BLBP-Immunoreactive Cells in the Primary Culture of Neural Precursors from Embryonic Mouse Brain

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The population of neurosphere cells was studied by staining for the radial glial marker BLBP protein. An immunohistochemical study of cultures showed that neurosphere cells proliferate and retain the ability for pluripotent differentiation over 9 passages. BLBP-immunoreactive cells were present in neurospheres at all passages. However, they did not belong to the population of proliferating multipotent precursors or GFAP-positive cells. BLPB expression was detected in S-100b-positive cells of astrocyte differentiation. Our results suggest that BLBP-immunopositive cells of the radial glia *in vitro* loss the state of multipotent precursors and differentiate into the astroglia. Otherwise, some heterogeneous cells of the radial glia include a previously unknown linear population that differentiates into S-100b-positive astrocytes.

Key Words: *BLBP; radial glial cells; neurospheres; immunohistochemistry*

During normal development of mammals, all regions of the central nervous system (CNS) contain a particular type of cells that are mainly typical of embryogenesis (radial glial cells). It was hypothesized that these cells have a guide function for radial migration of newborn neuroblasts, but then differentiate into astrocytes [13,16]. Recent *in vivo* and *in vitro* experiments showed that radial glial cells serve as precursor cells for the majority of nerve cells [9,11].

Radial glial cells originate from the epithelium of the neural tube and generate a variety of nerve cells in all regions of CNS during early embryogenesis. During the perinatal period, they loss the apical process and migrate into the brain parenchyma (typical differentiated astrocytes) or die [16]. In adult mammals, the radial glia is preserved only in the hippocampal dentate gyrus. Otherwise, this structure exists in the form of specialized cells of the Bergman and Muller glia in the cerebellum and retina [7].

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Previous studies revealed that radial glial cells express molecular markers of multipotent precursors (vimentin, nestin, RC2, and BLBP) and differentiated astrocytes (GFAP, except for rodents and GLAST) [7,8,12]. BLBP (brain lipid-binding protein; FABP7, B-FABP) has a particular place among these markers. This lipid-binding protein is specific for the nervous system. BLBP belongs to a family of fatty acid-binding proteins (FABPs).

The time of BLBP expression in radial glial cells *in vivo* correlates with neuroblast generation and migration along radial processes [4]. Similar results were obtained in *in vitro* experiments with the culture of cerebellar glial cells from newborn mice. BLBP expression was stimulated during cocultivation with the population of low-differentiated granular neurons [5]. A major function of BLBP is the regulation of neuroblast adhesion to cell processes of the radial glia. Hence, this protein is involved in radial migration [4]. The *BLBP* gene is a direct target for the Notch-activated signal pathway [2], which has a role in the maintenance of radial glial cells in the developing brain [6]. Nearly all neurons in various regions of the brain originate from BLBP-positive cells of the radial glia [1].

These data indicate that stem and progenitor cells of the embryonic brain constitute the population of radial glial cells [3,10]. This population can proliferate during cultivation in serum-free medium with epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF-2). It occurs in the form of free-floating aggregates, or neurospheres [15]. Much attention was paid to stem cells in the culture of neurospheres. However, radial glial markers were not used to study these cells.

Here we studied the population of immunohistochemically stained cells for the radial glial marker BLBP protein. Experiments were performed with long-term passage of cultures from neural precursor cells of embryonic mouse brain.

MATERIALS AND METHODS

Cell cultures were obtained from the embryonic forebrain of C57Bl/6 mice (day 14 of intrauterine development). Brain tissues were minced with scissors and maintained in Accutase dissociation regent (Sigma) at 37°C for 20-30 min. The samples were carefully pipetted for 5-10 min. The suspension was washed 2 times with Hanks solution (Sigma). The cells were inoculated to a mattress in serum-free medium (500,000 cells/ml). The culture medium consisted of DMEM/F12 (ratio 1:1, Gibco), 2 mM L-glutamine (Sigma), 8 mg/ml heparin (Sigma), N2 addition (1:100, Gibco), EGF (20 ng/ml, Sigma), and FGF-2 (20 ng/ml, Sigma). The medium was replaced (50%) at 2-3-day intervals. Passage of growing neurospheres was performed at 4-6-day intervals. Neurospheres were passaged in Accutase regent (Sigma) with dissociation into the suspension of individual cells and further inoculation to a mattress. The effectiveness of dissociation was evaluated in a microscopic study of the mattress. The cells were subjected to 9 passages. The first inoculation was considered as passage 0. The time of treatment was 48 days. Some neurospheres were taken at passages 0-4, 8, and 9 (*i.e.*, initial and final stages) and placed in a petri dish (diameter 35 mm) with 7 cover glasses (diameter 10 mm). The medium did not contain growth factors, but included 10% fetal bovine serum (Gifco). Adhesion of neurospheres to cover glasses was followed by the migration and differentiation of cells. The glasses with samples of adhered neurospheres were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) at room temperature for 10-15 min. The samples were thoroughly washed with PBS (pH 7.4). Immunohistochemical staining was performed immediately after this treatment. Otherwise, the samples were embedded in glycerol (Sigma) and stored at -20°C.

A double immunohistochemical treatment was performed by the standard method according to the recommendations of antibody manufacturers. The samples were incubated in a solution of primary antibodies from host animals (corresponding concentration) at 4°C for a night. The solvent for primary antibodies consisted of 0.3% Triton X-100 (Sigma), 0.05% NaN3 (Sigma), and 10% normal goat serum (Sigma) in PBS. Experiments were performed with mouse primary antibodies to nestin (1:200), mouse antibodies to vimentin (1:5), mouse antibodies to β-tubulin III (1:400), mouse antibodies to PCNA protein (proliferating cell nuclear antigen, 1:200), mouse antibodies to O4 (1:50), rabbit antibodies to GFAP protein (1:400, Chemicon), mouse antibodies to S-100b protein (1:50), and rabbit antibodies to BLBP protein (1:100, Abcam). The samples were washed 3 times with PBS and incubated in a solution of secondary antibodies to rabbit and mouse immunoglobulins. These immunoglobulins were labeled with fluorochromes Texas Red (JacksonImmuno) and Alexa 488 (Invitrogen), respectively. Incubation was performed at room temperature for 2 h. The solvent for secondary antibodies consisted of 0.3% Triton X-100 (Sigma) and 0.05% NaN3 (Sigma) in PBS. When required, the samples were additionally stained with a solution of DNA-binding fluorescent dye Hoechst 33342 (Sigma) for the identification of cell nuclei. The prepared samples were embedded into glycerol and examined under an Opton-3 luminescence microscope. The images were obtained using an Olympus SP-350 digital camera.

RESULTS

Similar data were obtained previously in *in vitro* studies with the culture of neural precursor cells. However, there are some discrepancies between the results of experiments of various research groups. Therefore, the growth and differentiation of cells were studied during cultivation of neural precursors from embryonic mouse brain.

In primary cultures of dissociated cells from embryonic mouse brain, cell growth was accompanied by the formation of free-floating colonies (neurospheres; Fig. 1, *a*, *b*). Studying the passages showed that neurospheres are formed due to aggregation of several cells from the initial suspension and further proliferation of cells in the composition of colonies. Otherwise, the formation of neurospheres was related to division of individual cells. The study of passage 0-9 cells showed that the growth rate of neurospheres remains practically unchanged at various passages. Passage 9 was used

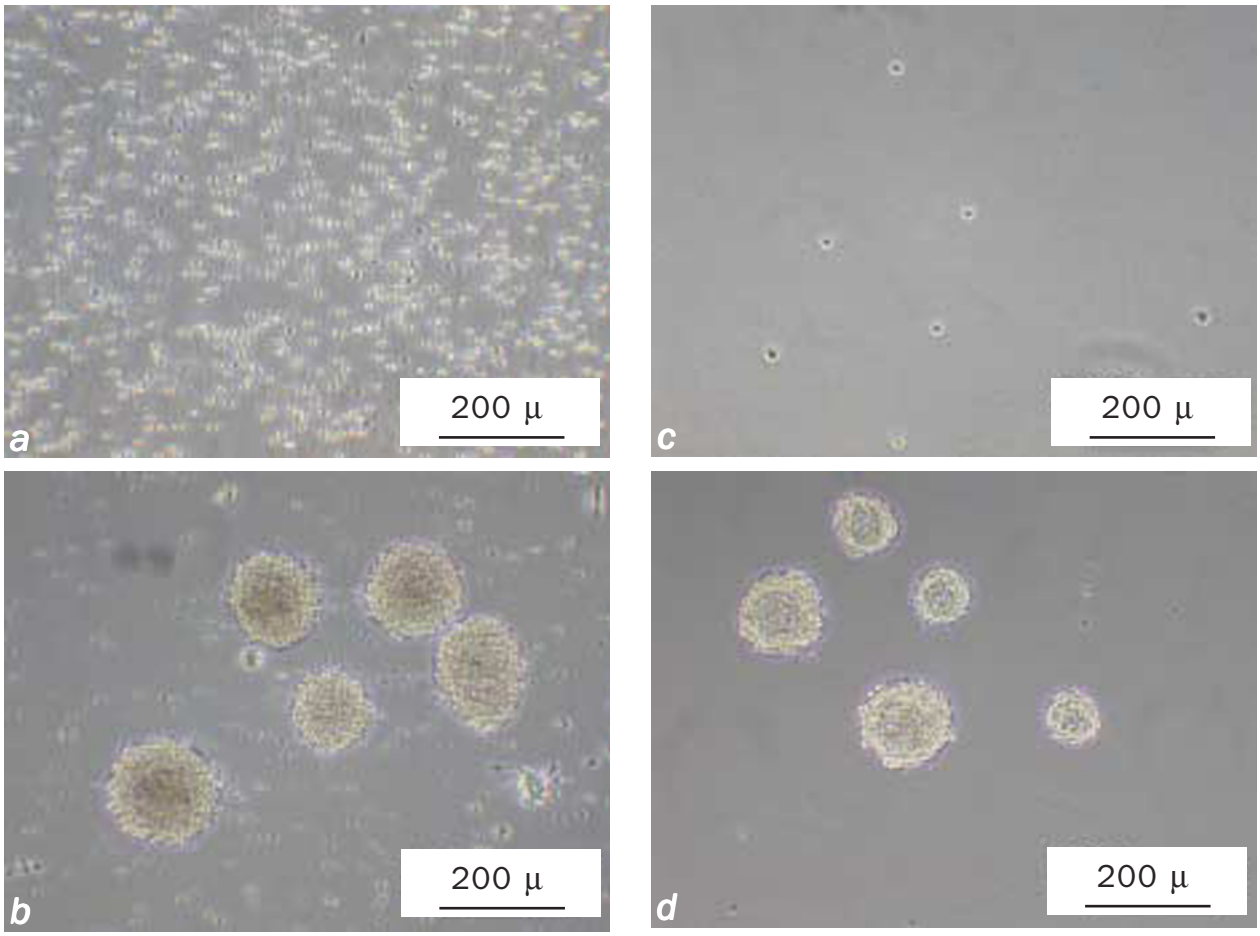

Fig. 1. Neurospheres in primary cultures of neural precursor cells (phase contrast). Initial suspension and neurospheres at passage 1 (*a*, *b*); neurosphere growth from the cell suspension inoculated at clonal density during passage 9.

to evaluate the ability of neurosphere cells to proliferate under conditions of clonal density. Studying the growth of neurospheres in a clonal culture (cell density in the initial suspension ~2000-3000 cells/ml) revealed that not less than one-third of inoculated cells differentiate into new neurospheres (Fig. 1, *c*, *d*).

The differentiation of adherent cells on cover glasses of neurospheres was studied in various stages of cultivation. All passages were shown to include the undifferentiated cells with staining for nestin and vimentin (population of stem/progenitor cells). There were also the following cells, which differentiated into three types of CNS cells: neurons (stained for β-tubulin III), astrocytes (stained for GFAP and S-100b), and oligodendrocytes (stained for O4, Fig. 2). Significant differences in histological characteristics were revealed during staining of neurospheres with antibodies to β-tubulin III at passage 0 and further passages. A considerable number of β-tubulin III-immunopositive neuroblasts were found in all neurospheres at passage 0. They were probably presented by surviving cells of the native brain. The cells had long and branching processes, which formed a specific network covering the adherent neurospheres. These features were not observed at further passages. At passages 1-9, individual β-tubulin III-immunopositive cells or clusters of ten cells were revealed only in some neurospheres. Hence, the culture has a constant small number of multipotent precursor cells that may generate neuroblasts.

The cells of astroglial differentiation were studied using antibodies to GFAP and S-100b. All passages contained the cells expressing these proteins. However, none of the cells coexpressed these markers. The data indicate that cultured cells of astroglial differentiation were presented by at least two different populations (Fig. 2, *c*).

No changes were found in the cellular composition (types of cells with immunohistochemical markers of differentiation) and morphology of cells at passages 1-9. We conclude that the differentiation properties of cells in primary cultures of neural

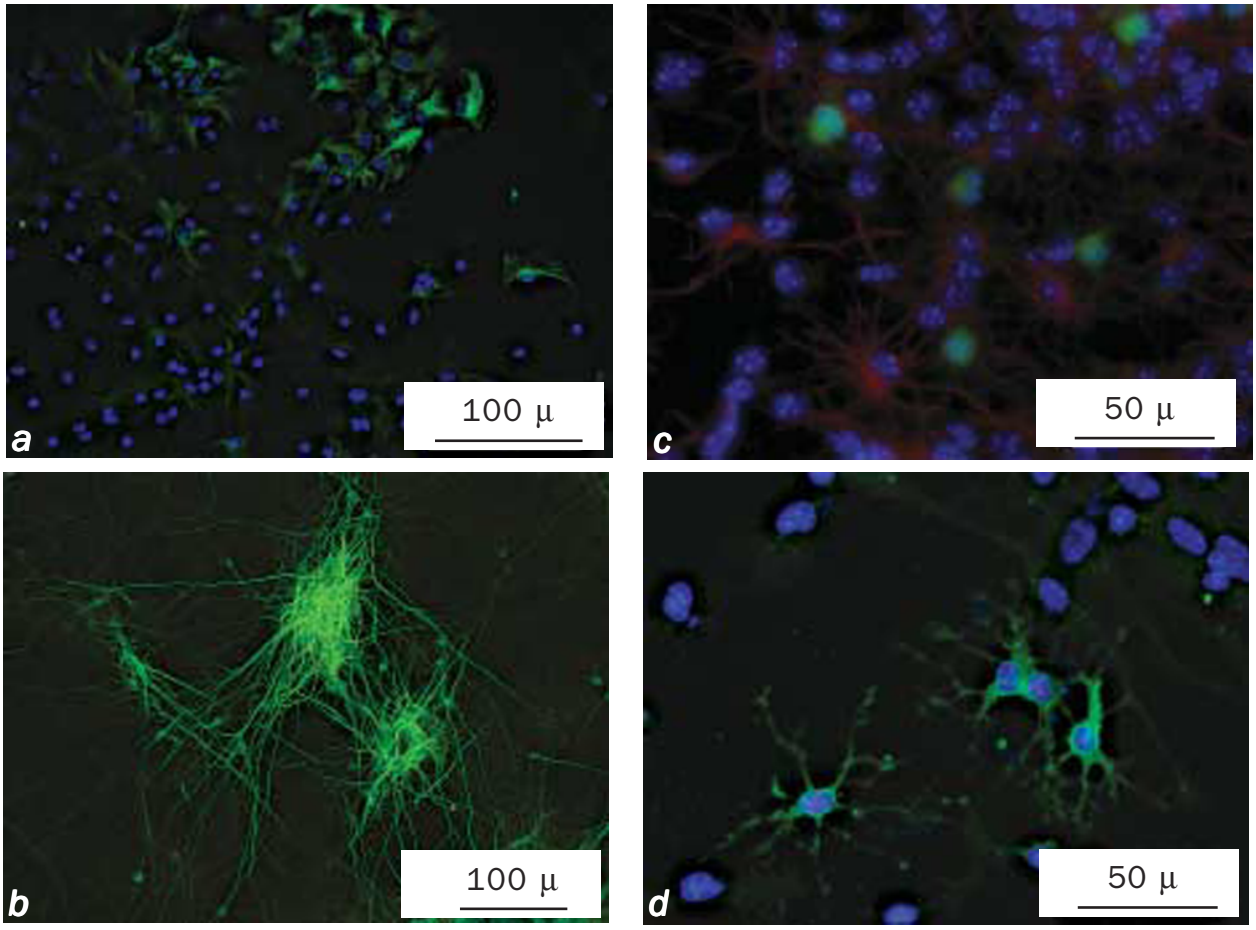

Fig. 2. Cell differentiation in adherent neurospheres at various passages. Multipotent nestin-immunoreactive stem/progenitor cells (passage 9, *a*); b-tubulin III-positive cells differentiating into neurons (passage 0, *b*); GFAP-immunopositive (red) and S-100b-immunopositive (green) astrocytes (passage 0, *c*); oligodendrocyte staining for O4 (passage 1, *d*). Additional staining of cell nuclei with Hoechst 33342 (blue).

precursors from embryonic mouse brain remain unchanged (at least up to passage 9).

The expression of BLBP (protein marker for radial glial cells) was studied in cultures with these characteristics. Staining with antibodies to BLBP showed that all passages contain the cells immunopositive to this protein (Fig. 3). There were two morphological types of cells, including a considerable number of multipotent cells and individual bipolar cells. Morphologically, they differed from nestin-immunoreactive, vimentin-immunoreactive, GFAP-immunoreactive, and β-tubulin III-immunoreactive cells. These cells were located on other cells. None of the cells were individual or adhered to the glass surface.

Similarly to the above-described culture, histological characteristics of the samples stained for BLBP were different at passage 0. The majority of passage 0 cells had long and branching processes, which formed a network on neurospheres. Due to weak staining of cell bodies, only BLBP-immunopositive processes were often found. At further passages, BLBP-positive cells did not have long processes. The bodies and processes of these cells were strongly stained.

We analyzed the coexpression of BLBP with other immunohistochemical markers. The proliferation of BLBP-immunopositive cells was studied in the culture. The samples of neurospheres were subjected to double staining with antibodies to BLBP protein and cell proliferation marker PCNA. Coexpression was detected only in individual cells. The majority of BLBP-positive cells were PCNA-negative cells (Fig. 4). These data indicate that the population of BLBP cells is not characterized by strong proliferation.

BLBP expression was undetected in the culture of stem/progenitor cells stained for protein markers nestin and vimentin. Nestin and vimentin serve as the markers of radial glial cells in the developing brain [7]. Moreover, they can coexpress nestin and BLBP [1]. However, these features were not observed in our experiments (Fig. 5, *a*, *b*).

Previous studies revealed that BLBP-immunoreactive cells have no proliferative activity. These

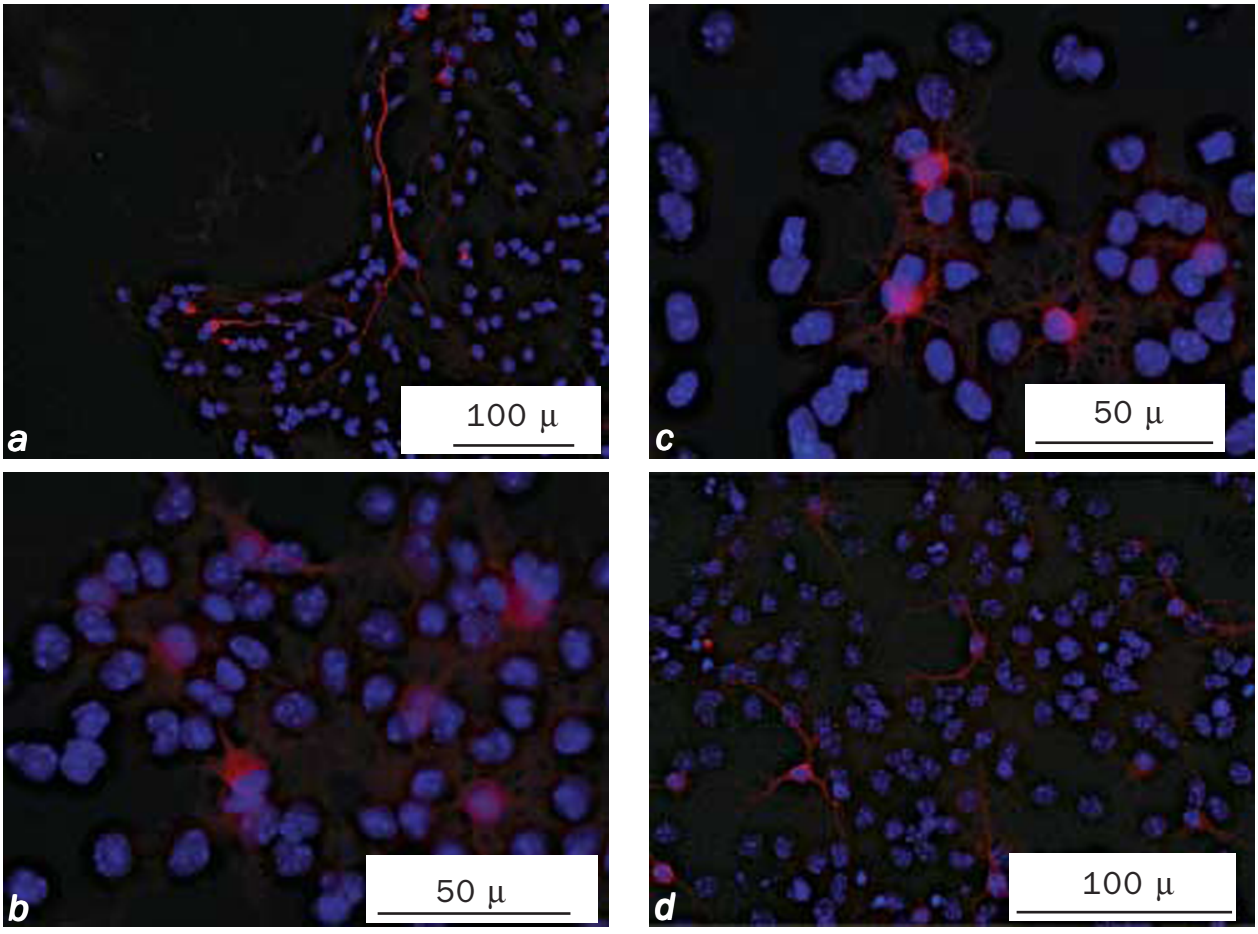

Fig. 3. BLBP-immunoreactive cells. Passage 0 (*a*); passage 3 (*b*); passage 4 (*c*); passage 9 (*d*). Additional staining of nuclei with Hoechst 33342 (blue).

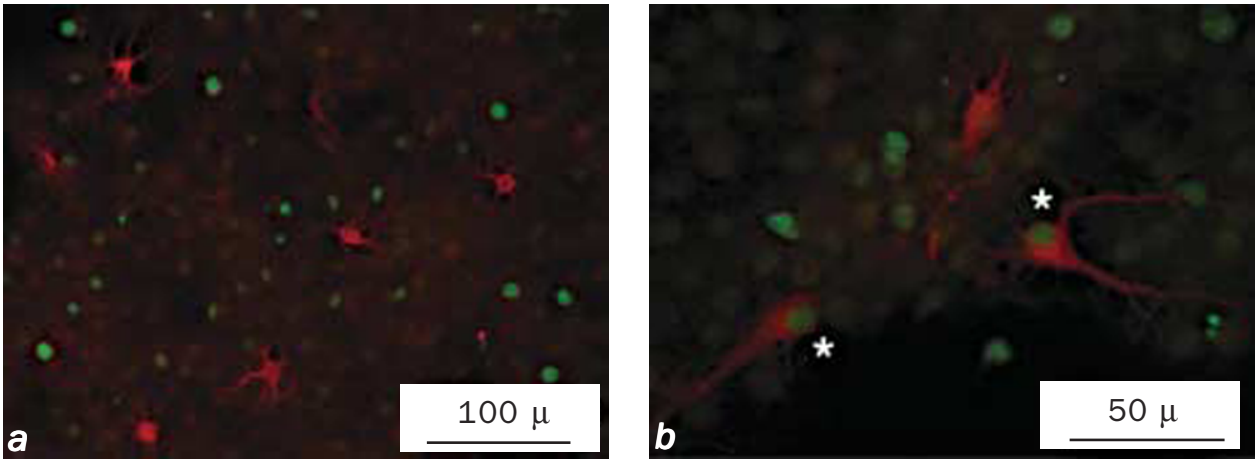

Fig. 4. Proliferative activity of BLBP-immunoreactive neurosphere cells at passage 8. Among BLBP-immunoreactive cells (red), there are individual cells stained for the cell proliferation marker PCNA (green). *Double-labeled cells.

data and results of our experiment suggest that BLBP-immunoreactive cells *in vitro* loss the property of pluripotency and do not function as neural precursors. A further study showed that BLBP-expressing cells are committed to astroglial differentiation. Double staining of adherent neurospheres with antibodies to BLBP and S-100b (astrocyte differentiation marker) revealed that nearly all BLBP-immunoreactive cells are stained for S-100b. Individual cells were shown to express only one of the

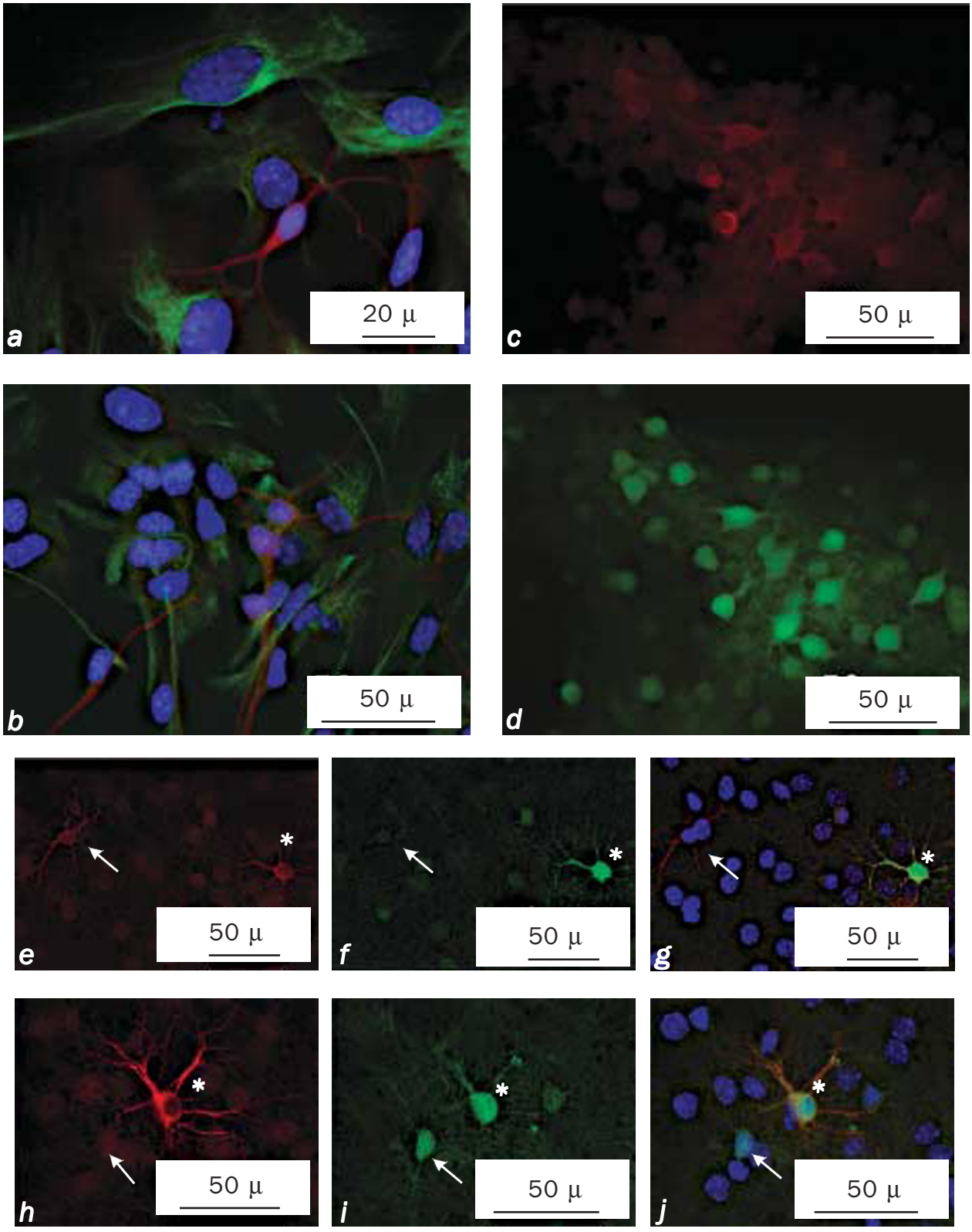

Fig. 5. Coexpression of differentiation markers in BLBP-immunoreactive cells. *a*, *b*: BLBP-positive cells (red) are not stained for stem/progenitor cell markers nestin (*a*, green) and vimentin (*b*, green) even at passage 0; *c*, *d*: beginning from passage 1, nearly all BLBP-positive cells (*c*) express the astrocyte differentiation marker S-100b (astrocyte differentiation marker, passage 9, *d*); *Neurosphere cells coexpress BLBP and S-100b. Arrow: individual expression of these proteins in cells (passage 8, *e-j*). Staining for BLBP (*e*, *h*); staining for S-100b (*f*, *i*); and superposition of micrographs (*e*, *f*) and (*h*, *i*), respectively (*g*, *j*). Additional staining of cell nuclei with Hoechst 33342 (blue).

proteins (BLBP or S-100b; Fig. 5, *c-j*). Coexpression of BLBP and S-100b was observed at passages 1-9.

An immunohistochemical study of neurospheres after staining for BLBP showed that the cells immunoreactive to this protein are present at various passages. The expression of BLBP and morphological characteristics of BLBP-expressing cells undergo significant changes after passage 1. Studying the coexpression of BLBP with markers of proliferation and differentiation revealed that BLBP does not serve as a marker for the population of neural precursor cells under *in vitro* conditions (*e.g.*, radial glial cells). Glial markers GFAP and S-100b were not coexpressed in our experiments. This fact is probably associated with the inhibition of S100-b expression in astrocytes due to the effect of EGF [14]. Our results indicate that BLBP protein is expressed in S-100b-immunopositive cells (highly differentiated cells).

These data suggest that BLBP-immunopositive radial glial cells loss the pluripotent status and differentiate during *in vitro* cultivation. It cannot be excluded that some heterogeneous cells of the radial glia (*e.g.*, multipotent cells and linear neural progenitor cells [8]) include a previously unknown population, which differentiates into S-100b-positive astrocytes.

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