Neurogenic Induction of Human Mesenchymal Stem Cells in Fibrin 3D Matrix

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We studied the potential of neurogenic induction of human bone marrow mesenchymal stem cells in fibrin-based 3D matrix. The best results were obtained after incubation of mesenchymal stem cells in fibrin 3D matrix in the presence of neurogenic medium containing EGF and bFGF growth factors. Under these conditions, most cells formed numerous branching processes and expressed neural cell marker βIII-tubulin. Optimal combination of 3D matrix and neurogenic factors of the medium can provide new insight into neurogenic potential of mesenchymal stem cells, which can be used for the therapy of neural traumas and neurodegenerative diseases.

Key Words: *mesenchymal stem cells; 3D culture; fibrin; neurogenic induction*

We previously showed that culturing of mesenchymal stem cells (MSC) from human bone marrow under neurogenic conditions leads to the appearance of neuron-like phenotype in some cells, which is accompanied by the expression of some molecular and immunocytochemical markers (NSE, MBP, MAP-2, βIII-tubulin, *etc.*) [1]. These results agree with some previous reports [10,12,13]. Analysis of the expression of markers specific for the nervous tissue in induced MSC, electrochemical properties of cells, and secretion of neurotransmitters drove some authors to a conclusion on the capacity of MSC-derived neuron-like cells to perform functions of nerve cells [10,14]. On the other hand, the possibility of terminal neurogenic differentiation of MSC was not proven and requires further investigation [3,6].

Creation of a 3D microenvironment simulating biological properties of tissues in the organism is a promising way for improving the efficiency of neurogenic differentiation of stem cells. It was found that culturing in 3D matrix promoted differentiation of neural and embryonic stem cells [5,15]. Matrigel, collagen, alginate, fibrin, hyaluronic acid, and other materials are used for creation of 3D matrixes [5]. Chemical composition, architecture, and biomechanics of 3D microenvironment provide signals essential for cell differentiation and function. In particular, experiments with differentiation of MSC in a 3D matrix showed that differentiation lineage can be determined by changes in structural and biomechanical properties of the matrix [8]. Differentiation and function of stem cells largely depend on the composition, biomechanical properties, and factors of microenvironment.

The aim of the present study is to evaluate the potentialities of neurogenic induction of MSC.

MATERIALS AND METHODS

For isolation of MSC, BM aspirate was separated by centrifugation in Ficoll gradient, the mononuclear fraction was collected, washed twice with PBS, and seeded in α-MEM medium (Gibco BRL) supplemented with 10% FBS (HyClone) [7]. MSC selected by adhesion to plastic were immunotyped using antibodies to CD90, CD105, CD45, and CD34. MSC were

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cultured in a CO_2 incubator (5% CO_2) at 37°C; the growth medium was replaced every 2 days.

Fibrin 3D disks (volume 100 μl) were polymerized in wells of a 96-well plate. Fibrin concentration was 8.5 mg/ml, proteases were neutralized with 20 U aprotinin. After adding the cells and polymerization with thrombin, the 3D disks were transferred to wells of a 24-well plate for incubation in a liquid medium under the specified conditions. The disks were cultured in DMEM medium (Gibco BRL) without serum or containing 10% FBS and in proliferative medium NeuroCult (Stemcell Tech.) supplemented with 20 ng/ml EGF, 10 ng/ml bFGF, and 10 ng/ml heparin.

Immunocytochemical analysis of MSC for the expression of neuronal marker βIII-tubulin was carried routinely as described elsewhere [1]. Primary antibodies to βIII-tubulin (1:1000) and secondary antibodies conjugated with Texas Red (1:100) were used. Fluorescence was detected on a Carl Zeiss Axio Imager M1 microscope. Cell viability was evaluated by trypan blue staining.

RESULTS

For optimization of conditions for neurogenic induction, human bone marrow MSC were embedded into a fibrin 3D matrix providing structural support for neural phenotype development.

Polymerized fibrin disks (volume 100 μl) were placed in nutrient media differing by the absence or presence of serum and neurogenic growth factors. The best results were obtained after incubation of MSC in fibrin 3D-matrix in the presence of proliferative NeuroCult medium containing EGF and bFGF factors. As soon as after 1-week incubation, most cells formed numerous branching processes and had a clear-cut soma typical of neurons (Fig. 1, *b*). The length of processes and their growth pattern depended on the number of cells embedded in the matrix: the most pronounced neural phenotype was observed at a seeding concentration of 5000 cells per 100 μl, while at a concentration of 30,000 cells per 100 μl the length of processes and intensity of their growth somewhat decreased. Immunocytochemical analysis showed that cells with neural morphology express βIII-tubulin, a classical marker of immature neurons [11].

The percent of cells acquiring the neuron-like phenotype during induction in fibrin 3D matrix considerably surpassed the percent of neuron-like cells after induction with the same medium (NeuroCult with EGF and bFGF) on the plate. On day 20 of culturing, 57.5±5.3% cells in fibrin 3D matrix were neuron-like cells (*vs*. 18% after induction on the plate) [1]. The cells not forming processes in the matrix retained round shape and did not acquire morphology typical of MSC in the control fibrin disk in DMEM with 10% FBS (Fig. 1, *a*).

DMEM with 10% FBS was used as a control variant for detection of phenotype typical of MSC in 3D culture in the absence of neurogenic induction. It was previously shown that MSC seeded on polyacrylamide hydrogels can undergo neurogenic differentiation under the effect of substrate elasticity irrespective of the presence of inducing factors [8]. For evaluation of inductive potential of fibrin 3D matrix in the absence of neurogenic factors, some disks were cultured in serum-free medium. Substantial portion of MSC (38.0±5.9% on day 20 of culturing) also acquired neuron-like phenotype, but less pronounced than after induction with NeuroCult medium with EGF and bFGF (Fig. 1, *c*). Thus, under conditions of neurogenic differentiation of MSC in 3D matrix the medium and growth factors probably play a secondary role relative to the structure and biomechanical properties of 3D microenvironment.

The cues from matrix mechanics are thought to occur by the cell "pulling" on the matrix and then generating signals based on this force [5]. According to previously proposed model, axon initiation is a result of amoeboid motion on the neuron surface leading to generation of a potential growth cone with tensile force surpassing a threshold value [9]. Another model, a model of neurite formation, also implies the existence of tension created in the growth cone by actin polymerization for overcoming the mechanical barrier created by the 3D matrix [2]. It is evident that optimization of the matrix structure and properties can essentially promote effective neuron formation from stem cells of different origin.

The importance of stem cell interaction with microenvironment manifests itself in not only the responses to biomechanical signals from the matrix, but also in the formation of 3D cell structures. It is known that neural stem cells form 3D spherical cell conglomerations during culturing, so-called neurospheres [4]. These floating spherical aggregates carrying microprocesses typical of neurospheres (Fig. 1, *d*) were obtained by us during culturing of MSC from the bone marrow and umbilical cord blood under low-adhesion conditions. However, subsequent culturing of these neurospheres on plastic in NeuroCult medium with EGF and bFGF revealed no differences in the yield of cells with neuronal morphology compared to the standard induction protocol [1], whereas more than half cultured cells acquired neuronal phenotype after induction in fibrin 3D matrix. The cells in fibrin 3D matrix remained viable for more than 50 days of incubation.

The recent achievements in stem cell biology aimed tissue engineering at the creation of biologically

adequate microenvironment promoting cell growth, differentiation, and effective functioning. These problems require comprehensive study of all aspects of various 3D matrixes and their interaction with the cell, which will help to determine and simulate (if necessary) the properties essential for cell differentiation and assembly into bioartificial organs and tissues. In cell therapy of the central and peripheral nervous system, 3D matrixes are very important as a potential material for creation of "bridges" in damaged zones of the nervous tissue. We showed that fibrin-based 3D matrix considerably promotes acquisition of the neural phenotype by human MSC, which attests to advantage of 3D culture for the studies of the potentialities of neurogenic differentiation of MSC. Adequate combination of 3D matrix and neurogenic factors of the medium can provide new insight into neurogenic potential of MSC as a source of autologous cells for the therapy of neural traumas and neurodegenerative diseases.

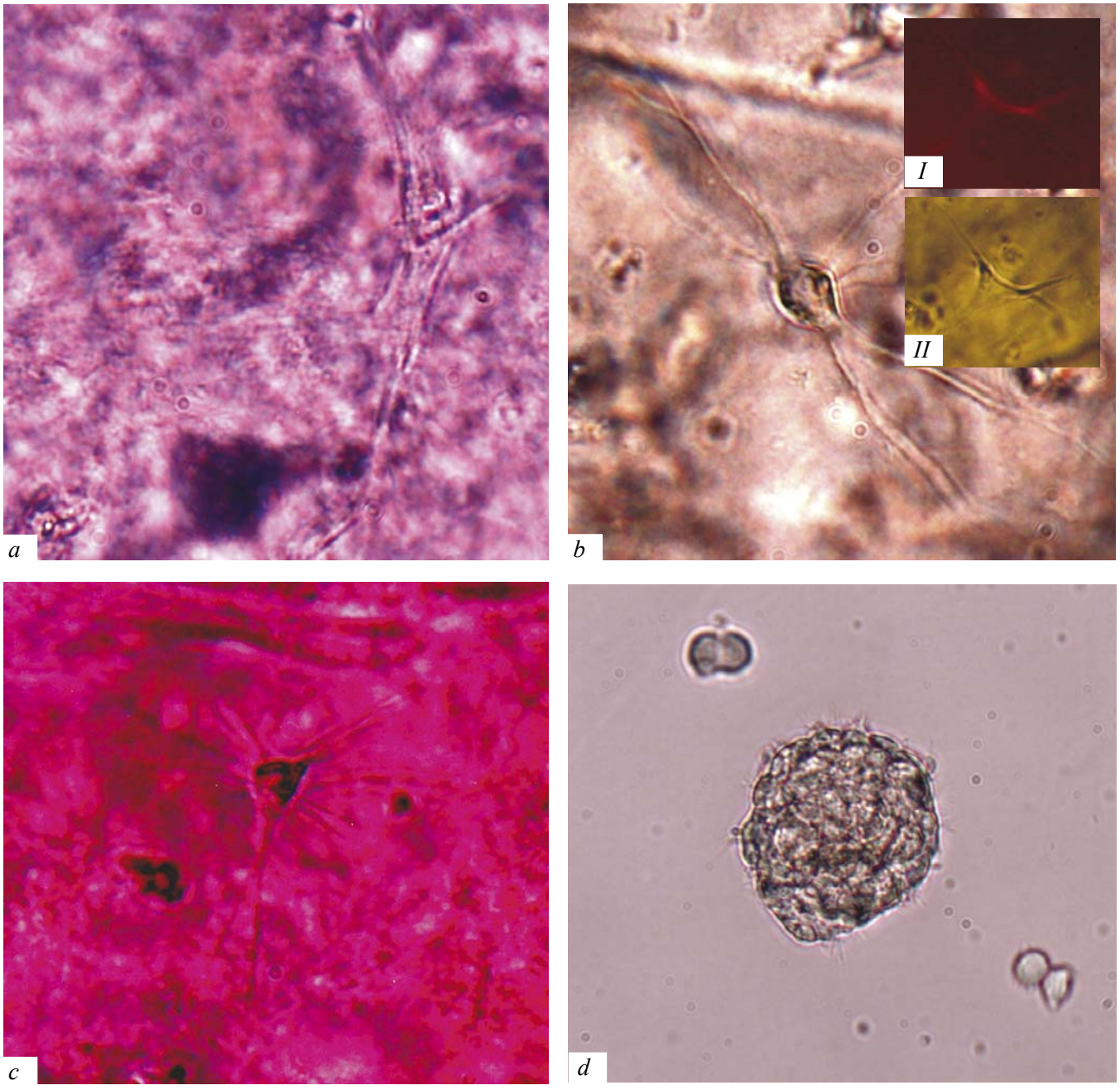

Fig. 1. MSC in 3D culture, ×200. *a*) control MSC, fibrin disk in DMEM with 10% FBS; *b*) MSC in 3D matrix in neurogenic NeuroCult medium with EGF and bFGF (*I-II*: fibrin disk section stained with antibodies to βIII-tubulin (×100); *I*: fluorescence, *II*: visible light); *c*) MSC in 3D matrix in serum-free DMEM; *d*) MSC aggregate with signs of neurosphere in neurogenic medium under low-adhesion conditions.

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