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Neurogenesis of Adult Circulating Blood Mononuclears in Vitro

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ABSTRACT
Cellular therapy of injuries of the nervous system with autologous cellular material of the patient requires the development of methods of neurodifferentiation of stem and progenitor cells of the adult organism. The use of autologous cells is the most acceptable from the point of view of biosafety and compatibility. In our work, we investigated the possibility of directed neurogenesis of circulating blood precursors adhered fraction, obtained from the patient with spinal cord injury. These cells were cultured for 2 weeks using neuroinducers (retinoic acid, BDNF, NGF). During the first 3 days of cultivation, the adherent fraction of cells consisted of round cells of small and medium size with a large nucleus and a narrow rim of the cytoplasm, which gradually stratified and formed an oval and granular shape. Subsequent cultivation showed that the cells acquire morphological characteristics of different types of cells of the nervous system. At the end of this period, the predominant cell population was Nest+. The patient's own cells after stimulation of neurogenic differentiation in vitro were removed, washed from medium components and injected into the spinal canal. Intracisterial and paraventricular administration of this fraction to the patient significantly improved impaired function.

Keywords: circulating blood precursors; neuroinduction; retinoic acid; BDNF; NGF; Nest + cells

INTRODUCTION
Finding cell sources for cell therapy of neurological diseases is a key issue in the development of this branch of reparative medicine. Stem and progenitor cells of the nervous system of the adult body are located in hard-to-reach areas of the CNS, the largest of which is the subventricular zone IV of the ventricle, which makes their use impossible. An alternative is embryonic nerve cells, the use of which raises many ethical questions. The use of autologous cells is the most acceptable from the point of view of biosafety and compatibility. In an adult, their source can be CM, adipose tissue, skin and blood. The use of any autologous stem and progenitor cells for neuro repair requires mandatory prior neurogenic differentiation.

The neuroreparative properties of BMSC are determined by their ability to neurogenic differentiation and synthesis of neurotrophic factors that stimulate the proliferation of Schwann cells [1, 2, 3, 4]. To date, researchers have demonstrated 2 types of neurogenic BMSC differentiation into neurons and Schwann cells. Neurogenic differentiation was performed directly in the fibrin matrix of Shakhbazau A.V. with co-authors (2011) using mitogenic factors EGF and FGF, as well as a neurogenic environment containing retinoic acid, and was confirmed by the appearance of βIII-tubulin positive cells [5]. Directed differentiation of BMSC into Schwann cells is also widely used in scientific and clinical experiments, is carried out under the influence of cytokines and is confirmed by the appearance of appropriate markers - S100, P75, GFAP and others [1, 5, 6, 7].

The neuroreperative properties of ADSC are primarily attributed by researchers to their ability to differentiate into Schwann cells [8, 9], which has been experimentally confirmed by the expression of S100, P75 and other markers [8]. Directed differentiation is carried out in vitro by two-stage cultivation using neuroinducers and myogens - retinoic acid, PDGF, bFGF, etc. [9, 10]. Another source of neural progenitors can be skin-derived precursor cells, wich are neural crest stem cells that persist in certain adult tissues, particularly in the skin [11]. There are also some data on the neurogenic potential of circulating blood stem cells [12]. This biological material is the most readily available and promising in terms of direct clinical use. The aim of our work was to investigate the possibility of neuronal differentiation of blood progenitors in culture and the possibility of their practical application.

MATERIALS AND METHODS
Obtaining the adhered fraction of blood cells
To obtain a suspension of mononuclear cells, the patient's peripheral blood was collected in sterile containers using heparin (2500 IU) as an anticoagulant. It was then transferred to culture flacs and kept for 72 hours in a CO2 incubator under standard culture conditions (t - 37 ° C, 5% CO_2) to fix the adherent cell fraction. At the end of this period, the non-adherent fraction was removed, and the fixed layer of cells was washed 3 times with fisiologcal saline.

Cells culturing
After that_cell suspension was culturing in the medium, which consist of RPMI (Biowest, France), 20% FCS (Biowest, France), enreached by 80 ng/ml NGF (Sigma, USA), 40 ng/ml BDNF (Sigma, USA), 1mM retinoic acid (Sigma, USA). Visual control and photography were carried out with using of an inverted microscope (Leica DMi8). The medium was changed once weekly.

Immunohystochemistry
Nest-positive cells were stained using monoclonal antibody and secondary antibody (Dako, Denmark) according to manual protocol. Visualization of positive cells was made by Multivision Polymer Detection (Dako, Denmark).

Clinical application
Patient NA The 29-year-old suffered a spinal injury three years ago. He was diagnosed with a ruptured spinal cord with vertebral displacement.

The following clinical manifestations were observed: quadriplegia, urinary incontinence and defecation disorders.

Cell removing

Removal of cells from the surface of the vials was carried out according to the following method: the cells were washed 2 times with 0.25% trypsin solution in a mixture with saline in a ratio of 1: 1. Then 4 ml of the above mixture was added to the vials and incubated at 370C for 5-10 minutes. (bottle - 75 cm2). The cells were transferred to the suspension by shaking; this method was able to remove about 50% of fixed cells, the other 50% after treatment with trypsin mixture was rounded, which significantly weakened their contact with the surface, so it was quite easy, without injury to cells, to remove them for the 2nd scraping, using a scraper, in saline with a volume of 4-5 ml per bottle of 75 cm2; to consolidate the effect after 2 scrapings - did another rinsing / rinsing with saline, resulting in the surface of the vials under the magnification of the microscope was as clean as possible; the entire collected wash mixture with detached cells was centrifuged 2 times for 5-10 minutes. at 500 rpm (2nd centrifugation was in saline for washing). The resulting precipitate was filled with the required volume of saline and packaged in ampoules for further injection (6 x 1.5 ml ampoules were packaged for this patient).

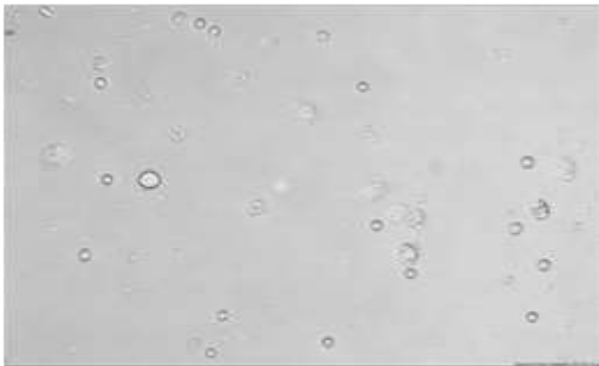
Cell residues were not left for further cultivation: all patient cell material was transferred for injection.

RESULTS

During the first 3 days of cultivation, the adherent fraction of cells consisted of round cells of small and medium size with a large nucleus and a narrow rim of the cytoplasm, which gradually stratified, acquiring an oval and granular shape (Figure 1,2).

Subsequent cultivation showed that the cells acquire morphological characteristics of different types of cells of the nervous system. We observed the formation of neuronal cells: polygonal, pear-shaped, and spindle-shaped with axons of the type of uni-, bi- and multipolar neurons (Figure 3-6). Numerous cells had the characteristics of astrocytes in vitro: large cytoplasm, often asymmetric nucleus, typical "stratification", the formation of the vascular stalk in the terminal parts of the processes (Figure 3-6). We also observed small cells with numerous short processes with characteristic dichotomous branching, which are typical features of oligodendrocytes (Figure 6).

After 7 to 14 days in a culture, we observed father processes elongation and formation of cell-cell connection (Figure 7,8).



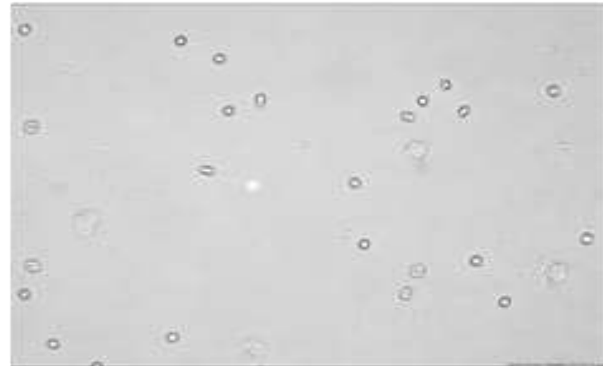
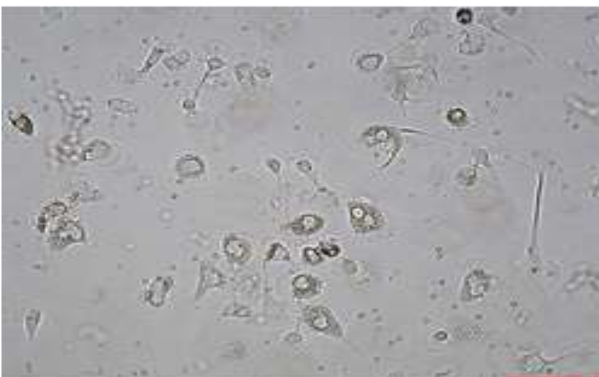


FIGURE1, 2: Adhered fraction of human circulation blood progenitors, neurogenes stimulation 3 days in culture x100.



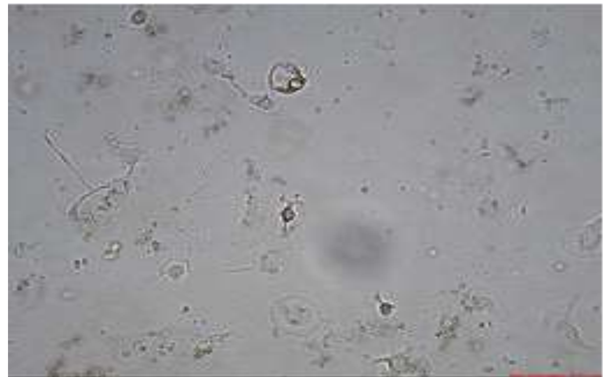


FIGURE 3, 4: Adhered fraction of human circulation blood progenitors, neurogenes stimulation, 5 days in culture x100.

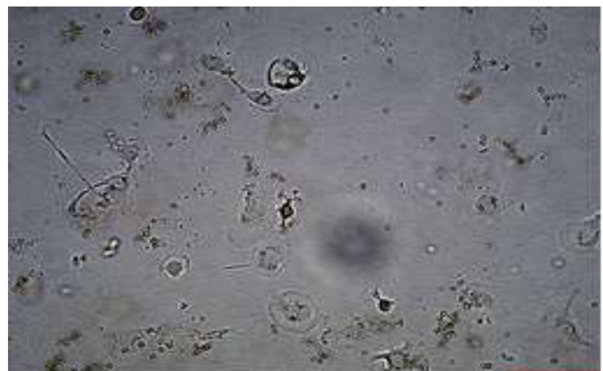


FIGURE 5, 6: Adhered fraction of human circulation blood progenitors, neurogenes stimulation, 7 days in culture x100.

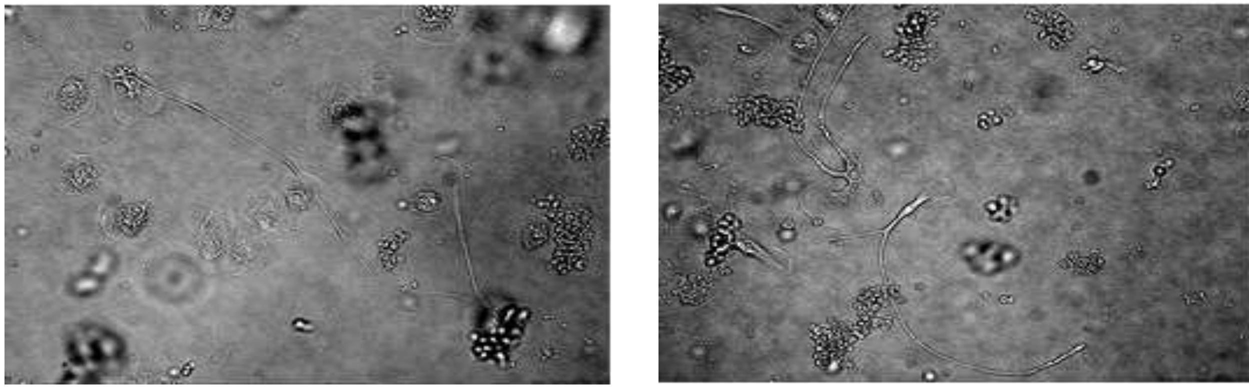


FIGURE 7, 8: Adhered fraction of human circulation blood progenitors, neurogenes stimulation, 14 days in culture x100.

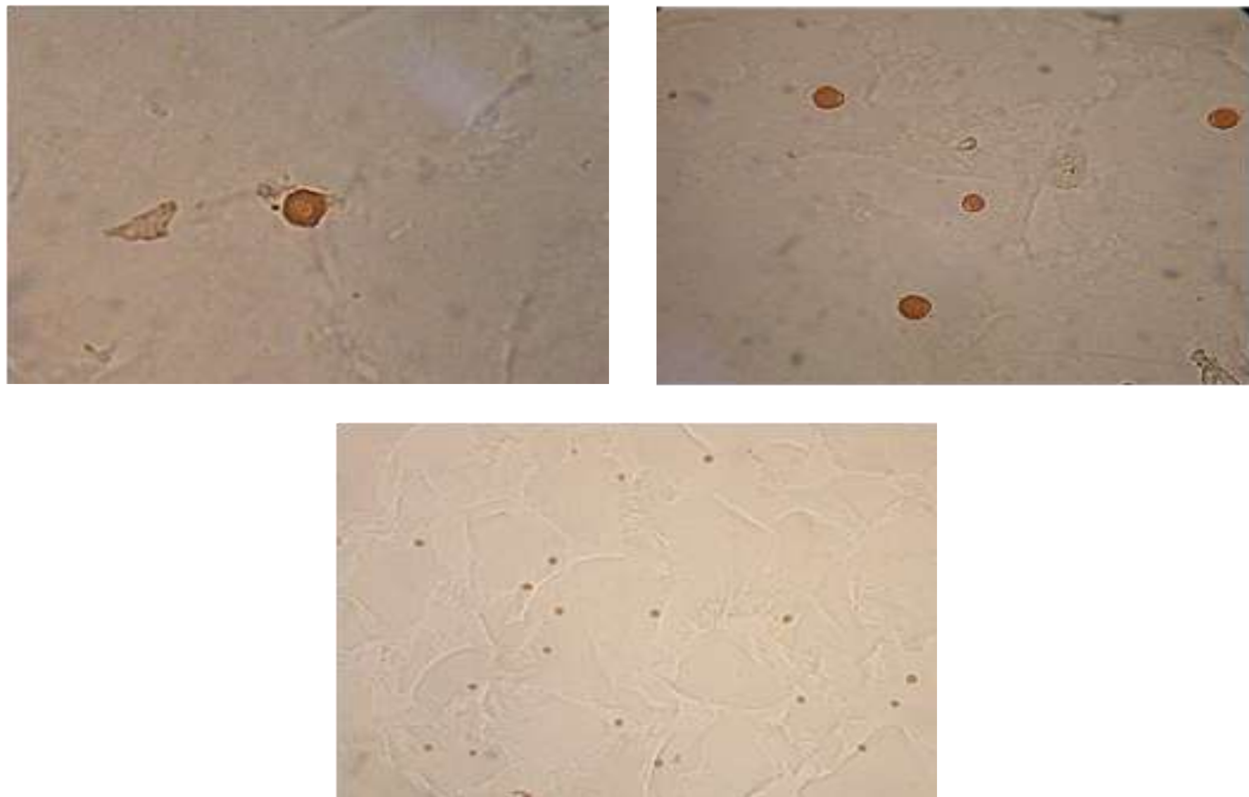


FIGURE 9, 10, 11: Nest+ cells in the adhered fraction of human circulation blood progenitors, neurogenes stimulation, removing after 14 days in culture x400, x100.

The patient's own cells after stimulation of neurogenic differentiation in vitro were injected into the spinal canal in three portions (10 x 106 cells each): At the level of C-4, T-11, L-2, and also at the level of C2-T in the paravertebral zone. No adverse reactions to the introduction were observed. Currently, after 3 months the patient controls defecation and urination and improved movement of the upper and lower limbs.

DISCUSSION

The directional influence in the direction of differentiation in a neurogenic way revealed by us is possible thanks to the existence of the corresponding receptors. Thus, it is known that human CD34 + cells, the population of which is a component of MSCs, express Trk A (tyrosine kinase), Trk B, Trk C and p75NTR, which are receptors for NGF, BDNF, NT-3 (neurotrophyn-3), NT- 4 [7, 13]. The presence of the retinoic acid receptor RAR has been confirmed in MSCs [14]. It was also found that the activation of low-affinity NGF receptors in MSCs blocks their differentiation by osteo-, adipo-, chondro- and myogenic pathways [15]. At the same time, blocking neurotrophin receptors inhibits the neurogenic development of stem and progenitor cells [16].

The possibility of differentiating MSCs by neurogenesis has been repeatedly confirmed by scientists. So Xie S.T. (2013) and colleagues showed that human bone marrow MSCs treated with retinoic acid for 1-2 weeks showed expression of Tuj1, GFAP and GalC, while the levels of typical MSC markers CD44 and CD90 are significantly reduced [3]. The use of retinoic acid together with mitogenic factors EGF (epidermal growth factor) and FGFb (fibroblast growth factor basic) promotes transdifferentiation of bone marrow MSCs in oligodendrocytes, in particular the number of Olig 2-positive cells in the population is (52.1 ± 1.74) % [17]. Stimulation of retinoic acid neurogenesis has been associated with stabilization of the HRas protein (retrovirus-associated DNA sequences). 90% of Hras-positive cells are also Tuj1-positive [18]. Hras blockade inhibits neurogenesis without affecting the differentiation of astrocytes and oligodendrocytes [18].

It is known that RAR (retinoic acid receptor) α and β are nuclear receptors, binding of the ligand to them leads to the formation of the transcription complex RA-RAR-PXR (pregnan X receptor), which binds to RARE (retinoic acid receptor elements) , localized in the promoter of the Oct4 pluripotency gene, and causes its suppression [19].

Instead, the complex of genes of neurogenesis is activated [19]. In addition to RAR, retinoic acid binds to PPAR (peroxisome proliferator activated receptor) [20]. RAR and PPAR are associated with CRABP-II (cellular retinoic acid bind protein) and FABP5 (fatty acid binding protein) lipoproteins [20]. Depending on which receptors are present in the cell, different pathways of neurogenesis are involved. The CRABP-II / RAR pathway stimulates neurogenic differentiation in stem cells and early progenitors, and FABP5 / PPAR activates the formation of mature neurons from determined neuronal progenitors [20].

The neurogenic effect of NGF and BDNF on MSCs is due to the activation of a complex complex of kinases (Fig. 2.6.6). Binding of growth factor to a receptor with tyrosine kinase activity activates RAS or PI3K (phosphoinozitide kinase 3) signaling pathways [21]. Activation of RAS and complex NORE1 (novel Ras effector), RASSF1A (Ras association domain family members) and MCT1 (mammalian sterile 20-like kinase) causes JNK (jun N-terminal kinase) - stimulation of cell cycle arrest (Fig. 2.6.6) , which in our case the use of MSC is defined as the cessation of mitosis and the beginning of cell differentiation. RAS-stimulated activation of RAF (rapidly accelerated fibrosarcome) / MEK (methyl ethyl ketone) and ERK (extracellular receptor kinase) signaling pathways leads to the survival of neurogenic precursors, their proliferation and neurite outgrowth [21]. Also, MAPK (mitogen-activated protein kinasa) is involved in this metabolic pathway [21, 22]. It is believed that this pathway with RAS activation regulates the fate of carrier-positive stem cells of the nervous system, both in the early stages of its formation and during regeneration in adulthood [22].

Another signaling pathway involved in stem and progenitor cells, including MSCs, in the binding of trophic factor to the receptor, begins with PI3K (phosphoinozitide kinase 3) -stimulated differentiation of ACT kinase (serine / threonine kinase 1). dissociation of TSC (tuberose sclerosis complex) with lysosomes and activation of RHEB (Ras homdog enriched in brain) [23]. The consequence is the suppression of autophagy of cells, maintaining their viability, neuronal differentiation and neurite outgrowth. Also, PI3K indirectly stimulates PKC (protein kinase C), resulting in the formation of neuronal morphology due to a specific cytoskeleton and synaptic plasticity [23].

Thus, NGF, BDNF and retinoic acid can stimulate neurogenesis in different ways, depending on the types of receptors present in the cell, covering a wide pool of potential nerve progenitors.

CONCLUSION

Using an adhesive fraction of circulation blood progenitors into neurodifferentiative culture condition during 2 weeks we have produced autologic population of nerve stem cells. It's a perspective way of nerve injury treatment.

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