Formation of Neuroepithelial Structures in Culture of Neural Stem Cells from Human Brain

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Dissociated fetal brain cells in a floating culture form clusters and then neurospheres, some of which contain structures shaped as cell "rosettes". The cells in these "rosettes" are arranged radially around the central cavity, in which their apical processes form desmosome-like contacts. Mitotic division of cells in the "rosettes" is associated with migration of the nuclei, similarly to division of neuroepithelial cells in the neural tube during normal embryogenesis. These cells express nestin, a marker of neural stem cells. The cells in "rosettes" found after transplantation have similar characteristics.

Key Words: *human brain neural stem cells; neurospheres; immunohistochemistry*

Culturing of dissociated human fetal brain cells leads to the formation of spherical structures, neurospheres [13]. Two ways of neurosphere formation are possible in cultures of dissociated brain cells, in contrast to cultures obtained from single cells. One way is aggregation of dissociated cells; the other is development from single stem cells, whose descendants remain closely connected to each other. True neurospheres "grow" from single stem cell.

It was previously shown that irrespective of their origin, neurospheres consist of cells at different stages of neural differentiation: stem and progenitor cells, neuroblasts and glioblasts [1,2,5,10]. The data on the organization of neurospheres and distribution of certain cell phenotypes in them are scanty. It is only known that nestin-positive stem cells are located on the surface of neurospheres [2,6]. On the other hand, there are data on the formation of spheroid aggregations in cultures of dissociated fetal retinal cells [12], cultures of fetal stem cells directed toward neural differentiation [7,15], and cultured explants of human fetal neocortex [3]. Multilamellar structures, closed around the central cavity, were found inside these aggregations. These structures were called "rosettes", due to their characteristic appearance on sections. Histotypical organization of normal retinal layers was found in "rosettes" forming in fetal retinal cultures [12]. Partially or completely formed tubes from neuroepithelial cells developed in cultured neocortical explants [3]. Culturing of fetal stem cells in collagen gel and transplantation of these cells into fetal brain led to the appearance of neuroepithelial formations shaped as complete "rosettes". Cells in these rosettes possessed mitotic activity and intensely expressed nestin, a marker of neuroectodermal stem cells [7,15].

Examinations of sections of neurospheres forming in human fetal brain tissue culture showed that some of them contained rosette-like structures. In some cases "rosettes" were seen after transplantation of cell culture into adult rat brain.

We studied the origin of cells forming rosette-like structures in neurospheres *in vitro* and after transplantation into adult rat brain.

MATERIALS AND METHODS

Human fetal brain tissues were derived from 9-week embryos obtained during medical abortion. Tissue

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fragments were isolated from periventricular area of fetal brain, put into F-12 medium, and cell suspension prepared by repeated pipetting was then cultured in DMEM/F12 growth medium with N2 supplement containing epidermal growth factor (EGF) and basal fibroblast growth factor (bFGF). Neurospheres formed in this culture were examined by histological, immunohistochemical, and electron-microscopic methods during different periods after the start of culturing (on days 1-65). The material for immunohistochemical analysis was fixed in 4% paraformaldehyde in PBS (pH 7.2), treated with 30% sucrose, and sliced on a cryostat. For electron microscopy the neurospheres were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.3), treated with 2% osmium tetroxide solution in the same buffer, dehydrated in alcohols, and embedded in epon. The resultant blocks were sliced on an LKB ultratome. Semithin sections (1-2 μ) were stained with methylene blue and examined under a light microscope. Ultrathin sections for transmission electron microscope were applied onto copper lattices and contrasted with uranyl acetate and lead citrate.

The behavior of cultured cells was studied after their transplantation into rat brain. Female Wistar rats (250-300 g) subjected to acute hypoxia in a special pressure chamber (the animals survived for 3 min at atmospheric pressure of 180 mm Hg) served as the recipients. After 24 h the animals were narcotized with chloralhydrate (300 mg/kg). Suspension of mechanically dissociated neurospheres cultured for 65 days was stereotactically injected into the hippocampal area (coordinates A-3.5-4.0 mm, L-2.5 mm, V-5 mm). Twenty-seven days after transplantation narcotized animals were perfused with 4% paraform in PBS, the brain was removed and treated with 30% sucrose; the sections were prepared on a freezing microtome.

Immunohistochemical studies of cryostat sections of neurospheres and rat brain sections were carried out with primary antibodies to nestin (anti-Human Nestin, Chemicon, 1:50), human nuclear proteins (anti-Human

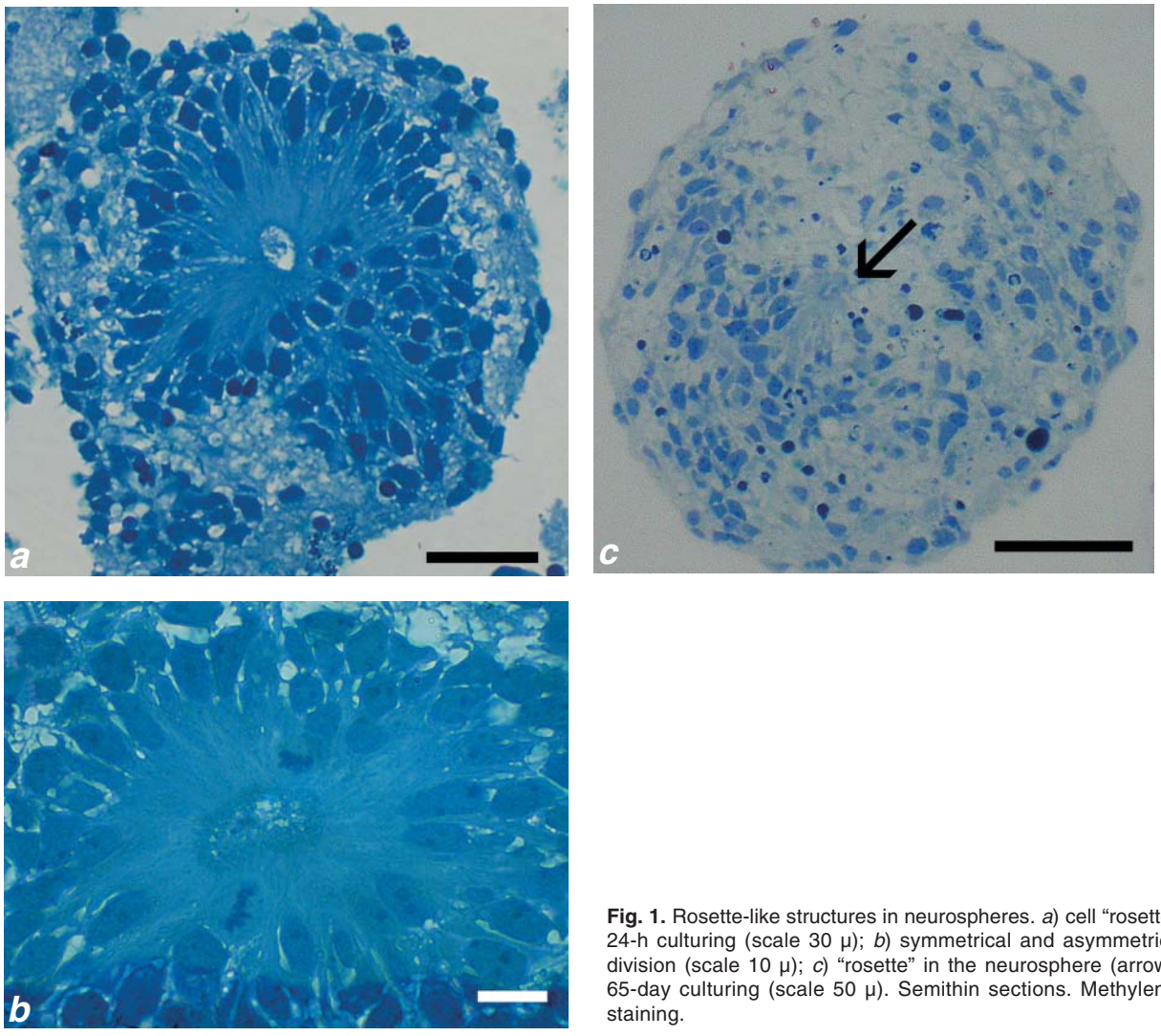

Fig. 1. Rosette-like structures in neurospheres. *a*) cell "rosette" after 24-h culturing (scale 30 μ); *b*) symmetrical and asymmetrical cell division (scale 10 μ); *c*) "rosette" in the neurosphere (arrow) after 65-day culturing (scale 50 μ). Semithin sections. Methylene blue staining.

Nuclei, Chemicon, 1:30), β-tubulin-III (anti β-tubulin-III, Abcam, 1:100), and glial fibrillar acid protein (anti-GFAP, Chemicon, 1:100). The material was then treated in solution of secondary antibodies labeled with Texas Red and Cy-2 fluorescent stains (Jackson, 1:100). The preparations were clarified with 50% glycerol in PBS, embedded in glycerol under slides, and examined under Opton-3 fluorescent microscope and laser scanning confocal microscope (Leica).

RESULTS

Morphological analysis of semithin sections of neurospheres stained with methylene blue indicated the presence of groups of cells organized into "rosettes" among chaotically distributed poorly differentiated cells (Fig. 1). The cells in these "rosettes" had uni- or bipolar shape and were arranged radially at some distance from the center close to each other. Cell nuclei were arranged in several layers. The center of the rosette had a cavity; apical processes of cells constituting the rosette came together in this cavity. Some neurospheres contained up to 3 "rosettes". Degenerative and dead cells were sometimes seen at the periphery, but not inside the "rosettes". Presumably, some cells migrating to the periphery undergo apoptosis.

Mitotically dividing cells were often seen in "rosettes". The nuclei of dividing cells lay closer to the center than the nuclei of other cells in the rosette, which was similar to the proliferative behavior of neuroepithelial cells *in situ* associated with migration of the nuclei and mitoses near the neural tube lumen (in the ventricular zone). During mitosis in the "rosette" the chromosome separation axes are oriented either parallel to the "rosette" radius? or perpendicularly to it, which seems to correspond to asymmetrical and symmetrical mitoses, respectively. In symmetrical mitosis the daughter cells remained in the "rosette", while after asymmetrical division one daughter cell remained in the "rosette" and the other migrated to the periphery. Presumably, the orientation of the division spindle axis in neuroepithelial cells in embryogenesis and in adult brain subependymal zone is a factor determining further development: whether

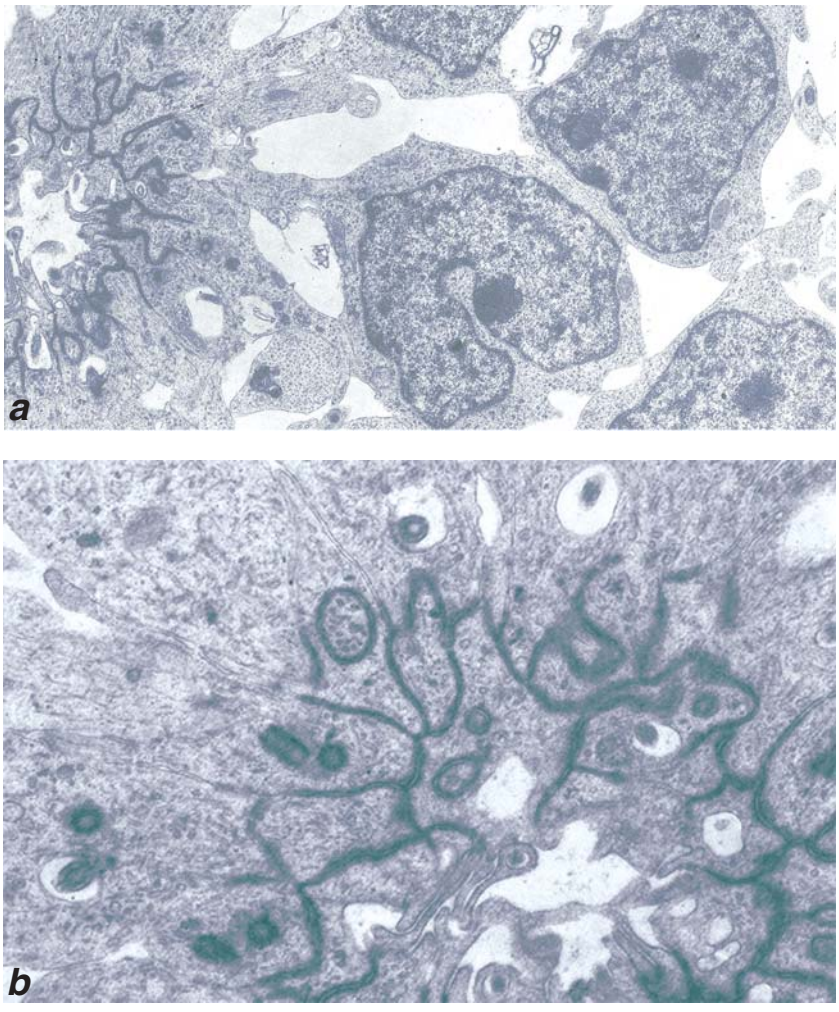

Fig. 2. Ultrastructural organization of a "rosette". *a*) "rosette" sector; close contacts of cells around the central cavity, processes and cell nuclei at the periphery, ×4800; *b*) cell processes and contacts in the center of "rosette", ×10,000.

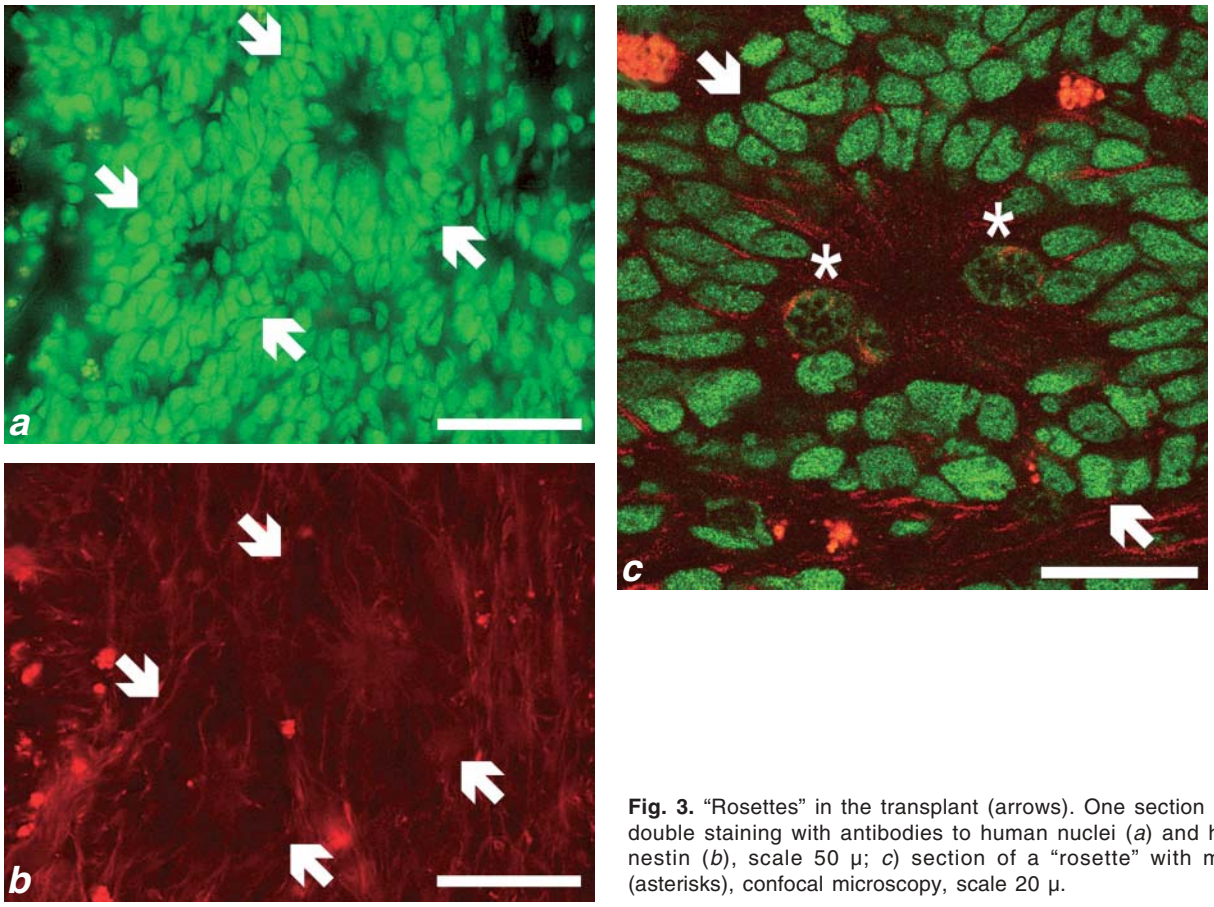

Fig. 3. "Rosettes" in the transplant (arrows). One section shows double staining with antibodies to human nuclei (*a*) and human nestin (*b*), scale 50 μ; *c*) section of a "rosette" with mitosis (asterisks), confocal microscopy, scale 20 μ.

both daughter cells continue mitotic division or one of them enter the next phase of development (migration from the ventricular zone and differentiation) [4].

Electron microscopy showed that "rosettes" consisted of cells of the same type with round or elongated electron-dense nuclei, sometimes lobed. Structures of the Golgi complex, cisterns of the smooth endoplasmic reticulum, rare formations of rough endoplasmic reticulum, numerous polysomes, some ribosomes, and solitary large mitochondria were seen in the perinuclear cytoplasm. Cytoskeleton components were presented by rare microtubules and filaments. Apical processes typically contained cisterns of smooth endoplasmatic reticulum, multiple microtubules, filaments, centriole, and ciliary complex. Apical processes near the central cavity were connected via long desmosome-like contacts (Fig. 2) similar to those in ependymal cells.

Immunohistochemical studies of neurosphere specimens showed that cells in "rosettes" expressed nestin, a marker of neuroectodermal stem cells.

Completely formed "rosettes" were detected as early as after 24-h culturing. This suggests that "rosettes" are either a result of autoassembly from dissociated neuroepithelial cells, or form due to closure of neuroepithelial fragments retained even after long repeated pipetting during cell inoculation into the culture.

The "rosettes" are highly stable, because they were detected in neurospheres after 65 days of culturing, despite regular mechanical dissociation of cells during replacement of the medium. Moreover, 27 days after transplantation of these cultures into adult rat brain similar structures were found in the solid core of transplanted cells. "Rosettes" were situated among chaotically distributed transplanted cells, which were well preserved, without necrotic changes. The transplants are usually vascularized during this period, but the "rosettes" were not associated with vessels.

The morphology of rosette-like structures in the transplants was similar to that in neurospheres. The cells proliferated, and mitoses were associated with migration of the nuclei. Immunohistochemical analysis showed intense expression of nestin in cells also during the division phase (Fig. 3). Neuroblasts were situated outside the "rosettes", they were detected by antibodies to β-tubulin-III. No glial cells either in "rosettes" or among other cells of the transplant were detected by staining for glial acid fibrillar protein. On the other hand, rare processes of the recipient glial cells growing into the transplant were clearly seen.

The findings indicate that rosette-like clusters of cells with morphological organization resembling the neural tube in normal embryogenesis are formed in floating cultures of human neural stem cells from the start of culturing. Cells constituting the "rosettes" are ependymal or neuroepithelial by many signs (nestin expression, presence of cilium, and characteristic migration of the nuclei during mitosis). These cell structures are retained for a long time in tissue cultures from fetal human brain growing in a medium with specific mitogens. Moreover, the rosette-like structures were detected after culture transplantation into rat brain, where their cells divided and retained undifferentiated phenotype. The "rosettes" in the transplants were histotypically similar to neoplastic cells, both types of cells expressed nestin. However, cells in "rosettes" differed greatly from tumor cells by many ultrastructural and phenotypical signs, and no characteristic vascularization and necrosis were observed in the transplants [13]. The presence of "rosettes" in the transplants was due to the fact that suspension of mechanically dissociated cells was used for transplantation, and it seemed that "rosettes" or neurospheres containing "rosettes" were not broken and got into the transplant intact. So long (up to 3 months) persistence of cell "rosettes" indicates their high mechanical strength, which can be due to cell connection via desmosome-like contacts. Presumably, unique cell microenvironment essential for long-term maintenance of neural stem cells in culture is retained in "rosettes" due to specific neuroepithelial organization. In addition, the capacity of dissociated stem cells of different origin to aggregate *in vitro* with the formation of histotypical structures is now regarded as a perspective trend in the development of tissue engineering [8,9,11,15]. Studies of cellular and molecular mechanisms, underlying this spontaneous aggregation, are extremely important for this direction, as it will help develop methods for directed histotypical organization of cells under conditions of culturing.

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