Evaluation of progenitor cell cultures from human embryos for neurotransplantation

R.A. Poltavtseva\textsuperscript{a}, M.V. Marey\textsuperscript{a}, M.A. Aleksandrova\textsuperscript{b,\,*}, A.V. Revishchin\textsuperscript{c,d}, L.I. Korochkin\textsuperscript{b,d}, G.T. Sukhikh\textsuperscript{a}

\textsuperscript{a}Institute of Biological Medicine, Moscow, Russia
\textsuperscript{b}Institute of Development Biology, Russian Academy of Sciences, Moscow, Russia
\textsuperscript{c}Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia
\textsuperscript{d}Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia

Accepted 2 January 2002

Abstract

Human neural stem cells (HNSCs) are used in studies of neural development and differentiation, and are regarded as an alternative source of tissue for neural transplantation in degenerative diseases. Selection and standardization of HNSC samples is an important task in research and clinical approaches. We evaluated embryonal brain matter obtained from human 8–12-week-old fetuses by means of flow cytometry on a panel including: nestin; vimentin; NeuN; GFAP; \(\beta\)-tubulin III; CD56; N-Cad; OB-Cad; HLA-ABC; HLA-DR; CD34, and annexin. Samples from embryos of even the same gestation differ dramatically regarding neural cell development, their phenotype and viability. The samples containing the highest proportion of stem cells and multipotent progenitors of neural types, and the least of definitive cells and antigens of histocompatibility, were selected for further expansion in serum-free medium. Secondary phenotyping 14 days later revealed again a marked heterogeneity of the cultures. For the final culturing for 24 h in a serum-containing medium we selected only samples having following phenotype: nestin\(^{+}\), and vimentin\(^{+}\) no less than 25%; HLA-DR\(^{+}\) and CD34\(^{+}\) no more than 5%; GFAP\(^{+}\) no more than 10%; \(\beta\)-tubulin\(^{+}\) no more than 20%; CD56\(^{+}\), N-Cad\(^{+}\), OB-Cad\(^{+}\), HLA-A,B,C\(^{+}\), and annexin\(^{+}\) no more than 15%; cell viability no less than 60%. Immunocytochemical study of selected samples proved that numerous neural stem cells, and neuro- and glioblasts necessary for transplantation were present. Our results demonstrate that the flow cytometry phenotyping allows the screening and standardization of HNSC samples for further expansion and transplantation.

1. Introduction

Numerous diseases of the central nervous system of a metabolic, genetic or inflammatory nature are accompanied by dysfunction or degeneration of a significant proportion of neuronal population. A promising approach for curing such diseases is transplantation of neural cells. Recent neurobiological researches demonstrate distinctly that the brain stem cells which possess a wide spectrum potential for differentiation are best candidates for such replacement cell therapy [1,4,11,12].

Stem and multipotent cells exist in germinative (ventricular and subventricular) brain structures not only in embryonal and early postnatal period of ontogenesis [8,10,12], but during the whole life span of human and other mammals [11,14,16,18,22]. Nowadays researchers are concentrating on the problems of isolation, propagation and cloning of the neural stem cells [6,9,11,23], as well as on the investigation of their differentiation in culture and after transplantation into the recipient brain [2,15,20,27].

The propagation of human neural stem cells has important potential clinical application as material for re-
placement therapy in CNS degenerative diseases. More recently, some groups have expanded human progenitor cells in the presence of mitogen factors [3,7,9,19,21,24–26].

In this work we studied the processes of development and differentiation of first-trimester human embryonic neural stem cells under cultivation in vitro. The investigation was aimed at the elaboration of some standard parameters on the standard antibody panel proved that cell viability was determined by means of trypan blue and anti-GFAP (Dako, 1:250); anti- and density of cells in samples were evaluated, and their Antibodies used included: anti-nestin (Biogenesis, 1:20); anti-vimentin (NeoMarkers, 1:100). To this end the viability of samples was measured by means of trypan blue. On the 7th and 14th days of cultivation, the samples were phenotyped again. A part of the developed neurospheres would permit us to evaluate stem cell samples in order to unify those for cultivation and further transplantation.

2. Materials and methods

Human embryonic brain tissue samples were obtained from medically aborted fetuses at 8–12 weeks of gestation in accordance with guidelines and permission of ethics committee of Russian State Medical University.

Brains were extracted and the cover tissues removed. Neural tissue was suspended by means of careful pipetting medium, and the pattern of differentiation of neural stem and progenitor cells. Cell suspensions were cultivated at 37°C under 5% CO₂. Each 2 days the culture was pipetted many times and the medium partially (1:1) refreshed. The viability of cells was measured by means of trypan blue. After which the samples were ready for fluorimetric analysis. The viability of cells was defined as the ability to exclude trypan blue.

The cytofluorometry was performed involving an array parameters on the standard antibody panel proved that cell viability was determined by means of trypan blue. The viability of cells was measured by means of trypan blue. On the 7th and 14th days of cultivation, the samples were phenotyped again. A part of the developed neurospheres was transferred to a full nutritive serum-containing medium, and the pattern of differentiation of neural stem cells was investigated by immunocytochemical methods. Antibodies used included: anti-nestin (Biogenesis, 1:20); anti-GFAP (Dako, 1:250); anti-β-tubulin III (ICN, 1:100), anti-vimentin (NeoMarkers, 1:100). To this end the spheres attached to the dish bottom were fixed in 4% paraformaldehyde in a phosphate buffer, washed, and processed by 0.3% Triton X-100 solution, and the primary antibodies added overnight. Then they were processed for 1 h in a solution of biotinylated secondary antibodies (Vector Laboratories) diluted to 1:200. Staining was performed using a solution of streptavidin labeled by the fluorescent dye, DiI (Molecular probes). For all immunohistochemical procedures, adjacent sections served as negative controls and were processed using identical procedures, except for incubation without the primary antibody in each case.

3. Results

The outcome of such phenotypic analysis of human neural stem cell population with flow cytofluorimeter demonstrated a significant variability of embryonal material available for cultivation (Table 1). This heterogeneity of parameters on the standard antibody panel proved that cell populations obtained from fetuses of even the same gestational age may differ significantly regarding their development, phenotype and viability. This difference may be due to the difference in individual embryo development, or result from medicamental pathogenesis, or due to mechanical damage during abortion.

Keeping in mind this heterogeneity of the initial embryonal matter, we selected the best samples for cultivation. In order to enable comparative analysis of different experiments, the cell samples to be used for further experiments.
Table 1
Representative results of cytofluorimetric phenotype analysis of human fetal neural cells before cultivation (on day 0 of culturing)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cells immunopositive to marker (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95°</td>
</tr>
<tr>
<td>Nestin</td>
<td>15.6 7.4 17.5 8.9 16.9 7.9 6.5 9.5 5.8</td>
</tr>
<tr>
<td>Vimentin</td>
<td>20.1 11.6 18.9 20.8 24.8 12.8 19.7 12.4 14.9</td>
</tr>
<tr>
<td>GFAP+</td>
<td>3.9 4.8 3.1 3.9 3.6 4.8 4.9 4.6 3.6</td>
</tr>
<tr>
<td>NeuN+</td>
<td>7.0 8.2 8.9 10.4 8.5 11.6 9.6 11.8 8.6</td>
</tr>
<tr>
<td>CD56</td>
<td>10.3 5.2 7.7 5.7 12.4 5.8 5.8 6.4 6.3</td>
</tr>
<tr>
<td>N-CAD+</td>
<td>5.2 7.4 4.7 8.3 4.8 7.8 7.5 6.5 6.6</td>
</tr>
<tr>
<td>OB-CAD+</td>
<td>5.3 9.6 5.4 8.5 4.7 7.9 7.4 7.8 6.7</td>
</tr>
<tr>
<td>HLA-A,B,C+</td>
<td>7.2 13.9 10.7 13.7 6.4 12.8 12.6 11.6 12.6</td>
</tr>
<tr>
<td>HLA-DR+</td>
<td>3.4 5.5 3.8 5.6 3.0 5.8 5.7 5.8 9.8</td>
</tr>
<tr>
<td>CD34+</td>
<td>3.0 6.8 3.8 5.7 3.6 5.9 6.8 5.6 5.8</td>
</tr>
<tr>
<td>β-Tubulin+</td>
<td>7.1 6.3 6.8 6.9 12.8 23.8 7.9 11.6 11.2</td>
</tr>
<tr>
<td>Annexin+</td>
<td>4.0 8.6 4.5 9.8 5.0 6.0 9.6 3.7 8.8</td>
</tr>
<tr>
<td>Viability</td>
<td>84 62 88 58.5 82 68 44 78 59</td>
</tr>
</tbody>
</table>

*a* These samples were taken for cultivation.

Neurotransplantation should be as uniform as possible. Obviously the initial samples must contain the highest proportion of stem and multipotent progenitors of neural type, and the lowest proportion of differentiated cells and cells bearing antigens of histocompatibility. Hence, we accepted the following demands regarding cell samples: proportion of Nestin-positive cells: no less than 15%; proportion of Vimentin-positive cells: no less than 15%; proportion of HLA-DR positive cells: no more than 4%; viability: no less than 60%; proportion of Annexin-positive (apoptotic) cells: no more than 10%.

Twenty-seven cell population samples fitting these restrictions were selected. On the 7th day of cultivation in selective serum-free medium, cells formed true neurospheres (Fig. 1), that gradually grew in size during cultivation. During the first week of cultivation the number of cells decreased, but it was restored to the initial level by the end of the second week. Samples were cultivated for 14 days, after which the cells were phenotyped again by flow cytometry with the same array of markers.

The outcome of the secondary cytofluorimetry again revealed a pronounced heterogeneity of the obtained cell cultures. In some cultures, human neural stem cells changed their phenotypic features significantly, while in others they remained unchanged. For further immunocytochemical analysis of cell differentiation (and further neurotransplantation) we selected the cultures which contained highest proportion of stem and multipotent cells and showed highest viability. These cultures had the following features

- Nestin+ cells: no less than 25%;
- Vimentin+ cells: no less than 25%;
- GFAP+ cells: no more than 10%;
- NeuN cells: no more than 15%;
- CD56+ cells: no more than 15%;
- N-Cad+ cells: no more than 15%;
- OB-Cad+ cells: no more than 15%;
- HLA-A,B,C+ cells: no more than 15%;

Fig. 1. Formation of neurospheres in human neural progenitor cell culture. (A) Cell suspension of fetal brain tissue on day 0 of culturing. (B) Neurospheres are formed on day 7 of culturing in selective serum free medium. Scale bar, 100 μm.
HLA-DR+ cells: no more than 5%;
CD34+ cells: no more than 5%; β-tubulin+ cells: no more than 20%;
Annexin+ cells: no more than 15%.
Viability was no less than 60%.

Cell samples fitting these restrictions had been seeded on plastic dishes without cover in a serum-containing medium. After 24 h they were fixed in 4% paraformaldehyde and studied immunocytochemically by means of antibodies against Nestin, Vimentin, β-tubulin and GFAP.

During the 24 h the sedimented neurospheres became more or less flattened on the dish surface. Small neurospheres gave rise to monolayered cell groups of different shape, while the big ones transformed into more or less multilayered cell aggregates having a round, convex form.

Staining for Nestin reveals the presence of nestin-immunopositive cells (Fig. 2A) in neurospheres of any size.

Such cells are distributed all over the aggregate surface. Staining for Vimentin demonstrated that the cells which express this protein, possess thin processes and are distributed uniformly over the neurosphere surface (Fig. 2B). Cell differentiation into neurons and glia starts in sedimented neurospheres even before the migration of their cells along substrate. Staining with antibodies for β-tubulin showed that on the surface of both small and big neurospheres, bright coloured cells having peculiar processes can be observed at the borders of big multilayer formations (Fig. 3A), or they were rather uniformly distributed over whole surface of smaller ones, monolayer formations (Fig. 3B). Finally, staining for glial fibrillar protein showed that the cells of astroglial array are formed in sedimented aggregations and groups of different sizes. Astrocytes, bearing as well their peculiar processes, are distributed not uniformly, but in clusters containing different amount of cells (Fig. 4).
Vimentin-expressing cells (revealed during the second phenotyping), hold their viability or even increase it. If cultivated on a serum-containing medium, they demonstrate differentiation of both neural and glial types, thus proving their perspective use for further neurotransplantation.

Acknowledgements

This work was supported by Russian Basic Science Foundation, A-T Parents to Save Children Foundation, and MedBioComp Inc. We are grateful to Professors V.S. Repin and I.V. Vicktorov for stimulating discussion and advice.

References


