New Method of *In Vitro* Culturing of Pigment Retinal Epithelium in the Structure of the Posterior Eye Sector of Adult Rat

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We propose a new method of organotypic roller 3D-culturing of the posterior sector of the eye. The method allows maintaining tissue viability *in vitro* for 14 days (which considerably surpasses the capacities of stationary culturing) and studying of the behavior of pigment retinal epithelial cells and choriocapillary membrane. Using this method we demonstrated phenotypic transformation, migration, and proliferation of pigment retinal epithelial cells under conditions of roller organotypic culture. In the absence of the retina, these cells exhibit properties of scavenger cells (phagocytes) both within and outside the layer. Under conditions of roller culturing *in vitro*, cells of the pigment retinal epithelium undergo changes similar to those observed in various retinal pathologies *in vivo*, including age-associated changes. Here we discuss the possibility of using the proposed method for evaluation of the effect of various factors added to the culture medium on the pigment epithelium, for modeling of processes developing in damaged pigment epithelium or under conditions of various pathologies, and for the study of regeneration responses in cells of pigment retinal epithelium in adult vertebrates.

Key Words: *roller culturing; rat; pigmented epithelium; choroid*

Many eye diseases in humans and mammals are associated with pathology of the retinal pigmented epithelium (RPE), Bruch's membrane, and choroid [8,16,24,30]. Some of these diseases, *e.g.* macular degeneration and proliferative retinopathy, are associated with aging. In adult mammals, accumulation of lipofuscin granules in PRE [4,26] and the formation of druses between the basal membrane of PRE cells and Bruch's membrane are observed during aging [3,7,20]. Accumulation of lipofuscin in the choroid, increased permeability of Bruch's membrane, and translocation of choroidal endothelial cells were also described. Evaluation of various pathological states of PRE and study of the effect of some factors and preparations require simple and adequate animal models *in vitro*.

An important aspect is evaluation of the regeneration capacities of the retina in mammals. High regeneration capacity is typical of the retina in some low vertebrates, *e.g.* in fishes and amphibians, but in birds and mammals it is lost [1]. However, it was found that PRE cells in adult rat can exhibit properties of neural cell precursors [6], while human PRE cells can differentiate into neurons *in vitro* [2]. An *in vitro* organotypic model of PRE culture is required for systemic study of the proliferation and differentiation potencies of PRE cells from adult rats, evaluation of their regional differences by these properties, as well as controlled modulation of re-

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generation capacities of these cells with regulatory factors.

The properties of PRE from human and mammalian eye are usually studied on primary cell cultures or cell strains derived from this tissue *in vitro* [10,15,18]. Organotypic culturing is used for evaluation of the neural retina from eyes of adult mammals and embryos [19,23,25,28,29]. High viability and maintenance of the basic layer-by-layer structure of the neural retina in neonatal and early postnatal rodents and the possibility of its further development *in vitro* were reported.

The role of PRE in the development of neonatal retina in rats [22] and chicks [14] was studies in some works. However, there are no data on organotypic *in vitro* culturing of PRE tissue in the structure of posterior eye wall (PEW) from adult mammals. At the same time, this *ex vivo* model can be very useful for the evaluation of regeneration capacities of PRE cells and the effect of various factors on these cells and for understanding of the processes occurring in PRE at the cellular and molecular levels in various pathologies of the retina in adult mammals and humans.

We present data that PRE in the structure of PEW of adult albino Wistar rats survived for 2 weeks under conditions of roller culturing *in vitro* despite the death of some cells. Proliferation of PRE cells and changes in their behavior and phenotype were observed under these conditions. Considerable changes were also observed in the choroid: translocation of some endothelial cells in the vitreal direction against the background of preserved cytoarchitectonics. Many changes observed in PRE and choroid were similar to those observed in various eye pathologies in adult mammals and humans.

MATERIALS AND METHODS

Freshly isolated eyes from normal albino Wistar rats (from vivaria of N. K. Kol'tsov Institute of Developmental Biology and Moscow State University) were used for culturing of PRE in the structure of rat PEW after removal of the neural retina. The rats were narcotized, the eyes were removed, washed with 70% ethanol, placed in cold DMEM on ice, and transferred into sterile box. Under conditions of sterile box, the eyes were placed in Perti dishes with culture medium and the anterior sector containing the cornea-iris complex and the lens was isolated under a binocular magnifier. The PEW (PRE—choroid—sclera complex) was incubated with medium with EDTA for 5 min and the neural retina was completely removed using glass tubes with ball-shaped ends. To this end, the retina was separated by the periphery (near *ora serrata*) going deep into the eye center, the optic nerve was carefully cut, and the retina was separated. After that PEW was completely freed from the retina and external tissues and included pigment epithelium, choroid, and sclera. PEW preparations (a total of 12) were placed into special 20-ml vials (Wheadon) with 5 ml culture medium, closed with sterile cups and sealed with Parafilm. Sterile culture medium contained DMEM with phenol indicator (Institute of Poliomyelitis), L-glutamine (300 mg per 500 ml medium), 10% FCS (10 ml per 100 ml medium), and antibiotic. The tissues were cultured with constant rotation (60 rpm) on a miniroller RM-1 (Elmi) in darkness at 35.5°C for 7, 10, and 14 days. During 14-day culturing, the medium was replaced once (after 7 days). During rotation PEW samples were suspended in the medium and did not adhere to flasks, which prevented their deformation and improved nutrition. After the end of culturing, the roller was stopped, the preparations were removed from flasks, washed with medium, fixed in individual tubes in Bouin fluid, washed in 70° ethanol, dehydrated, and embedded in paraffin. Serial cross-sections (7 μ) were prepared for histological analysis.

For morphological analysis of initial PRE (before culturing), freshly isolated PEW were fixed in Bouin fluid, processed and sectioned as described earlier. The sections were stained with hematoxylin and eosin, embedded in balm under coverslips, examined and photographed at different magnifications.

For stationary culturing of PRE in the structure of PEW, free from neural retina PEW were transferred into 35-mm glass Petri dishes with 2 ml DMEM and placed into a CO_2-incubator (37°C, 6% CO_2); the medium was carefully replaced with a fresh portion every 24 h. After 3 days the preparations were carefully transferred into tubes with Bouin fixative. After 24 h the fixative was washed with 70° ethanol, the preparations were dehydrated, embedded into paraffin, and processed for morphological analysis as described earlier.

For the study of intact PRE of normal eyes of adult (2-3-month-old) rats, whole amount preparations of the retina were isolated using dissociating media. Immediately after enucleation, the eyes were placed into cold DMEM, freed from muscles and connective tissue, and transferred into cold Ca^{2+}-free Hanks solution containing 10 mM EDTA (pH=7.4). The anterior and posterior sectors of the eyes were separated under a binocular magnifier. Then, a microsurgical cut was made outside the limbus and the growth zone of the retina. Neural retina remained within PEW. Eyecups with the neural retina were washed 3-fold with cold Hanks saline with EDTA,

after that PEW were incubated in Hanks saline containing hyaluronidase IV (220 U/ml) and collagenase (65 U/ml) for 12 min at 37°C. Then PEW were again transferred into cold Hanks saline with EDTA and the retina was completely removed with glass sticks. PEW were one more time incubated with all three dissociating factors for 8 min at 37°C, transferred into cold Hanks saline with EDTA, and washed twice from collagenase and hyaluronidase. Incubation for 30 min at room temperature led to separation of PRE from Bruch's membrane. Then, the epithelium was separated along the edge and then into the depth of the eyecup with glass sticks or fine forceps, collected with a 200-μ pipette, and carefully transferred to clean albumin-coated coverslips. Moisture excess around the tissue was soaked and the coverslips were treated with formalin vapor for soft fixation of the preparations. After 1-day fixation, PRE preparations were washed, stained with Karacchi hematoxylin, poststained with eosin, and embedded into balm under coverslips.

Stained sections of PRE in the structure of PEW before and after culturing and stained layers of PRE from eyes of adult rats were analyzed under Jenaval (Carl Zeiss, JENA) and Olimpus AH-3 microscopes. The images were recorded using Olimpus AH-3 microscope, digital camera, and computer equipped with Studio Lite and Viewfinder Lite software. Cell viability was evaluated by counting the percent of pyknotic nuclei in PRE layer on 100-150 cross-sections (7 μ) from the peripheral and central areas of PEW from each eye. The number of transformed PRE cells was counted on 100-150 sections (the number of PRE cells lost epithelial morphology and leaving the layer, but remaining in immediate proximity to it).

RESULTS

On preparation of PEW, PRE is seen as a single-row layer of non-pigmented binuclear cells and few mononuclear elongated cells with poorly discernible lateral boundaries (Fig. 1, *a*, *b*) lying on Bruch's membrane adjacent to the choroid. These structures tightly adhere to each other and represent a single complex adjacent to the sclera. In rat eye, the choroid has a developed nonuniform structure, vascular network consisted of blood vessels of different diameter. Cells of the vascular network are presented by vascular endotheliocytes, dendritic melanophores (lacking pigment in albino rats), fibroblasts, mast cells, and macrophages. Dense fibrous sclera lying behind the choroid is normally responsible for mechanical tension and maintenance of the topology of PRE and choroid.

A layer of PRE cells isolated from eyes of albino rats was formed by densely packed hexagonal cells (Fig. 1, *c*). Most of them had 2 nuclei of similar size containing 1-2 nucleoli. This typical feature (presence of 2 nuclei) can be a specific marker of PRE in adult rats. Apart from binuclear cells, PRE

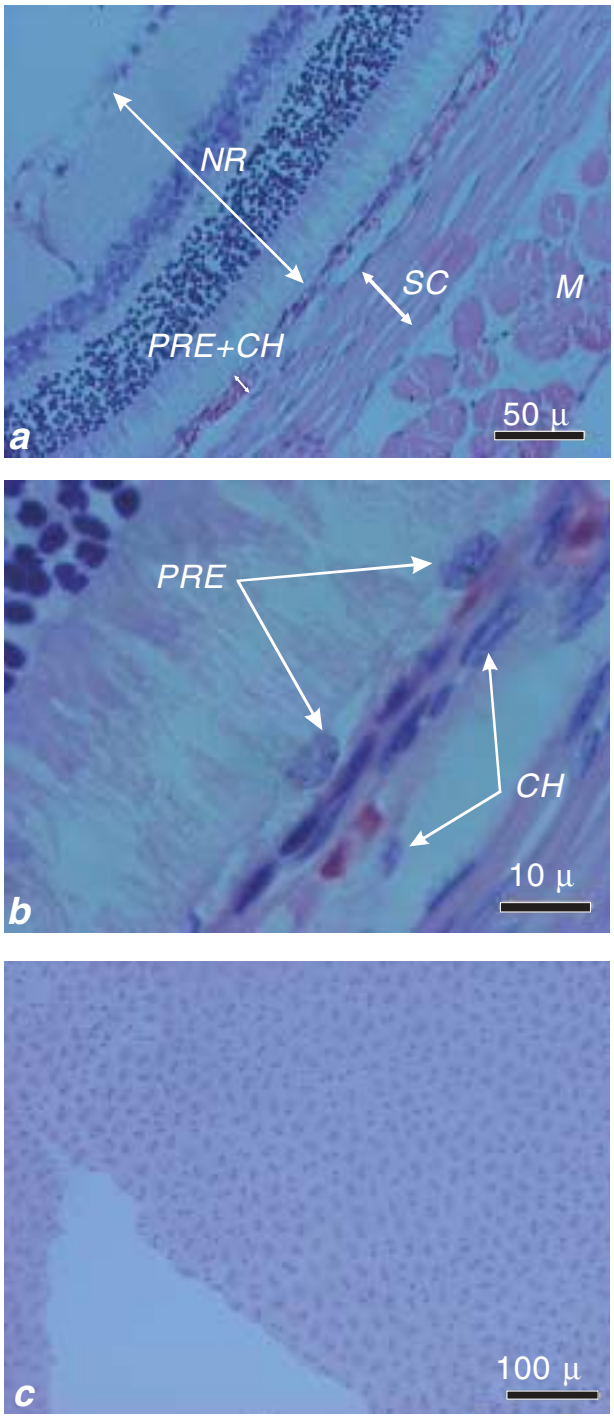

Fig. 1. Pigment retinal epithelium in the structure of PEW (*a, b*) and PRE layer (*c*) isolated from normal eye of adult albino rat. Here and on Figs. 2-6: NR: neuroretina; CH: choroid; SC: sclera, M: muscles.

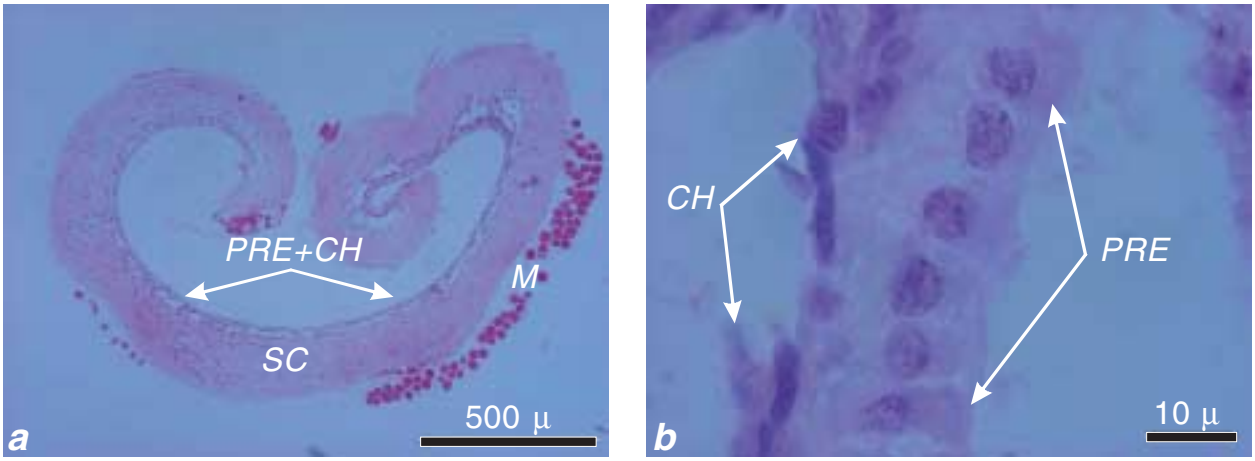

Fig. 2. Pigment retinal epithelium in the structure of posterior eye sector of adult rat during roller culturing *in vitro*. *a*) general appearance or PEW *in vitro*; *b*) pigment retinal epithelial layer.

contained few mononuclear cells with larger nucleus (compared to binuclear cells). The cytoplasm of PRE cells in albino rats contained no melanin granules and was evenly and uniformly stained, which allowed visualization of lipid inclusions accumulated during phagocytosis. Comparison of central and peripheral PRE areas revealed no morphological differences between PRE cells and their displacement outside the PRE layer. The study of 8 eyes obtained from 4 animals revealed no mitotic figures.

The PEW tissues retained their basic structure and location under conditions of 3-day stationary culturing *in vitro*, but many cells died. Counting of viable and dead (pyknotic nuclei) cells on preparations showed that on day 3 in culture cell death attained 57%. On day 7 of culturing we observed exfoliation of PEW tissues, disorganization and massive death of cells in PRE and choroid.

Soon after the start of roller culturing, PEW preparations changed their shape because of invagination of their edges and looked as spheres with edges turned inward (Fig. 2, *a*). Morphological study showed that PRE in these structures did not degrade and was presented by an intact layer adhering to the choroid and sclera (Fig. 2, *b*), which made possible not only analysis of PRE cells, but also preliminary quantitative study.

On day 7 of roller culturing of PEW preparations we observed relatively low rate of cell death: cells with pyknotic nuclei constituted not more than 10% of the total number of PRE cells. In three PEW tissues, the number of pyknotic nuclei was maximum in small vessels, which can be explained by the presence of blood cells remaining after eye enucleation. Pronounced macrophage reaction was also observed in PEW; this reaction is characteristic of both PRE cells and true monocyte-derived macrophages. Conversion of differentiation of some PRE cells towards macrophages manifested in changes in their shape and localization: hexagonal cells flattened on Bruch's membrane leaving the PRE layer acquire oval or round shape (Fig. 3, *a*). Morphology of these PRE cells and true macrophages is different (Table 1, Fig 3, *a*, *b*), and the behavior of PRE cells can be evaluated independently of histiocyte populations. Direct counting of PRE cells at the state of macrophage transformation on serial sections showed that the number of displacements in the peripheral and central areas of PEW was 6-14 and 11-30 cells per section, respectively. One more feature is the presence of solitary mitoses primarily at the periphery of the epithelial layer (5 per 1.5 mm^2 PEW). There were no mitotic cells among cells displaced from PRE.

TABLE 1. Morphological Differences of PRE Cells Transforming towards Macrophages and Resident Macrophages

Morphological unit	PRE cells	Resident macrophages
Cell shape	Oval or round	Round
Nucleus	One or two, regular round shape, loosely stained	One, displaced to the periphery, sickle-shaped, densely stained
Cytoplasm	Evenly stained, light blue	Vesicular, with yellowish or gray shade

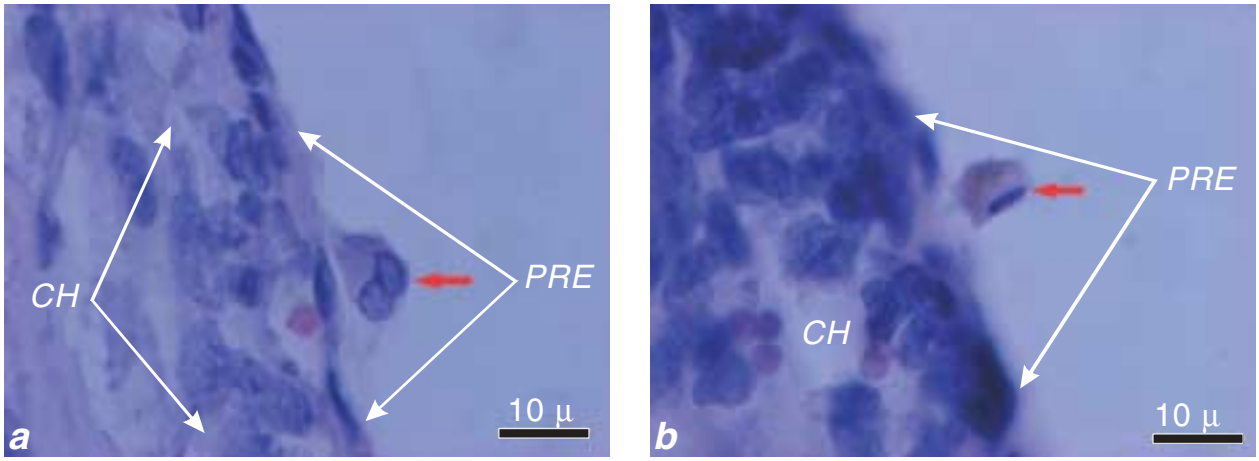

Fig. 3. Displacement (arrow) of pigment epithelial cell (*a*) and resident macrophage (*b*) inside beyond PEW during roller culturing *in vitro*.

Morphological analysis of PEW after 7-day roller culturing revealed also transmigration of choroidal cells: they migrated towards the PEW cavity through Bruch's membrane and PRE layer, which led to mixing of endothelial and PRE cells and blurring of the boundaries between the layers (Fig. 4, *a*). Mitoses of endothelial cells were somewhere seen in the choroid (Fig. 4, *b*).

Analysis of serial sections of PEW after 10-day roller culturing revealed disturbed organization of PRE layer in some areas manifested in irregular arrangement of cells and their nuclei relative to each other. Similarly as on day 7, we observed pyknotic nuclei; different cells sometimes contained 1-2 pyknotic nuclei. The percentage of pyknotic cells was 15-20% of total number of cells on serial sections, which somewhat surpassed the corresponding parameter after 7 days of roller culturing, but was almost 3-fold lower than after 3 days of stationary culturing. On day 10, the number of mitoses increased compared to the 7th day. Mitotic figures (24 per 1.5 mm^2 of PEW area) were regularly seen, primarily at the periphery of PRE. Similarly as on day 7 of roller culturing, migration of PRE cells from the layer and their morphological transformation into cells of macrophage phenotype were observed on serial sections of PEW. The displaced cells can pass long distance towards the PEW cavity. Direct counting of these cells near the inner surface of PRE showed that their number in peripheral and central areas of PEW was 3-5 and 7-15 per section, respectively. Despite the number of displaced cells on day 10 was lower than on day 7, macrophage transformation of PRE cells in the central zones of PEW was still more pronounced than in the peripheral zones. These cells contained either one, or two nuclei, but there was no correlation between the number of nuclei and capacity of PRE cells to displacement from the layer. In some cases, the number of nucleoli increases to 3-4, which attests to enhanced synthetic activity of these PRE cells. It was also noted that one nucleus of PRE cells was often displaced towards the lumen of choroidal vessels. Pronounced changes in the choroid were also seen after 10-day roller culturing of PEW: its thickness increased, choroidal cells initially located around blood vessels were loosened and oriented in different directions, and migrated in the vitreous direction. Some endothelial cells of the choroid were displaced through Bruch's membrane and PRE layer and choroidal cells and PRE cells were mixed in some areas.

After 14 days of roller culturing of PEW preparations, changes in PRE and choroid observed on days 7 and 10 progressed and became pathological. All PEW tissues were still present, but the complex was thinned due to cell loss and separated from the sclera. The number of cells decreased in both tissues of the complex, apparent organization of the choroid disappeared, cell contacts in PRE were impaired. We observed a great number of macrophages derived from epithelial cells (PRE) and monocytes (resident macrophages). The population of macrophages increased primarily at the expense of PRE cells, because under *in vitro* conditions migration of histiocytes from the circulation is absent. PRE cells left the layer and migrate not only towards the inner cavity of PEW (in the vitreal direction), but also to the space between the choroid and sclera, which confirmed degradation of Bruch's membrane. Disintegration of PRE layer due to macrophage transformation of cells and changes in Bruch's membrane on day 14 in culture were more pronounced than at earlier terms of culturing; this led to local (sometimes extensive) loss of PRE cells (Fig. 5). Groups of cells seen in the immediate pro-

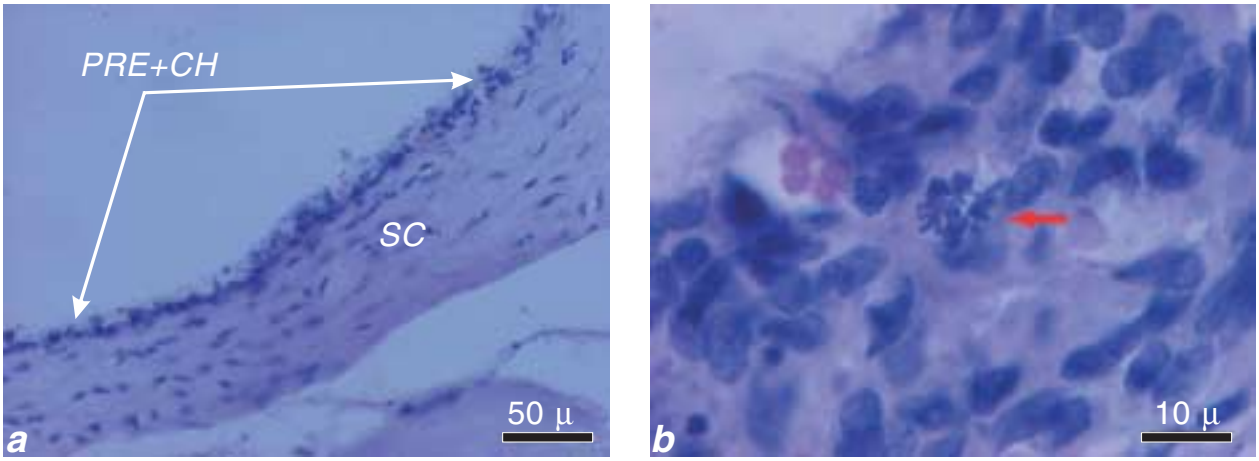

Fig. 4. Transmigration of endothelial cells of the choroid across Bruch's membrane into PRE layer towards arbitrary eye cavity (*a*) and mitosis (red arrow) of endothelial cell in choroid (*b*) under conditions of roller culturing *in vitro*.

ximity to the destructed areas were transformed cells previously residing in the layer; the content of phagocytes increased due to division of these cells (Fig. 6, *a*). On day 14 of *in vitro* culturing, a characteristic feature of PRE was accumulation of phagocytized red-grayish lipid material in the cytoplasm of PRE cells, which was similar to the material observed in macrophage cytoplasm (Fig. 6, *b*). Apart from accumulation of phagocytized material, we observed enlargement of cells remaining in PRE layer, the volume of their cytoplasm also increased, and they filled the defects in the epithelial layer.

Our study of PRE in the structure of PEW of adult rats under conditions of roller culturing *in vitro* demonstrated obvious advantages of the applied method over routine stationary culturing in dishes. Indeed, the number of pyknotic cells in PRE during roller culturing in the structure of PEW without medium replacement did not exceed 10-20% until day 10, whereas during stationary culturing with daily medium replacement this parameter was by 3-fold higher (57%) as soon as on day 3 (maximum life time of PRE in the structure of PEW under these conditions). Studies on the retina isolated from rats [25,28] and mice [19,29] showed that rotation provides not only constant access of fresh medium to the preparation, but also leads to the formation of closed spheres from tissue or its sections. This, in turn, preserves layer-by-layer organization of the retina and cell-cell interactions.

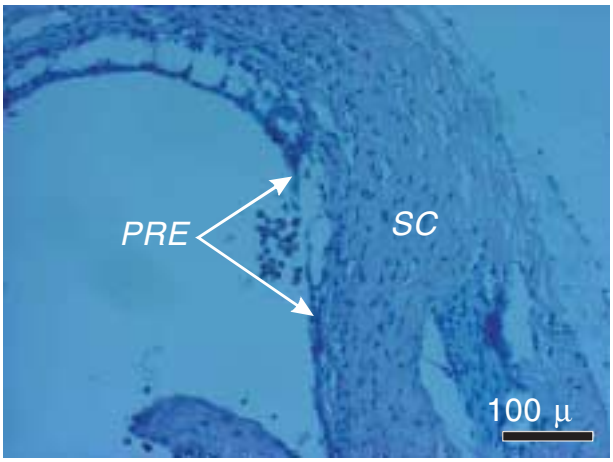

Fig. 5. Area of PRE destruction due to massive migration of cells from the layer and transformation towards macrophages during long-term roller culturing *in vitro*.

As soon as on day 7 of culturing of PEW preparations from adult albino rats we observed displacement of some cells from PRE layer and their morphological transformation towards macrophages. These displaced and transformed cells contained two nuclei; therefore, they undoubtedly originated from PRE. Modulation of the phenotype of PRE cells in mammals is thought to be a reversible phenomenon induced by pathological cell environment. For instance, induction of macrophagic phenotype is associated with the absence of extracellular matrix components of Bruch's membrane after displacement of the cell into the eye cavity [12]. In our experiments on culturing of the PRE—choroid—sclera complex, displacement of PRE cells was accompanied by activation and migration of monocyte-macrophages located in PEW membranes. This behavior of PRE cells and histiocytes (most active on day 7) was induced by cell debris formed due to degeneration and death of some cells. It can be also hypothesized that transformation of PRE cells can be additionally stimulated by resident macrophages; the role of these cells in modulation of the behavior and phenotype of PRE cells was demonstrated on animal model of proliferative retinopathy [17].

Detachment of the retina can also be accompanied by modulation of the behavior and pheno-

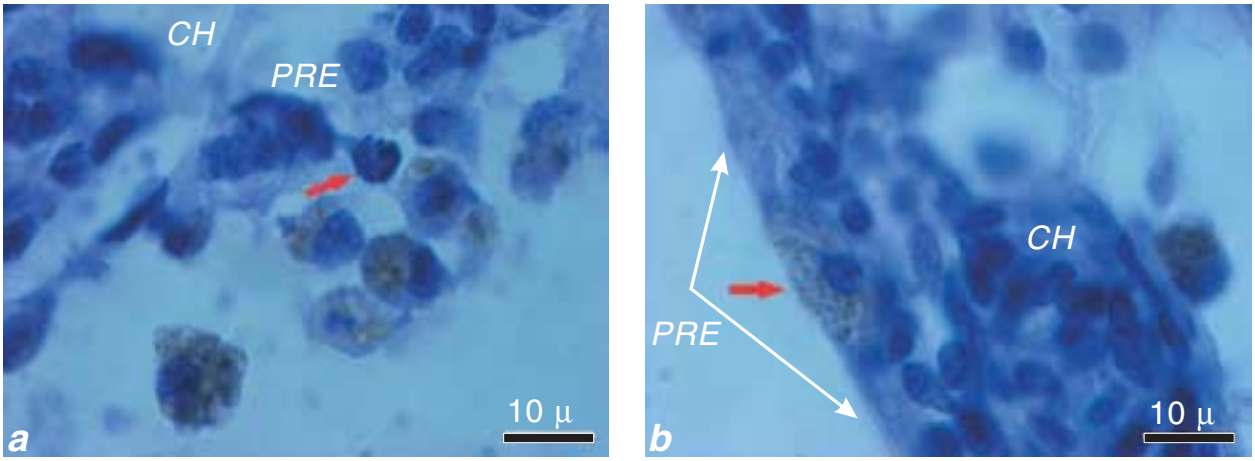

Fig. 6. Phagocytizing and mitotic PRE cells near (*a*) and within (*b*) the layer during long-term roller culturing *in vitro*.

type of PRE cells. In this case, PRE cells separate from Bruch's membrane due to changes in adherent properties or action of proteolytic enzymes, lose epithelial properties and gain macrophage-like features [8]. These events underlie the condition known as proliferative vitreoretinopathy (PVR) and characterized by transformation of PRE cells into macrophage-like and fibroblast-like phenotypes [8]. Pleomorphic and accelerated changes in PRE underlie age-related eye pathologies, *e.g.* macular degeneration [4].

On day 14 of roller culturing of PEW preparations from adult albino rats, cell displacement from PRE layer (at this stage in both vitreal and scleral directions) and their transformation into macrophage-like cells continued and were followed by partial destruction of the layer. Moreover, we observed further accumulation of phagocytized material in cells remaining in the PRE layer. These cells were enlarged and flattened as if they filled lost fields in the PRE layer. Similar changes in the retina and PRE were described in *in vivo* experimental photodynamic damage and photothrombosis in albino rats [5]. It was shown that survived PRE cells actively phagocytize, partially digest, and accumulate debris of dead cells. In should be emphasized that accumulation of remnants of destructed cells is a cause of long-term local inflammation and formation of druses between the basal membrane of PRE and Bruch membrane, which, in turn, are the cause of age-related eye pathologies [3].

It is clear that transformation of PRE cells towards macrophage phenotype can be explained by the fact that these cells are destined for ingestion and degradation of spent photoreceptor disks detached from the apical part of outer photoreceptor segments. High capacity of PRE cells to transformation into macrophages is observed in not only mammals, but also in lower vertebrates. Thus, roller culturing of adult newt eye was accompanied by massive death of neuroretinal cells and transformation of almost 100% PRE cells into macrophage-like cells.

We observed no mitoses in PRE from eyes of adult albino rats. However, on days 7 and 10 of roller culturing of PEW few mitoses were seen in PRE layer, and on day 14 they were also observed in transformed PRE cells displaced from the layer. The presence of mitoses in postmitotic differentiated cells is a pathological process similar to that observed in pathologies of the retina in mammals and humans. For instance, successful surgical restoration of retinal apposition terminates migration and proliferation of PRE cells, but only partially restores vision, which can be explained by changed phenotype of PRE cells and their and readiness to division.

During roller culturing of PEW preparation, pathological processes in PRE developed rapidly (within 2 weeks). This can be determined (apart from culturing conditions) by the absence of pigment granules in PRE of adult albino rats. Melanin protects PRE cells from damaging light exposure and accumulation of lipofuscin (melanosomes participate in lisosomal degradation of lipofuscin) [21]. Our previous experiments showed that PRE of 2-month-old albino rats contains lipofuscin, which promotes displacement and transformation of PRE cells [9].

In retinal pathologies, *e.g.* AMD, PRE layer should be considered in its interaction with choriocapillary network and Bruch's membrane [16]. Under conditions of roller culturing of PEW, migration of endothelial cells of the choroid led to blurring of the boundaries between PRE and choroid and mixing of these layers. The described transmigration

is observed in some eye pathologies. For instance, in retinal diseases transmigration of endothelial cells leads to neovascularization in the neural part of the retina and to exudative processes, so-called wet AMD [27]. It is accepted that transmigration depends on production of vascular endothelial growth factor (VEGF) by PRE cells; this factor acts on endothelial cells as a chemotactic stimulus in response to damage [11,13].

There are another similar feature in events occurring in PEW during roller culturing and degenerative processes in AMD. The macula, central area of human eye fundus, is most vulnerable to the development of pathological states. This is also true for AMD: changes in PRE and choroid, abnormality of the extracellular matrix, and chronic inflammatory response resulting in atrophy of PEW tissues [30]. Under conditions of roller culturing, some pathological processes (displacement and transformation of PRE cells into macrophage-like cells, reactivity of histiocytes, inflammation and transmigration of choroidal cells) were more pronounced in the central zone of PEW.

We believe that the proposed model, PEW complex from adult albino rats consisting of PRE, choroid, and sclera and new methodical approach (roller culturing of this complex) can be useful for the solution of some experimental and practical tasks. For instance, they can be used for evaluation of the effect of various factors and pharmacological preparations on PRE, for modeling of processes in PRE induced by damage or accompanying pathological states of the neuroretina, and for evaluation of regeneration responses of PRE cells in developing and adult higher vertebrates.

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