Comparative Analysis of Differentiation and Behavior of Human Neural and Mesenchymal Stem Cells *In Vitro* and *In Vivo*

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Comparative analysis of differentiation of human neural and mesenchymal stem cells in tissue culture and after transplantation into the brain was carried out using the same antibody set. Neural stem cells differentiated into all types of neural cells, are retained after transplantation, migrate, and form reciprocal relationships with the recipient brain. Mesenchymal stem cells were incapable of neural development under conditions of common culturing or after transplantation and retained the fibroblast-like status. Recipient filaments grew into mesenchymal stem cell transplants containing no neural cells due to local changes in the extracellular matrix at the site of transplantation.

Key Words: *neural stem cells; mesenchymal stem cells; differentiation; filament growth; immunohistochemistry*

Detection of neural stem cells (NSC) in the brain was a priority discovery of the last century for neurobiology. This discovery necessitated revision of classical assumptions of neuronal theory and problems associated with regeneration. NSC are present in the brain during the entire life span of mammals and humans. They can be isolated from fetal and adult brain, multiply in tissue culture, and differentiate into all three types of neural cells. These characteristics of NSC make them extremely perspective for cell therapy of neurodegenerative diseases and injuries of the CNS. On the other hand, the problems of immunological compatibility of allogenic NSC remain unsolved, as are the ethical problems associated with derivation of initial material. One of approaches to solution of these problems is the search for autologous cell sources with neurogenic potential.

Studies of stem cells have changed the classical concepts on linear limitations of tissue-specific stem cells, capable of differentiating into cell types specific of the organ or tissue from which they were isolated. Many studies of recent years have shown that tissue-specific stem cells presumably can overcome the barrier of committed development of a certain embryonal leaflet (stem cell plasticity phenomenon) and differentiate in culture or after transplantation into cells of another tissue. The possibility of neural differentiation of bone marrow, skin, muscle cells, adipocytes, *etc.*, under conditions of *in vitro* culturing with various biochemical agents was demonstrated. The best studied of these cell sources are bone marrow cells, of which stromal mesenchymal stem cells (MSC) seem to be characterized by maximum plasticity. It was shown that some cells express neural markers in native

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MSC population [11,19,23]. Compositions of culture media and factors and coculturing with NSC or differentiated cells are used for stimulating MSC to neural differentiation [6,9,18,23]. These studies demonstrated the development of glial and neuron-like cells in cultures, which, according to immunohistochemical and molecular analysis, express specific markers. After neurotransplantation MSC react to the brain microenvironment signals and differentiate into glia and neurons [1,13]. Moreover, numerous studies showed that after intravenous or intracerebral transplantation MSC promote restoration and regeneration of the nervous tissue in injuries to the brain and spinal cord, though the mechanisms of their effect are virtually unknown [3,12, 13,25].

Along with the above mentioned reports demonstrating high plasticity of MSC, there are papers whose authors doubt the possibility of real neural differentiation of MSC [4]. It is hypothesized that under conditions of culturing MSC can become morphologically neuron-like cells due to modification of the cytoskeleton under the effect of environmental factors [7,14]. According to other data, MSC did not develop in the neural direction after transplantation into the brain [8] and in many cases transformed into microglial cells [21]. MSC express many growth factors and cytokines [5,10, 22,24], which presumably maintain regeneration after transplantation even without differentiation into the neural phenotypes.

Some of these data prove or deny the possibility of bone marrow cell differentiation in the neuronal direction, but these results require further studies and thorough verification. Therefore, the aim of our study was comparative analysis of phenotypical differentiation and behavior of human NSC and MSC under conditions of culturing and after transplantation into the brain of intact rats and rats pre-exposed to acute hypoxia. Our task was to use similar conditions of xenotransplantation and universal pool of antibodies and methods for detection (conditions when stained neural cells served as a clear-cut control of MSC differentiation).

MATERIALS AND METHODS

NSC culturing. Tissue fragments were taken from cerebral periventricular area of 9-week human fetuses, thoroughly suspended, and cultured in DMEM/F12 with N2 supplement with epidermal growth factor, main fibroblast growth factor, and leukemia inhibitory factor. Neurospheres formed in this culture were studied by immunohistochemical methods after 65 days of culturing. Some neurospheres were totally stained with antibodies, another part was stained after precipitation onto plastic in a medium with fetal serum (FCS), and the third part was dissociated to suspension of solitary cells and used for transplantation.

MSC culturing. Bone marrow cells were derived from adult human pelvic bone crest. Cell suspension was mechanically dissociated and placed into plastic flasks (80 ml) in a concentration of 5×10^5 cells/ml medium. Culturing was carried out in α-MEM with 10% autologous serum and 10% FCS at 37°C and 5% CO_2. On days 10-12 of culturing the cells were removed from plastic with 0.25% trypsin and placed into fresh medium in new flasks. Cells cultured for 30 days (3 passages) were used for transplantation. On the day of transplantation some cells from the culture were transferred onto slides. After 2 days they were fixed in 4% paraform in PBS (pH 7.4) and used for immunohistochemical studies.

Transplantation of human NSC and MSC into the brain of intact rats and rats exposed to acute hypoxia. Before transplantation of human MSC and NSC culture into the brain, female Wistar rats (200-250 g) were narcotized with chloralhydrate intraperitoneally (300 mg/kg). Animals exposed to hypoxia were transplanted the cells the next day after hypoxic exposure. Human MSC suspension was injected with a microsyringe into the striatum (0-0.5 mm from the bregma, 2.5 mm laterally into the right hemisphere to the depth of 4 mm). NSC culture was transplanted into the hippocampus (3.5-4.0 mm from the bregma, 2.5 mm laterally into the right hemisphere to the depth of 4-5 mm). No immunosuppression was carried out after transplantation. Ten (n=6) and 12 (n=6) days after transplantation of MSC and 27 days (n=7) after transplantation of NSC the animals were perfused with 4% paraform in PBS (pH 7.4), the brain was removed, postfixed overnight on the cold, and impregnated with 30% sucrose. Rat brain sections were sliced on a freezing microtome and applied onto gelatin-coated slides.

Immunohistochemical and histological studies of human NSC and MSC cultures and transplant preparations. Immunohistochemical study of cells from NSC and MSC cultures and brain sections from animals transplanted the cells was carried out using primary antibodies to marker proteins: nestin (monoclonal murine, Abcam, 1:100; polyclonal rabbit, Chemicon, 1:200), vimentin (monoclonal murine, Chemicon, undiluted), β-III-tubulin (monoclonal murine, Abcam, 1:100), acid glial fibrillar protein (AGFP; polyclonal rabbit, Chemicon, 1:200), Ki67 (monoclonal murine, Abcam, 1:50), fibronectin (polyclonal rabbit, Santa Cruz, 1:100), neurofilaments

(monoclonal murine, ICN, 1:10), and human nuclei (monoclonal murine, Chemicon, 1:50). The preparations were incubated overnight at 4°C in solution of primary antibodies, after washing in PBS the material was treated for 2 h at ambient temperature in solution of second goat antibodies to rabbit Ig (Jackson, 1:100, conjugated to Texas Red fluorochrome) and mouse Ig (Jackson, 1:100, conjugated to Cy-2 fluorochrome). Double immunohistochemical staining was carried out by simultaneous incubation with host animal primary antibodies and fluorochrome-labeled second antibodies. If necessary, the material was post-stained with Hoechst 33342 fluorescent dye (5 μg/ml; Sigma). All preparations were embedded under slides in glycerol. In addition, brain sections from rats receiving transplantation were stained with Cresyl Violet, thionin, and after Giemsa. Ready preparations were examined under an Opton-3 microscope in mercuric and incandescent lamp light. The preparations were photographed using a Nikon CoolPix 4500 digital camera.

RESULTS

NSC in culture and after transplantation into rat brain. The formation of spherical aggregations from stem cells starts in suspension NSC cultures after about 1 week; these aggregations increase in size with culturing and form typical neurospheres. Immunohistochemical analysis of whole neurospheres showed their heterogeneous cell composition. Groups of small nestin-positive axonal cells were as a rule located near the surface or completely covered the neurosphere. Their number gradually decreased with neurosphere growth. Glial precursors, AGFP-positive cells, formed small groups; they had brightly stained bodies with short axons. Cells expressing β-III-tubulin were less brightly stained, with long filaments. Glial AGFP-positive and neuronal β-III-tubulin-positive cells were usually situated in the inner areas of neurospheres.

Neurospheres put into medium with 10% FCS precipitated to the bottom, adhered, spread, and

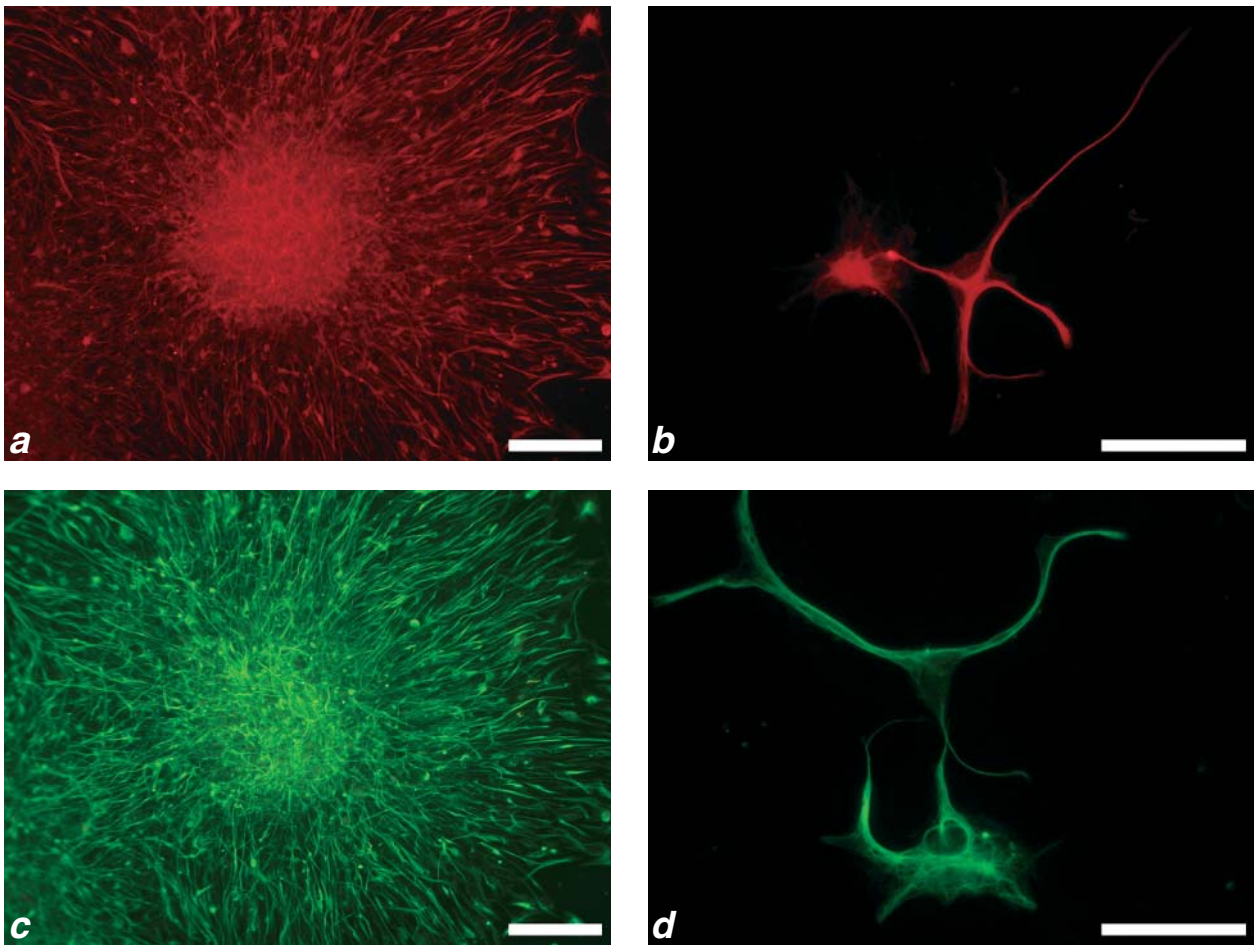

Fig. 1. Human neural stem cell (NSC) culture, staining of adhesive neurospheres (immunohistochemical analysis). *a*) stem cells express nestin; *b*) morphotypes of nestin-positive cells; *c*) progenitor cells express vimentin; *d*) types of vimentin-positive cells. Scale: *a*, *c*) 200 μ; *b*, *d*) 50 μ.

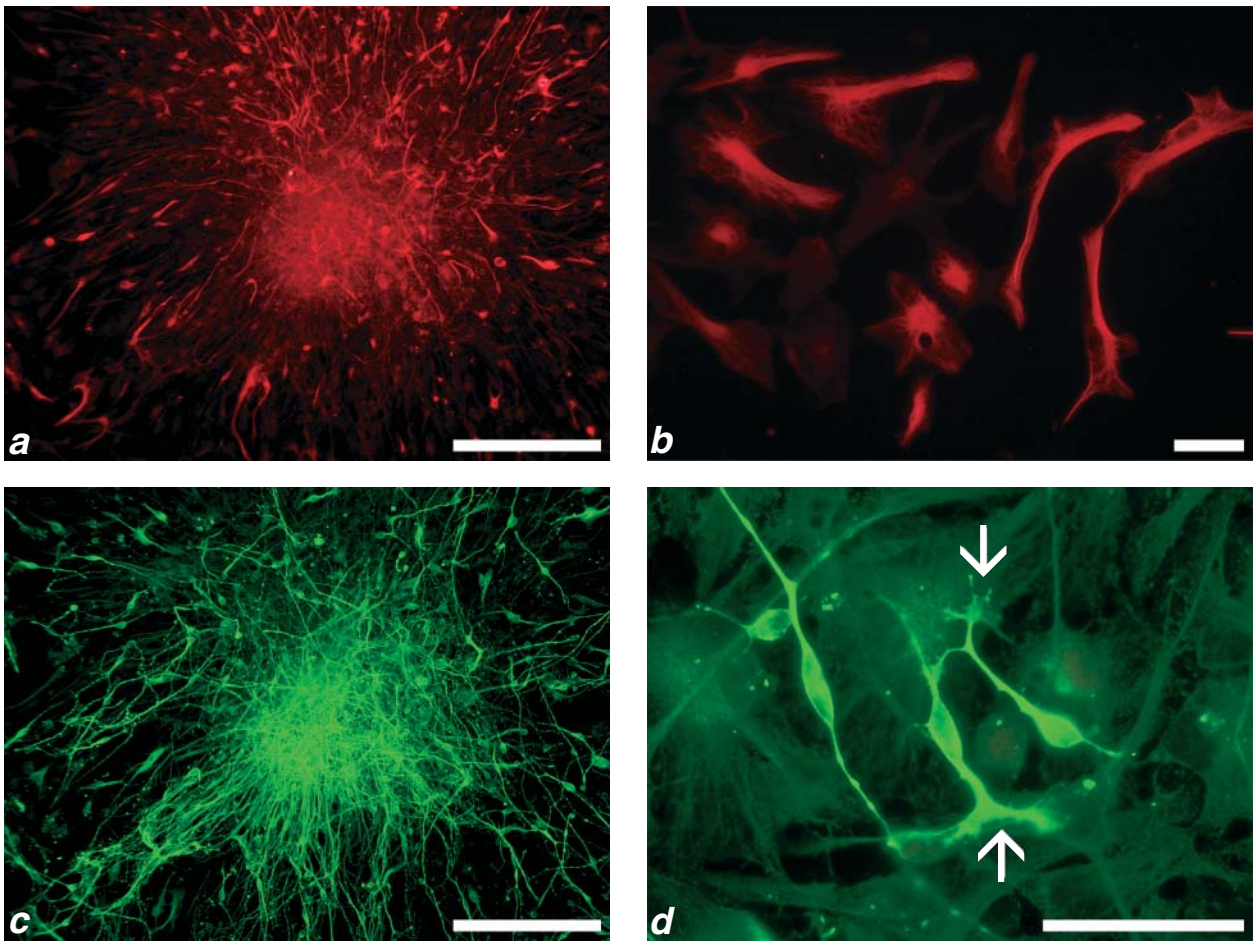

Fig. 2. Human NSC. Neural differentiation cells (immunohistochemical analysis). *a*) glial cells express AGFP; *b*) types of glial cells; *c*) neuronal cells express β-III-tubulin; *d*) neuroblasts with growth cones. Scale: *a*, *c*) 200 μ; *b*, *d*) 50 μ.

cells migrated from their marginal zones. An appreciable number of nestin-positive stem cells, numerous vimentin-positive cells, and cells with glial and neuronal differentiation were present in the migration zone (Fig. 1, 2). Nestin- and vimentin-positive cells were morphologically similar (Fig. 1), were often characterized by co-expression of nestin and vimentin. Cells differentiated in the glial direction expressed AGFP, some had long processes, like the radial glial cells (Fig. 2, *a*, *b*). Migration on the surface of other cells was characteristic of numerous cells differentiated in the neuronal direction. These cells had the typical neuroblast shape with clearly seen long axons forming growth cones and were brightly stained with antibodies to β-III-tubulin (Fig. 2, *c*, *d*).

Hence, by the moment of transplantation NSC cultures had all types of cells characterizing the neuronal differentiation: stem, progenitor cells, neuroblasts, and glia.

Histological analysis carried out after NSC transplantation into the brain showed transplanted cells in the brain of all recipients after 27 days. The transplants did not change the structure of the recipient brain; they were not separated by the glial barrier and virtually did not provoke macrophage invasion (Fig. 3, *a*).

Transplanted cells detected by staining with antibodies to human nuclei were located in the cerebral cortex, hippocampus, and partially in the thalamic structures (Fig. 3, *b*). The majority of cells formed compact groups, though some of them migrated to different distances from the track. Staining for nestin visualized an appreciable number of well surviving stem cells in all series of transplantations. Nestin-positive cells had small round bodies and long thin processes stretched along the transplant or growing deep into the recipient brain tissue (Fig. 3, *c*). Staining with antibodies to vimentin showed that the transplants contained a great number of vimentin-positive cells.

Neuronal cells stained with antibodies to β-III-tubulin were evenly spread in the transplants, had a bipolar shape with processes. Many cells migra-

ted outside the transplants and were detected in recipient brain parenchyma.

Some transplanted cells differentiated into astrocytes. Double staining with antibodies to human nuclei and AGFP showed separate groups consisting of several tens of cells expressing both markers. Moreover, staining with antibodies to AGFP demonstrated the reaction of the recipient brain glial cells to human NSC xenotransplants. Glial reaction was weak, no solid glial cicatrix formed at the host-transplant interface in any case (Fig. 3, *b*).

Staining with antibodies to neurofilaments showed no expression of this protein in transplanted cells. However, recipient axons were clearly stained with these antibodies, which indicated their growth into NSC transplants (Fig. 3, *d*).

Hence, NSC effectively differentiate in culture into all types of neural cells. They survive under conditions of xenotransplantation, migrate, and do not provoke pronounced immune reaction. Cells of all stages of neuronal differentiation are detected in the transplants: from stem to neural cells and glia. Reciprocal growth of filaments between the transplanted cells and recipient brain was observed.

MSC in culture and after transplantation into rat brain. Cells on slides formed a more or less even monolayer without dense accumulations. The culture contained fibroblast-like cells with different morphology. Spread and spindle cells with long thin axons were seen. Immunohistochemical analysis of differentiation showed that all cells were immunopositive to vimentin (Fig. 4, *a*). Among these cells, there were few detected with antibodies to nestin (Fig. 4, *b*). Staining with antibodies to fibronectin showed that almost all cells in the culture expressed this protein, with but a solitary exclusion (Fig. 4, *c*). Cells stained with antibodies to nestin were also detected among fibronectin-positive cells (Fig. 4, *d*). Total overlapping of the markers indicated that cells co-expressing vimentin and

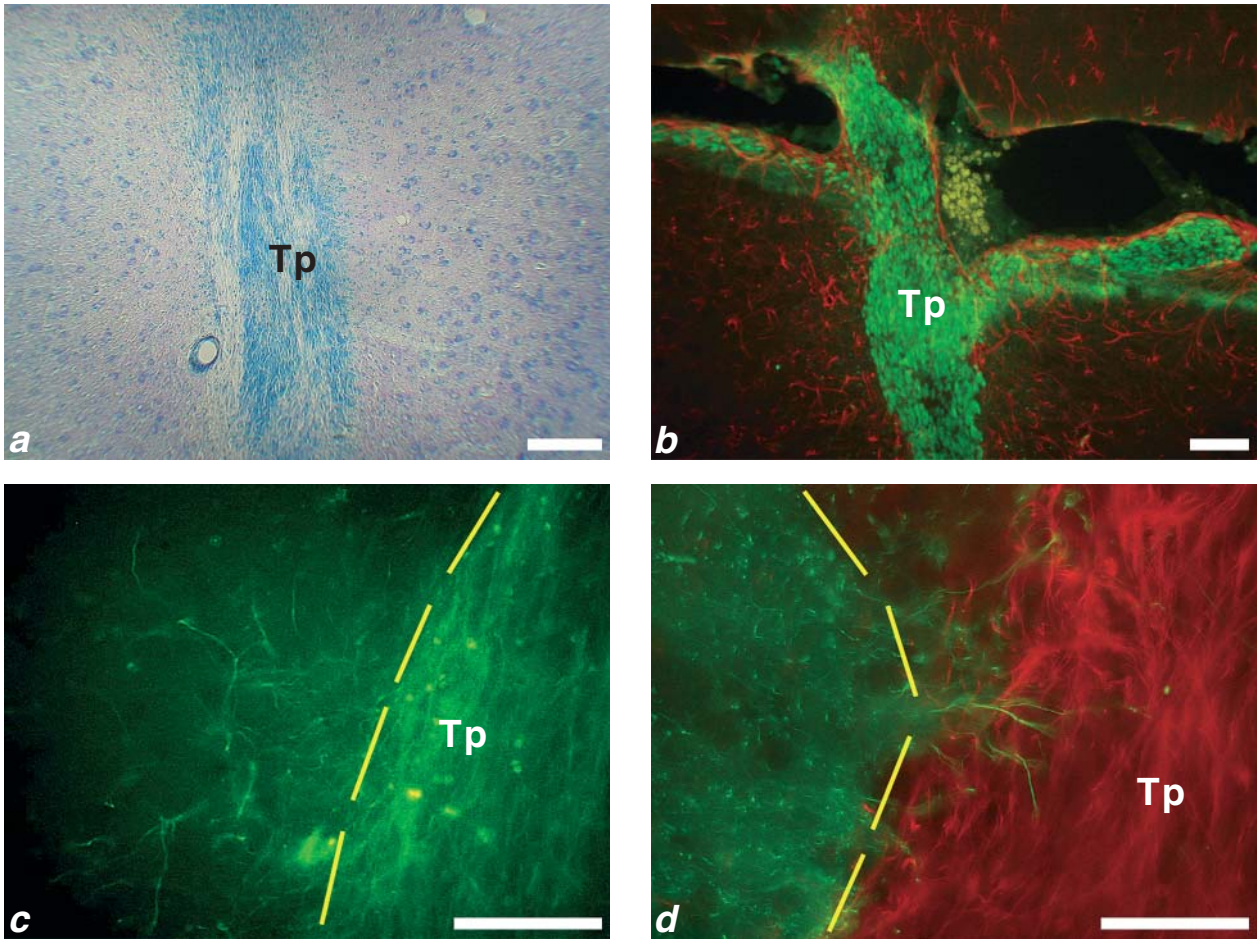

Fig. 3. NSC transplanted into adult rat brain, 27 days posttransplantation (morphological analysis). *a*) NSC transplant (Giemsa staining); *b*) double staining for human nuclear protein (green) and by antibodies to AGFP (red); *c*) filament growth from the transplant into recipient brain, staining for nestin (interface shown by interrupted line); *d*) growth of recipient axons into NSC transplant (antibodies to neurofilaments: green; to nestin: red). Scale: *a*) 200 μ; *b-d*) 50 μ.

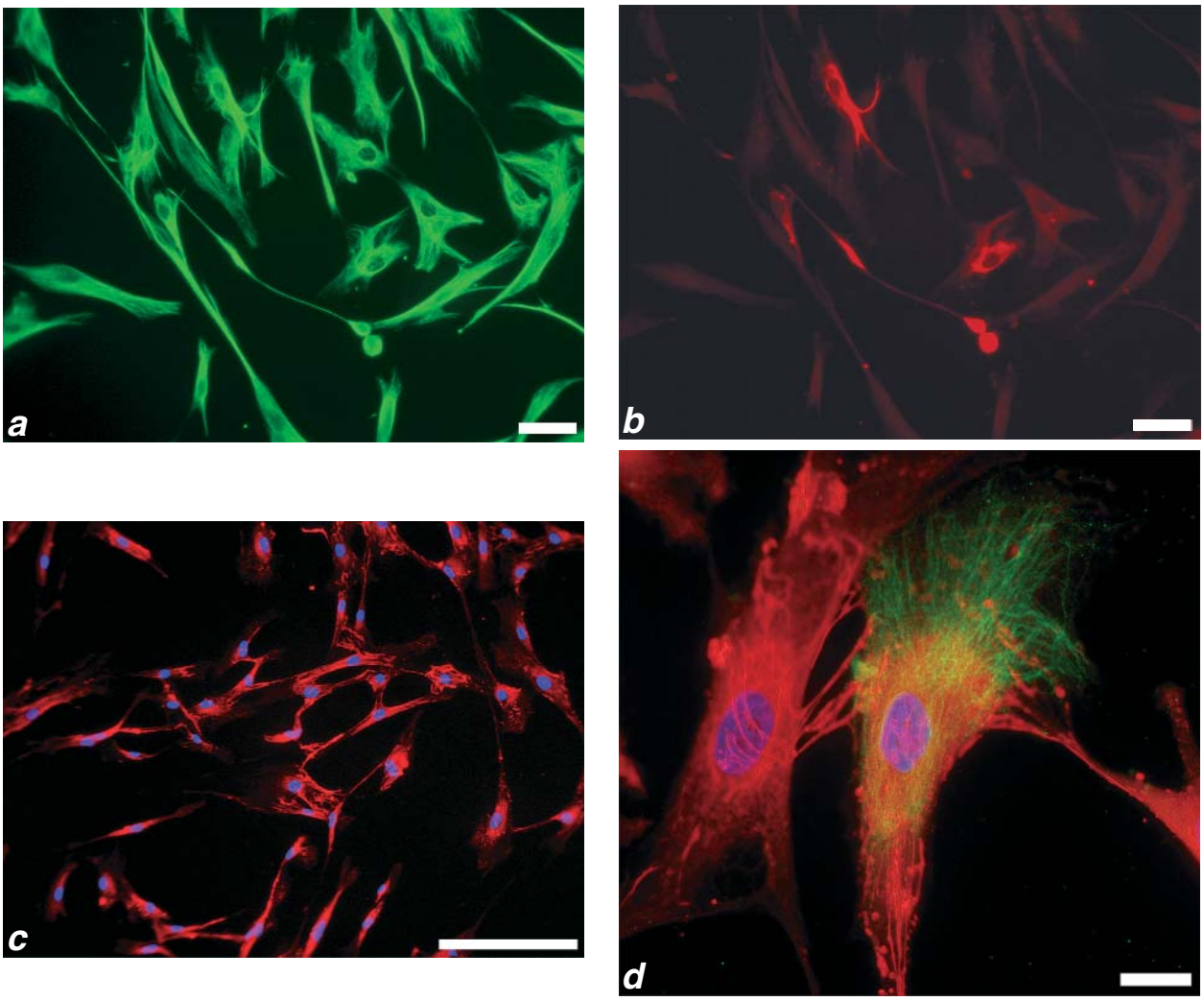

Fig. 4. Human mesenchymal stem cell (MSC) culture (immunohistochemical analysis). *a*) vimentin expression; *b*) nestin expression; *c*) fibronectin expression; *d*) fibronectin and nestin co-expression. Scale: *a*, *b*) 50 μ; *c*) 200 μ; *d*) 20 μ.

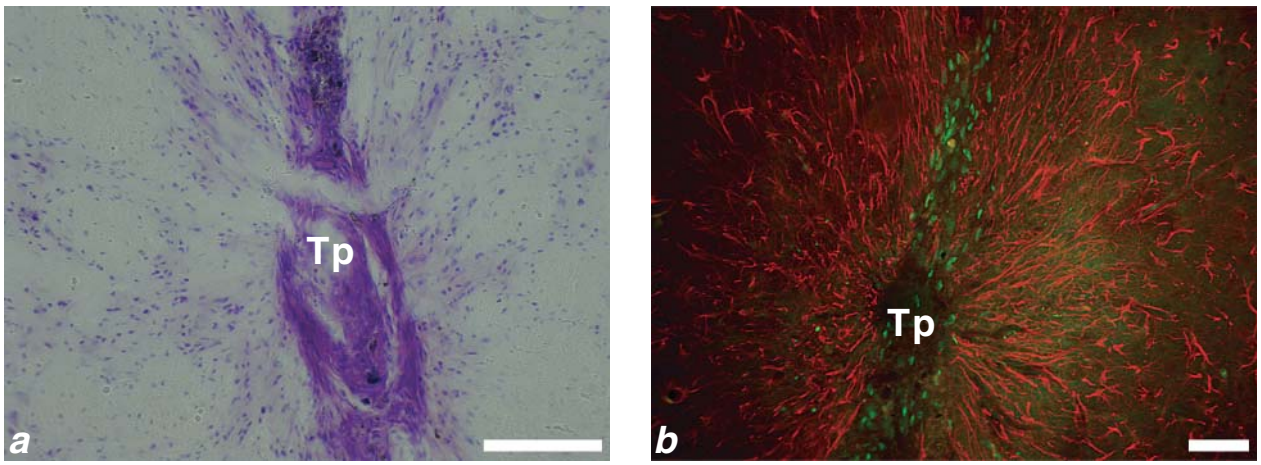

Fig. 5. MSC transplanted into adult rat brain, 10 days posttransplantation (morphological analysis). *a*) MSC transplant, Cresyl Violet staining; *b*) reactive gliosis around MSC transplant, double staining for human nuclear proteins (green) and AGFP (red). Scale: *a*) 100 μ; *b*) 50 μ.

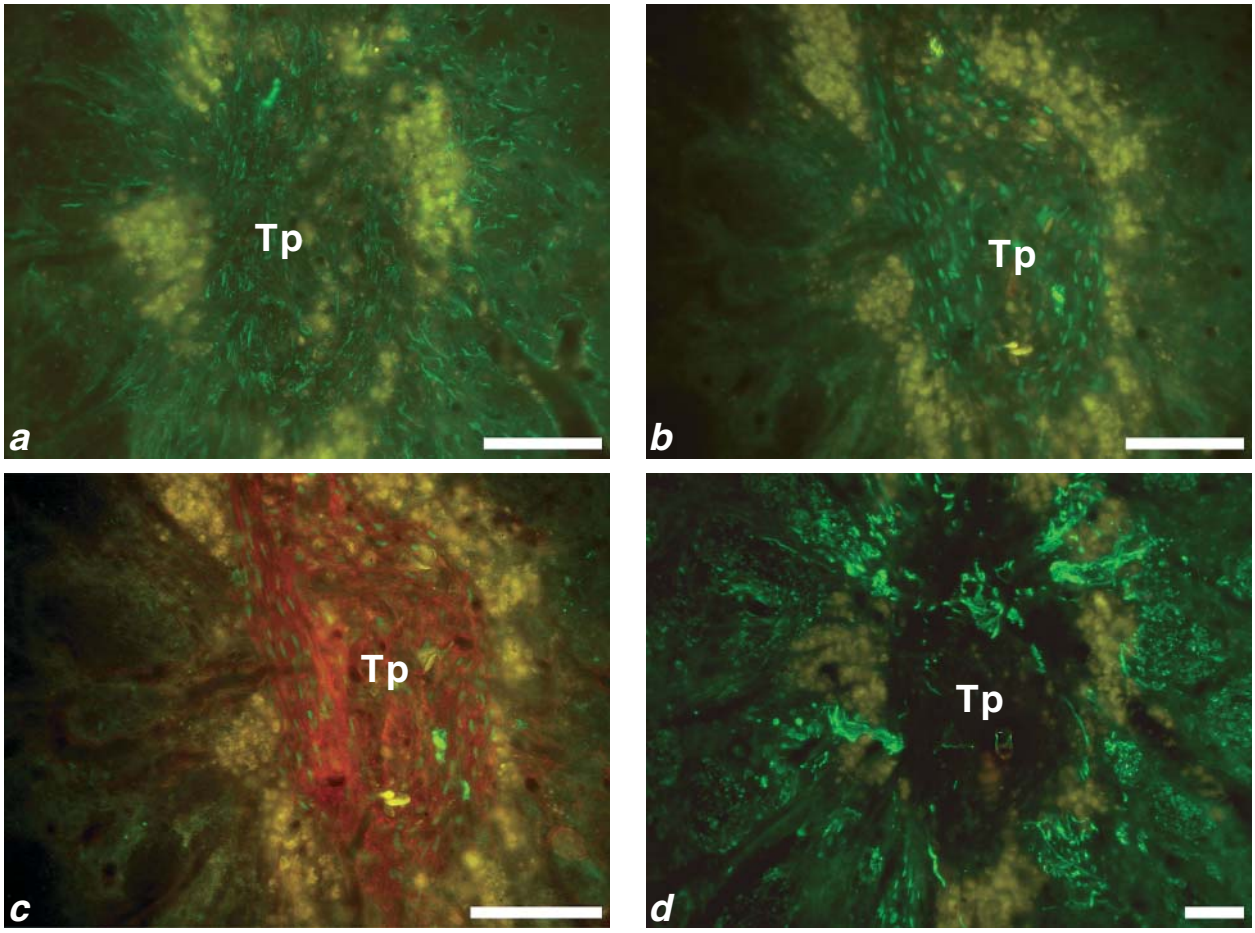

Fig. 6. MSC transplants in rat brain (immunohistochemical analysis). *a*) MSC in transplant express vimentin (green); macrophage roll around the transplant (yellow); *b*) MSC stained by antibodies to human nuclear proteins (green); *c*) double staining for human nuclear proteins (green) and fibronectin (red); filament growth from recipient brain into MSC transplant, staining by antibodies to neurofilaments (green). Scale: *a-c*) 100 μ; *d*) 50 μ.

fibronectin predominated in the culture; some cells also expressed nestin.

Staining with antibodies to markers of neuronal and glial differentiation (β-III-tubulin and AGFP, respectively) detected no cells immunopositive to these proteins. High proliferative activity of cells, part ($^1/_3$) of them expressing Ki67 protein, was detected in human MSC culture.

Hence, by the moment of transplantation MSC culture was presented by fibroblast-like cells expressing vimentin and fibronectin, just few cells co-expressed nestin.

The study of human MSC transplanted into the brain of intact rats and rats exposed to hypoxia showed that the track of the injection needle passed through the neocortex, white matter, and ended in the striatum in all cases. Histological analysis of preparations of the transplants showed a sort of collagen cicatrix, resulting in significant morphological modification of the adjacent tissues (Fig. 5, *a*).

Pronounced glial reaction of host astrocytes was seen around human MSC transplants; it was detected by double staining with antibodies to AGFP and human nuclear protein (Fig. 5, *e*). Reactive astrocyte filaments were oriented radially towards the transplants. Gliosis was disseminated to an appreciable distance from transplants. The morphology of adjacent tissues in the recipient brain was also changed in this case.

Ten days after transplantation MSC were detected (with antibodies to human nuclear protein) in all animals — intact (n=3) and exposed to hypoxia (n=3). The bulk of transplanted cells were located in the striatum, solitary cells were detected in the neocortex and white matter. No cell migration in the recipient brain tissues was detected. Transplanted cells in all preparations were surrounded by macrophage accumulations, detected by macrophage autofluorescence. Twenty days after transplantation solitary cells with staining for human nuclei were detected in one intact rat and one rat with acute

hypoxia. Similarly as after 10-day survival, there was a significant accumulation of macrophages at the site of transplantation. Hence, by day 20 transplanted cells were virtually completely eliminated.

Analysis of cell differentiation showed positive staining of cells with antibodies to vimentin inside the transplant 10 days after transplantation (Fig. 6, *a*), that is, MSC retained the expression of this protein after transplantation. On the other hand, vimentin-positive cells were detected around the transplants, this indicating the development of reactive gliosis in the recipient tissue. In addition, transplant cells were stained for fibronectin, which was detected by double staining with antibodies to human nuclei and fibronectin. The entire transplant area was immunopositive to fibronectin, which formed a solid extracellular matrix here (Fig. 6, *b*, *c*).

Transplanted cells exhibited no positive staining for human nestin, β-III-tubulin, and AGFP, this indicating no differentiation of cultured human MSC in the neuronal direction after transplantation.

No differentiation of transplant cells in the neuronal direction was detected. However, staining with antibodies to neurofilaments showed that host neuron filaments deeply grew into the transplant area in ruptures between macrophage accumulations (Fig. 6, *d*). It seems that fibronectin of the extracellular matrix, developing in human MSC transplants, promoted this growth.

Hence, NSC in tissue culture spontaneously differentiate into all types of neural cells in serum-free medium with mitogens and after removal of mitogens and addition of serum. MSC, as was shown previously, initially contain the neural pool cells [11,19,22,23]. However, in our experiments MSC were presented by fibroblast-like cells expressing vimentin and fibronectin, among which a very little part co-expressed nestin and none had markers of neuronal and glial differentiation (β-III-tubulin and AGFP). It is noteworthy that nestin is not an evidence of cell neurogenic potential, because it was detected in tissue cells of other than neural genesis [17]. Presumably, expression of nestin in MSC cultures reflects cell function, but does not indicate its potentiality for neural differentiation.

Xenotransplantation into the brain showed that NSC virtually did not provoke immune reaction, survived, and retained the neurogenic potential. Adult brain microenvironment factors maintained the differentiation of various types of neural cells from NSC, their migration, reciprocal growth of filaments, and even the formation of synaptic contacts [2].

In contrast to NSC, MSC transplanted into the brain provoked strongest gliosis, macrophage invasion, and were rapidly resorbed, which is in disagreement with previous data [1,13]. Recipient brain microenvironment did not stimulate neural differentiation of MSC, though it was shown not once [13], and in our experiments the cells retained their characteristic expression of fibronectin and vimentin. No migration of transplanted human MSC culture cells in the recipient brain tissues was detected.

It was found that recipient neurofilaments stained with antibodies to neurofilaments deeply grew into the transplants. Presumably, active filament growth was stimulated by local changes in soluble and insoluble components of extracellular matrix at the site of MSC transplantation. MSC release trophic, growth factors and cytokines [5,10,22,24] and express fibronectin maintaining axonal growth and stimulating vascularization [16,20]. Similar data on filament growth were obtained in experiments with human MSC transplantation into the site of spinal injury, when the authors noted significant individual variability of MSC cultures [15].

The findings indicate obvious advantages of NSC due to their capacity to differentiate into characteristic neural cell types, which are retained after xenotransplantation and form reciprocal contacts with the recipient brain. MSC used in our study failed to differentiate in the neural direction under conditions of culturing or after transplantation into the brain. On the other hand, filaments from recipient brain grew into MSC transplants containing no neural cells, which was most likely due to local changes in the extracellular matrix at the site of transplantation. These features of MSC are interesting and deserve further investigation.

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