Study of Morphofunational Peculiarities of Fibroblasts Isolated from Human Vertebral Growth Plates during *In Vitro* Culturing

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We studied structural and functional peculiarities of growth zone chondroblasts isolated from human fetal spines on gestation week 12 and cultured *in vitro* over 4 passages. The morphology of chondroblasts of different differentiation degree was described. It was found that the population of chondroblasts had certain features determined by changes in the relative content of cells of different differentiation degree. The data suggest that culturing conditions affect cell differentiation and the possibility of using primary human chondroblast culture as the experimental model of differentiating human vertebral growth plate chondroblasts *in vitro*.

Key Words: *cell culture; chondroblasts; differentiation; light and electron microscopy*

Correction of damage to the cartilaginous tissue caused by traumas or hereditary disease is an important problem of modern medicine [6]. The use of primary culture of human chondroblasts is a promising method for correction of these damages [2,3,5]. Previous studies demonstrated high efficiency of application of autologous chondroblasts in acute and chronic traumas [7]. Embryonic (fetal) chondroblasts can be used as a universal allogeneic material for the treatment of acquired defects and hereditary metabolic pathologies of the cartilaginous tissue [4,8], which necessitates comprehensive study of their biological properties. Here we studied morphofunctional properties of cells obtained from human fetal vertebral growth plates during *in vitro* culturing.

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MATERIALS AND METHODS

The vertebra were isolated from human fetuses (gestation weeks 10-12) obtained during medical abortions from 15 healthy women in licensed medical institutions (Federal Agency for Health Care and Social Development). The material was taken in accordance with ethic norms; informed consent was obtained from all women.

All procedures for cell isolation and culturing were performed in special rooms under aseptic conditions. Hyaline cartilage was isolated from vertebral growth zones, washed in Hanks solution containing 0.3-0.4 g/liter kanamycin for 15 min, crushed to 0.5-mm^2 fragments in a Petri dish with minimum volume of PRMI-1640, placed into siliconized vials with 1.5% collagenase, and incubated for 5-8 h at 37^{o}C on a shaker. The obtained suspension was filtered through Nylon filters and centrifuged at 1500 rpm for 10 min. The cells were then resuspended in DMEM/F12 medium mixture (1:1, Gibco) supplemented with 20% FCS, brought

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to a concentration of 3×10^5/ml, and cultured at 37°C. The cells were subcultured every 3-4 days.

Cells of passages 1-4 were examined under a light microscope. To this end, the cells were fixed with cold methanol and stained with hematoxylin and eosin; glycogen and glycoproteins were visualized using PAS reaction after McManus with amylase control; total glycosaminoglycans (GAG) were detected in the reaction with alcian blue after Steedman [2]. Sulfated GAG, chondroitin sulfate (CS) and keratan sulfate (KS), were visualized with toluidine blue at pH 1.5 and 2.5 [1]. Type 2 collagen was identified by the immunoperoxidase method using primary rabbit antibodies to type 2 collagen (Biermann, Bad Nauheim). The data were processed using AxionVision 3 software.

For ultrastructural analysis, the cells were harvested with a mixture (1:1) of trypsin (0.02%) and Versene (0.025%), the suspension was centrifuged, and the sediment was fixed in 4% paraform, postfixed in 1% OsO_4, dehydrated, and embedded in epon-araldite. Ultrathin sections were contrasted with uranyl acetate and lead citrate. The preparations were examined under an H-600 electron microscope (Hitachi).

RESULTS

Cell culture during passage 1 looked loosened and was presented by 3 cell types (Fig. 1, *a*). Type 1 cells (79% population, 60.90±3.72 μ) had round shape, contained round nucleus with high content of heterochromatin occupying almost the entire cell volume and moderately basophilic cytoplasm. Type 2 cells (18% population, 98.50±2.31 μ) had oval shape, moderate oxyphilic cytoplasm, and oval or kidney-shaped nucleus with high heterochromatin content. Type 3 cells (3% population) were the largest cells (196.20±13.07 μ); they had oval nucleus with high euchromatin content. In contrast to types 1 and 2 cells, the cytoplasm occupied greater area, was homogenously stained with eosin, and formed few short processes on the cell surface.

Cells of all types had fine glycogen granules in the cytoplasm. The intensity of reaction was maximum in type 3 cells. Reaction for total GAG revealed finely dispersed substance moderately stained with alcian blue and filling the entire volume of the cytoplasm. Type 2 and 3 cells contained large alcian-positive granules diffusely spread in the cytoplasm. Histochemical reaction revealed CS and KS granules localized at the periphery of cells of all three types.

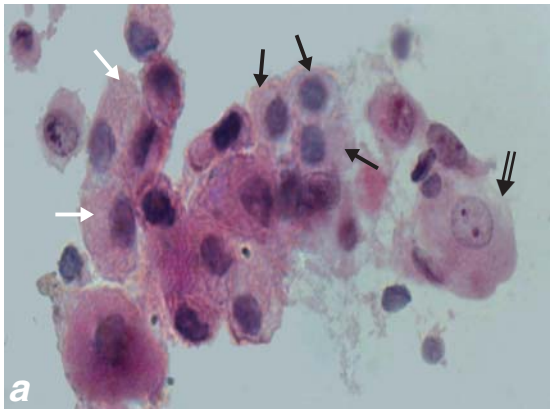
Passage 2 cells formed a uniform dense monolayer. The content of type 1 cells decreased to 59%, while the number of type 2 and type 3 cells increased to 34 and 7%, respectively. Dividing cells with mitotic figures and cells with signs of apoptosis were detected.

All cells contained much glycogen. The intensity of reaction for total GAG did not differ from that in passage 1 cells. The reaction for sulfated GAG in the cytoplasm of type 1 cells also remained unchanged. Small KS granules in type 2 and type 3 cells were located mainly at cell periphery, while large CS granules occupied the entire cell volume. A peculiar feature of passage 2 cells was the appearance of extracellular matrix (Fig. 1, *b*) containing type 2 collagen (detected immunochemically, Fig. 1, *c*).

The character of culture growth changed during passage 3. The cells formed a monolayer with typical islets of dense growth. The center of the islet was formed by compactly packed cells of all three types, while peripheral monolayer was loosened. The content of type 1 cells further decreased to 3%, while the number of type 2 and type 3 cells increased to 60 and 37%, respectively. Morphohistochemical characteristics of type 1 and type 2 cells did not differ from those during passage 2. Type 3 cells (Fig. 1, *d*) had oval central nucleus with high euchromatin content, eosinophilic cytoplasm with optically clear vacuoles, and large and long processes. Histochemical reaction for sulfated GAG revealed small CS and KS granules filling the entire cytoplasm; other characteristics did not differ from those observed during passage 2.

Cell culture during passage 4 formed dense monolayer with cell-free round loci (Fig. 1, *e*) presented by homogenous moderately oxyphilic substance; accumulations of type 2 cells and degenerating cells were seen at the periphery. In passage 4 cultures, the content of type 1 and type 3 cells decreased to 1 and 12% of the total number of cells, while type 2 cells predominated (~87% population). Reaction for total GAG revealed finely dispersed alcian-positive substance spread diffusely in the cytoplasm of type 2 cells and in the extracellular matrix (Fig. 1, *f*). The intensity of the reaction increased in the perinuclear area and at the periphery of cell cytoplasm. Type 2 cells were characterized by high glycogen content (Fig. 1, *g*) and the presence of glycoproteins. Immunochemical staining revealed type 2 collagen in the extracellular matrix (Fig. 1, *h*).

Ultrastructural study of the primary chondroblasts culture during passages 1-3 revealed cells of different differentiation degree. Low-differentiated cells (Fig. *a*) had large nucleus surrounded by a thin cytoplasmic rim of medium electron density with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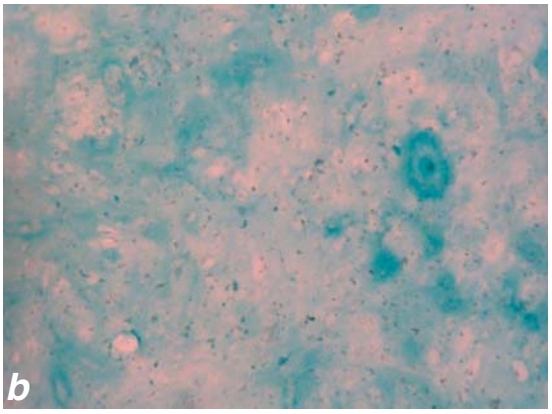
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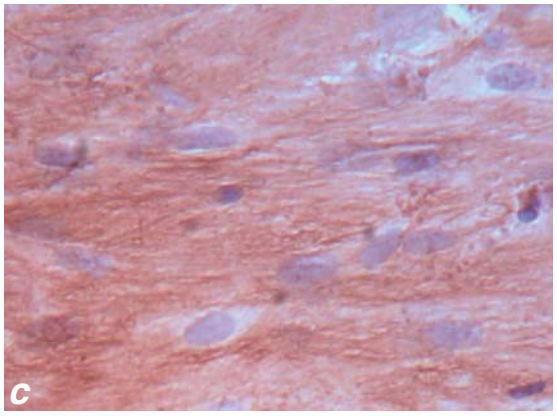
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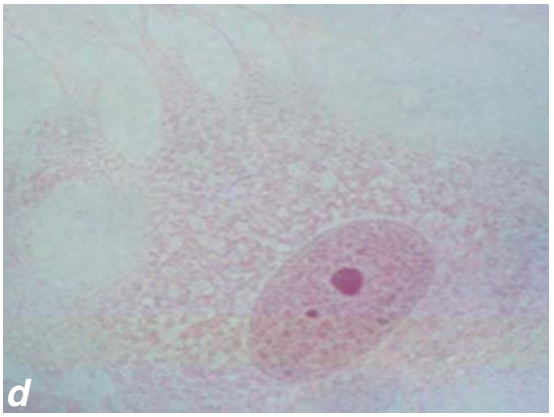
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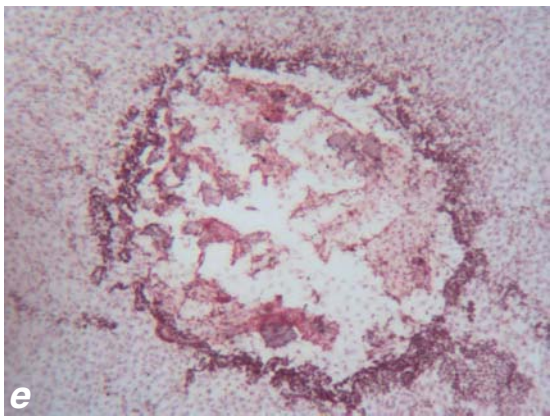
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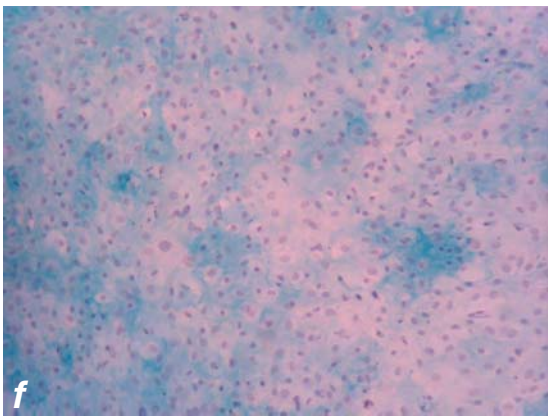
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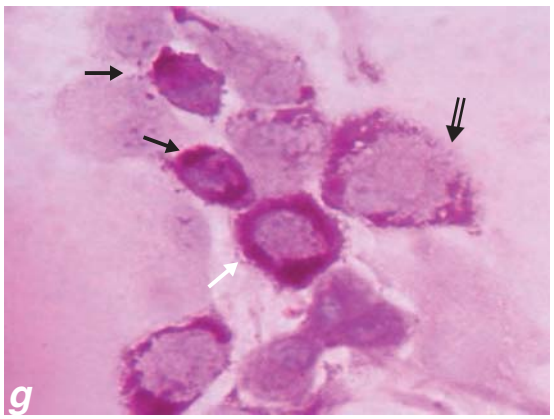
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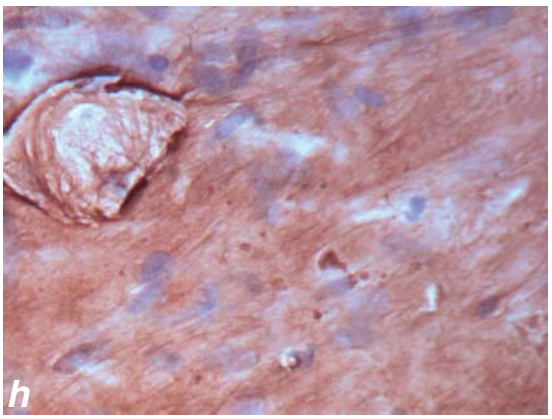
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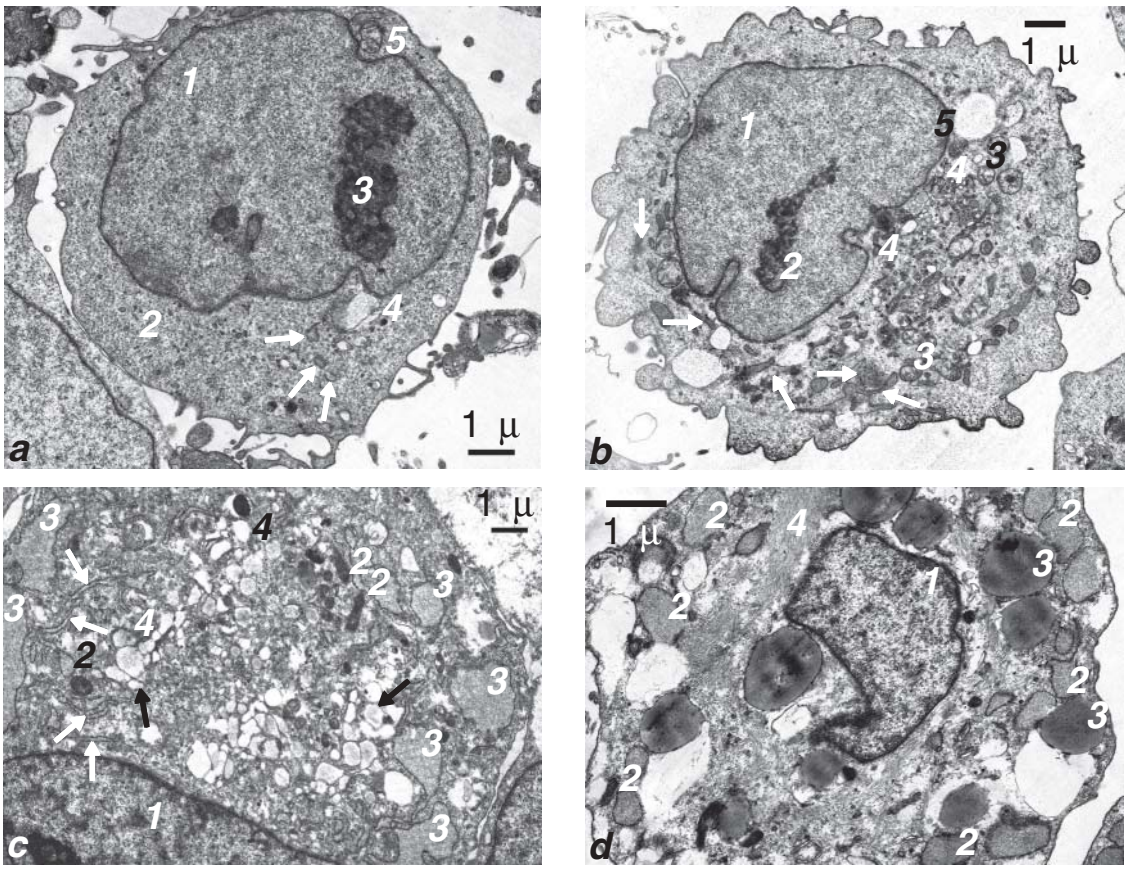

Fig. 2. Primary chondroblasts isolated from fetal human vertebra. *a*) low-differentiated chondroblast, passage 1: *1*) nucleus; *2*) cytoplasm; *3*) nucleolus; *4*) poorly developed Golgi apparatus; *5*) mitochondrion. Arrows show short profiles of EPR; *b*) medium-differentiated chondroblast, passage 1: *1*) nucleus; *2*) nucleolus; *3*) mitochondria; *4*) Golgi apparatus; *5*) lipid inclusion. Arrows show EPR profiles; *c*) fragment of highly differentiated chondroblast, passage 3: *1*) nucleus; *2*) mitochondria; *3*) widened cisterns of EPR; *4*) elements of Golgi apparatus. Arrows show narrow EPR profiles (light arrows) and secretory granules (dark arrows); *d*) chondroblast with signs of degenerative changes, passage 3: *1*) nucleus; *2*) widened cisterns of EPR; *3*) lipid inclusions; *4*) accumulation of filaments in cytoplasm.

numerous free ribosomes. The nucleolus contained well discernible granular and fibrillar components, which attested to intensive synthesis of ribosomal components. Solitary mitochondria and short profiles of the rough endoplasmic reticulum (EPR) were seen, Golgi apparatus was presented by 2-3 short cisterns. The area of the cytoplasm, the number of mitochondria, the length of rough EPR profiles and area of Golgi apparatus increased during cell differentiation (Fig. 2, *b*), numerous transport vesicles appeared in the zone of Golgi apparatus. Signs of high synthetic activity were observed in cells: accumulation of a homogenous substance of medium electron density in cisterns of rough EPR and appearance of granules of medium electron density in the zone of Golgi apparatus (Fig. 2, *c*). Glycogen inclusions sometimes occupying large area of the cytoplasm were found in some cells during passage 3. Some cells contained lipid inclusions (Fig. 2, *d*).

Highly differentiated cells containing widened EPR cisterns, secretory granules, and lysosomes predominated in the population of passage 4 chondroblasts. The area of Golgi apparatus and the number of transport vesicles considerably decreased, which attested to decreased synthetic activity of these cells. The number of cells with lipid inclusions increased. Numerous degenerating cells and cell detritus were seen on sections, signs of apoptosis were observed in some cells.

Fig. 1. Primary cell culture isolated from developing human vertebra on gestation week 12. *a*) type 1 cells (light arrows), type 2 cells (dark arrows), and type 3 cells (double arrows). Hematoxylin and eosin staining, ×200. *b*) passage 2: reaction for total GAG in the extracellular matrix, alcian blue staining, ×100); *c*) passage 2: immunochemical reaction for type 2 collagen in extracellular matrix, ×400; *d*) passage 3: type 3 cell, hematoxylin and eosin staining, ×1000; *e*) passage 4: node formation in the monolayer, hematoxylin and eosin staining, ×40; *f*) passage 4: reaction for GAG in cells and extracellular matrix, alcian blue staining, ×100); *g*) passage 4: intensive reaction for glycogen in type 1 cells (dark arrow), type 2 cells (light arrow), and type 3 cells (double arrows). PAS reaction, ×400; *h*) passage 4: positive immunochemical reaction for type 2 collagen matrix, ×400.

The results of ultrastructural analysis showed that the content of cells with signs of high synthetic activity accumulating great amounts of synthesized material considerably increased during passages 1-3. During passage 3, secondary lysosomes containing fragments of membranes and cytoplasm and secretory granules appeared in highly differentiated cells. Cell structure during passage 4 attested to inhibition of vital processes in cultured chondroblasts.

Our findings suggest that the primary culture isolated from human vertebral growth plate consists of chondroblasts; under conditions of monolayer primary culture these cells undergo successive stages of genetically determined differentiation and produce specific secretory products. Fetal chondroblasts can be cultured in a monolayer over 4 passages without addition of special growth and differentiation factors. Further culturing led to chondroblast death and their replacement with fibroblasts. In our experiments, we did not observe signs of chondroblast differentiation described for monolayer culture of these cells [10].

Thus, human fetal vertebral growth plate can be a source of primary chondroblast culture, which can be used as an experimental model for testing of various factors affecting chondroblast differentiation and for the development of methods of cell therapy.

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