purified to homogeneity using standard biochemical techniques such as ion exchange and size exclusion chromatographies.[4] As can be seen in lane D (Fig 1), the band of the purified protein corresponds in position with the major secreted band in the conditioned medium and often appears as a "doublet". More recently, the purification procedure has been refined and extended to PEDF present in the bovine interphotoreceptor space.[5]

Y-79 cell differentiation

It was important to determine if this secreted protein exhibited neurotrophic properties. For this purpose, the protein in the narrow band depicted in lane B of Fig 1 was electroeluted and added to cultures of Y-79 cells maintained in serum-free medium. Similarly, the purified protein (lane D) was added to Y-79 cells and the cells monitored for morphological changes. Typical

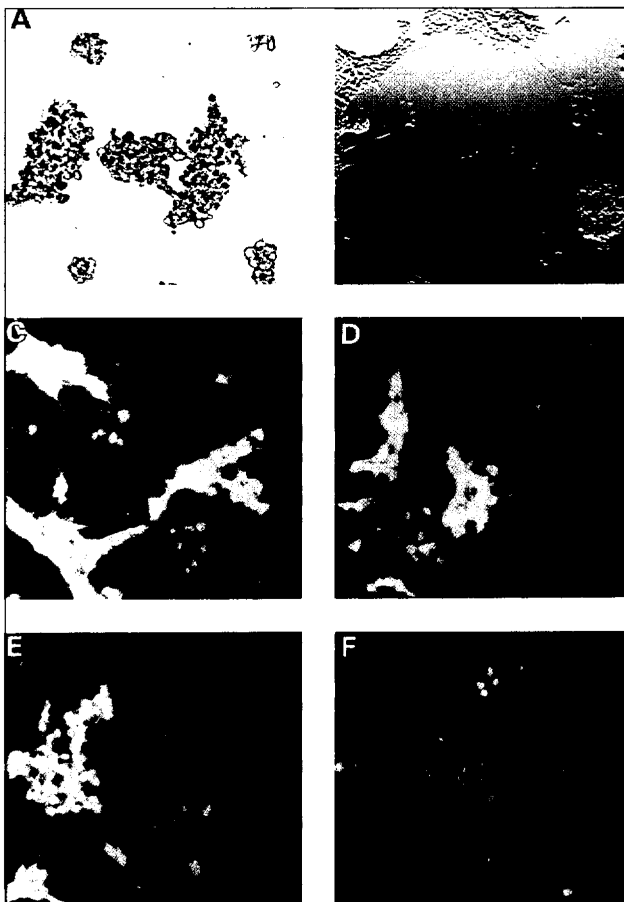

Fig 2. Effect of PEDF on Y-79 cell differentiation. 2a. Control attached cells. 2b. Cells with 50 ng/ml PEDF. 3c,d NF-200 immunofluorescence in PEDF-treated cells. 3e,f NSE immunofluorescence in PEDF-treated cells (x 94).

Source: Tombran-Tink J, et al. Exp Eye Res 1991.[4]

changes are depicted in Fig 2. In both cases, the cells elaborated numerous neurite-like processes, demonstrating the neuronal inductive effect of the 50 kDa protein. Fig 2a shows the typical undifferentiated nature of the untreated Y-79 cells in attachment culture. Panel B shows that the addition of 50 ng/ml of PEDF into the culture medium caused long neurite-like processes to be induced. It is important to highlight the fact that a recombinant form of PEDF (rPEDF) induces identical changes in Y-79 cell morphology.[6]

Panels C and D also show the neuritic mesh elaborated by the cells in response to PEDF. They also show intense immunostaining by an antibody raised against a neuron-specific marker, neurofilament protein-200. Similarly, panels E and F demonstrate intense immunostaining with an antibody to another neuronal marker protein, neuron-specific enolase, both in the cell bodies and the neuritic processes. In addition to these biochemical changes, Klaidman et al[11] have shown that conditioned medium from foetal human RPE cells has a striking effect on the expression of neurotransmitter systems in cultured Y-79 cells. They demonstrated a change from a serotonergic to a dopa-like system after treatment, concomitant with the switch to a more differentiated morphological phenotype induced by PEDF.

It was apparent that RPE cells in culture produce a potent neurotrophic protein, PEDF, which causes retinoblastoma cells to morphologically differentiate and to biochemically change in several respects. Is PEDF actually present in vivo in the normal adult mammalian IPM between the neural retina and the pigment epithelium? To address this question, an IPM "wash" (a crude mixture of soluble proteins obtained from lavage of the IPM space of the bovine eye) was added to Y-79 cells to see if it induced a neuronal phenotype.

The effects on Y-79 cells were identical to those seen with the RPE-conditioned medium or with purified PEDF (Fig 3). Panels A and B show the typical undifferentiated morphology of cells maintained in suspension and attachment culture, respectively. Treatment with IPM wash resulted in most of the attached cells assuming a highly differentiated neuronal appearance (panel C), including the extension of long, neurite-like processes that appeared to exhibit varicosities and growth cones. In addition, IPM wash (like PEDF) caused most of the cells to aggregate in circular, "corona-like" structures that typically had a central, cell-free "lumen-like" area and neurite-like processes extending radially. These structures were similar in

appearance to the rosettes and fleurettes that are seen in retinoblastoma tumours in vivo and may be attempts by the cells to differentiate and organise in a more normal manner. Importantly, PEDF seems to be the only such neurotrophic protein in the IPM since an antibody specific to PEDF effectively blocks the differentiative effect of IPM wash on Y-79 cell differentiation.

Neuron-survival properties of pigment epithelium-derived factor

Although we had shown native PEDF and rPEDF to be neurotrophic in the Y-79 tumour cell system, it was of interest to determine whether PEDF had an effect on normal neurons in primary culture. For this purpose, we used cultures of normal CGCs prepared from the eight-day-old rat. We have found the CGCs treated with rPEDF do not respond to treatment by exhibiting a more neuronal morphological appearance since they naturally tend to differentiate in culture. We were surprised to see that PEDF had a marked effect on CGC survival.[7] Since CGC neurons are "normal", i.e. they do not originate from tumours and have not been transformed or immortalised, they have a finite life in culture. As seen in Fig 4, the sensitive MTS assay for cell viability demonstrates that only approximately 40% of the neurons originally seeded survive for 10 days in culture. However, rPEDF-treated cultures consistently demonstrated an approximately two-fold greater number of cells than the control (untreated) cultures throughout the 10-day experimental period. These results were confirmed by direct microscopic observation and cell counting.[7] It is also evident that PEDF increases the survival of CGCs and does not affect their proliferation rate. Hence, PEDF should be added to the short list of newly-emerging "neuron survival" proteins.

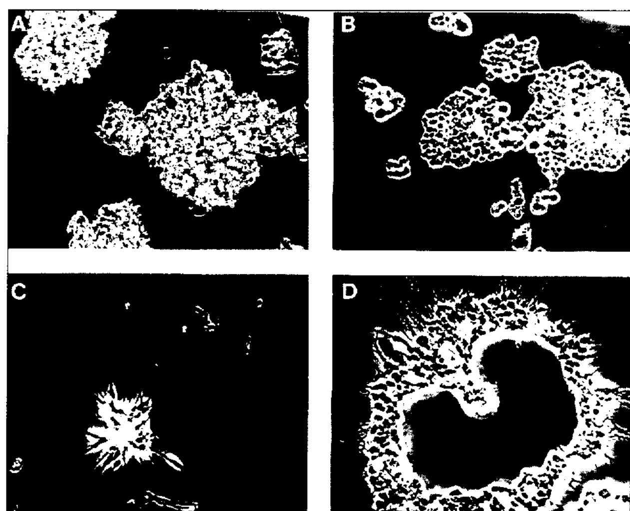

Fig 3. Effect of IPM wash on Y-79 cell differention. A) Control cells in suspension culture. B) Control cells in attachment culture. C) Cells treated with IPM wash for seven days in suspension culture and 11 days in attachment culture (x 200).

Source: Tombran-Tink J, et al. Invest Ophthalmol Vis Sci 1989.[2]

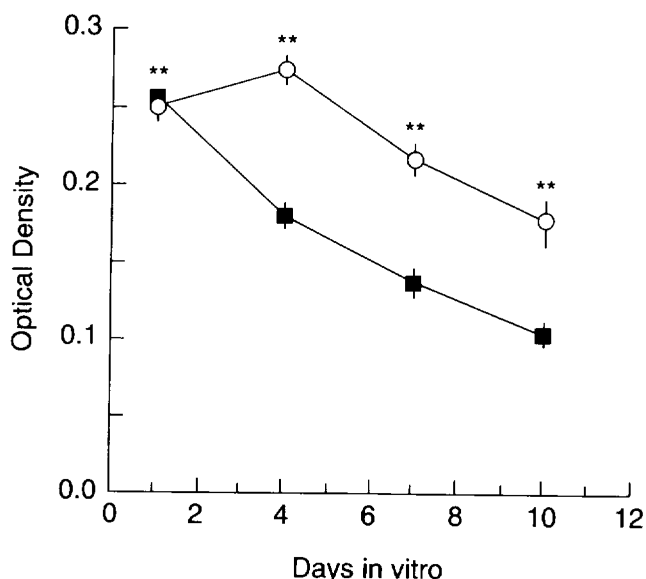

Fig 4. Effect of rPEDF on CGC viability. Data are means ± SEM (bars). ** = control vs experimental differences at a significance of $P < 0.0005$.

Source: Taniwaki T, et al. J Neurochem 1995.[7]

Discussion

Peptide sequencing of the purified protein has allowed for synthesis of specific oligonucleotide probes and cloning of the PEDF cDNA.[12] Sequence analysis shows that PEDF belongs to the serine protease inhibitor supergene family, an ancient family of proteins whose archetype is the serum (α-1-antitrypsin. The PEDF gene has been shown to be active very early on in human RPE cells, at least by 17 weeks of gestation.[13] Hence, this protein is a prime candidate for inducing retinal differentiation in early human development.

The gene for PEDF has been localised to 17p13.1 using both in situ hybridization and analyses of somatic cell hybrid panels.[14] This is very close to the p53 tumour suppressor gene as well as to the chromosomal localisation of a number of hereditary cancers (e.g. medulloblastoma, glioma, breast, colorectal) shown to be unrelated to mutations in the p53 gene product. Along with differentiation, PEDF virtually

abolishes mitotic activity and replication of the Y-79 retinoblastoma tumour cells. The PEDF gene is thus a candidate for study in these cancers and for possible use in stopping retinoblastoma cell growth. Of final interest, is its chromosomal location because a new type of autosomal dominant retinitis pigmentosa (ADRP) has been mapped to the 13.1 region of 17p.[15] Thus PEDF is a prime candidate gene in the study of this form of ADRP.

Several studies have demonstrated that PEDF expression is related to the cell cycle. The protein is expressed by dividing Y-79 cells and there is little or no expression in their quiescent counterparts.[14] Pignolo et al showed the synthesis of PEDF in WI-38 fibroblast cells to be restricted to the G_0 stage of the cell cycle in young cells.[16] Moreover, in senescent WI-38 cells, PEDF mRNA is absent. We have also shown the disappearance of PEDF protein and mRNA in senescent monkey RPE cells in culture[13]—a change that temporally correlates with dramatic alterations in cell morphology. Hence, PEDF seems to be a cell cycle-associated protein and to also be intimately involved in the ageing process. The possible relationship between loss of PEDF expression and ageing in the retina should be a fertile area for investigation in the future.

The term "neurotrophic" usually refers to any substance that promotes the mature functioning and survivial of neurons. The data summarised in the results section indicate that PEDF appears to perform both functions. In retinoblastoma cells, characteristics of mature neurons are enhanced by the presence of PEDF. Apart from the morphology of the cell, the basic biochemical properties of the blast cells are affected by PEDF. In CGC cultures, the addition of PEDF maintains cell viability over a much longer culture period. As these are single examples in specific cell types, more work will need to be done in order to determine if these effects are generally applicable to a variety of neurons. In the retina, several systems are available for such study. Adler et al have described methods for the culturing of specific retinal neurons from chicks and mice in glial-free monolayer culture and the enrichment of photoreceptor-like elements in the cell population.[17,18] A more simple system would involve the use of mixed cultures of undifferentiated retinoblasts from chick embryo retinas as described by Redfern et al.[19]

The basic pathway by which PEDF induces neuronal differentiation has yet to be elucidated. The Y-79 retinoblastoma cell culture system will facilitate the search for PEDF cell surface receptors, intracellular second messengers, and possible effects at the nuclear level. It will also be important to learn by which mechanism CGC neurons die in normal, longer-term culture and how PEDF blocks the process. For example, it is quite possible that the death of the CGCs in culture is by the process of apoptosis, i.e. programmed cell death. If this is the case, PEDF could be an effective inhibitor of apoptosis, at least under these conditions.

Clinically, it is thought that transplantation of neurons may cure certain pathologies. For example, in Parkinson's disease, transplantation of specific foetal brain cells into patients could alleviate or cure the problems associated with the disease. However, major problems to contend with include the need to prolong the life of the transplanted cells and to keep them differentiated and secreting the proper substances. Pretreatment of the cells with PEDF prior to transplantation might aid in both of these areas. Similarly, transfection of cells with the PEDF gene before implantation could provide a good long-term source of PEDF at the transplantation site.

Current research activity includes attempts to transplant neural retina and photoreceptor cells to help cure blindness, in particular, forms of retinitis pigmentosa and age-related macular degeneration. Studies to date have not been fruitful due to non-differentiation and rapid death of the grafts. Again, PEDF may help in both regards. Specifically, photoreceptor neurons to be transplanted could be pretreated with PEDF or the PEDF gene transfected into the cells before surgery. Alternatively, PEDF could be transfected at high levels into adjacent RPE cells which could then serve as a supranormal source of the protein. Several investigators have now shown that cultured RPE cells survive very well after transplantation into the interphotoreceptor space of test animals. Transfection of human RPE cells in vitro with the PEDF gene and then use of them in retinal transplantation seems feasible.

Many neurodegenerative diseases and other long term insults to the CNS (brain and retina) are typified by the slow death of specific populations of neurons. Alternatively, rapid increases in neurotoxin concentrations and loss of potassium or oxygen, etc. can lead to acute neuronal cell death; PEDF might be used effectively in these conditions to prolong the life and functioning of the primary neurons. In the retina, there is no direct evidence that PEDF prolongs retinal cell survival in vitro or in vivo. Experiments in these directions are currently underway. It will also be of in-

terest ultimately to examine the effects of PEDF in conditions such as retinal detachment, diabetes, etc. as well as in hereditary and environmentally-induced retinal degenerations.

Pigment epithelium-derived factor is a protein that is expressed by the RPE and secreted into the retinal interphotoreceptor space. We now know the general structural features of the PEDF molecule and its gene and are beginning to understand how these relate to the function of the protein. Functionally, PEDF is a neurotrophic protein that appears to be the sole component of the IPM that promotes neurite outgrowth from Y-79 retinoblastoma cells and also inhibits cell growth in these tumour cells. We hope to determine the shortest sequence of the protein that promotes neurotrophic activity so that a smaller peptide can be used clinically. The protein and its peptides have potential uses in a number of clinical situations, notably, development, transplantation, cancer, and the control of neurodegenerative diseases in both the neural retina and other CNS areas.

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