IMMUNOLOGY AND MICROBIOLOGY

Structure of Cell Clusters Formed in Cultures of Dissociated Human Embryonic Brain

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Cell clusters in a culture of dissociated brain from human fetuses at 8-12 weeks gestation in a serum-free growth medium were studied by immunohistochemical methods and electron microscopy. Heterogeneity of cell population in culture was demonstrated. Despite the influence of proliferation-stimulating factors, cell clusters contained not only nestin-immunopositive stem cells, but also β-tubulin-, vimentin-, and GFAP-positive cells differentiating by the neural pathway. Stem cells were localized on the surface of clusters. The percentage of stem cells in large clusters was lower than in small clusters.

Key Words: *stem cells; cell clusters; cultured embryonic cells; human*

Cultures of embryonic brain cells enriched with neural stem cells and precursors of neurons and neuroglia hold much promise for the development of new approaches to clinical and experimental neurotransplantation [3,4,7]. Multipotent stem and precursor cells possess high proliferative activity *in vitro*, which allows obtaining considerable amounts of these cells for intracerebral transplantation [3,8]. Previous studies demonstrated the development of stem and precursor cells in cultures of human fetal brain. A positive effect of growth factors and neurotrophins on the formation of proliferating stem cell pool was also shown [1-3,5,6,8]. Dissociated cells from fetal brain form floating cell aggregates in suspension cultures. Long-term culturing and dissociation during passages induced division of some cells and the formation of neurospheres. The relationships between various cells in aggregates and neurospheres, as well as localization of cells differing in the degree of differentiation after culturing in selective serum-free media with proliferative factors remain unclear. Here we performed histological, immunocytochemical, and electron microscopic examination of neurospheres and aggregates in cultures of dissociated brain cells from human fetuses. Since it was impossible to distinguish neurospheres from aggregates, all tissue fragments in this work are named cell clusters (as it was accepted in previous report [3,8]).

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MATERIALS AND METHODS

Human fetuses were obtained after abortions at 8-12 weeks gestation. Brain tissue fragments were dissociated by repeated pipetting in medium F-12. The obtained cell suspension was washed 2 times and centrifuged at 600*g* for 3 min. The cells were counted in a Goryaev chamber and their viability was estimated by flow cytofluorometry using trypan blue and propidium iodide.

The cells were phenotyped by flow cytofluorometry using antibodies to nestin (intermediate filament

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protein), vimentin (protein expressed in precursors of neuro- and glioblast and radial neuroglia cells), NeuN (nuclear marker in developing neurons), GFAP (glial fibrillary acidic protein of astrocytes), PLP (major protein of oligodendrocytes), β-tubulin III (protein expressed in neuroblasts), CD56 (neuronal adhesion protein), N-Cad (intercellular adhesion protein expressed in neural tube), cadherin (OB-Cad, mesenchymal cell adhesion protein), HLA-ABC (major histocompatibility complex class I factor), HLA-DR (major histocompatibility complex class II factor expressed on antigen-presenting cells), and CD34 (protein of hemopoietic precursor cells, endothelium, and tissue fibroblasts). Phenotyping was performed 2 times during culturing.

The cells were cultured in a serum-free NPBM medium (Clonetics) containing basic fibroblast growth factor, epidermal growth factor, and neuronal survival factor for 3-12 days. Some cultures were fixed for histological, electron microscopic, and immunocytochemical assays of floating cell clusters and paraffin slices of these structures. Other cultures were transferred into a complete nutrient medium containing fetal bovine serum and cultured in 50-ml flasks for 1 week. The cultures were consecutively fixed on days 1-7. Immunocytochemical assays were performed using antibodies to nestin (Biogenesis, 1:20), GFAP (DAKO, 1:250), β-tubulin III (ICN, 1:100), and vimentin (Neo-Markers, 1:100). The cells (floating and adherent to plastic) were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4), washed, and treated with 0.3% Triton X-100. Cultures or slices of cell clusters were incubated with primary antibodies for 18-20 h, treated with biotinylated secondary antibodies for 1 h (dilution 1:200, Vector Laboratories), and stained with DiI-labeled streptavidin (Molecular Probes). Primary mouse and rabbit antibodies were used for double staining. Bound primary mouse antibodies were detected using secondary fluorescein-labeled anti-mouse immunoglobulin antibodies (Sigma). Rabbit antibodies after treatment with secondary biotinylated anti-rabbit antibodies were stained with DiI-labeled streptavidin. This method allowed visualization of 2 antigens in one preparation (*e.g.*, GFAP and β-tubulin).

RESULTS

Forty-one cell cultures of dissociated human fetal brain were studied by flow phenotyping. Phenotyping showed that cells cultured in a selective serum-free medium retained their heterogeneity for a long time. However, the percentage of nestin-positive cells increased by 1.5 times. Examination of cultures transferred into a complete nutrient medium revealed cells adherent to plastic at various stages of differentiation. One day

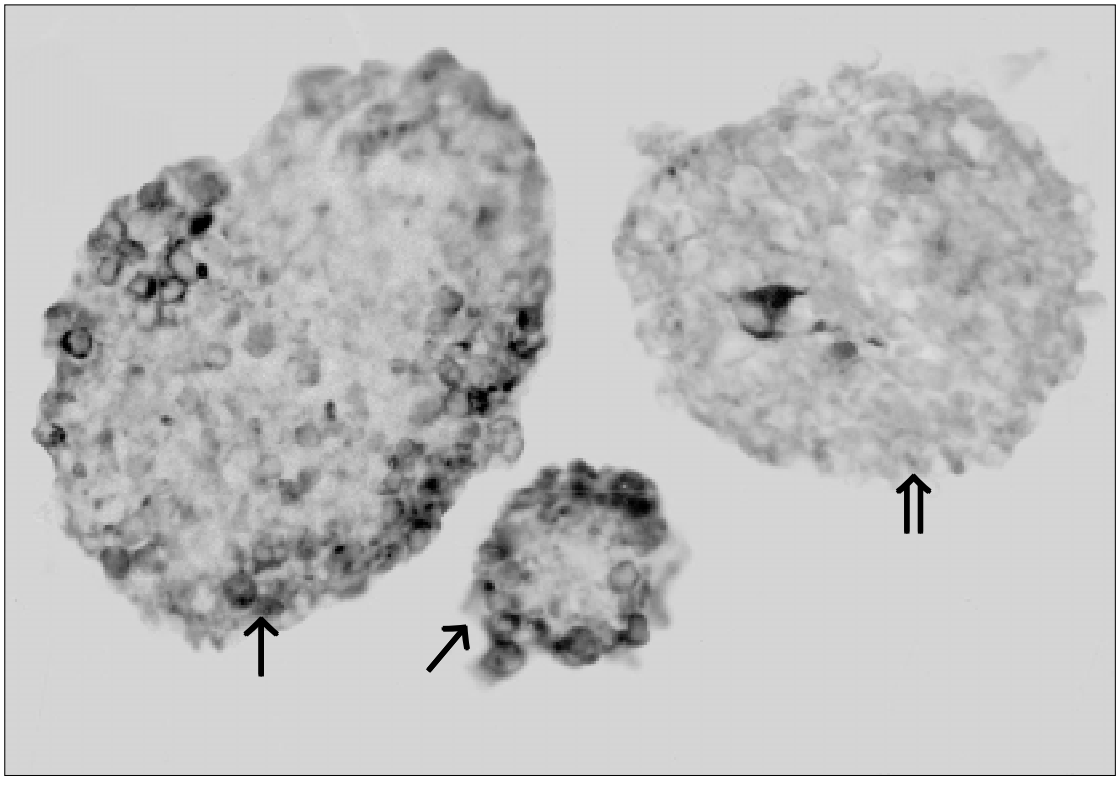

Fig. 1. Localization of nestin- (arrows) and GFAP-immunopositive cells (double arrows) in clusters. The smaller cluster contains a greater number of nestin-positive cells in the surface layer. Paraffin section. Immunocytochemical staining for nestin and GFAP (×400).

after transition into a complete nutrient medium, the cell clusters adherent to flask bottom contained not only nestin-positive stem cells, but also β-tubulin-, vimentin-, and GFAP-immunopositive cells. No cell migration from clusters was observed at this stage. After long-term culturing in a complete nutrient medium, cells migrating from adherent clusters formed surrounding migration zones. Migrating cells were presented by undifferentiated nestin-positive (stem) cells and cells containing β-tubulin (nerve cell progenitors), neurofilaments (neuroblasts), GFAP (astroblasts), and PLP (oligodendroblasts). Few bipolar cells containing neurofilaments had signs of migrating neuroblasts and were localized on the surface of clusters. β-Tubulin- and GFAP-immunopositive cells in the migration zone had several processes. β-Tubulin-immunopositive cells were densely positioned.

Immunocytochemical staining of floating clusters cultured in a serum-free medium revealed nestin-, β-tubulin-, and GFAP- immunopositive cells. Nestin-immunopositive cells with small round bodies had no processes. A thin layer of immunopositive cytoplasm surrounded unstained nuclei in these cells. Examination of paraffin sections revealed the presence of nestin-positive cells in the marginal zone of clusters (Fig. 1). Nestin-positive cells were localized on the surface of small clusters. Their ratio in small clusters was much higher than in large clusters (Fig. 1).

GFAP-immunopositive cells had large and intensively stained bodies, probably due to thicker cytoplasmic layer compared to nestin-positive cells (Figs. 1 and 2). Long and branched processes of GFAP-immunopositive cells penetrated the core of small clusters. β-Tubulin-positive cells were less intensively stained and had shorter processes.

Examination of semithin sections and electron microscopy of clusters cultured in a serum-free medium revealed no peculiar structural organization typical of embryoid bodies. These clusters were pierced by thin undifferentiated cell processes containing various amounts of the cytoplasm. Cells with dark nuclei and dark cytoplasm differed from cells with relatively light nuclei and light cytoplasm. Many cells had blade-shaped nuclei (Fig. 3, *b*) surrounded by thin cytoplasmic rim with minimum content of organelles. Some cells underwent mitotic division. However, no specific localization of mitotic cells in clusters were found. We found no specialized cell-cell contacts. In some cells the cytoplasm was well developed and contained a great number of mitochondria and cisternae of the granular and rough endoplasmic reticulum, which did not form the compact Golgi apparatus (Fig. 3, *a*). Some processes contained vacuoles typical of growth cones. The cytoplasmic membrane of some cells formed outgrowths with growth vesicles (Fig. 3, *a*). We revealed no structural signs of cell degeneration even in relatively large clusters (80 μ).

Cytological examination and flow fluorometry show heterogeneity of cell populations from dissociated human brain cultured in a serum-free medium with proliferative factors inhibiting cell differentiation. The cultures contain differentiated cells, which is probably related to the heterochronic development of neural cells from human fetal brain and the presence

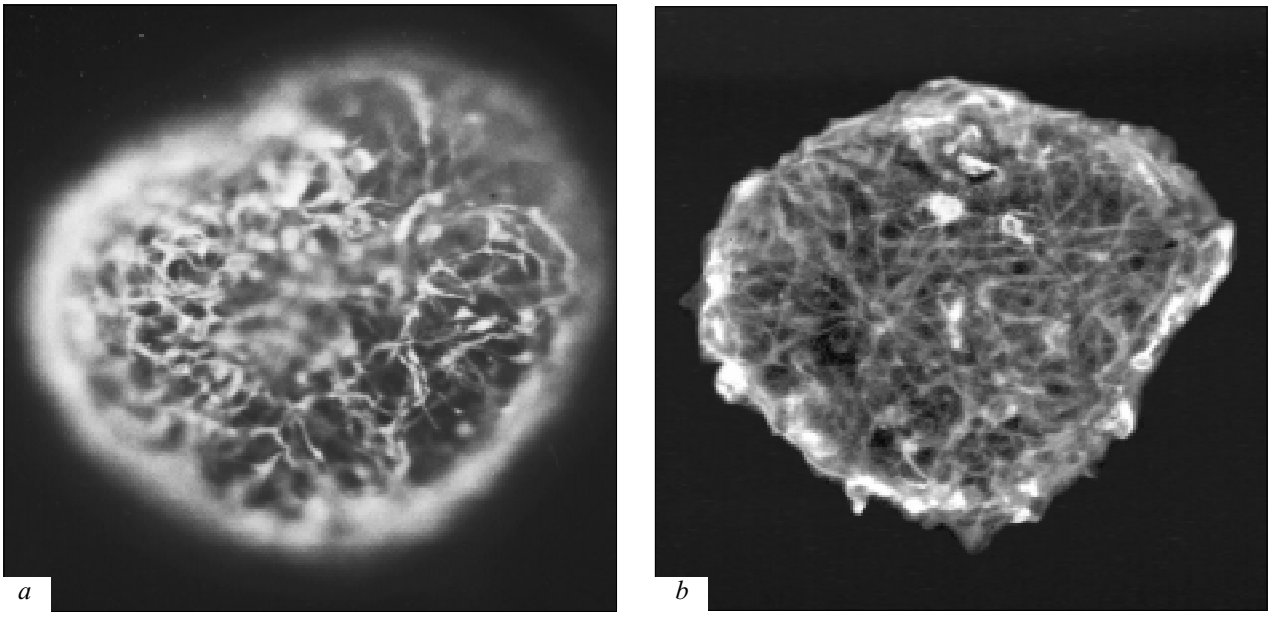

Fig. 2. GFAP- (*a*) and β-tubulin immunopositive cells (*b*) in intact floating cluster. Immunocytochemical staining for glial fibrillary acidic protein and β-tubulin (×500).

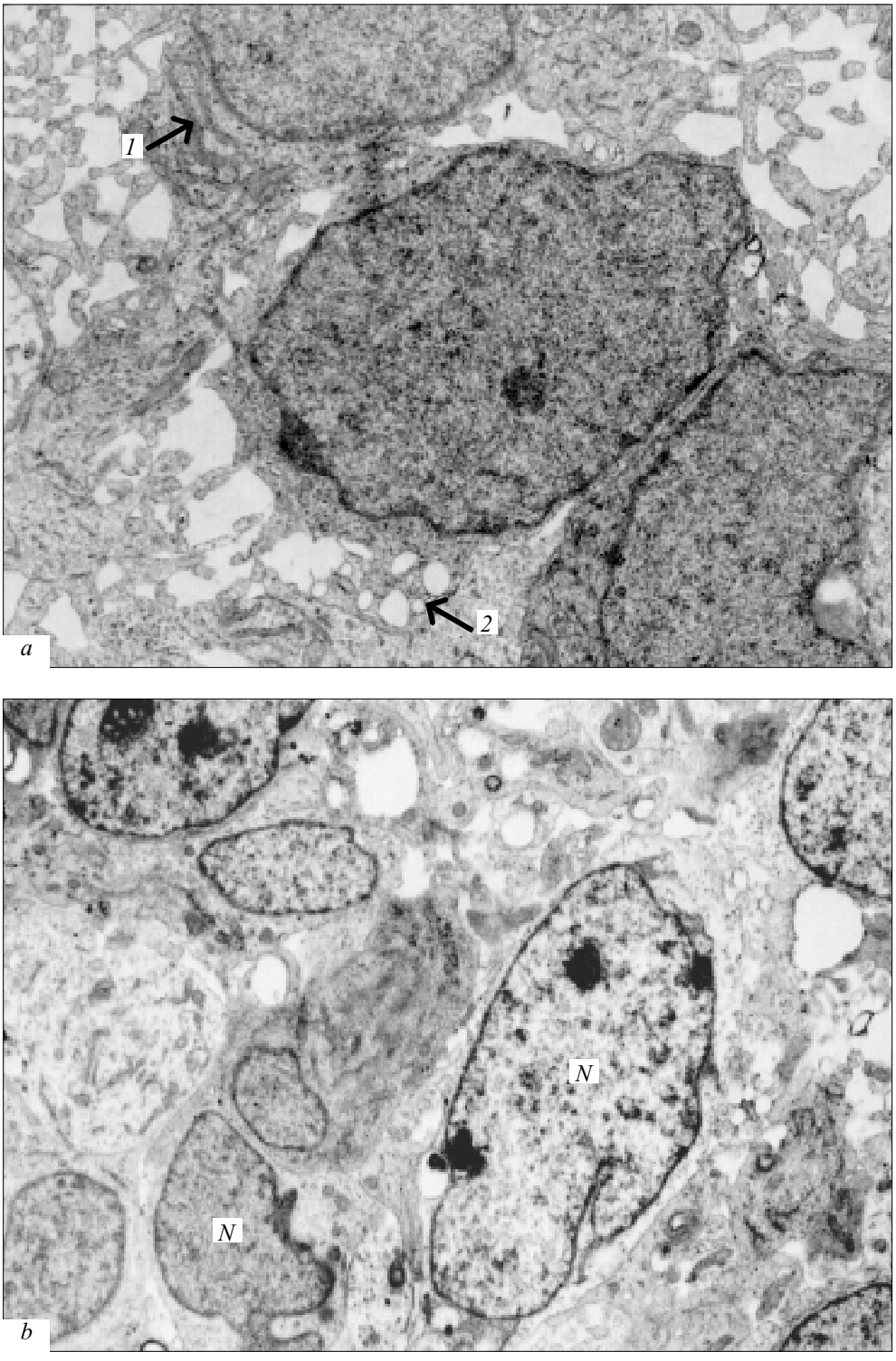

Fig. 3. Electron microphotographs of cluster core (×80,000). Dark cells with granular endoplasmic reticulum (*1*) and cytoplasmic membrane outgrowths (*2*, *a*); dark and light cells with blade-like nuclei (*N*, *b*).

of more differentiated cells. The effects of proliferation-stimulating factors in the culture medium are manifested in the formation and differentiation of nestin-positive cells. High content of these cells on the surface of cell clusters is associated with their migration, sorting and, probably, suppressed effects of proliferative factors in deep layers of these clusters. The decreased content of nestin-positive cells in large clusters is related to cell-cell interactions, which modulates differentiation of these cells and formation of mature neural phenotypes.

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