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Adult human retinal pigment epithelial cells capable of differentiating into neurons

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Abstract

We investigated the ability of adult human RPE cells to differentiate into neurons. Two cell lines were evaluated. The cells were cultured in medium with 8% serum, transferred to a neural stem cell maintenance culture, and induced to differentiate with retinoic acid. The cells were immunocytochemically examined at each step. The cells showed epithelial-like morphology with 8% serum and all were immunoreactive for β-III tubulin. After transfer to the stem cell maintenance culture, they changed morphologically and became immunoreactive for MAP5 and neurofilament200 after inducement with retinoic acid. The ratio of MAP5 positive cells was higher in the young adult RPE cell line. No GFAP or rod-opsin immunoreactive cells were observed. Adult human RPE cells even from old person are capable of differentiating into neurons, although the ratio of mature neurons was greater in the young than in the old cell line in this condition.

Keywords: Adult human retinal pigment epithelium cell; Differentiation; Neuron; Glia

Stem cells of various tissues have recently gained interest because of their potential use as sources of cell transplantation. Furthermore, the possibility of transdifferentiation of stem cells even beyond germ layers has been reported, such as blood cell differentiation from neural stem cells and neuronal differentiation from hematopoietic cells [1], although this remains controversial [2].

Retinal progenitor cells are present in embryonic retinas [3] and are a potential retinal cell transplantation source [4]. It also has been shown that even in adult mice retinal neural progenitor cells reside in ciliary epithelium and can generate photoreceptors [5].

We previously reported that adult rat brain-derived neural stem cells could integrate and survive after transplantation into the retina [6] and that they differentiated into neurons even in adult host retina, although they did not show any retinal neuronal marker expression [7].

We therefore investigated the potential of progenitor cells in other parts of the eyes such as embryonic retinal progenitor cells [8], adult rat iris-derived neural progenitor cells [9] and, in the study presented here, adult retinal pigment epithelium cells.

Early in the 1970s, the word 'transdifferentiation' was already used for the ability of retinal pigment epithelial cells to dedifferentiate to an immature status and redifferentiate into another type of cell [10]. Since then it has become well known that RPE cells of adult newts can transdifferentiate into lens epithelial cells. Moreover, these cells can completely regenerate the neural retina after its removal [11,12].

Retinal pigment epithelial (RPE) chick embryo cells also can transdifferentiate into neural cells with acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) [13].

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As for mammals, in the presence of bFGF, cultured early embryonic rat RPE cells that have not acquired pigment yet develop to form a retina-like multilayer structure containing neuronal cells that express markers of retinal ganglion, amacrine, and rod photoreceptor cells. However, the ability of rat RPE cells to transdifferentiate is restricted to a certain age range [14].

In this study, we investigated whether adult human RPE cells have the ability to differentiate into neurons and evaluated the difference between young adult RPE cells and the RPE cells of an elderly man.

Materials and methods

Human retinal pigment stem cells

Two human adult retinal pigment epithelial cell lines were used in this study, H80HrPE (80-year-old human differentiated pigmented epithelial cells)-6 and ARPE-19.

H80HrPE-6 was established by Eguchi et al. and harvested from the eye bank eye of an 80-year-old male, while ARPE-19 was harvested from a young eye.

Establishment of the human RPE cell clone

Eyecups were made from the eye, after which the neural retina was removed from the eye cups and placed in PBS. The eyecups were incubated in 0.05% EDTA, and only then the pigmented epithelial layer was peeled off. After trypsinization of the pigmented epithelium, dissociated cells were cultured in a collagen coated dish. Cells were passaged using trypsin EDTA, and after several passages the cells were cultured at low density and clones of the cells were obtained by means of colony rings. We used one clone that appeared healthy (H80HrPE-6) [12,15].

Monolayer cell culture

Epithelial culture. First, the cells of these two cell lines were cultured in Eagle's minimum essential medium (MEM; Gibco, Rockville, MD) supplemented with 8% heat inactivated fetal bovine serum, 1% penicillin–streptomycin, and, at 37 °C, with 5% CO_2. The medium was changed every 3 days.

Stem cell culture. Next, we cultured the cells under the same condition as used for the maintenance of neural stem cells. They were cultured on laminin/poly-L-ornithine-coated dishes containing Dulbecco's modified Eagle's medium-Ham's F12 (DMEM/F12; Gibco) supplemented with N_2 (Gibco) 20 ng/ml basic fibroblast growth factor (bFGF; Genzyme, Cambridge, MA) and REC Human epidermal growth factor (EGF; Genzyme), and incubated at 37 °C in humidified 5% CO_2 in air. The medium was changed every 3 days.

Differentiation culture. After having been cultured for more than 2 weeks under these conditions, the cells were induced to differentiate in the DMEM/F12 by the addition of N_2, 0.5% FBS and 0.5 mM retinoic acid for 10 days.

Immunocytochemistry. The monolayer culture cells were fixed in 4% paraformaldehyde (Wako Pure Chemical, Osaka, Japan) in PBS for 30 min at 4 °C and blocked with 20% Block Ace (Dainihon–Seiyaku, Osaka, Japan) in PBS containing 0.005% saponin (Sigma, St. Louis, MO) for 30 min. After removal of the blocking solution, the cultured cells were incubated with primary antibodies for 60 min at room temperature. Primary antibodies were used at the following concentrations: mouse monoclonal anti-pancytokeratin (1:400; Sigma) as the retinal epithelium cell marker, rabbit polyclonal anti-ZO-1 (1:200; Zymed Laboratories, South San Francisco, CA), as the tight junction marker, rabbit polyclonal anti-musashi (1:400; Chemicon) as central neural stem cell marker, mouse monoclonal anti-β tubulin isotype III (β-III tubulin; 1:500; Sigma), mouse monoclonal anti-microtubule associated protein (MAP) 5 (1:1000; Chemicon, Temecula, CA) and mouse monoclonal anti-neurofilament 200 (1:1000; Sigma) as neuronal markers, rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) as the glial cell maker, and mouse monoclonal anti-rod-opsin (RET-P1, 1:10,000; Sigma) as the rod photoreceptor cell marker. After washing with PBS, the cells were incubated with the appropriate secondary antibodies for 60 min at room temperature. Secondary antibodies were used at the following concentrations: Alexa Fluor 488 goat anti-mouse immunoglobulin (Ig, 1:500; Molecular Probes, Eugene, OR) and Alexa Fluor 546 goat anti-mouse immunoglobulin (Ig, 1:500; Molecular Probes). Cell nuclei were counterstained with 4′,6′-deamino-2-phenylindole, dihydrochloride (DAPI) (1 μg/ml; Molecular Probes) added to the secondary antibody solution. All antibodies were diluted in PBS containing 0.005% saponin and 5% Block Ace. The cells were then washed with PBS and mounted with glycerol/PBS (1:1).

Results

Characteristics of human retinal pigment cell line

H80HrPE-6 cells and ARPE19 cells showed a flat and polygonal epithelial-like morphology without pigment in epithelial culture medium with fetal bovine serum (Fig. 1A). They had remained healthy after freezing and thawing. Cells of either line contained no visible pigment until after 5 months' culture, when some cells of ARPE19 showed visible pigment in their cytoplasm.

The cells cultured for 2 weeks in the epithelial culture medium were examined immunocytochemically. Most of the cells showed immunoreactivity for pancytokeratin, a marker for epithelial cells, and some parts of the cells for ZO-1, a marker for tight junction (Fig. 1B). Moreover, all the cells showed β-III tubulin (Fig. 1C), but no MAP5 positive cells were observed (Fig. 1D).

Culture under conditions used for neural stem cell maintenance

We cultured the adult human RPE cells in the MEM with fetal bovine serum for several weeks (epithelial culture) and then transferred them to serum-free medium with bFGF and EGF on laminin/poly-L-ornithine coated dishes (stem cell culture). The shape of the cell bodies of H80HrPE-6 changed to spherical or spindle-like after 2 months of culture (Fig. 2A). ARPE-19 cells became elongated after several days and then spherical or spindle-like.

In this condition we could not observe pancytokeratin, ZO-1, MAP5, nor NF200 positive cells.

Differentiation induced for 10 days with retinoic acid

After 10 days in a differentiation culture, the cells extended multiple processes into the medium with

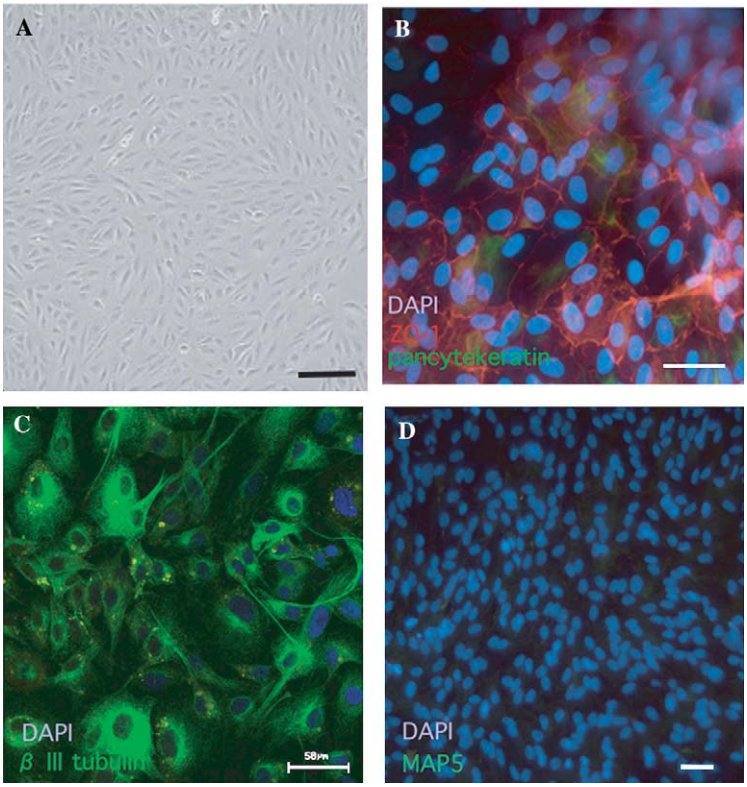

Fig. 1. RPE cells cultured in Eagle's minimum essential medium supplemented with 8% fetal bovine serum. (A) Phase contrast photograph. The cells show flat, epithelial-like morphology. The bar = 100 μm. (B–D) Immunocytochemistry for pancytekeratin (B, green) and ZO-1 (B, red), for β-III tubulin (C, green), and for MAP5 (D, green). Nuclei were stained with DAPI. The bar = 50 μm (B,C). The bar = 100 μm (D).

retinoic acid. All the cells were β-III tubulin-positive (Fig. 2B), and a few cells in the H80HrPE-6 culture and some in the ARPE-19 culture showed immunoreactivity for MAP5 (Figs. 2C and D). Some of the cells were also NF200-positive (Fig. 2D). However, we could not detect any GFAP- or rhodopsin-positive cells (Figs. 2E and F), and even when we tried to induce glial differentiation with a high concentration of FBS, no GFAP-positive cells were observed in either cell line.

Discussion

The RPE cells of many vertebrate species can dedifferentiate and transdifferentiate in response to changes in their environment. It is well known that in amphibians RPE cells transdifferentiate not only into lens cells, but also into retinal neurons [10,11,16]. It was reported that growth factors and extracellular matrix components are found to be important in the control of the transdifferentiation process of vertebrate pigmented epithelial cells [15].

In embryonic rat, RPE cells were seen to transdifferentiate into neural retina [11], although this ability was restricted until age E13 [14]. These findings show that environmental factors regulate the expression of neuron-associated genes in RPE cells.

To evaluate the potential of adult mammalian RPE cells for transdifferentiation into neural cells, we used two adult human RPE cell lines. ARPE-19 was harvested from the eye of a 19-year-old young adult. H80HrPE-6 is one of the cell lines which we isolated from an 80-year-old person, and the cells were cloned from a single cell. The original primary cells were shown to be capable of transdifferentiating into lens. It was reported that H80HrPE-6 cells became lens cells when they were cultured in hard agar and the other on MATRIGEL which contained growth factors, and this cell line expressed key genes of lens development and regeneration, Pax-6 and six-3 [15,17].

ARPE-19 is a retinal pigment epithelial (RPE) cell line derived in 1986 by Amy Aotaki-Keen from the normal eyes of a 19-year-old male. This cell line has been widely used in studies of RPE function as well as gene expression [18].

Under light microscopy, both ARPE-19 and H80HrPE-6 showed flat and polygonal epithelial shapes in medium with FBS. They proliferated constantly and could be passaged with the aid of trypsin EDTA. Cells of both lines remained healthy after freeze-and-thaw manipulation. These were common characteristics of the two cell lines. While neither cell line showed pigmentation during short-time culture, ARPE-19 cells contained pigment after several months' culture.

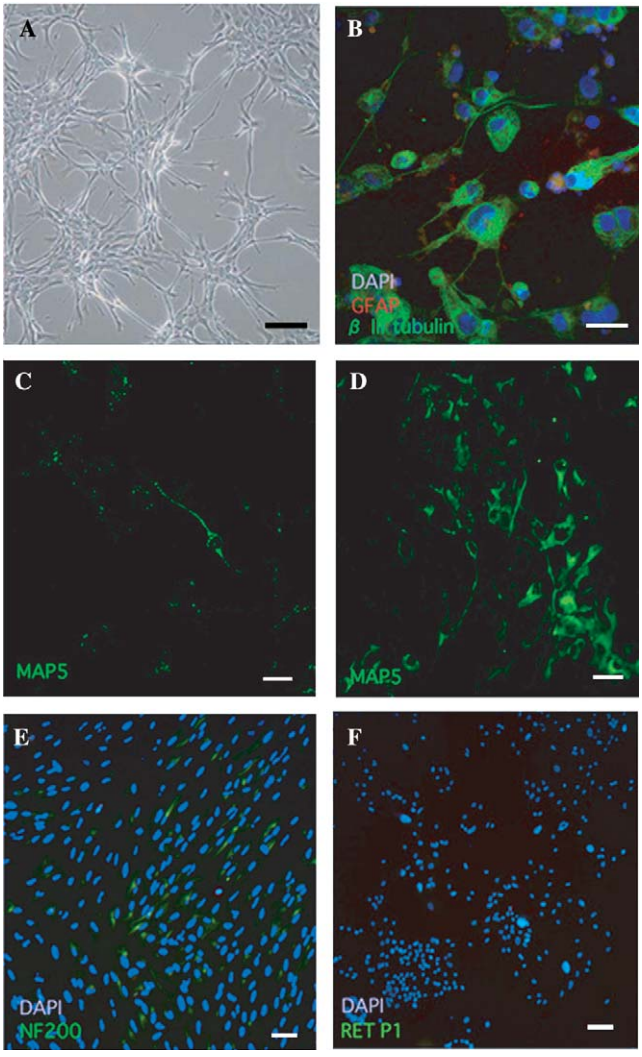

Fig. 2. (A) Phase contrast photograph of RPE cells cultured in serum free DMEM/F12 with bFGF and EGF on laminin/poly-L-ornithine-coated dishes. The bar = 100 μm. (B–F) RPE cells after induction of differentiation with retinoic acid without bFGF. Nuclei were stained with DAPI. (B) Immunocytochemistry for β-III tubulin (green) and GFAP (red). The bar = 50 μm. (C,D) Immunocytochemistry for MAP 5 (green) in ARPE-19 (C) and H80HrPE-6 (D). The bar = 100 μm. (E) Immunocytechomistry for neurofilament 200 K (green). The bar = 100 μm. (F) Immunocytochemistry for rod-opsin (RET P1) (green). The bar = 100 μm.

In a previous study, it was reported that once RPE cells were moved from an in vivo to an in vitro culture system, they began to express the class III isotype of β-tubulin [19], which is normally restricted to neuronal cell phenotypes in the retina [20,21]. In the same study, all the cells of both lines expressed β-III tubulin in a medium in which pigment epithelial cells can proliferate. From this result it appears that the RPE cells are closely related to neuronal lineage cells and easily acquire their characteristics in vitro.

Palmer et al. succeeded in isolating the neural stem cells from adult rat hippocampus tissue on laminin/poly-L-ornithine coated dishes with a bFGF containing medium. To induce differentiation, the cells were cultured in a medium with 0.5% serum and 500 nM all-trans retinoic acid [22,23]. We used the same methods for the RPE transdifferentiation by culturing the RPE cell lines on laminin/poly-L-ornithine coated dishes and in a medium with bFGF and EGF. After culturing under stem cell maintenance conditions, the shape of most cells changed to spherical or spindle-like, and the cells no longer expressed the RPE marker or ZO-1.

After the cells were cultured in the medium with retinoic acid to induce neural differentiation, some of them extended processes and not only β-III tubulin—but also MAP5-positive cells were detected. A few cells expressed neurofilament 200, which is a mature neuronal marker. These adult human RPE cells therefore have the potential to differentiate into mature neurons. The ratio of MAP5-positive cells was much higher in ARPE-19 than in H80HrPE-6, which means the younger RPE cells could more easily differentiate into neurons. However, it is remarkable that the RPE cells from an 80-year-old human could still maintain the ability to differentiate into neurons.

Another interesting point is that we did not observe any GFAP-positive cells even with 10% fatal bovine serum in culture medium. Neural stem cells usually differentiate into only glia with this high concentration of serum (unpublished data). Together with the fact that all of the cells were positive for β-III tubulin, it can be postulated that these dedifferentiated RPE cells attained the characteristics of neuronal progenitor like cells, so that they could not differentiate into glial cells. Moreover, it appears that we could not use β-III tubulin as a mature neuronal marker because the cells with a flat, epithelial-like morphology also proved to be immunoreactive for it in this study. We therefore consider β-III tubulin to be a neuronal lineage marker.

In summary, we showed that adult human retinal pigment epithelium cells are capable of differentiating into neurons. The ability to differentiate was greater in the young than in the old human RPE cell line, although the latter could still differentiate. Since we could not observe any photoreceptor marker or glial marker positive cells, conditions making it possible to obtain retinal neuronal cells from RPE cell lines should be examined further.

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