Int. J. Dev. Biol. 45: 753-758 (2001)

Original Article

A unique aged human retinal pigmented epithelial cell line useful for studying lens differentiation *in vitro*

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ABSTRACT Lens regeneration occurs in some urodeles and fish throughout their adult life. Such an event is possible by the transdifferentiation of the pigment epithelial cells (PECs) from the dorsal iris. Studies of this event at the cellular level have been facilitated owing to the ability of PECs to become lens cells even when they are placed in culture, outside of the eye. In fact, PECs possess the capacity for transdifferentiation regardless of the origin of species or age. However, studies at the molecular level are still hindered by the intrinsic problems of primary cultures, namely storage, reproducibility and genetic manipulation. In an attempt to establish an ideal model system for lens transdifferentiation, we have analyzed the ability of a human dedifferentiated PEC line to differentiate into lens. We have found that this cell line can indeed be induced to synthesize crystallin and morphologically differentiate to three- dimensional structures resembling lentoids under controlled treatment *in vitro*. Gene expression studies also provided important insights into the role of key genes. This human cell line can be used for detailed genetic studies in order to identify the key factors involved in lens transdifferentiation from PECs.

KEY WORDS: *human, pigment epithelium, differentiation, lens, regeneration*

Introduction

Only a few urodeles can regenerate their lens throughout their adult life, following lentectomy (Stone, 1967). This has also been shown in a teleost (Sato, 1961). After the lens is surgically removed through an opening in the cornea, a lens vesicle is formed as a budding process on the tip of the dorsal iris. The cells that produce this vesicle are those of the pigmented epithelium (PECs) of the iris. These cells become dedifferentiated (they lose their pigments), grow and then redifferentiate to produce a new lens (Eguchi, 1963; Eguchi, 1964; Dumont and Yamada, 1977; Yamada, 1977; Tsonis, 2000).

However, the potential of PECs for transdifferentiation into lens *in vitro* is not restricted to some urodeles, nor is to the dorsal iris. When ventral iris or retina pigmented epithelium is removed from eyes and cultured *in vitro*, it undergoes lens transdifferentiation (Eguchi *et al.*, 1974; Abe and Eguchi, 1977). Pigmented epithelial cells (PECs) derived from higher species, including human, are able to carry out this process as efficiently (Eguchi, 1976; Eguchi and Okada, 1973; Yasuda *et al.*, 1978; Eguchi, 1993, 1998).

Transdifferentiation is a switch from one cell type to another, and such a switch can be easily studied if *in vitro* systems are established. Systems using primary cultures of retinal pigmented epithelial cells (PECs) from chick and newt have been well established (reviewed in Eguchi, 1993, 1998). Past research with PECs from embryonic chick retina has shown that when these cells are cultured in modified medium, cells become dedifferentiated pigmented epithelial cells (dePECs). When cells are left without passing, they grow without contact inhibition of growth and become multilayered. Multilayered cells gradually start lentoid formation (Itoh and Eguchi, 1986b). Although culturing of the retinal PECs from chick embryos has been well established, there exist some technical problems. First, isolation, storage and propagation of primary cells can lead to variation of results in different experiments. As a consequence reproducibility is problematic. Second, molecular biology experiments, which could lead to isolation of key genes and genetic manipulation to study the function of these genes, are not possible with primary cells. The solution to these problems is the creation and availability of cell lines especially those of dedifferentiated PECs.

In this study, we used an established human dedifferentiated pigmented epithelial cell line in an attempt to characterize its potential for lens differentiation and to develop a model for systematic study of the process of lens transdifferentiation in human cells. We have found that this cell line can be manipulated to synthesize crystallin and redifferentiate into lens-like structures. In addition,

Abbreviations used in this paper: PEC, Pigment Epithelial Cells;

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0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

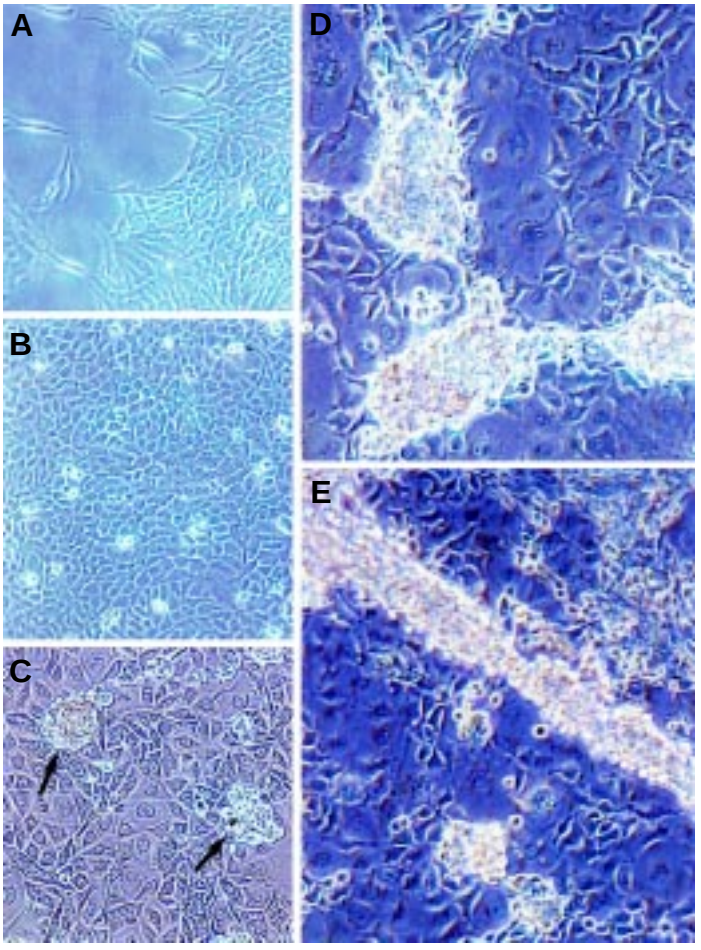

Fig. 1. The H80HrPE-6 cell line in conventional culture. (A) *3 days after plating. Note that as the cells have more room to expand they are elongated.* **(B)** *Cells at confluence 5-7 days after seeding.* **(C)** *Three weeks after seeding. Note the formation of aggregates (arrows) with distinguishable individual cells.* **(D)** *Individual aggregates fusing to form larger ones.* **(E)** *Part of a long aggregate, which has participated in the network formation.*

expression of *pax-6, six-3* and FGFR-1 was examined, in an attempt to correlate their expression with the ability for lens transdifferentiation from the iris PECs. These genes are paramount regulators of crystallin synthesis and lens differentiation and transdifferentiation (Del Rio-Tsonis *et al.,*1995,1997, 1998; Cvekl *et al.,* 1995; Richardson *et al.,* 1995; Altmann *et al.,* 1997; Chow *et al.,* 1995; Oliver *et al.,* 1996; Robinson *et al.,* 1995). This is the first report showing strong possibility of redifferentiation from a human dedifferentiated PE cell line into lentoids and lens-like structures and obviously this system will provide the means to fathom and assess the effect of various genes and factors on lens transdifferentiation and regeneration.

Results

Establishment of an in vitro *lens differentiation model system*

In the present study, we followed the behavior of the H80 cell line under various culture conditions. At first we examined how this cell line behaved in conventional culture where no particular treatment was implemented (see Materials and Methods). After plating, the cells proliferated and produced a confluent culture within 5-7 days. When sparse, the cells looked elongated (Fig. 1A), but after they reached confluence their shape did not remain elongated, but it resembled the rather characteristic hexagonal shape of pigment epithelial cells (Fig. 1B). Nearly two weeks after plating, the cells started to form aggregates. These aggregates were not very clear or transparent and the individual cells that composed them were easily distinguishable (Fig. 1C). These aggregates continued to grow and started to connect with each other (Fig. 1D). Finally, five weeks into the culturing these connected aggregates had formed an extensive network (Fig. 1E). The aggregates never became transparent to resemble lentoids, but their formation in culture was a characteristic behavior of H80HrPE-6 cells, despite the duration or the passage of the cells.

The inability of H80HrPE-6 cells to form transparent lentoids in conventional cultures, prompted us to examine their behavior under different conditions. One of them was on hard agar and the other on MATRIGEL. Two days after the cells were cultured on hard agar, they formed aggregates, which in 2-3 weeks became compact and transparent (Fig. 2 A,B). Note that the aggregates in Fig. 2A are similar with the ones seen in Fig. 1C where individual cells can be distinguished. However, in contrast with what occurred in conventional cultures the aggregates in agar cultures did become compacted and transparent.

This effect and the formation of transparent structures resembling lentoids *in vitro* (Eguchi and Okada, 1973; Eguchi, 1993, 1998) were more impressive when cells were cultured on MATRIGEL. Embedded in such a matrix the H80HrPE-6 cells formed clear lentoid-like aggregates within 4 days (Fig. 2C). This was much faster when compared with the cells cultured on agar, indicating that the use of MATRIGEL was the most efficient in driving the H80HrPE-6 cells to express lens specificities.

To verify the status and quantitate the effect of lens-specific expression, we examined the cell cultures for betaB2 crystallin synthesis. Western blot analysis (Fig. 3) showed that the production of crystallin was greatly enhanced in H80HrPE-6 cell line during the differentiation processes, and this, in conjunction with the morphological features indicate that the aggregates are

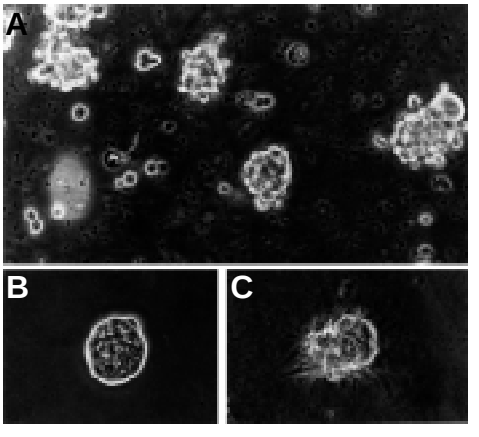

Fig. 2. H80HrPE-6 cells cultured in 3% agar (A) and (B) and in MATRIGEL (C). (A) *Aggregates with distinguishable cells at day two after seeding.* **(B)** *The cells have formed, by day 22, a compact and transparent lentoid.* **(C)** *Cells have formed a lentoid 4 days after seeding.*

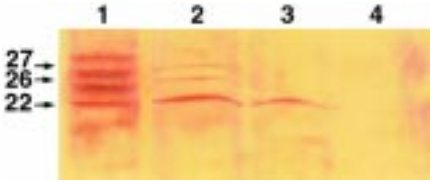

Fig. 3. Western analysis for crystallin synthesis in cultures of H80HrPE-6 cells using the betaB2 antibody. *Lane 1, protein extracts from whole lens indicating reaction of the antibody with the beta crystallin fraction ranging from 22-29 kDa; Lane 2, protein extracts from H80HrPE-6 cells cultured in MATRIGEL; Lane 3, protein extracts from H80HrPE-6 cells cultured in hard agar; Lane 4, protein extracts from H80HrPE-6 cells cultured conventionally without any treatment. Note the apparent induction of crystallin synthesis in the treated cultures, especially the 22 kDa betaB2 crystallin.*

differentiated lentoid bodies. H80HrPE-6 cells grown under conventional culture conditions did not show any significant levels of crystallin synthesis, even though large amounts (100ug) of protein were loaded. The western analysis showed that the betaB2 antibody detected three bands of 22-27 KDa in size, which are the right sizes for the beta crystallin fraction. The 22 Kda band is the most dominant. In fact this 22 Kda betaB2 crystallin has been shown before to be increased in human lens epithelial cells undergoing differentiation to lentoid bodies in culture (Blakely *et al,* 2000). The 22 and 26 Kda betaB2 crystallins are very similar in size with beta crystallins detected with other beta antibodies that are commonly used to determine differentiation to lens cells (Sawada *et al.,* 1993; Fleming *et al.,* 1998). Note also that in cells plated on agar only the 22 Kda protein of the beta crystalline fraction is expressed. This indicates a difference between the treatments in the induction of lens specificities.

To further verify that crystallin synthesis is induced after treatment with MATRIGEL we examined the aggregates with two other methods, by staining them with crystallin antibodies and by RT-PCR. Indeed, the aggregates were shown to synthesize crystallin. When sections of the aggregates were stained with aplhaA, betaB2 and beta6 antibodies, we observed presence of these proteins (Fig. 4). While the architecture of the tissue in the aggregates is not well preserved due to the isolation procedure, the sections were clearly positive, when compared with the negative control, which lacked the treatment with the primary antibody. Likewise we detected the mRNA for *alphaB* by RT-PCT (Fig. 5). These analyses of crystallin expression by three different methods clearly demonstrate that the aggregates do synthesize crystallins and therefore the H80HrPE-6 cells can be used to study lens differentiation *in vitro*.

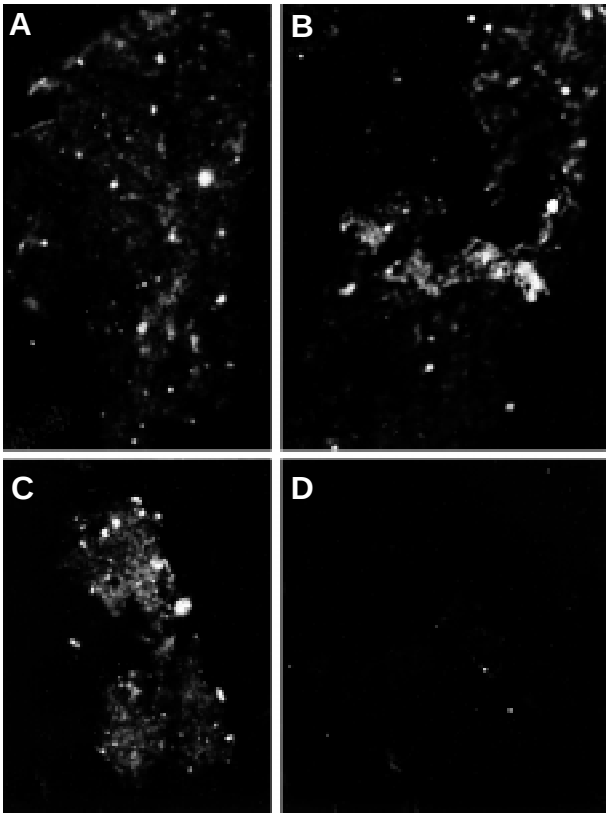

Fig. 4. Detection of crystallins in aggregates treated with MATRIGEL by immunofluorescence (A) *A section stained with anti alphaA crystallin.* **(B)** *A section stained with anti beta6 antibody.* **(C)** *A section stained with anti betaB2 antibody.* **(D)** *A negative control showing background levels.*

Gene expression

Expression of key genes, which are implicated during lens development and regeneration, was examined. Since the cells showed certain behavior and different morphologies in culture (i.e. aggregates), we preferred to localize expression by *in situ* hybridization or antibody staining. *In situ* hybridization results showed that *Pax-6* and *six-3* were expressed in H80HrPE-6 cell line. The expression was seen in virtually all cells and it was not associated with the formation of aggregates (Fig. 6). Immunocytochemistry analysis did not show FGFR1 staining in H80HrPE-6 cell line at early stages in culture as monolayer. However, as cells started to form aggregates later, expression was very high in those cells involved in the formation of aggregates (Fig. 7). The reader should note here that while expression of *pax-6* and *FGFR1* is not restricted only to lens, both of these genes are important regulators for lens fiber differentiation. Therefore, it is important that their maintenance is shown in the cell line.

Discussion

In this study we have examined the behavior of a human dedifferentiated PE cell line and its ability to initiate crystalline synthesis and therefore its potential for the study of lens differen-

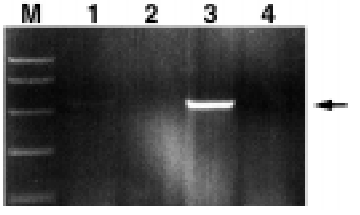

Fig. 5. RT-PCR detection of alphaB sequences amplified from mRNA isolated from MATRIGEL-treated aggregates. *The arrow indicates the correctly amplified band of 540 bp. Lane 1, untreated cells; lane 2 negative control with the RT reaction omitted; lane 3 cells from MATRIGEL treatment and lane 4, negative control with the RT reaction omitted. Some very low levels of amplification can be seen in the untreated cells. However, the levels are considerably higher in the treated cells. M, markers: 1,000, 750, 500, 300, and 200 bases.*

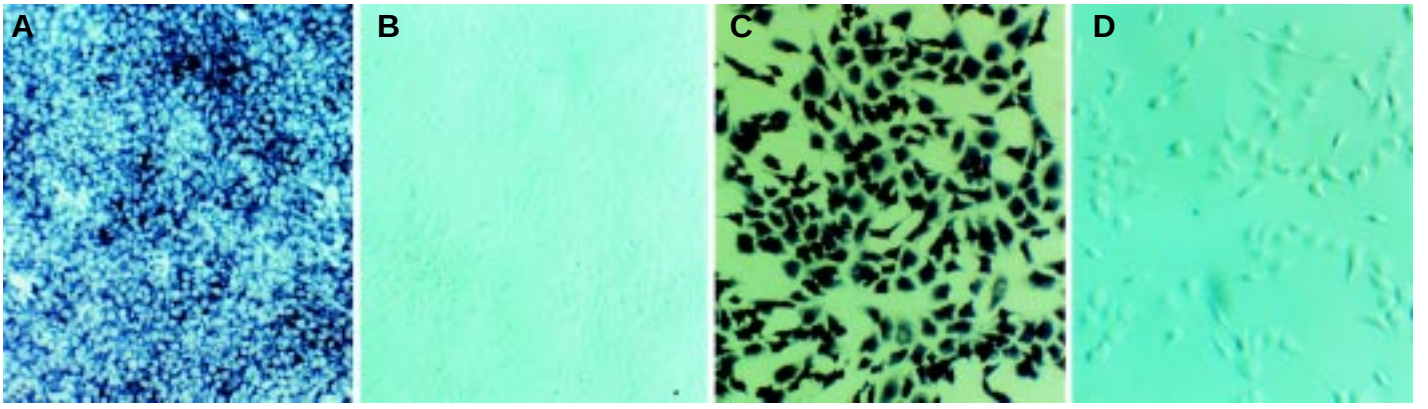

Fig. 6. *In situ* hybridization using *Pax-6* and *six-3* probes, labeled with digoxigenin. (A) *Expression of Pax-6 in H80HrPE-6 cells.* **(B)** *Negative control, using pax-6 sense probe.* **(C)** *Expression of six-3 in H80HrPE-6 cells.* **(D)** *Negative control, using six-3 sense probe.*

tiation. We have indeed shown that under certain experimental conditions this cell line can be induced to synthesize crystallin and to form lens-like or transparent structures resembling lentoids *in vitro*. The most effective procedure was the culturing of these cell lines in MATRIGEL. This is significant because MATRIGEL contains growth factors, such as FGF and TGF-beta and is rich with components of basement membranes. These factors can now be studied more systematically to identify the important molecule(s) responsible for the initiation of lens differentiation. The present results might also indicate a multi-step process for lens differentiation where the transition from pigment epithelium to lentoids might be marked by different stages. Indeed in our experiments, we observed that our cell line underwent differentiation in different steps depending on the treatment. Without treatment our cell line does not form clear lentoids but it does form aggregates with specific gene induction. At the same time our untreated cells do not seem to synthesize crystallin. We were not able to detect crystallin synthesis even when 100ug of protein was loaded on the gel. With hard agar or MATRIGEL treatments H80HrPE-6 cells proceeded to differentiate, but not in the sma fashion. For cells in agar it took nearly 20 days to achieve lentoid-like appearance while for cells in MATRIGEL this occurred within four days. H80HrPE-6 seems unique in its behavior. Another dedifferentiated PE cell line, the RPE-28 (from a 3-month old human fetus; Coriell Institute for Medical Research) was not able to form any clear aggregates in MATRIGEL (Jang and Tsonis, unpublished observations).

Our expression results are in fact very informative in regard to the behavior of H80HrPE-6 cells and the formation of aggregates in conventional cultures. The fact that FGFR1 expression was associated with the formation of aggregates indicates that this is a discrete step marked by specific gene regulation. The aggregates are, thus, not an artifact of the culturing of the cells, but a definite intermediate step demarcating their potential for lens differentiation. Since *pax-6, six-3* and FGFR1 regulate lens crystallin expression and fiber differentiation, their expression might explain the “readiness” of the H80HrPE-6 cells to redifferentiate to lens cells, which are able to otganize a lens under appropriate conditions. Our results suggest that *pax-6, six-3* or FGFR1 is involved in lentoidgenesis only as part of an orchestrated scenario, which includes other factors as well. While *in vivo* these factors are available (and these genes can induce lens formation when ectopically expressed *in vivo*), *in vitro* they must be supplied. This help is obviously provided by an environment created by agar or MATRIGEL. This could include the addition of extracellular matrix factors or changes in cell shape and organization, important parameters when cells grow in three dimensions.

The division of the process of lens transdifferentiation in different stages and the availability of cell lines with discrete properties, provide a unique and ideal system for the study of regulation of the different stages and of transdifferentiation. Since it seems that gene expression of key regulatory genes, known to regulate lens differentiation and regeneration, is associated with the ability of the H80HrPE-6 cell line to differentiate, this cell line can be used

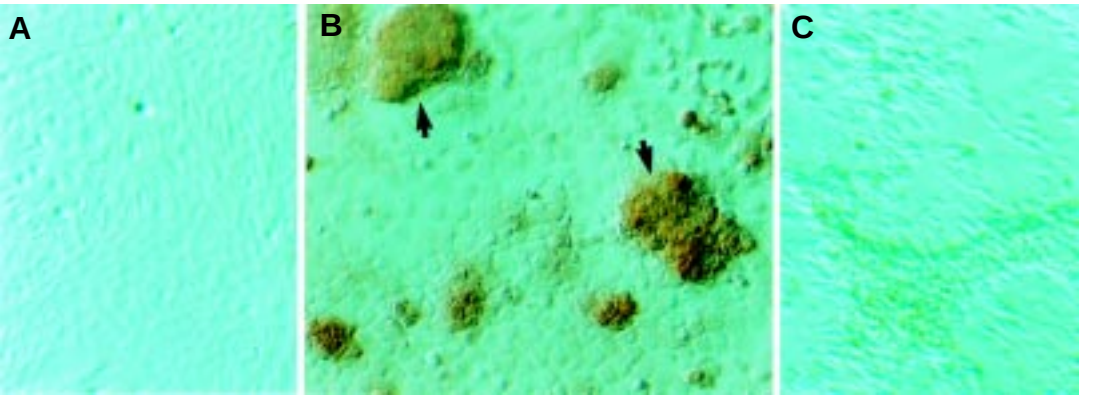

Fig. 7. Immunocytochemistry using anti-FGFR1 antibody on H80HrPRE-6 cells. (A) *Lack of expression of FGFR1 in confluent H80HrPE-6 cells.* **(B)** *Expression of FGFR-1 in H80HrPE-6 cells involved in aggregate formation.* **(C)** *Negative control (no primary antibody).*

as an ideal and much needed system for molecular biology. This will open new avenues in the study of lens differentiation with obvious applications in *in vivo* studies as well. In addition this cell line might also provide a highly useful appropriate system for studying the reconstitution of lens by human iris or retina PECs for clinical applications.

Materials and Methods

Cells

A human dePEC cell line (H80HrPE-6) has been created using primary retinal PECs from an 80-year old person. The original primary cells have been shown to possess the ability to transdifferentiate into lens (Eguchi, 1993; Kodama and Eguchi, 1995; Eguchi, 1998).

Conventional cell culture

Cells were cultured in Eagle's minimum essential media supplemented with 8% heat inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamate and 1% amphotericin B, and incubated at 37°C, 5% CO_2 in saturated air. For *in situ* hybridization and immunocytochemistry, coverslips containing cells were collected at the desired density by fixing in 4% paraformaldehyde in phosphate-buffered-saline (PBS), pH 7.2, and stored in 70% ethanol in diethyl pyrocarbonate (DEPC from Acros Organics, Princeton) at 4°C until needed. For western blot, cells were collected at the appropriate stage to extract proteins.

Hard agar culture

Sterilized 3% agar in PBS (pH 7.2) was set on culture dish 30 minutes before seeding cells. Cells were shaken at 125 rpm for 1.5 hours to promote aggregation. Then cells were incubated in their conventional culture media at 37°C, 5% CO_2 in saturated air until desired stage.

MATRIGEL basement membrane matrix culture

MATRIGEL Basement Membrane Matrix (Becton Dickinson Labware, Bedford) was diluted 1:1 ratio with culture medium and incubated at 37°C for 30 minutes to solidify before seeding cells. Cells were shaken at 125 rpm for 1.5 hours to promote aggregation. Then they were incubated in their normal media at 37°C, 5% CO_2 in saturated air until desired stage.

In situ *hybridization*

In situ hybridization was performed to determine the patterns of gene expression. We employed nonradioactive *in situ* hybridization using digoxigenin incorporated UTP. Plasmids containing *pax 6* and *six 3* (gifts from Dr. van Heyningen and Dr. G. Oliver respectively) were digested with the appropriate restriction enzymes. Riboprobes (both antisense and sense) were transcribed by using either 2 units/µl of T7, T3 or Sp6 RNA polymerase and labeled using RNA labeling kit from Boehringer-Mannheim (Indianapolis). Cells on the coverslips that were stored in 70% ethanol-DEPC were rehydrated in ethanol series (50%, to 0% ethanol in PBS). Then cells were prehybridized in hybridization solution (50% Formamide, 2X standard saline citrate (SSC), 5 mM NaH_2PO_4) for 20 min at room temperature. Subsequently the samples were hybridized with probe at 1ng/µl concentration in hybridization solution at 50°C overnight. The next day, cells were first washed in 50% formamide, 2x SSC at 50°C for 30 min., and in 1X SSC three times. The cells were incubated in buffer I (0.1 M Tris pH. 7.5, 0.15 M NaCl) for 1 min. Then they were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase in 1% BSA, buffer I, washed 2 times in buffer I at room temperature for 15 min. each. Then the cells were incubated in buffer III (0.1 M Tris pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) for 1 min. at room temperature. NBT/BCIP (nitroblue tetrazolium/ bromochloroindoyl-phosphate, Boehringer-Mannheim, Indianapolis) solution was then added until the purple color developed. Deionized water was added to stop the color reaction. Crystal clear was used to mount the surface.

Antibodies

A polyclonal antibody against betaB2 was used for the western analysis and this antibody along with a polyclonal antibody against alphaA-crystallin (gifts from Dr. Andley (Andley *et al.,* 1994) and a monoclonal antibody directed against beta6 (Sawada *et al.,* 1993) were used for the immonofluorescence studies. These antibodies were used at a dilution of 1:1000. A polyclonal antibody for human FGFR1 detection was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). This antibody was made in rabbit. The epitope corresponds to an amino acid sequence mapping at the carboxy terminus of the precursor form of FGFR1 receptor of human origin. This antibody was used at a dilution of 1:50 for immunocytochemistry.

Immunocytochemistry

Cells on coverslips that were fixed and stored in 70% ethanol were rehydrated and blocked in 1.5% normal goat serum (NGS) in PBS for 1 hour at 37°C. Then they were incubated with anti-FGFR1 polyclonal antibody, diluted in PBS/NGS by 1:50, for 1 hour at 37°C. Cells were then washed in PBS for three times, 5 min. each. Secondary antibody kit (Santa Cruz Biotechnology, Santa Cruz) was used to reveal the location of bound antibodies. Cells were incubated with goat anti-rabbit biotinylated antibody for 1 h at 37°C, washed twice in PBS, 5 min. each. Then the cells were incubated with avidin-biotinylated horseradish peroxidase for 30 min. at room temperature, and washed twice in PBS, 5 min. each. Diaminobenzidine tetrahydrochloride (DAB) solution (50 µg/ml DAB, 0.0003 % H_2O_2 in PBS) was used to reveal the signal and the reaction was continued until the brown color was visible.

Immunofluorescence

Aggregates from conventional and MATRIGEL cultures were analysed for immunofluorescence. The cultures were treated with 25 units/ml of dispase for 2 hours at 37°C to remove MATRIGEL. The aggregates were recovered by centrifugation and embedded in OCT. Cryosections were blocked with 1.5% NGS or horse serum and then treated with the different primary antibodies against crystallins (see above). Following this, the sections were treated with the appropriate fluorescein-conjugated secondary antibody (Vector laboratories).

Reverse transcription Polymerase Chain Reaction

The aggregates in MATRIGEL were also examined for crystallin synthesis by RT-PCR. The aggregates were collected as described above and RNA was isolated by the TRIZOL reagent from GIBCO, BRL. We used primers to detect expression of human *alphaB* crystallin. For *alphaB* the following primers amplify a 540 bases long fragment (Gonzalez *et al.,* 2000): 5'-TCACCTAGCCACCATGGACATCGCCA-3'. The PCR markers from PROMEGA (sizes 50-1,000 bases) were used.

Protein extraction

Cells in conventional cultures were washed twice in PBS. Then the culture flask was placed on ice, then 0.5 ml RIPA buffer (0.5 M Tris, 0.15 M NaCl, 0.1 M EDTA, 1 % v/v NP40, 0.5 % w/v deoxycholic acid, 0.1 % w/v SDS) was added to 25 cm^2 flask, and incubated for 10 min. Cells were scraped off and collected into microcentrifuge tubes. For cells cultured on MATRIGEL, cells were recovered by adding dispase, 25 units in 1 ml PBS, and incubated for 2 hours at 37°C. Cells were collected into microcentrifuge tube and centrifuged at 1000 rpm for 1 min. Pellets were washed twice with PBS and resuspended in 0.5 ml RIPA buffer. For cells grown on hard agar, culture media was collected into microcentrifugetube and centrifuged to recover cell aggregates. Aggregates were treated like those recovered from MATRIGEL culture. Protease inhibitor cocktail (Sigma, St. Louis) was added to the cells from all cultures, and the tube was incubated for 30 min. on ice. Cells were disrupted by passing the solution 10 times through 21'' gauge needle. Then the lysates were centrifuged at 10,000 rpm for 30 min. at 4°C in microfuge. The supernatants were collected and stored in –20°C until needed. Protein concentration was determined by using Bradford reagent (Sigma, St. Louis).

Western Blot analysis

Proteins (100 µg was loaded for all samples) were separated by electrophoresis in 15% polyacrylamide gel, electrophoretically transferred to nitrocellulose membrane (Biorad, Hercules). For markers we used the rainbow marker kit ranging from 14.3 to 220 KDa. The membrane was blocked overnight in 7.5 % casein in PBS (pH 7.2), washed twice in PBST (0.005 % tween-20 in PBS) 10 min. each. Then it was probed with the betaB2 anti-crystallin antibody for 1 h at 37°C, and washed in PBST twice. A secondary antibody kit (Santa Cruz Biotechnology) was used to reveal the presence of crystallin protein on the blot. The membrane was incubated with goat anti-rabbit biotinylated antibody for 1 h at 37°C, washed twice in PBS, 5 min. each. Then it was incubated with avidin-biotinylated horseradish peroxidase for 30 min. at room temperature, and washed twice in PBS, 5 min. each. DAB was used to reveal the reaction of the antibody, which it was continued until the brown color was visible. For negative control the primary antibody was omitted.

Acknowledgments

This work was supported by NIH grant EY10540 to PAT. This research was part of the fulfillment of the requirements for W. Jang's Master degree. This work was also supported in part by a Grant-in-Aid for Scientific Research (B), Project No 10044212 from JSPS to G.E.

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Received: December 1999
Reviewed by Referees: February 2000
Modified by Authors and Accepted for Publication: July 2001