Phenotypic Plasticity of Retinal Pigment Epithelial Cells from Adult Human Eye *In Vitro*

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Phenotypic plasticity of retinal pigment epithelial cells from adult human eye was studied by immunohistochemical methods under different culturing conditions. It was found that retinal pigment epithelium in adult human eye is a heterogeneous population of cells demonstrating different behavior *in vitro*. Some cells retain epithelial morphology for a long time in culture, while others are rapidly transformed into fibroblast-like cells and synthesize proteins typical of proneural, neural, glial, and photoreceptor cells. However, irrespective of initial morphological features differentiation of retinal pigment cells can be modulated by varying culturing conditions.

Key Words: *retinal pigment epithelium of human eye; transdifferentiation; cell culture; immunohistochemistry*

Retinal pigment epithelium (RPE) represents a monolayer of highly polarized and highly pigmented hexagonal rarely dividing cells located between the retina and choroid. RPE participates in the formation of the blood-retina barrier and plays a very important role in vision [17].

In many vertebrate animals, RPE cells can transdifferentiate into retinal cells at early stages of embryonic development [18,26,27]. In some amphibians this capacity is retained in an adult state. In caudated amphibians (*Urodela*), damage or removal of the retina triggers transdifferentiation of RPE cells into neural and glial cells leading to complete retina reconstruction [13,14].

In adult humans, RPE is presented by highly differentiated weakly proliferating cell population [16]. However, damage or pathological states of the eye can induce enhanced proliferative activity RPE cells and their migration to subretinal space. The migrating cells can transdifferentiate into macrophage- and fibroblast-like cells. These pathophysiological events are underlain by processes accompanying transdifferentiation in lower vertebrates (proliferation, displacement from the layer, and migration). Activation of progenitor cells presumably located in RPE cannot also be excluded [7,9]. *In vitro* study of RPE cells from human eye brings us closer to understanding of fundamental mechanisms of phenotypical modification of these cells which can underlay a number of retinal pathologies.

Here we studied the capacity pf RPE cells from adult human eye to phenotypic changes *in vitro*. To this end, we studied the behavior and morphological changes in RPE cells and analyzed the obtained cultures using a wide set of antibodies, in particular, markers of neural differentiation, under different culturing conditions.

MATERIALS AND METHODS

Autopsy material (eyeballs from adult cadavers, 24-73 years) obtained from Moscow forensic morgue was used in the study. The material was obtained and processed within 12-48 h after death. Additionally,

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specimens obtained during surgeries for traumatic eye injuries were used. This material was processed within 5 h after eye excision. RPE cells were isolated from the eyeballs under a binocular lens. To this end, the posterior sector of the eye was separated, the vitreous body and the retina were removed, and cold Hanks saline with EDTA (Sigma) was poured in the eyeball and incubated for 10-30 min. The cells detached from the choroid were thoroughly collected with a pipette. Enzyme treatment of RPE cells was not carried out.

The cells were cultured at 37°C and 5% CO_2 in the following two media: DMEM/F12 (1:1, Sigma), 10% FBS (Sigma), 2 mM L-glutamine (Sigma) and DMEM/F12 (1:1, Sigma), 1% FBS (Sigma), 2 mM L-glutamine (Sigma), N2 additive (1:100, Invitrogen), 20 ng/ml FGF-2 (Sigma), 20 ng/ml EGF (Sigma), 8 μg/ml heparin (Sigma). The latter medium containing growth factors is used for maintenance and accumulation of neural SC and progenitor cells. In both media, the cells grew in the form of adherent cultures. After attaining confluence, the cells were subcultured with trypsin (Sigma).

For obtaining suspension cultures, the cells were cultured in serum-free medium containing growth factors: DMEM/F12 (1:1, Sigma), 2 mM L-glutamine (Sigma), N2 additive (1:100, Invitrogen), 20 ng/ml FGF-2 (Sigma), 20 ng/ml EGF (Sigma), 8 μg/ml heparin (Sigma). Seven days after formation of spherical floating structures, the cultures were transferred to a medium with 10% serum for induction of differentiation.

For immunohistochemical analysis, the cultured cells and eyeballs from adult human cadavers were fixed in 4% paraformaldehyde on 0.01 M PBS (pH 7.4). After fixation, the samples were 3 times washed with PBS, incubated in 5, 10, and 20% sucrose in PBS, placed in Tissue Freezing Medium (Leica Microsystems Nussloch GmbH), and frozen in liquid nitrogen. Serial 12-μ cryosections of eyes were placed on gelatin-coated slides.

After washout with PBS, the samples (eye sections and cell cultures) were subjected to permeabilization in a solution containing 0.1% Triton X-100 and 0.25% Tween on PBS (30 min) and then nonspecific binding was blocked in 3% BSA and 0.25% Tween on PBS (30 min). Then the samples were incubated for 1 h at 37°C in PBS containing 3% BSA and 0.25% Tween with primary antibodies to the following proteins: RPE65 (marker of retinal pigment epithelium, Abcam, 1:250), CRALBP (marker of retinal pigment epithelium, Abcam, 1:400), connexin-43 (Cx43, gap junction marker, Sigma, 1:200), N-cadherin (marker of cell contacts typical of neural cells, Sigma, 1:200), nestin (marker of neural SC, Chemicon, 1:200), βIII-tubulin (early neuroblast marker, Abcam, 1:200), recoverin (marker of photoreceptors, antibodies are kindly provided by Prof. P. P. Filippov, 1:50), Ki-67 (marker of proliferating cells, Abcam, 1:100), and fibronectin (extracellular matrix protein, Abcam, 1:500). After washout, the specimens were treated with fluorescence-labeled secondary antibodies. Nuclei were stained with Hoechst 33342. Stained preparations were embedded in glycerin and analyzed under an OLIMPUS luminescent microscope with a DP70 digital camera.

RESULTS

Characteristics of intact RPE. Heterogeneity of RPE cells in adult human eye was observed even in the total preparation of intact eye tissue. Phase contrast microscopy revealed layer heterogeneity: the cells differed by size, shape, pigmentation degree, and number of nuclei (Fig. 1, *a*). Heterogeneity of the population was also confirmed by the results of immunohistochemical analysis (Fig. 1, *b, c*): not all RPE cells contained CRALBP, a specific marker of RPE cells (Fig. 1, *b*). CRALBP-negative cells were highly pigmented. Solitary medium-pigmented cells were stained with antibodies to nestin (Fig. 1, *c*). Moreover, these cells showed immunopositive reaction to Cx43. This protein is involved in the formation of gap junctions ensuring the integrity and barrier function of the epithelium. However, N-cadherin, a protein of adherens junctions of neural cells, was absent. Intact RPE was Ki-67 negative, which attests to the absence of proliferation. No staining after treatment with antibodies to neural differentiation markers of RPE cells was observed, while the retina was immunopositive. Thus, nestin-positive cells (presumably Muller glial cells), βIII-tubulin-positive cells (ganglionic cell layer), and recoverin-positive photoreceptors were found in the neural retina.

Characteristics of adherent RPE cultures. RPE cells from adult human eye adhered and grew in DMEM/F12 with 10% serum. Two main cell types were revealed: flat polygonal epithelial cells in the center of colonies and elongated or flattened fibroblast-like weakly or non-pigmented cells at their periphery (Fig 1, *d, e*). Similar morphotypes of RPE cells *in vitro* were described by other authors [5,6,17,21,27]. Some researchers believe that fibroblast-like morphology appears due to loss of contacts [4] and vast space for growth [16]. This phenotype is not constant, but transitory [20]. Others think that acquisition of fibroblast-like morphology is related to de- and transdifferentiation of RPE cells [5,26].

Cell contact proteins Cx43 and cadherins are specific markers for most cells of epithelial morphology. In RPE culture these markers did reveal epithelial cells and were located exactly in sites of cell-cell contacts (Fig. 2, *a*). At the same time, only solitary cells among

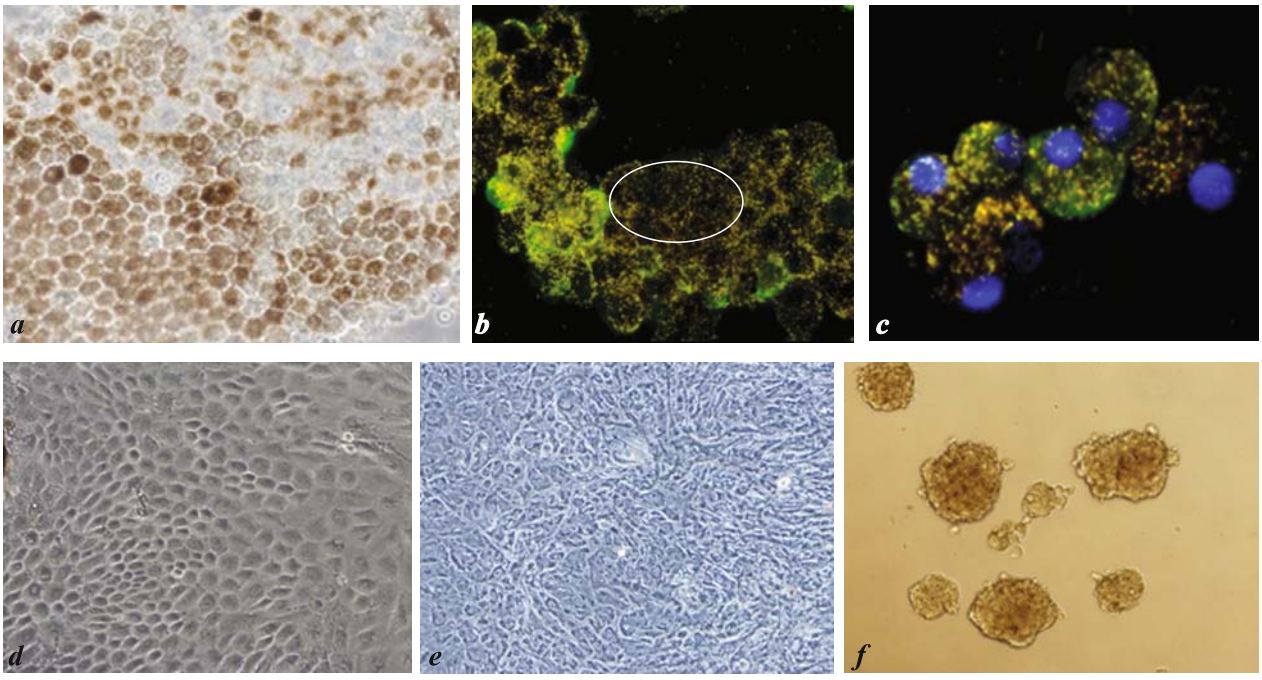

Fig. 1. Heterogeneity of retinal pigment epithelial cells from adult human eye: intact specimen (*a-c*) cell culture (*d-f*). *a*) cells differ by the size, shape, pigmentation, and number of nuclei (×200); *b*) CRALBP-negative cells (encircled area) among CRALBP-positive cells (green; ×400); *c*) solitary nestin-positive cells (green; ×400); *d*) cells with epithelial morphology in culture grown in DMEM/F12 with 10% serum (×200); *e*) cells with fibroblast-like morphology in culture grown in DMEM/F12 with 10% serum (×200); *f*) freely floating spherical aggregates cultured in DMEM/F12 with FGF-2, EGF, and N2 (×200). *a*, *d-f*: phase contrast; *b*, *c*: immunofluorescence.

fibroblast-like cells produced Cx43 and contained N-cadherin in the cytoplasm (Fig. 2, *b*). It is known that Cx43 plays an important role in the formation of RPE cells and its expression indicates their differentiated state [10,12]. The absence of this protein suggests that the cells are in a state of partial dedifferentiation (at least by this marker). According to published data, N-cadherin is a dominant cadherin in cultured RPE cells. Its expression in the epithelium can attest to transition from epithelial to mesenchymal phenotype and acquisition of migration ability [3].

During culturing, many cells were stained with antibodies to Ki-67 (Fig. 2, *c*). Proliferating cells were more incident among fibroblast-like than among epithelial cells. However, the number of Ki-67-positive proliferating cells decreased from passage to passage.

In adherent culture, RPE cells demonstrated behavior and phenotypic characteristics differing from those *in vivo*. They actively proliferate and lost pigmentation. The number of pigment granules in cells rapidly decreased with increasing the number of cell divisions and after attaining confluence the monolayer was presented by depigmented cells. In RPE cells, the synthesis of marker protein RPE65 was terminated as soon as at passage 0, which was shown immunochemically by using RPE-65 antibodies. Specific staining for RPE marker CRALBP was found in solitary cells of primary and subcultured cultures (Fig. 2, *d*).

Considerable changes in synthetic processes in RPE cells *in vitro* were detected immunohistochemically using neural markers. The culture contained numerous nestin-positive (marker neural precursor cells) elongated bipolar cells (Fig. 2, *e*). Double staining with antibodies to Ki-67 and nestin showed that nestin-positive cells proliferated. In many clusters of fibroblast-like cells, βIII-tubulin (early neuroblast marker) was detected. Fibroblast-like βIII-tubulin-positive cells had different morphology. Among them, both flattened and elongated bipolar cells were seen, some cells contained solitary melanin granules. Positive staining for Pax6 was found in RPE cells of passages 1 and 2 (Fig. 2, *f*). Pax6-positive cells had primarily fibroblast-like morphology and only solitary cells demonstrated epithelial phenotype. The presence of Pax6 attests to dedifferentiation of RPE cells in culture, because this factor is known to be expressed in neuroepithelial precursor cells [15] and mature neural retina, but is absent in differentiated RPE [12,13]. We hypothesized that Pax6-positive cells can differentiate towards neuronal lineage, because the culture simultaneously contained a large population of nestin- and βIII-tubulin-positive cells (Fig. 2, *e*).

However, after passage 3 the culture lost the signs of proneuronal differentiation and was primarily presented by fibroblast-like cells actively synthesizing extracellular matrix component fibronectin (Fig. 2, *h*).

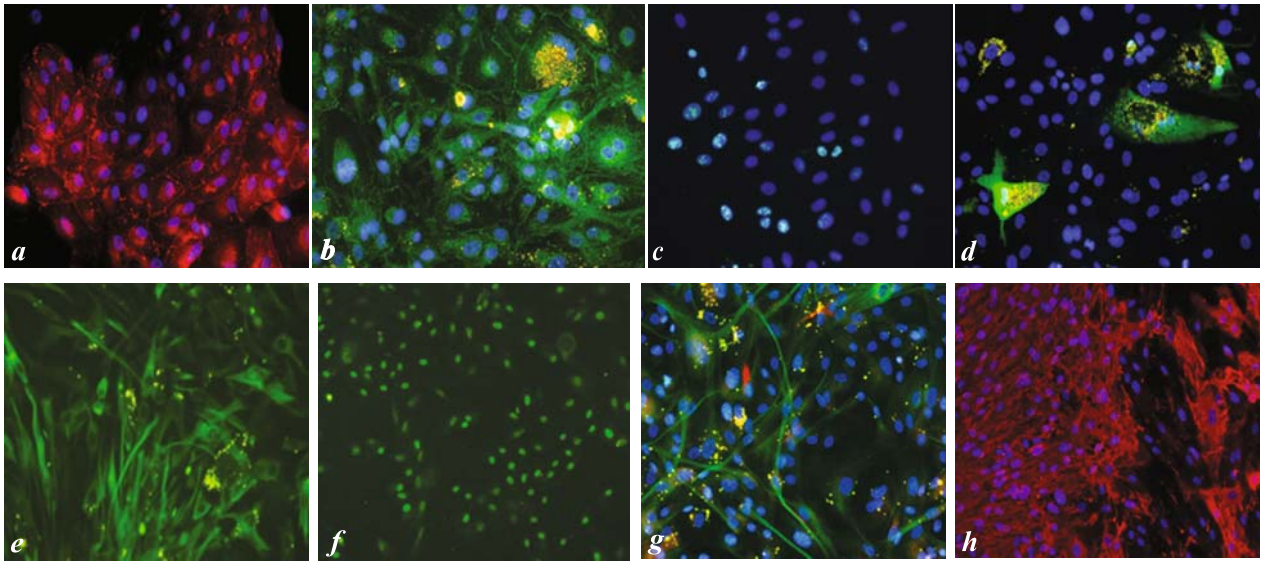

Fig. 2. Immunohistochemical analysis of adherent cultures of retinal pigment cells from adult human eye grown in DMEM/F12 with 10% serum (*a-f, h*) and DMEM with 1% serum, N2, FGF-2, EGF (*g*). *a*) preserved gap junctions (Cx43, red) in cells with epithelial morphology in passage 2 culture (×200); *b*) adherens junction protein (N-cadherin, green; ×200); *c*) proliferating cells (Ki-67, green; ×200); *d*) solitary passage 1 cells stained with antibodies to CRALBP (green; ×200); *e*) nestin-positive cells in passage 3 culture (×100); *f*) Pax6-positive cells in passage 1 culture (green; ×40); *g*) neuroblasts (βIII-тубулин, green) and GFAP-positive cells (red) in passage 1 culture (×100); *h*) fibronectin-positive cells (red) in passage 3 culture.

This suggests that RPE cells only temporary exhibit properties of neuronal cells and these signs disappear during long-term culturing under the specified conditions. Active synthesis of fibronectin can attest to transition from epithelial to mesenchymal phenotype and cell development towards myofibroblasts [8].

Experiments showed that RPE cells cultured in DMEM/F12 meium with 10% serum formed two morphologically different cell types, epithelioid and fibroblast-like. Both populations contained proliferating cells; their content among fibroblast-like cells was considerably higher than among epithelial cells. Cell proliferation was accompanied by their depigmentation and expression of differentiation features not characteristic of RPE. This phenomenon was confirmed by the results of other researchers [2,16,19,23]. Expression of βIII-tubulin attests to cell ability to neuronal differentiation, but under the specified culturing

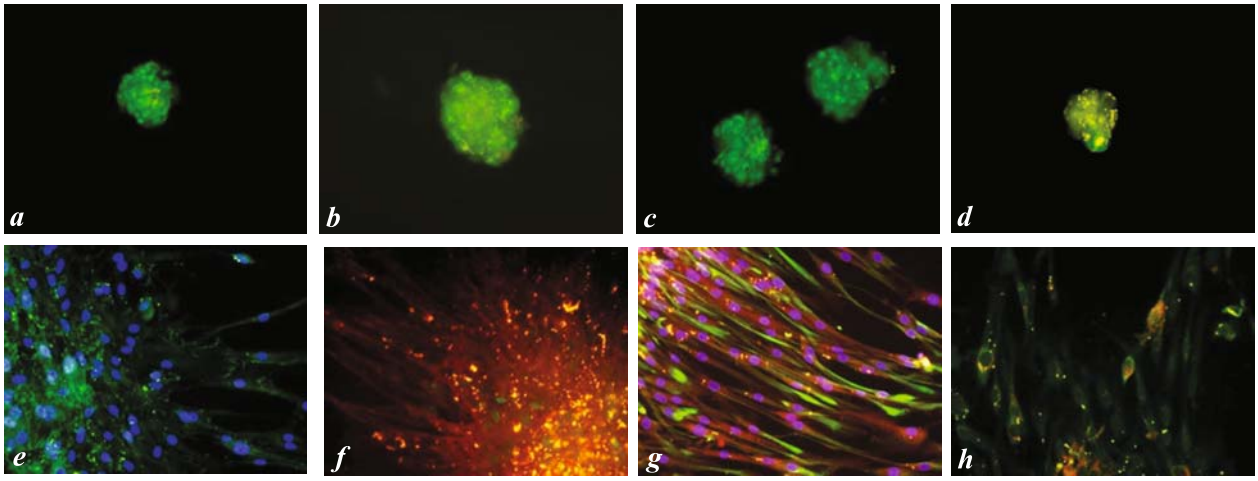

Fig. 3. Immunohistochemical analysis of retinal pigment epithelial cells from adult human eye: freely floating spheres in serum-free medium (*a-d*) and differentiated spheres in DMEM/F12 with 10% serum (*e-h*). Double staining (*f-h*). *a*) Ki-67-positive cells (green; ×100); *b*) Pax6-positive cells (green; ×100); *c*) cells stained with antibodies to nestin (green; ×100); *d*) solitary cells stained with antibodies to βIII-tubulin (green; ×100); *e*) N-cadherin (×200); *f*) fibronectin-positive migrating cells (red) and soliotary Pax6-positive cells (green; ×200); *g*) neuroblasts (βIII-tubulin, green) and glial cells (GFAP, red) among migrating RPE cells (×200); *h*) solitary recoverin- (red) and βIII-tubulin-positive cells (green; ×200).

conditions this capacity was preserved only before passage 3.

An attempt was undertaken not only to stimulate, but also to maintain neuronal differentiation by culturing RPE cells in a medium containing growth factors (DMEM/F12 with 1% serum, N2, FGF-2, EGF). In this medium, cell growth corresponded to the above-described regularities. The cultures contained weakly pigmented and depigmented epithelial and fibroblast-like cells capable of proliferation. Epithelial cells and some fibroblast-like cells produced Cx43 and N-cadherin. Dedifferentiation was confirmed by the presence of Pax6-positive cells and large population of nestin- and βIII-tubulin-positive cells. However, an important peculiarity was revealed in differentiation of RPE cells. Expression of GFAP, a marker of glial cells, was noted in some fibroblast-like cells cultured in a medium with growth factors as soon as during passage 1 (Fig. 2, *h*). Nevertheless, despite the observed signs of glial differentiation under the specified culturing conditions, the cell ability to neural differentiation disappeared after passage 3.

Comparison of adherent cultures grown in different media showed that some RPE cells underwent neural differentiation irrespective of culture medium composition. At the same time, factors FGF-2 and EGF stimulated RPE cells to differentiation towards a greater number of lineages, which was seen from the presence of βIII-tubulin- and GFAP-positive cells, although this property was only temporary.

Characteristics of suspension RPE cultures. At the final stages of the experiment we studied floating spherical aggregates of RPE cells formed in response to transfer of an adherent culture to a serum-free medium containing FGF-2, EGF, and N2. These formations consisted of pigmented and partially depigmented cells (Fig. 1, *f*). Immunochemical analysis showed that cells in spheres differed from initial adherent RPE cells by the absence of gap junction marker protein Cx43, and hence, did not exhibit epithelial properties. Moreover, the spheres contained a large number of dedifferentiated cells, which was revealed by staining with antibodies to Pax6. Pax6-positive cells in some spheres actively proliferated (Fig. 3, *a*, *b*). Some cells were able to neural differentiation: these cells were stained with antibodies to nestin and solitary cells expressed βIII-tubulin (Fig. 3, *c, d*).

For stimulation of cell differentiation, the culture medium was replaced with the so-called differentiation medium (without factors and with 10% serum; a method used for stimulation of differentiation of SC from the brain). Under these conditions, the neurospheres attached to the bottom of the culture flask and their cells started active migration. On day 2, a sharp decrease in proliferation and initiation of cell differentiation were observed. The cells from spherical aggregates differentiated into neurons and glia, while some cells demonstrated positive staining for GFAP and/or βIII-tubulin and for βIII-tubulin and/or recoverin (Fig. 3, *g*, *h*). In cells of spheroid cultures, expression of recoverin (photoreceptor marker) was noted. It was seen in perinuclear and peripheral cytoplasm in the form of small and large granules; cells differed by recoverin distribution in cytoplasm areas.

Examination of *in vitro* formed cells showed that they consisted of weakly pigmented cells that lost their epithelial properties. They included numerous non-differentiated Pax6-positive cells. Hence, conditions permissible for the formation of spherical aggregates lead to destabilization of differentiation of all RPE cells irrespective of their initial properties. Spheres obtained from RPE are similar to spheres of ciliary area [7], but differ from neurospheres from the brain by the structure and passageability [1,15,25]. RPE cells form spheres demonstrated multipotency, which manifested in their neuronal, glial, and photoreceptor differentiation.

Thus, our experiments showed that RPE of adult human eye is initially presented by a heterogeneous cells population consisting of at least two cell subtypes differing by adhesion properties, migration, phenotypic changeability, and responses to microenvironmental factors. In culture, some cells retain epithelial properties, while others demonstrate phenotypic plasticity. Nevertheless, culturing under targeted conditions can induce dedifferentiation of all RPE cells irrespective of their initial phenotype. It can be assumed that stable neuronal differentiation of RPE cells can be ensured by using media and factors promoting these phenotypic manifestations.

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