Int. J. Dev. Biol. 37: 319-326 (1993)

Original Article

Basic fibroblast growth factor as one of the essential factors regulating lens transdifferentiation of pigmented epithelial cells

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ABSTRACT ***In vitro*** **transdifferentiation of retinal pigmented epithelial cells of the chick embryo into lens cells can be markedly enhanced by culture in the presence of testicular hyaluronidase and phenylthiourea. Since the commercial preparations of hyaluronidase that had previously been used were very crude, a search for the actual effective molecule(s) enhancing lens transdifferentiation was conducted. First, we purified the enzyme and tested the effect of the purified hyaluronidase. Highly purified hyaluronidase itself did not enhance lens transdifferentiation. The crude hyaluronidase was then separated according to affinity with heparin, considering the possibility that the fibroblast growth factor (FGF) is contained in the crude hyaluronidase. Transdifferentiation-enhancing activity was detected in the fraction which was bound to heparin and eluted with 2 M NaCl, where no hyaluronate-degrading activity existed. Analysis of the fraction by SDS-PAGE revealed the existence of an 18 kDa protein whose NH_2-terminal sequence was identical to that of basic FGF. The basic FGF derived from bovine brain also enhanced lens transdifferentiation of pigmented epithelial cells. These findings suggest that basic FGF must play a major role in enhancing transdifferentiation of pigmented epithelial cells to lens cells.**

KEY WORDS: *FGF, transdifferentiation, pigmented epithelial cell, lens cell, hyaluronidase*

Introduction

The pigmented epithelial cells (PECs) of the retina and the iris of the vertebrate eye provide good materials for studying the stability of the expressed phenotype of once differentiated cells. In newts, experimental manipulations and histological observations have shown many examples of the metaplasia of tissues where PECs of the retina and the iris can switch their phenotype and reconstruct a complete retina (Hasegawa, 1958; Keefe, 1973) and lens (for a review, see Reyer, 1977), respectively. However, observations on tissues within a whole organism or in organ culture give only limited information on the behavior of individual cells and environmental factors regulating these phenomena.

In vitro cell culture of uncontaminated PECs was attempted as one of the modern approaches to establish an experimental system more precise and powerful than *in vivo* systems, and also to analyze the metaplasia at the cellular and molecular level. Eguchi and Okada (1973) first demonstrated that the progeny of singly dissociated PECs from chick embryos can change their phenotype into that of lens cells, and termed this phenomenon «transdifferentiation» instead of «metaplasia,» which had been used to describe the phenomenon at the level of tissues. It was also shown that the ability of PECs to transdifferentiate is not limited to newts but is common among vertebrate species including humans (Eguchi, 1979, 1988).

As the original culture method required repeated culture passages and a lengthy culture period for transdifferentiation to occur, improved culture conditions were sought to promote transdifferentiation. Phenylthiourea, a potent inhibitor of melanogenesis, enhances transdifferentiation of PECs into the lens phenotype (Eguchi, 1976; Eguchi and Itoh, 1981, 1982; Eguchi *et al.*, 1982; Itoh and Eguchi, 1986a). Furthermore, the enhancing effect of phenylthiourea can be greatly amplified with testicular

Abbreviations used in this paper: PEC, pigmented epithelial cell; HUase, hyaluronidase; FGF, fibroblast growth factor; CMF-PBS, Ca^{2+}-Mg^{2+}- free Dulbecco's phosphate-buffered saline; MEM, minimum essential medium; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; EDGF, eye-derived growth factor.

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0214-6282/93/$03.00

Printed in Spain

Fig. 1. SDS-PAGE of purified ovine testis HUase. *HUase was purified from a commercial preparation of HUase (Boehringer Mannheim, Lot. 11366220-24) by a two-step column chromatography procedure, as described in Materials and Methods.* **(Lane P)** *Purified HUase with an apparent MW 62 kDa;* **(Lane C)** *commercial crude preparation of HUase. Note that 62 kDa band appears very faintly;* **(Lane M)** *molecular weight standards: myosin, 205 kDa; ß-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; egg albumin, 45 kDa; carbonic anhydrase, 29 kDa.*

hyaluronidase (HUase) (Itoh and Eguchi, 1986a). The introduction of phenylthiourea and HUase in PEC cultures permitted exact control of PEC transdifferentiation and preparation of homogeneous cell population at various stages during the process, making way for biochemical and molecular biological analyses of this phenomenon (Itoh and Eguchi, 1986b).

During long-term experience of PEC culture, we came to notice that commercial preparations of HUase having equal hyaluronate-degrading activity showed a large variation in the transdifferentiation-enhancing effect. Preliminary results showed that these commer-

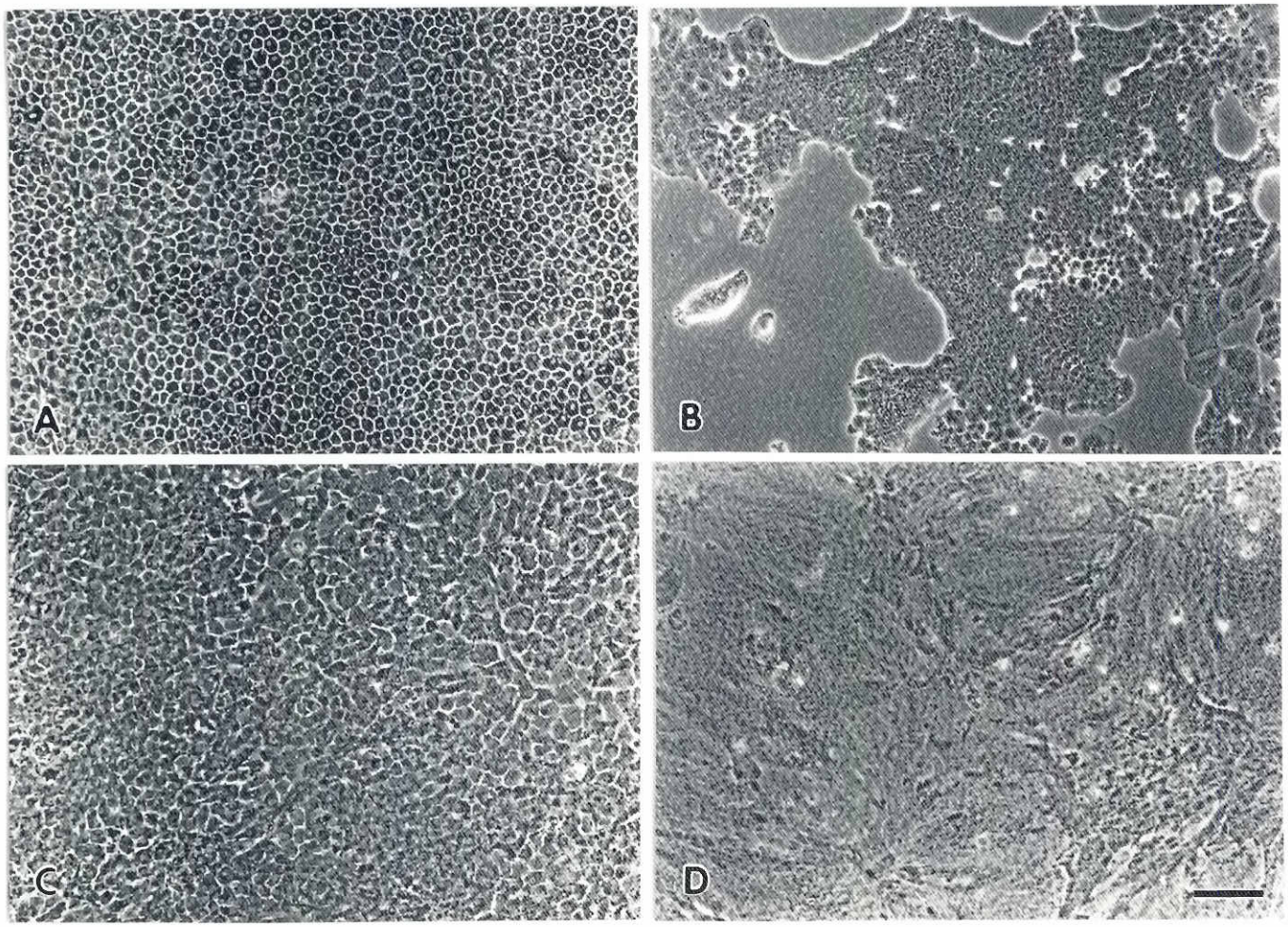

Fig. 2. Phase micrographs of various cellular patterns of PECs *which were maintained with EdF medium for 30 days* **(A)**, *EdFP medium for 30 days* **(B)**, *EdFP medium containing crude hyaluronidase (250 units/ml) for 20 days* **(C)**, *or EdFP medium containing purified hyaluronidase for 30 days (250 units/ml)* **(D)**. *Scale bar= 100 µm.*

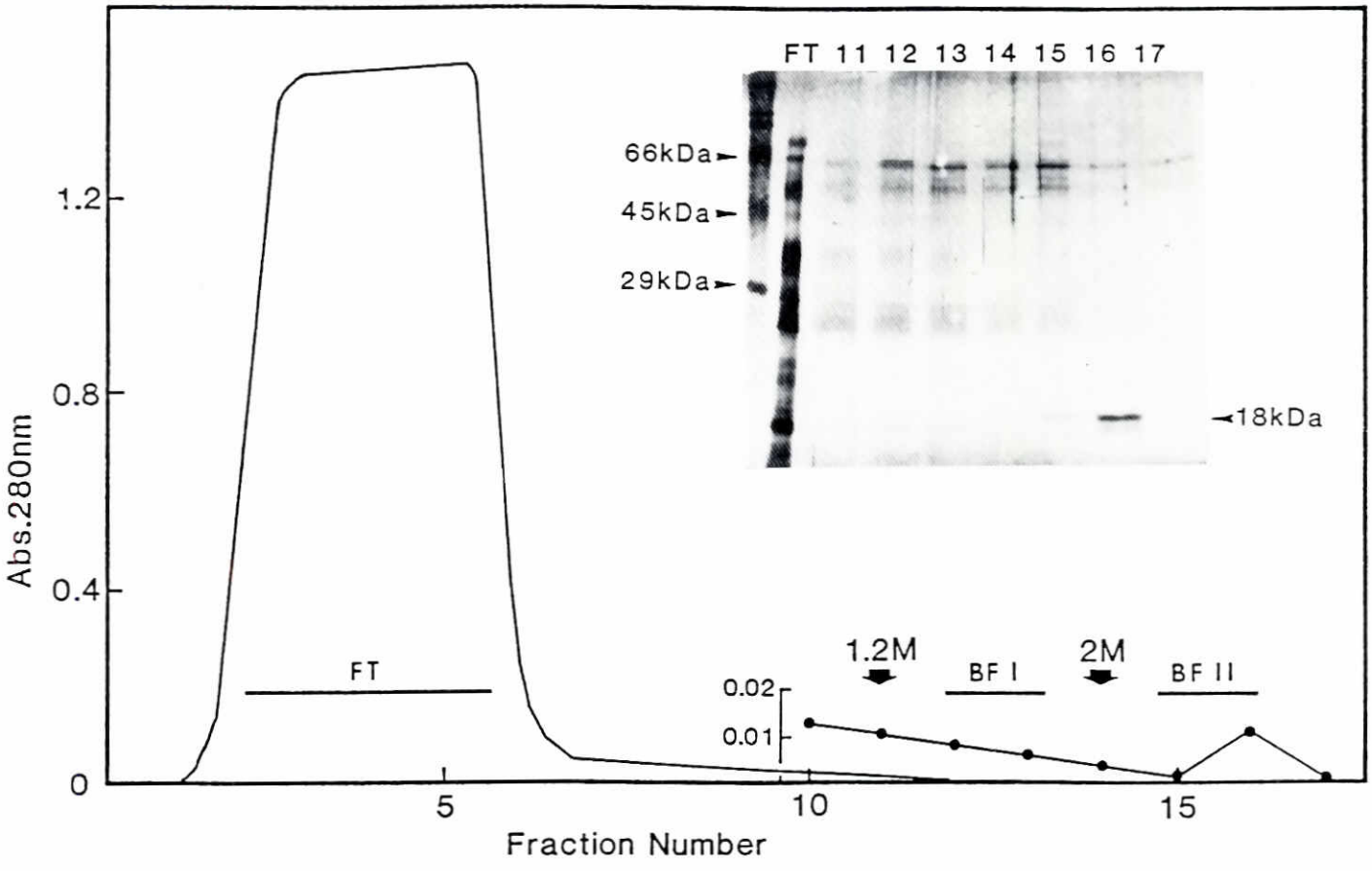

Fig. 3. Heparin-Sepharose chromatography. *One hundred mg of crude HUase (Sigma, Lot. 79F-0098) was applied to a heparin-Sepharose column (10 mm x 20 mm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 0.8 M NaCl. The column was washed with equilibration buffer and eluted stepwise with 1.2 M NaCl and 2 M NaCl in 10 mM sodium phosphate buffer (pH 7.4) at a flow rate of 10 ml/h. The eluates were monitored at 280 nm (- - -). Flow-through fraction (FT, fractions No. 3, 4 and 5), bound fraction I (BF I, fractions No.12 and 13) and bound fraction II (BF II, fractions No.15 and 16) were pooled for the bioassay (horizontal bar). Inset: SDS-PAGE of heparin-binding fractions. Lane FT, flow-through fraction; Lanes 11-17, heparin-binding fractions. The 18 kDa band (arrowhead on the right) were observed in lane 16 (bound fraction II). Molecular weight standards (arrowhead on the left) were as described in the legend for Fig. 1.*

cial preparations of HUase are very crude. These observations prompted us to analyze the mode of action of this enzyme preparation in transdifferentiation.

We report in this paper that the basic fibroblast growth factor (FGF), which is present as a contaminant in the crude HUase, must play, in combination with phenylthiourea, a major role in enhancing transdifferentiation of PECs to lens cells.

Results

Effect of purified HUase on lens transdifferentiation of PECs

First, we purified HUase and tested the effect of the enzyme on lens transdifferentiation of PECs (Fig. 1). When PECs were cultured with EdF medium, they showed a typical polygonal cellular pattern (Fig. 2A). When PECs were cultured with EdFP medium supplemented with crude HUase (250 units/ml), they grew vigorously, gradually piled up, and formed multicellular layers in many parts of the culture, with lentoid bodies developing approximately 25 days after seeding (Itoh and Eguchi, 1986b). Figure 2C shows the early stage of such changes, where cells have lost epithelial morphology and started to pile up. When PECs were cultured with EdFP medium supplemented with purified HUase (250 units/ml), PECs showed a more irregular pattern than that observed in EdFP medium (Fig. 2B and D). However, purified HUase did not promote growth nor piling up of PECs. Thirty days after the addition of purified HUase, no lentoid differentiation was observed in cultures, and δ-crystallin was not detected. Thus, highly purified HUase did not enhance lens transdifferentiation. These results suggest that factor(s) present as contaminant(s) in the crude HUase play an important role in enhancing transdifferentiation of PECs to lens cells.

Isolation of transdifferentiation-enhancing factor

We further searched for factors enhancing transdifferentiation, focusing mainly on FGF for reasons described in the Discussion below. The heparin affinity column has been widely used for the purification of FGF. The crude HUase was applied to a heparin affinity column equilibrated with phosphate buffer containing 0.8 M NaCl and eluted stepwise with 1.2 M NaCl and 2 M NaCl, and the eluates were designated as bound fractions I and II, respectively (Fig. 3). Hyaluronate-degrading activity appeared only in the flow-

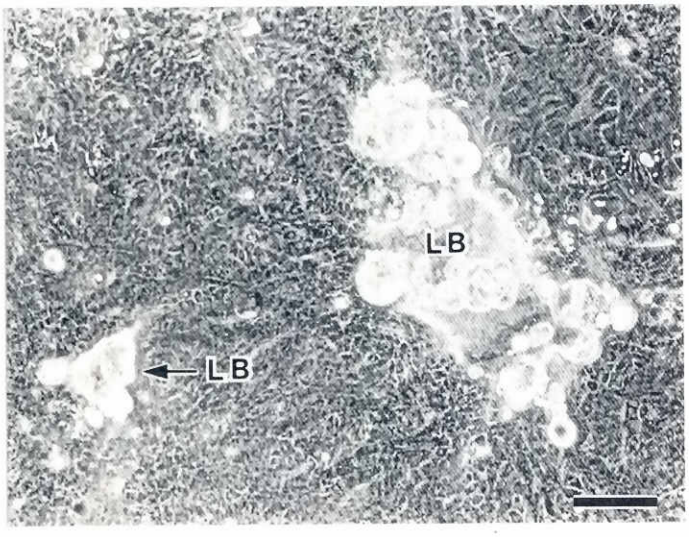

Fig. 4. A phase micrograph showing lentoid body (LB) in a culture of PECs *which were maintained for 30 days with EdFP medium containing bound fraction II. Scale bar= 100 μm.*

through fraction (data not shown). Analysis of eluates by SDS-PAGE revealed the existence of an 18 kDa protein in the bound fraction II (Fig. 3, inset).

Although the flow-through fraction and bound fraction I promoted proliferation of PECs, lentoidogenesis could not be detected even in prolonged cultivation for more than 30 days. Transdifferentiation-enhancing activity was found in bound fraction II (Fig. 4). The lens-transdifferentiation-enhancing activity of fractions is summarized in Table 1.

Identification of 18 kDa protein

The 18 kDa protein in the bound fraction II was further purified by reversed-phase HPLC. The purified 18 kDa protein showed a single band by SDS-PAGE (Fig. 5). Automated Edman degradation of the 18 kDa protein resulted in the identification of 15 amino acid residues in the NH_2-terminal region, which were identical to those of bovine basic FGF except for the tenth residue, which we could not identify. The sequence data are given in Table 2.

Effect of basic FGF on lens transdifferentiation

PECs were cultured with EdFP medium containing different concentrations of bovine brain basic FGF (1, 5, 10 or 30 ng/ml). Daily observation of cultures using an inverted phase-contrast microscope revealed that PECs grew vigorously and the *de novo* formation of pigment granules stopped completely at all concentrations tested. The depigmented PECs gradually piled up and formed multicellular layers in many parts of the culture dish (Fig. 6A). These features resembled «the dedifferentiated PECs» observed in the culture of PECs with EdFP medium containing crude HUase (Fig. 6B) (Itoh and Eguchi, 1986b). Irrespective of FGF concentration, the first lentoid body appeared approximately 25 days after seeding. The number of lentoid bodies was increased in a dose-dependent manner by basic FGF. The assay of β- and δ-crystallin content by ELISA (Fig. 7) showed this increase quantitatively.

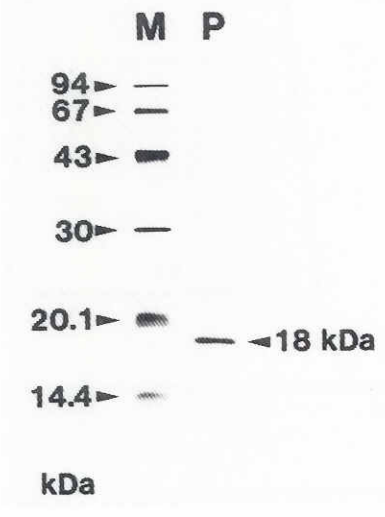

Fig. 5. SDS-PAGE of purified 18 kDa protein. *The 18 kDa protein in the bound fraction II from the heparin affinity column was further purified by reversed-phase HPLC.* **(Lane P)** *Purified 18 kDa protein, whose molecular weight was identical to that of bovine basic FGF.* **(Lane M)** *Molecular weight standards: phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa.*

TABLE 1

LENS TRANSDIFFERENTIATION-ENHANCING ACTIVITY OF FRACTIONS FROM HEPARIN AFFINITY CHROMATOGRAPHY

Fraction	Total protein (μg)	Bioassay [a] Protein [b] concentration (ng/ml)	δ-crystallin/ total protein (ng/mg)
Starting material	100,000	250,000	74,000
Flow-through	80,000	135,000	35
Bound I	40	100	7
Bound II	35	88	56,000

a) Aliquot of each fraction was added to cultured PECs at given protein concentration and the amount of δ-crystallin was assayed after 30 days. See Materials and Methods for details.
b) Each fraction was added according to the protein content ratio in the starting material. Concentration of flow-through is a little lower than calculated value because of specimen dilution.

When the phenylthiourea was omitted from the above culture medium, that is, when PECs were cultured with EdF medium containing basic FGF, they grew vigorously. However, PECs did not transdifferentiate to lens cells. Lens transdifferentiation of PECs can be markedly enhanced by co-treatment with phenylthiourea and basic FGF.

Discussion

We purified HUase and tested the effects of the purified enzyme. Highly purified HUase did not enhance transdifferentiation, sug-

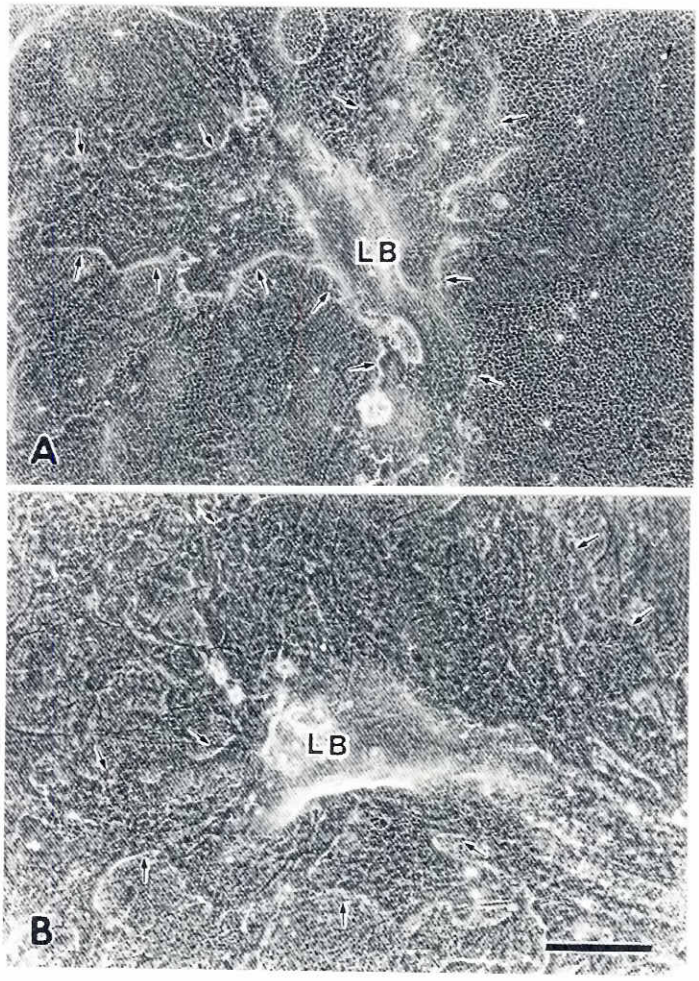

Fig. 6. A phase micrograph showing typical lentoid body (LB) in a culture of PECs *which were maintained for 30 days in EdFP medium containing basic FGF at 30 ng/ml* **(A)**, *or crude HUase (250 units/ml)* **(B)**. *Lentoid body differentiated in the multilayered portion (arrows). Scale bar= 200 μm.*

gesting that unknown factor(s) present as contaminant(s) in the crude HUase enhance lens transdifferentiation. One candidate for such factors was FGF, for the following reasons. 1) The factor should comprise basic protein(s), because our preliminary results showed that the basic fraction of crude HUase enhanced transdifferentiation (unpublished data). 2) Basic FGF was recently shown to influence *in vitro* cell differentiation (reviewed in Gospodarowicz *et al.*, 1986). It was further shown that basic FGF can elicit transdifferentiation of PECs into neural retina in an *in vivo* regeneration experiment (Park and Hollenberg, 1989). 3) Basic FGF had been isolated to homogeneity from bovine testis (Ueno *et al.*, 1987), while the crude HUase we used was prepared from ovine testis.

FGFs can be purified using their selective binding to heparin (Gospodarowicz *et al.*, 1986). FGF exists as two closely related forms, one basic and the other acidic. Acidic and basic FGF have been shown to elute from a heparin-Sepharose column successively with 1.2 M and 2 M NaCl, respectively (Conn and Hatcher, 1984). When the crude HUase was fractionated using a heparin-Sepharose column, transdifferentiation-enhancing activity appeared

TABLE 2

NH_2-TERMINAL AMINO ACID SEQUENCE ANALYSIS OF 18 kDa PROTEIN

Cycle No.	18 kDa protein	Yield (pmol)	basic[a] FGF
1	Pro	220	Pro
2	Ala	235	Ala
3	Leu	221	Leu
4	Pro	106	Pro
5	Glu	60	Glu
6	Asp	37	Asp
7	Gly	58	Gly
8	Gly	64	Gly
9	Ser	25	Ser
10	X[b]		Gly
11	Ala	38	Ala
12	Phe	27	Phe
13	Pro	38	Pro
14	Pro	44	Pro
15	Gly	26	Gly

a) The amino acid sequence of pituitary basic FGF reported in Esch *et al.* (1985).
b) Unidentified amino acid residue.

mainly in 2 M NaCl eluate (bound fraction II). Bound fraction II contained an 18 kDa protein, whose NH_2-terminal amino acid sequence was identical to that of basic FGF. We could not detect acidic FGF in bound fraction I, which is in agreement with the report of Ueno *et al.* (1987) in which they could not isolate acidic FGF from bovine testis. The basic FGF derived from bovine brain enhanced lens transdifferentiation. The promotion of lens transdifferentiation by FGF was also shown for neural retinal cells of the chick embryo (Karim and de Pomerai, 1990).

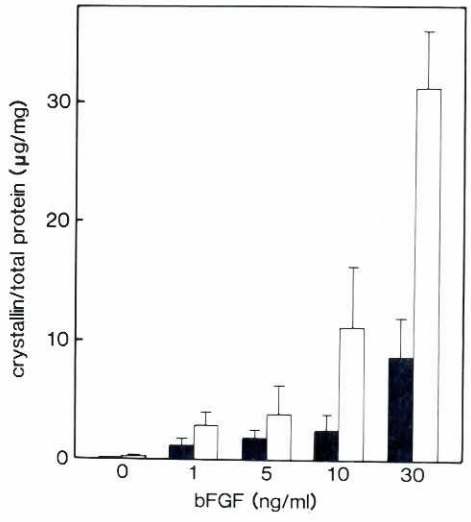

Fig. 7. Effect of basic FGF on lens transdifferentiation. *PECs were maintained for 30 days with EdFP medium containing basic FGF at a concentration of 1, 5, 10 or 30 ng/ml. The ß-crystallin (solid bars) and δ-crystallin (open bars) contents were determined by ELISA.*

The culture medium used for bioassay of the effect of FGF on transdifferentiation was «EdFP medium,» adopted from the work of Itoh and Eguchi (1986b). Itoh (1976) had shown that the dialysis of the serum resulted in the inhibition of differentiation of the neural retinal cells of the chick embryo and that this inhibition was released by the addition of ascorbic acid, suggesting that the effect of the dialysis was to remove the ascorbic acid from the serum. Eldridge *et al.* (1987) confirmed the effect of dialysis on decreasing ascorbic acid concentration of the serum by chemical quantification and showed that the differentiation of Schwann cells *in vitro* depends on the presence of ascorbic acid. Itoh and Eguchi (1986b) used dialyzed serum to promote the dedifferentiation of PECs and further added ascorbic acid to start differentiation into lens cells. We did not add ascorbic acid because 1) ascorbic acid is dispensable for lens differentiation if the culture period is long enough (Eguchi, unpublished observation), and 2) we wanted to simplify the culture method. The lower concentration of crystallins in our result (cf. Fig. 7) as compared to that reported by Itoh and Eguchi (1986b) may have resulted from the absence of ascorbic acid.

Although testicular HUase is a substance foreign to the eye, its active component, FGF, was reported to exist in the eye, and some of the roles which FGF plays in the eye were also suggested as follows. 1) The supernatant of homogenized bovine neural retina contains growth factors called eye-derived growth factors (EDGFs) (Courty *et al.*, 1985). EDGF I and II have similar biochemical characteristics to basic and acidic FGF, respectively (Baird *et al.*, 1985; Schreiber *et al.*, 1985). 2) Basic FGF can stimulate transdifferentiation of retinal PECs into neural retina *in vivo* in the chick embryo (Park and Hollenberg, 1989). The process of transdifferentiation from retinal PECs to neural retina was originally reported to be stimulated by the presence of a piece of neural retina (Coulombre and Coulombre, 1965, 1970). 3) Basic FGF is synthesized not only in neural retina but also in PECs (Schweigerer *et al.*, 1987; Noji *et al.*, 1990).

The molecular mechanisms of the effect of basic FGF on the transdifferentiation of PECs have not yet been elucidated; however, two possibilities can be pointed out. 1) Basic FGF is a potent mitogen for lens epithelial cells (Gospodarowicz *et al.*, 1977) and FGF, as a neural retinal factor, is shown to promote the differentiation of lens epithelial cells into lens fiber cells *in vitro* (McAvoy and Chamberlain, 1989). These results suggest that FGF promotes lens transdifferentiation by enhancing the growth of cells which have already acquired growth requirements similar to lens epithelial cells. Our observations that addition of basic FGF does not speed up the timing of the appearance of lentoid bodies but increases the number and the volume of lentoid bodies seems to support this possibility. 2) A collagen substrate inhibits transdifferentiation of pigmented epithelial cells into lens cells (Eguchi, 1976; Yasuda, 1979). Basic FGF is shown to enhance collagenase activity in cell culture through the induction of collagenase and also through the induction of a plasminogen activator, which in turn activates an inactive form of collagenase (Moscatelli *et al.*, 1986; Presta *et al.*, 1986; Edwards *et al.*, 1987). These data suggest that basic FGF may enhance transdifferentiation through the degradation of the collagen substrate by induced collagenase activity.

Basic FGF caused phenotypic change of PECs *in vivo* into neural retina (Park and Hollenberg, 1989). Pittack *et al.* (1991) recently confirmed this effect of basic FGF in organ culture of PECs. Under our culture condition, no differentiation towards neural retinal phenotypes was observed following addition of basic FGF to PECs. Several differences in the method can be pointed out which may explain this difference in results. The first is the stage of embryos from which materials were taken. Pittack *et al.* used stage 24 to 28 embryos (approx. 4.5 to 6 days), while we used 9-day (stage 35) embryos. Tsunematsu and Coulombre (1981) showed that the capacity of retinal PEC to form neural retina decreases as the embryo becomes older, and vanishes at stage 27 in the case of explant culture without basic FGF. The second one is found in culture method. Pittack *et al.* cultured PECs as a free-floating explant of an intact sheet together with adhering mesenchymal tissue, whereas in our culture, PECs were cleanly isolated, dissociated and cultured on a plastic dish. Pittack *et al.* also reported that dissociated PECs cultured on a plastic dish did not respond to basic FGF to form neural retina. The third difference which may be biochemically studied is that our culture medium contained phenylthiourea in combination with basic FGF. We observed that the addition of basic FGF alone enhanced only cell proliferation and cannot promote lens transdifferentiation.

The mode of action of phenylthiourea has yet to be clarified. Phenylthiourea has long been known to be an inhibitor of tyrosinase which catalyzes a key step in melanin synthesis (Lerner *et al.*, 1950), and the apparent effect of phenylthiourea on PECs is to inhibit melanogenesis. A naive assumption is that phenylthiourea promotes the transdifferentiation of PECs by inhibiting the expression of their own phenotypes through inhibition of melanogenesis. By comparing four thiourea derivatives, Masuda and Eguchi (1982) showed that the effects of these derivatives on the inhibition of melanogenesis and on the enhancement of lens transdifferentiation do not always parallel each other, and argued that there should be no causal relationship between the two effects of phenylthiourea. Phenylthiourea exhibits other effects on PECs including a drastic change in copper ion permeability (Masuda and Eguchi, 1984) and a change in the assembly of extracellular matrix components such as tenascin and laminin (Kodama, unpublished observation). Further investigation on the mechanism of the effect of phenylthiourea should be of great importance because our results show that phenylthiourea, rather than basic FGF, could be the key substance steering the differentiation of PECs in the direction of lens cells.

In conclusion, we have shown that the addition of basic FGF and phenylthiourea to pure cell cultures of PECs can enhance the phenotypic change of PECs to lens-like cells. Although the precise mode of action of these factors is still elusive, this paper contributes greatly to the simplification of the humoral requirements for the promotion of transdifferentiation. Further simplification, including the use of serum-free medium, is in progress at our laboratory (Kosaka *et al.*, 1992). These investigations should lead to a better understanding of the control mechanisms of cellular differentiation.

Materials and Methods

Reagents

Ovine testicular HUase was purchased from Boehringer Mannheim (Lot. 11366220-24) and Sigma (Lot. 79F-0098). Bovine brain basic FGF was purchased from R&D systems (Lot. RS007133).

Culture media

The following culture media were used: (a) EF medium: Eagle's MEM (Nissui Co., Tokyo) supplemented with 8% fetal bovine serum, 2 mM L-glutamine, and 26 mM sodium bicarbonate. (b) EdF medium: Eagle's MEM supplemented with 8% fetal bovine serum dialyzed against Hanks' saline, 0.1% glucose, 2 mM L-glutamine, and 26 mM sodium bicarbonate. (c) EdFP

medium: EdF medium supplemented with 0.5 mM phenylthiourea (Wako Pure Chemicals Co., Osaka).

Preparation of cells

Cell preparation was carried out according to the method described by Eguchi and Okada (1973). Nine-day-old chick embryos were used as the source of PECs. Pigmented epithelium of the retina was mechanically isolated without contamination by any other cell types after incubation in 0.05% EDTA in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (CMF-PBS) for 30 to 50 min at room temperature. Isolated epithelia were then dissociated into single cells by incubation in 0.1% to 0.2% trypsin in CMF-PBS for 10 min at 37°C. Approximately $5x10^6$ PECs were seeded into a 6-cm plastic dish (Corning, No. 25010) and maintained in a CO_2-incubator (5% CO_2/95% air, 100% humidity). Primary cultures were maintained in EF medium. The medium was replaced every two days. Confluent primary cultures were washed twice with CMF-PBS, treated with 0.05% EDTA in CMF-PBS for 10 min at room temperature and subsequently treated with 0.1% to 0.2% trypsin in CMF-PBS for 15 min at 37°C. After one washing with EF medium, $2x10^5$ dissociated PECs were seeded into a 3.5-cm dish (Corning, No. 25000), maintained in EF medium for 24 h and used for the bioassay as described below.

Bioassay for enhancement of lens transdifferentiation

We had used EdFP medium supplemented with 250 units/ml hyaluronidase to enhance lens transdifferentiation (Itoh and Eguchi, 1986a). Specimens were added instead of hyaluronidase to test whether they could enhance lens transdifferentiation. The medium was replaced every day. Enhancement of lens transdifferentiation was assessed by the appearance of the lentoid body and content of β- and δ-crystallin, which are marker proteins of the chicken lens. In order to detect the appearance of the lentoid body, cultures were observed daily by means of an inverted phase-contrast microscope.

Measurement of crystallin content

The β- and δ-crystallin contents were determined with an enzyme-linked immunosorbent assay (ELISA). Cultured cells were frozen and thawed several times, and supernatants were used to quantify crystallins. The monoclonal anti-β-crystallin antibody was raised in our laboratory (Sawada *et al.*, 1989). The polyclonal anti-δ-crystallin antibody was a gift from Dr. Watanabe (Himeji Institute of Technology) and Dr. Itoh (Aichi Medical University).

Measurement of hyaluronidase activity

Hyaluronidase was assayed by the method of Harisson (1988). This assay was based on the method of Gacesa *et al.* (1981), in which the rate of release of N-acetylglucosamine end groups from hyaluronic acid was measured.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). The proteins were stained with Coomassie brilliant blue R-250, or a silver staining kit (Daiichi Kagaku, Tokyo).

Purification of ovine testis hyaluronidase

Step 1 — The crude HUase was dissolved in 20 mM sodium phosphate buffer (pH 7.2) and applied to an S-Sepharose (Pharmacia) column (2.6 cm x 2.0 cm) equilibrated with the same buffer. The HUase was eluted by a linear gradient of NaCl from 0 to 0.2 M in the equilibration buffer. The fractions showing HUase activity were pooled and dialyzed against 20 mM ethanolamine-HCl buffer (pH 9.0).

Step 2 — The dialyzed fraction was applied to a Q-Sepharose (Pharmacia) column (1.6 cm x 15 cm) equilibrated with 20 mM ethanolamine-HCl (pH 9.0). The HUase was eluted by a linear gradient of NaCl from 0 to 0.5 M in the equilibration buffer. The fractions showing HUase activity were pooled. The fractions were analyzed by SDS-PAGE. The purified HUase appeared as a single major band on SDS-PAGE. The purified HUase was dialyzed against Hanks' saline and applied to the bioassay.

Heparin affinity column chromatography

The crude HUase was dissolved in 10 mM sodium phosphate buffer (pH 7.4), 0.8 M NaCl, and applied to a Heparin Sepharose (Pharmacia) column (10 mm x 20 mm) equilibrated with the same buffer. The column was washed with the equilibration buffer and eluted stepwise with 1.2 M NaCl and 2 M NaCl in 10 mM sodium phosphate buffer (pH 7.4) at a flow rate of 10 ml/h. The fractions were collected in siliconized test tubes. Aliquots of each fraction were used for the bioassay.

Reversed-phase HPLC

The eluate from the heparin affinity column was further purified by reversed-phase HPLC using an LKB 2150 HPLC pump and 2158 UVICORD SD detector. To the eluate from the heparin affinity column, trifluoroacetic acid was added to a final concentration of 0.1% and loaded on a C4 column (4.6 mm x 250 mm, BAKERBOND WP-C4) previously equilibrated with 0.1% trifluoroacetic acid. The elution was done by a linear gradient of acetonitrile from 0 to 60% in distilled water containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min, and the eluate was monitored at 226 nm.

NH_2-terminal amino acid sequence analysis

The NH_2-terminal amino acid sequence was determined by automated Edman degradation which was performed on a protein sequencer 477A and PTH analyzer 120A (Applied Biosystems).

Acknowledgments

We sincerely thank Dr. M. Mochii, Mr. K. Sawada and other colleagues in our laboratory, and Drs. K. Watanabe, K. Agata and H. Orii, Laboratory of Regeneration Biology, Department of Life Science, Faculty of Science, Himeji Institute of Technology, for technical advice and discussions. We also thank Ms. K. Sawada, NIBB, for her kind assistance in the cell culture work and Ms. H. Kajiura, NIBB, for amino acid sequencing. This study was supported in part by a Grant-in-Aid for Priority Areas (Project No. 02236103) from the Ministry of Education, Science and Culture to G.E., and by research funds granted by the Naito Foundation and the Cataract Institute to G.E. This study was also supported by the Joint Study Program of the Graduate University for Advanced Studies.

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Accepted for publication: April 1993