Regenerative Properties of Fetal n. Raphe Cells in Modeling Injuries of Serotonergic Neurite-Glial Fibers in Vitro

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Abstract: It is considered that renewal of growth in serotoninergic caudal fibers their sprouting to injury site and formation of outgrowth net is factor for locomotion recovery. The aim of our work was to study the influence of rat fetal neural cells enriched with serotonin-producing cells, on morphogenesis, gene expression of serotogenesis (Pet1, Nk2.2, Lmx1b, Tph1, Tph2, Sert) and serotonin level in organotypic n. raphe cultures in injury of neurite-glial fibers in vitro. Injury was modeled by the way of crossing neurite-glial fibers after their formation under long-term culture. The cell accumulation was observed in the injury zone when fetal n. raphe cells, previously enriched with serotonin-producing cells were added into organotypic culture of n. raphe with crossing fibers under the culture conditions. As a result, crossing site was filled with these cells after 14 days of culture. The activation of expression in cascade of regulatory genes-regulators of serotogenesis Pet1, Lmx1b, Tph1, Tph2, Sert and increased serotonin content in the culture material were also observed. The activation of gene expression of serotogenesis Pet1 and Sert, and increased serotonin content by 1.7 times were revealed when conditioned medium from culture of fetal rat cells was used. An eviction of single cells was observed in control samples of organotypic n. raphe culture after injury. Crossing site was remained without signs of cell filling in these samples at day 14 after culture. The activation of regulatory gene expression of serotogenesis Pet1, Nkx2.2, Lmx1b, Tph1, Tph2, Sert and restoration of serotonin content were also absent in the culture material.

Key words: Fetal rat nerve cells, serotonin, n. raphe, organotypic culture, suspension culture.

1. Introduction

In the last decade, neurobiology is focused on the issue of serotonergic system reparation due to the expansion of understanding of its significant role in the development of post-traumatic complications [1, 2]. Serotonin (5-HT)-indolamin which is synthesized by brain stem neurons, has a significant effect on the activity of spinal neurons responsible for locomotion. The neuronal pathways, binding serotonergic regulatory structures of the brain stemed with the spinal motor neurons are destroyed, causing a decrease in serotonin production after injury of the spinal cord [3]. This process is associated with the deregulation of 5-HT receptors, resulting in different locomotion destructions [4]. It is considered that renewal of growth in serotoninergic caudal fibers their sprouting to injury site and formation of outgrowth net is a positive prognostic factor for locomotion recovery [5]. Different strategies directed to restoring serotonergic neuromediator system are considered today. These strategies include pharmacological ones—5-HT receptor agonists; agents increasing the production of all types of serotonergic receptors [6]. Today, the scientific search is also focused on new therapeutic strategies, among which an important place is occupied by cell therapy, which consists in administration of progenitor cells into injury site. Studies of fetal serotonergic neuron transplantation [7, 8], prove namely the value of the serotonergic system in the recovery of locomotion after spinal cord injury. The effectiveness of the embryonic raphe cells transplantation into the injury site at body level was confirmed by many studies [5-8].

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At the same time, the analysis of obtained effects is complicated by the complicated mutual influence between the CNS structures and other systems. Therefore, the aim of our work was to study the effect of cell population from fetal n. raphe neurocells, enriched with serotonin-producing cells, under culture and CM (conditioned medium) from them on morphogenesis, expression of regulatory gene cascade of serotogenesis Pet1, Nkx2.2, Lmx1b, Tph1, Tph2, Sert and serotonin level in the culture material when modeling injury in vitro.

2. Materials and Methods

During the experiment, laboratory animals were kept in accordance with the rules adopted by the European Convention for the Protection of vertebrate animals that are used for experimental and scientific purposes (Strasbourg, 1986), in accordance with the principles of the Helsinki Declaration adopted by the General Assembly of the World Medical Association (1964-2000) and “General ethical principles of experiments on animals”, approved by the First National Congress on bioethics (Kyiv, 2001). Animal keeping and manipulations, which they received (taken), correspond to the law of Ukraine № 3447-IV of 21.02.06 that was established by Committee on Medical Ethics of State Institution “Institute of Neurosurgery named after acad. A.P. Romodanov of NAMS of Ukraine” (Protocol № 1 of 19.04.2013), developed in accordance with the current legislation.

2.1 Receiving the Population of Fetal Rat n. Raphe Cells Enriched by Serotonin-Producing Cells under Suspension Culture

The rat brain E16 stage was released from membranes with removing n. raphe zone. Tissue was suspended with Hank’s solution (PAA, Austria) by mechanical pipetting, with counting content of living cells adjusting their concentration to 1-3 × 10^6 per 1 mL. The suspension was poured into sterile petri dishes on glass slides coated with polyetilenimine. Samples were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (PAA, Austria) with the addition of 10% heat inactivated FCS (PAA, Austria) enriched by EGF and FGFb factors in a concentration of 40 ng/mL (Sigma, USA) within 1 week. Culturing was conducted on DMEM (PAA, Austria) with the addition of 10% normal FCS (PAA, Austria) for the next week. The addition of BDNF, 50 ng/mL (Sigma, USA) stimulated cell serogenesis [9, 10]. Medium replacement was performed in 3-4 days.

The total number and content of living cells were counted using supravital staining by trypan blue solution, 0.2% (Janssen Chemica, Belgium).

CM (conditioned medium)

CM was received from suspension culture of fetal n. raphe serotonin-producing cells, enriched with serotonin-producing cells. After culturing for 14 days, the culture was washed with Hank’s solution followed by the addition of nutrient DMEM, enriched with 10% FBS and incubated for 24 h under standard conditions. Then medium was collected and its 30% concentration was used to study the effect of n. raphe after trauma on serotogenesis in organotypic culture.

Organotypic n. raphe culture

The n. raphe zone was isolated from newborn rat brain. Sagittal sections of n. raphe were used for obtaining organotypic culture. Two identical samples from the same animal were formed in this way. One sample after culture was used to study the effect of FSPC or CM on indicators of serotogenesis in neurotrauma and the other ones that was subjected to injury, without adding FSPC or CM, served as a control. Culture was carried out on glass slides coated with polyetilenimine in DMEM (PAA, Austria) enriched with 10% FBS (PAA, Austria). Nutrient medium was replaced every 3-4 days for 5 weeks of culture.

Modeling of mechanical injury in vitro

In 5 weeks after culturing organotypic n. raphe culture, traumatic lesion was modeling by the C. Lööv method [11], by crossing neurite-glial fibers, followed
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by three time washing with Hank’s solution (PAA, Austria). Samples with traumatic n. raphe (n. raphe (t)) injuries were used to study the effect of FSPC (fetal serotonin-producing cells) and CM on serotogenesis.

The study of FSPC effect on serotogenesis in vitro

10 million of FSPC were added to each test sample of n. raphe (t), and control samples were left in the nutrient medium. The control and test samples were cultured for 2 weeks with observing morphogenesis in native culture. Genetic, histochemical studies and ELISA (enzyme linked immunosorbent assay) were realized after the end of culturing.

2.2 Determination of Serotonin Content by Enzyme Linked Immunosorbent Assay

Quantitative serotonin level was studied by concurrent solid phase methods of immunoenzyme analysis using diagnostic kits RE 59,121 (IBL, Germany).

2.3 Visualization of Cell Serotonin

Serotonin in the cells was visualized using the Falk-Hillarp’s histochemical reaction with 1% paraformaldehyde (Janssen Chemica, Belgium) and 2% glyoxylic acid (Janssen Chemica, Belgium) [12].

2.4 Genetic Studies

An expression of regulative genes of serotogenesis-Nkx2.2, Lmx1b, Pet1, Tph1, Tph2, Sert was studied by RT-PCR method in culture material. RNA was studied using RIBO-sorb kits (“Amplisens”, Russia). Reverse transcription reaction was carried out using a set of “RevertAidTM First strand cDNA synthesis kit” (“Fermentas”, Lithuania).

The specific pairs of SERT primers (Forward 5’
gcggagatgaggaataagatgtg 3’ Reverse 5’
aggaagaatagattgcaagagt 3’), Tph1 (Forward 5’
caaggagaacaagacatc 3’ Reverse 5’
egaagtccacaaattctca 3’), Tph2 (Forward 5’
tactgagcccaagactctc 3’ Reverse 5’
gctatgagcattgtgcgtt 3’), Lmx1b (Forward 5’
atgtgagggcatcaagat 3’ Reverse 5’
tcaagagtagcactcagetc 3’), Nkx2.2 (Forward 5’
gaegecccagtctctctc 3’ Reverse 5’
gccagagtctgtggttgtg 3’), and Pet1 (Forward 5’
agaagctaacatgaactc 3’ Reverse 5’
aagtaaagcgtactgcgc 3’) [2, 13-15], were used to determine the expression of genes Nkx2.2, Lmx1b, Pet1, Tph1, Tph2, Sert, obtained amplification products corresponded to 374, 492, 184, 117, 127 nucleotide pairs, respectively. The amplification products were visualized by electrophoresis in 2% agarose gel using the imaging system (Vitran, Biocom, Russia).

2.5 Statistical Analysis

Differences between groups were determined by Student’s t-test. Statistical analysis was performed using STATISTICA 6.0 for Windows (Stat Soft Inc., USA) and Microsoft Office Excel-2013.

3. Results and Discussion

Enrichment of Fetal n. Raphe Neuronal Cell Populations by Serotonin-Producing Cells in Culture

Enrichment of fetal n. raphe neuronal cells by serotonin producing cells under the effect of differentiating factors [9, 10] was confirmed by histochemical and immunoenzyme methods, as well as by study the expression of relevant regulatory genes of serotogenesis. So, the content of serotonergic neurons in suspension culture of n. raphe cell population was increased by 1.6 times (Table 1), and serotonin content—by 1.7 times (Table 1). Expression of genes of serotogenesis Pet1, Nkx2.2 and Tph1 was shown by the studies of gene expression (Fig. 1).

Culture of n. raphe (t) in the FSPC presence

The reparative properties of FSPC population were studied using tissue n. raphe culture after modeling injury [11] and receiving the n. raphe (t) sample. Interaction between FSPC and n. raphe (t) was under
Table 1  Content of serotonergic neurons and serotonin in suspension culture of n. raphe zone.

<table>
<thead>
<tr>
<th>Group</th>
<th>Content of serotonergic neurons in the field of view, %</th>
<th>Serotonin content, ng/10^6 cells</th>
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<tbody>
<tr>
<td>Control after culture</td>
<td>6.16 ± 0.91</td>
<td>13.4 ± 1.23</td>
</tr>
<tr>
<td>Addition of differentiation factors</td>
<td>9.88 ± 1.06*</td>
<td>21.3 ± 1.92*</td>
</tr>
</tbody>
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* P < 0.05.

Fig. 1  Electropherogram of gene amplification products Nkx2.2 (374 n. p.), Lmx1b (492 n. p.), Pet1 (109 n. p.), Tph1 (184 n. p.), Tph2 (117 n. p.), Sert (127 n. p.): 1-FSPC; 2-raphe(t); 3-n. raphe(t)+FSPC; 4-n. raphe(t)+CM; M-molecular weight marker (100-1000) n.p.

Fig. 2  Co-culture of n.raphe (mt) and FSPC zones (1-14 days). Filling of cavity formed as a result of the mechanical cell intersection. Native culture. Phase-contrast microscopy (a-g). (a) zone of injury × 200; (b) fetal cells in the zone of injury, day 1. × 200; (c) fetal cells in the zone of injury, day 5. ×200; (d) fetal cells in the zone of injury, day × 400 (contacts between cells); (e) fetal cells in the zone of injury day 5. × 200; (f) fetal cells in the zone of injury, day 14. × 200; (g) fetal cells in the areazone of injury, day 14. Staining by Falck-Hillarp’s method (h).

observation in native culture in the dynamics. As can be seen from the presented figures (Fig. 2a-d) the concentration of cells in the damaged zone happened for 1-5 days of post-traumatic period. The next stage (7-10 days) consisted in establishing links between damaged neurite-glial fibers and cells, localized in the
the newly formed neuritis was noted (Fig. 2g). According to our observations, zone of tissue defect was filled with cell layer at days 10-14 (Fig. 2f).

An eviction of single cells that do not establish contact was registered in control samples, which were subject to experimental injury in the zone of intersection. A number of these cells didn’t increase at days 7-10 (Figs. 3a and 3b). The signs of destruction were shown by cells of organotypic n. raphe (t) culture, where the intersection occurred (Fig. 3c).

Thus, addition of n. raphe (t) FSPC to culture helps to fill zones of intersection by the cells that form the contacts at the morphological level. These processes are little expressed in the control samples.

The available expression both progenitor genetic markers of serotogenesis (Pet1, Lmx1b), and markers of mature neurons (Tph1, Tph2, Sert) were shown in n. raphe (t) and FSPC co-cultures by genetic studies (Fig. 1). Serotonin level is higher by 1.4 times in co-culture than that of the control samples according to the ELISA data (Fig. 4). A complete absence of expression of regulatory genes of serotogenesis in culture samples subject to experimental injury was fixed (Fig. 1).

Thus, complex of regulatory genes of serotogenesis Pet1, Lmx1b, Tph1, Tph2, Sert is expressed in the nerve cells of n. raphe (t) and FSPC co-cultures and increased serotonin level was shown by genetic studies.

Studies of CM effect on organotypic n. raphe (t) culture

The aim was to compare the profile of gene expression of serotogenesis and serotonin level obtained by joint culturing n. raphe (t) and FSPC with the same indices when n. raphe (t) was cultured with the CM addition from FSPC culture. Decreased crossing zone was observed at the morphological level on days 10-14. However, the cell eviction was not occurred. The presence of gene expression Pet1 and Sert in experimental samples was not observed, in contrast to the control samples where gene expression of serotogenesis was demonstrated by genetic analysis.

The activation of neurons synthesizing serotonin are able to form synapses with its participation confirmed by the result of this study (Fig. 1). The serotonin level was increased by 1.7-fold according to the ELISA data (Fig. 4).

Our results revealed the presence of signaling molecules in conditioned medium that are carriers of information and can affect its metabolic processes of the studied cell population.

4. Conclusions

(1) The addition of fetal serotonin producing cells to organotypic culture n. raphe with crossing of neurite-glia fibers leads to filling the site of crossing cell that make contacts and produce serotonin. The gene expression of serotogenesis Pet1, Lmx1b, Tph1, Tph2, Sert is observed in cells of co-culture. This is cardinally differed from organotypic n. raphe (t)
culture without FSPC adding where filling of cell crossing was not noted, serotonin level was 1.4 times lower and gene expression of serotogenesis was not observed.

(2) The addition of CM (conditioned medium) from n. raphe (t) to organotypic n. raphe culture with crossing of neurite-glial fibers leads to activation of gene expression of serotogenesis Pet1 Sert and 1.7 time increase in serotonin level compared to control samples of organotypic n. raphe (t) culture.

References