In Vivo and *in Vitro* Proliferative and Differentiation Activity of Human Embryonic Retinal Cells

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Differentiation of human embryonic retinal cells (20-22 weeks' gestation) was studied using morphological, immunohistochemical, and biomolecular approaches. The retina included several regions differing by the degree of cell differentiation. Mitoses were rarely found in the marginal zone. This zone contained low differentiated cells. The central retinal area consisted of typical layers with differentiated cells. Culturing was accompanied by the formation of aggregates and neurospheres, where mitoses and progenitor or differentiated cells expressing markers of photoreceptors, neurons, and glia were found.

Key Words: *human retina; embryogenesis; pluripotent/progenitor cells; immunohistochemistry; transcripts*

The study of changes in proliferative and differentiation activity of retinal cells during the development of mammalian eye is of considerable theoretical and clinical significance [1,10]. Proliferating cells and cells at various stages of differentiation can be found in various zones of human retina over the first 5 months of gestation [3,6,13,16]. The development of retina is accompanied by variations and sharp decrease in proliferative activity of cells. It is related to intracellular and extracellular changes and interaction of these cells with surrounding differentiated cells [12]. Experimental studies of the retina in rats and mice showed that low differentiated cells can be identified by molecular markers and isolated. Proliferation, migration, and differentiation of cells are studied in tissue cultures and under conditions of transplantation into the eye. This treatment serves as a therapeutic procedure and allows evaluation of the cell function [2,4-11,14-16]. Study of neuroblasts in the developing human eye is of considerable clinical importance. However, this approach received little attention [8,11,16].

The present work involved histological, immunocytochemical, and biomolecular methods to study human fetal retina *in situ* and during culturing.

MATERIALS AND METHODS

Experiments were performed on eyes from aborted fetuses (20-22 weeks' gestation). They were obtained from licensed authorities (Russian Ministry of Health) according to the Legislation of the Russian Federation on People's Health Care and in compliance with the approved list of medicinal indications. The age of fetuses corresponded to pregnancy terms estimated by the obstetrician.

The eyes were removed 4-24 h after abortion. Enucleated eyes were fixed in Carnoy fluid, washed in 70% alcohol, routinely treated with alcohols and xylene, and embedded in paraffin. Serial transverse sections (5 μ) were stained with Karachi hematoxylin.

The retina was isolated under a binocular microscope. Initial viability of retinal cells was 78%. Some cells were cultured in DMEM/F12 medium with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

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Other cells were cultured in serum-free DMEM/F12 medium containing fibroblast growth factor (FGF-2), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), heparin, and penicillin/streptomycin. The cells were cultured for 20 days.

Adherent monolayer of retinal cultures was cultured on medium I. The cells were passed at 6-day intervals. To this end the cells were trypsinized and mechanically dissociated. In serum-free medium the cells formed freely floating spheroids.

For immunocytochemical analysis, native retina, monolayer cultures, and spheroids were fixed with 4% formalin in phosphate buffer. The retina was cut on a cryostat. Sections (12 μ) and cultures were washed and incubated with primary antibodies for 12 h. We used primary antibodies to human nestin (1:30, Chemicon), glial fibrillar acid protein (GFAP, 1:250, DAKO), β-tubulin III (1:200, Abcam), vimentin (1:100, NeoMarkers), neurofilament protein 200 (1:500, Serotec and Sigma), Ki-67 protein (1:50, Abcam) against photoreceptor protein recoverin (1:20, Russian State University), and antibodies to proliferating cell nuclear antigen (PCNA, 1:100, Sigma). The cells were stained with secondary antibodies and fluorescent dyes Cy-2 and Texas Red (Jackson) or FITC and TRITC (Sigma). Hoechst 42333 was used for nucleus staining. The preparations were examined under an Opton-III luminescence microscope equipped with a Coolpix 4500 digital camera or computerized Olympus AH-3 camera (Studio Lite and Viewfinder Lite softwares).

For molecular studies, total RNA was isolated from the prepared retina using RNAzol B (Tel-Test, Inc.). mRNA was isolated from total RNA with SileksM kit. The first chain of cDNA was synthesized on matrix mRNA with SuperScript reverse transcriptase (Gibco BRL). We used random hexanucleotide primers. The polymerase chain reaction (PCR) was carried out on matrix cDNA (Hybyid amplifier) using Taq-polymerase (SileksM), deoxynucleotide triphosphates, and reaction buffer (SileksM). To study gene expression, the samples were amplified under similar conditions: 94°C, 3 min; 40 cycles, 94°C, 45 sec; 56°C, 45 sec; 72°C, 45 sec; and 72°C, 5 min. Expression was estimated visually by the results of PCR product separation in 1% agarose gel and Tris-acetate buffer. PCR was performed using primers constructed by mRNA nucleotide sequences with DNAStar software. They were obtained from the international databanks BLAST and HGNC. Primers were taken from various exons to exclude the possibility of synthesis of the PCR product on genomic DNA.

RESULTS

Morphological characteristics of 20-22-week-old human retina. The sensory retina (*pars optica retinae)* in human fetuses at 20-22 weeks' gestation can be conventionally divided into marginal, peripheral, equatorial, and central areas (Fig. 1, *a*, *b*). The marginal zone extends from the *ora serrata* to the peripheral area. This small zone consists of the outer nuclear and inner anucleate layers. The nuclear layer includes densely positioned elongated cells with oval dark nuclei (neuroblasts). The inner anucleate layer is narrow and includes parallel fibers. These fibers are positioned closely to the inner terminal membrane. The next zone is the peripheral retinal area. A well-defined border is found between the marginal and peripheral area. The outer and inner nuclear layers can be identified in this zone. The inner nuclear layer includes alternate rows of elongated and round cells, intermediate small dark cells, and slightly larger light cells. This zone has no distinct inner and outer retinal layers.

Equatorial cells are characterized by higher degree of differentiation. The outer retinal layer is poorly developed and visualized between the photoreceptor and inner nuclear layer. The inner retinal layer is well developed and separates the inner nuclear layer from the layer of ganglionic cells. A thin layer of nerve fibers is located under the inner terminal membrane.

Large light nuclei of round shape are identified among elongated cells in the outer nuclear layer. Similar nuclei are revealed in the inner nuclear layer, which also includes dense dark nuclei. These nuclei are located near the inner retinal layer. Cells of the inner nuclear layer adjacent to the outer retinal layer are elongated and have dendritic processes. Round nuclei are present in the layer of ganglionic cells and morphologically similar to those in the inner nuclear layer.

All layers of the equatorial retinal area are clearly seen. We revealed the outer retinal layer, more developed layer of ganglionic cells, and layer of nerve fibers. Small blood vessels and fusiform cells are found in the layer of nerve fibers.

The central retinal area is presented by different layers. The outer photoreceptor segments are at the initial stage of development. The layer of ganglionic cells has dense dark nuclei. We identified nerve fibers extending from ganglionic cells. The layer of nerve fibers is thickened and includes blood vessels and spindle cells. Mitoses are rarely seen in the retina.

Immunohistochemical study of 20-22-week-old retina. Proliferation and differentiation of cells in the developing retina were studied with antibodies to PCNA, neurofilament protein (NF70 or NF200), photoreceptor-specific calcium-binding protein recoverin, immature neuroblast protein β-tubulin III, nestin (marker of progenitor cells), vimentin (marker of early neuronal and glial precursors), and GFAP (marker of Muller cells).

PCNA gene expression is detected only in individual cells of the central retinal area. Our results sug-

gest that cells of the central retinal area examined at this stage of development nearly completed the stage of reproduction.

Staining with antibodies to neurofilaments revealed specific immune reaction of perikarya and processes in all examined retinal cells. Fluorescence intensity increases significantly in the outer and inner retinal layer. It is associated with high density of dendrites and axons in retinal neurons. Fluorescence is maximum in the layer of ganglionic cell axons. They are arranged in bundles in the vitreal region of the retina behind the inner terminal membrane (Fig. 1, *c*). The intensity of fluorescence is lower in the peripheral area near the limbus (as compared to the central retina).

Staining with antibodies to recoverin revealed specific immune reaction in the outer nuclear, photoreceptor, and ganglionic layer (Fig. 1, *d*). The distribution of recoverin-positive cells in human retina at 20-22 weeks' gestation reflects nearly completed development of the retinal photoreceptor layer. Examination of antibody-stained sections demonstrated the presence of individual recoverin-positive cells in the vitreal and scleral zones of the retinal layer (Fig. 1, *e*, *f*). These cells can migrate from the ganglionic layer to the photoreceptor layer.

The inner retinal layer included β-tubulin III-expressing cells (Fig. 1, *g*). Vimentin-positive cells are present in various layers of the retina. Fluorescence intensity is maximum in the layer of nerve fibers adjacent to blood vessels (Fig. 1, *h*). Treatment with antibodies to nestin identified radial cell processes and individual perikarya in the outer nuclear layer (Fig. 1, *i*). We found no GFAP-expressing cells.

PCR analysis of the expression of recoverin, β-tubulin III, vimentin, and GFAP mRNA. PCR analysis on matrix cDNA synthesized on mRNA from the retina of fetuses at 20-22 weeks' gestation was performed using cDNA libraries. We quantitatively studied expression of genes that serve as the markers of cells differentiation (Fig. 2). PCR analysis of expression revealed small amount of recoverin, GFAP, and vimentin transcripts. These data reflect active differentiation of photoreceptors and Muller glial cells. The

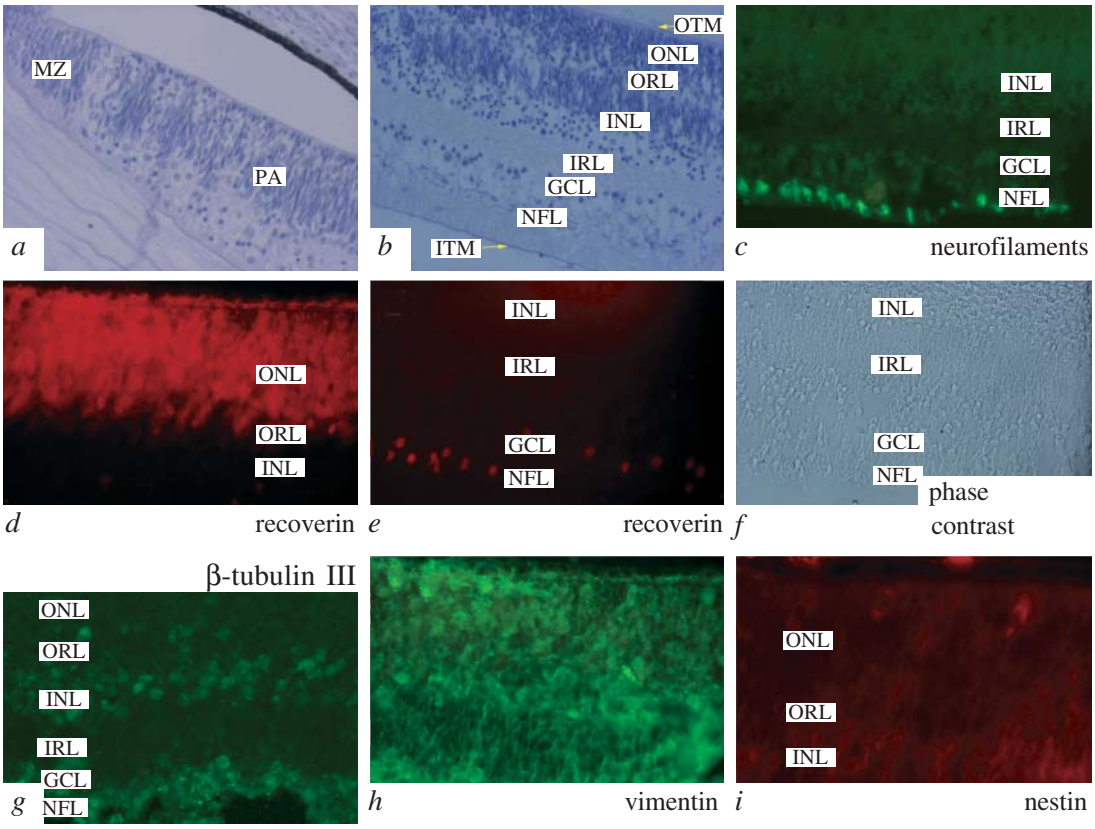

Fig. 1. Histological and immunohistochemical characteristics of human fetal retina at 20-22 weeks' gestation. Structure of the marginal, peripheral (*a*), and central retinal areas (*b*). Karachi staining with hematoxylin and eosin (×20). Immunohistochemical study: expression of neurofilaments in the axonal layer of ganglionic cells (*c*); recoverin-positive cells (*d*) in the outer nuclear, photoreceptor and ganglionic layer of the developing retina (*e*, *f*); distribution of immature neuroblasts expressing β-tubulin III (*g*); vimentin-positive and nestin positive cells in various layers of the retina (×20, *h, i*). MZ, marginal zone; PA, peripheral area; OTM, outer terminal membrane; ONL, outer nuclear layer; ORL, outer reticular layer; INL, inner nuclear layer; IRL, inner reticular layer; GCL, ganglionic cell layer; NFL, nerve fiber layer; ITM, inner terminal membrane.

ratio of β-tubulin III mRNA decreases, which is consistent with the results of immunohistochemical study (small number of cells in retinal sections specifically binding β-tubulin III). The number of recoverin transcripts is highest, which corresponds to intensive immunohistochemical staining of photoreceptors with antibodies to recoverin.

Immunohistochemical study of cultured retinal cells. The cells formed a more or less dense monolayer on glasses. Hoechst staining revealed cells with large and small nuclei. Macro- and micronucleated cells form a regular monolayer and dense clusters, respectively (Fig. 3, *a*). Among large cells, we revealed a considerable number of mitotic cells. However, mitoses were not found in micronuclear cells.

Large cells were stained with antibodies to nestin, vimentin, and GFAP. Small cells were stained with antibodies to recoverin and β-tubulin III.

Double staining with antibodies to nestin and vimentin suggests that the cultures contain mane nestin- and vimentin-positive cells (+). Vimentin-positive cells form a continuous layer, while nestin-positive cells are arranged in groups (Fig. 3, *b*, *c*). Visual examination showed that the number of nestin-positive cells is lower compared to vimentin-positive cells. Micronuclear cells are unstained and often adjacent to nestin-positive cells. Small cells do not express nestin and vimentin. Many nestin-positive cells express also vimentin. Nestin- and vimentin-positive cells are morphologically similar. These cells have large dense body with multiple processes or elongated body with 2-3 long processes. Some cells have single thin and long processes. The shape of nestin-positive cells varies; there are dendritic and small fusiform cells. Among cultured cells the number of mitoses is high in vimentin-positive cells, but low in nestin/vimentin-positive cells.

Staining with antibodies to GFAP and vimentin showed that GFAP+ (Muller) cells are often present in the culture. The number of these cells is low. They are arranged in groups between macronuclear cells. GFAP-positive cells differ in shape. We revealed spread cells with multiple processes or 2-3 processes (Fig. 3, *d*).

Neurofilament-expressing cells are elongated. We found individual cells with long fibers.

Staining with antibodies to recoverin and vimentin showed that clusters of recoverin-positive cells are located on a continuous layer of vimentin-positive cells (Fig. 3, *e*). These small micronuclear cells are strongly stained. Recoverin-positive cells are arranged in clusters or lie individually. Morphologically, these cells have processes and are characterized by a unipolar or bipolar shape (similarly to photoreceptors). Cultured recoverin-positive cells migrate along processes of vimentin-positive cells (Fig. 3, *f*).

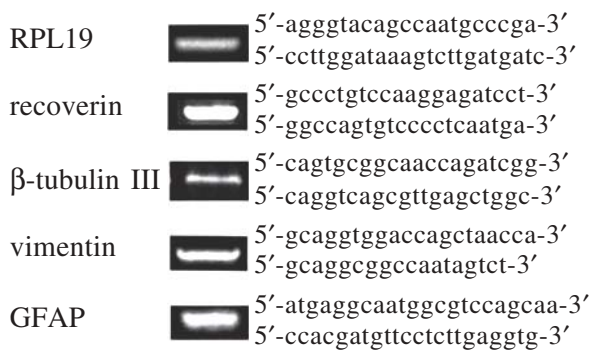

Fig. 2. PCR analysis of expression of recoverin, β-tubulin III, vimentin, and GFAP mRNA in human fetal retina at 20-22 weeks' gestation. Ribosomal protein serves as the control. Structure of the corresponding primers is shown on the right side.

Staining with antibodies to β-tubulin III revealed a small number of cells expressing this protein. Tubulin-positive cells are arranged in groups or lie individually (Fig. 3, *g*). They have small bodies with small nuclei and several processes. Most bipolar cells usually have long processes. Some cells have short processes.

Staining with antibodies to nestin and Ki-67 revealed numerous dividing Ki-67-positive cells. Some cells also express nestin (Fig. 3, *h*). We found no small recoverin-positive cells (Ki-67).

An immunohistochemical study of freely floating spheres grown on the selective medium involved staining with antibodies to recoverin and Ki-67. These spheres consist of numerous large recoverin clusters. Dividing Ki-67-positive cells are situated between clusters (Fig. 3, *i*). Recoverin-positive cells form spherical or elongated structures. No dividing cells are revealed among recoverin-positive cells.

Staining with antibodies to nestin and tubulin demonstrated the presence of numerous nestin-positive and tubulin-positive cells (Fig. 3, *j*, *k*). Some tubulin-positive cells are arranged in separate fields. Other cells form elongated or spherical structures that are similar to the photoreceptor layer. The shape, size, and location of tubulin-positive cells indicate that they enter the population of recoverin-positive cells. Polar cells are found in structures of tubulin-positive cells. Their apical processes are short and thickened. Very thin and long basal axonal processes form bundles (Fig. 3, *l*). Fibers of nestin-positive cells are situated between tubulin-positive cells. The number of nestin-positive cells and fibers is highest beyond the structures.

Staining with antibodies to GFAP showed that cultured Muller cells strongly express glial protein. Groups of GFAP-positive cells form structures that resemble clones on the surface of spheres (Fig. 3, *m*). Dense plaques of GFAP-positive cells are connected

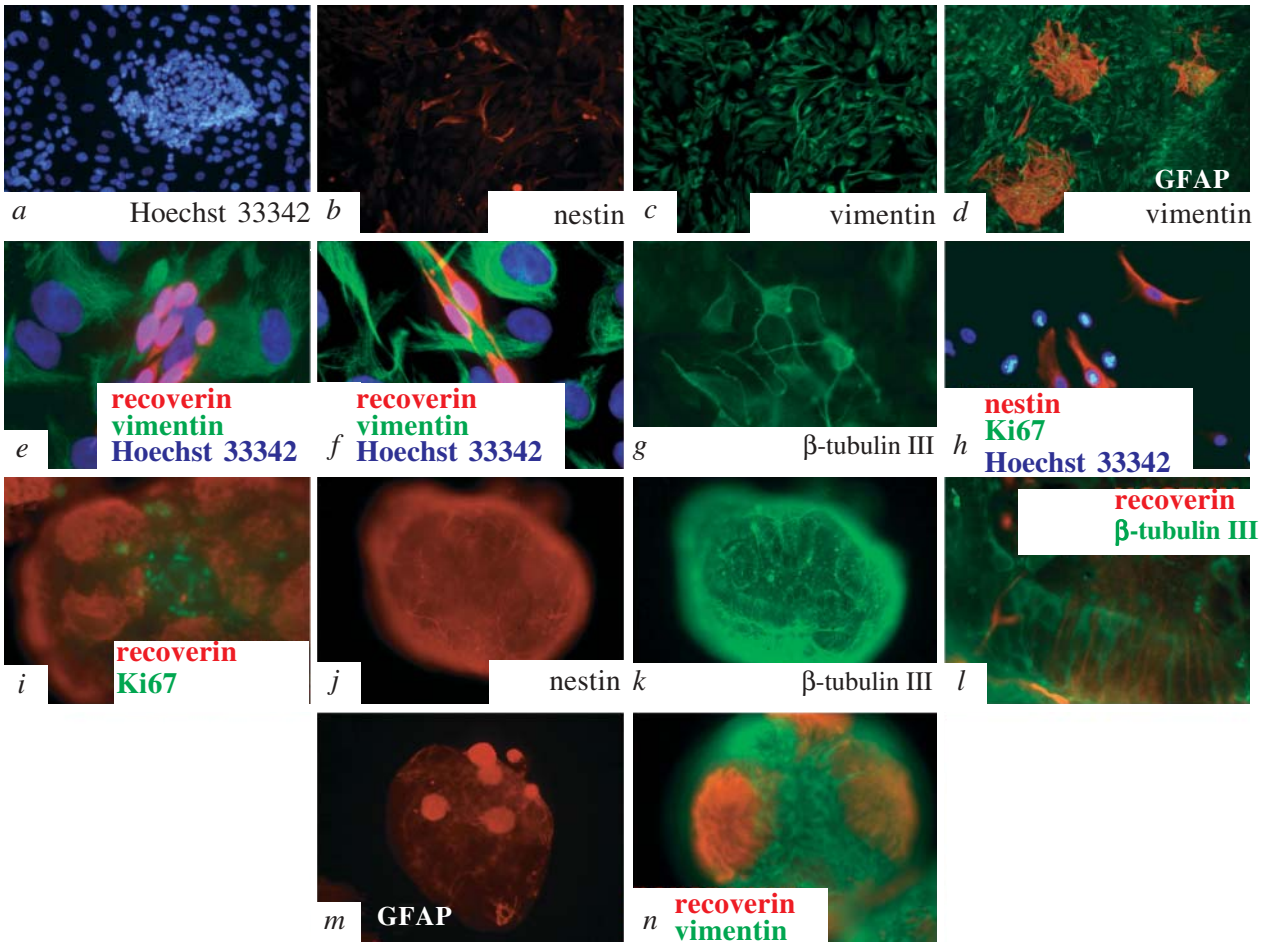

Fig. 3. Immunohistochemical characteristics of cultured human embryonic retinal cells at 20-22 weeks' gestation. Adherent cultures on a FBS-containing medium (*a-h*); monolayer of cells with large and small nuclei (×10, *a*); distribution of pluripotent and low differentiated cells during double staining with antibodies to nestin and vimentin (×10, *b*, *c*); cultured Muller cells expressing GFAP and arranged in groups (×10, *d*); (*e*) photoreceptor and recoverin-positive cells forming clusters and migrating along vimentin-positive cells (×40, *f*); β-tubulin III-expressing neuroblasts (adherent culture, ×40, *g*); culture with a considerable number of proliferating cells, some of which express nestin (×16, *h*). Retinal cells form spheroids in a serum-free medium with mitogenic factors (*i-n*). Spheres include numerous large recoverin-positive clusters and intermediate dividing cells (×16, *i*); double staining with antibodies to recoverin and Ki-67 (*j*, *k*). Double staining with antibodies to nestin and β-tubulin III (×10); reciprocal distribution of cells expressing nestin and β-tubulin III (×40, *l*); GFAP expression in retinal spheres (×10, *m*); spatial distribution of recoverin-positive and vimentin-positive cells on spheres (×16, *n*).

with each other by fibrous processes. Several similar structures are found on the surface of spheres.

Staining with antibodies to recoverin and vimentin showed that many fibers of vimentin-positive cells run between clusters of recoverin-positive cell. However, some fibers of vimentin-positive cells enter these clusters and form the radial skeleton (Fig. 3, *n*).

Histological study of human retina at 20-22 week's gestation indicates that this structure does not complete the histotopic development. By the degree of development, it can be divided into several areas. The marginal zone includes low differentiated cells. These cells are partially retained in the inner nuclear layer of the peripheral area (Fig. 1). Our results indicate that the retina continues to develop in this period of observations. An increase in the count of retinal cells is mainly associated with proliferation of cells in the peripheral area. Mitoses are sometimes revealed in this zone.

The results of morphological, immunohistochemical, and biomolecular studies complicate each other. A correlation was revealed between expression of vimentin, β-tubulin III, and recoverin in undifferentiated precursors, immature neuroblasts, and differentiating photoreceptors, respectively. Differentiating photoreceptors were strongly stained with specific antibodies to calcium-binding protein recoverin and expressed recoverin mRNA (Fig. 1, *d*; Fig. 2). Study of GFAP by means of PCR analysis and immunohistochemical staining produced different results. These data indicate that this protein is not synthesized in Muller glial cells at this stage of development.

We evaluated the possibility to maintain retinal cells under conditions of culturing. Study was per-

formed with 2 different culture media (complete nutrient medium with FBS and selective serum-free medium with growth factors).

We performed a comparative immunohistochemical study of adherent cultures and spheroids grown on a FBS-containing medium and selective medium, respectively. Embryonic retinal cells underwent adhesion in a complete nutrient medium. We found a considerable number of dividing cells, progenitor vimentin-positive and nestin-positive cells, and photoreceptor cells. The count of neuroblasts and glioblasts was much lower. Photoreceptor (recoverin-positive) cells are arranged in clusters. They are often situated near nestin-positive cells and migrate along vimentin-positive cells. Photoreceptor cells undergo differentiation and, therefore, do not divide.

All cells in spheroids grown on the selective medium have long processes. They form specifically located fiber tracts on the surface and in deep layers of spheroids. Photoreceptor cells continue to express tubulin and, therefore, are characterized by the early stage of differentiation. Photoreceptor tubulin-positive cells have long axon-like processes that form bundles. Fibers of nestin-positive and vimentin-positive cells penetrate clusters of photoreceptors and form a well-developed network of fibers in the intermediate zones. These zones also include proliferating cells stained with antibodies to Ki-67. The behavior of Muller cells is of interest. They are clearly identified in both culture media (as differentiated from the native retina). These cells form dense clone-like agglomerates. Bridges of glial processes are found on the surface of spheroids between these agglomerates.

The cells not only survive, but also undergo proliferation and differentiation in both systems of culturing. This conclusion is derived from the presence of mitoses. Expression of nestin and expression of proliferating cell protein Ki-67 serve as markers for the presence of low differentiated cells and proliferative activity of cultured human embryonic cells at 20-22 weeks' gestation. Cultured proliferating cells can be used as a source of progenitor cells for clinical studies [8]. Our results are consistent with published data on the development and culturing of human embryonic retinal cells [6,14,16].

Further studies are required to identify human embryonic retinal cells giving rise to proliferating and differentiating cells during culturing. These cells probably develop from low differentiated pluripotent cells and neuroblasts that are present in the peripheral retinal area and inner nuclear layer, respectively.

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