

Adult cerebrospinal fluid does not support neurogenesis from fetal rat neural stem cells

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Abstract The purpose was to study the effect of human cerebrospinal fluid (CSF) on differentiation of rat neural stem cells (NSCs), and thus explore the feasibility of transplanting stem cells via lumbar puncture clinically. Rat NSCs derived from fetal brain were divided into two groups, and cultured in DMEM/F12 supplemented with 10 % FBS and human CSF, respectively. Cellular growth was observed with an inverted microscope, and immunostaining was used to analyze differentiation of NSCs in both groups. Cells of fetal brain showed shapes of spindle or star with minor sprouts at fifth day post-culture, and stained with nestin. NSCs in the control group differentiated into neurons, with positive staining to NSE, when cultured further in DMEM/F12 supplemented with 10 % FBS. While NSCs in the experiment group, cultured in CSF, differentiated into astroglia on eighth day, with positive immunostaining to GFAP. The new neurons dissolved rapidly when they were cultured in CSF. Human CSF cannot promote NSCs to differentiation toward neuron, nor support newborn neurons survival. It seems an inappropriate approach to transplant stem cells through CSF.

Keywords Neural stem cells (NSCs) · Cerebrospinal fluid (CSF) · Transplantation · Differentiation

Introduction

Neuron necrosis and axon breakage contribute predominantly to the poor prognosis in patients with spinal cord injury (SCI). Currently, there is no effective therapy to reverse this disabling condition. Transplantation of stem cells, such as embryonic stem cells, cord blood stem cells and neural stem cells (NSCs) into the spinal cord circuitry is the key in designing potential therapies for patients suffering from SCI so far. In vivo analyses of intrastrially transplanted rodent and human fetal NSCs show long-term survival, extensive distribution, and morphological maturation in rats [1, 2]. Transplanted NSCs also formed synapses, suggesting integration into adult rodent CNS [3]. It has also been reported that transplantation of NSCs leads to a reduction of functional impairment in animal models of different neurodegenerative disorders such as Huntington's disease Parkinson's disease and amyotrophic lateral sclerosis (ALS) [4–6].

So far, NSCs have mainly been transplanted directly into the parenchyma of rodent CNS, which makes it far from clinical application in case of aggravating primary SCI. Nontraumatic transplantation via cerebrospinal fluid (CSF) demonstrated in recent reports provided a promising approach for clinical transplantation. Interestingly, CXCR4 expressed on the surface of stem cells helps them to recruit toward injury site under the direction of SDF-1, which is secreted locally following SCI [7, 8]. In different studies, an intensive invasion, migration, and integration of transplanted stem cells into the damaged CNS have been detected after transplantation via CSF [9, 10]. Given the fact that CSF plays an important role not only in brain development but also in survival, proliferation, and differentiation of neuroectodermal stem cells, it remains unclear how CSF influences those stem cells when they are

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infused into and transported in CSF. Can they still survive, proliferate, and even differentiate into neurons? NSCs of rats were isolated and cultured in adult CSF in the present study, and their growth and differentiation were observed, so as to explore the feasibility of intrathecal transplantation clinically. It was not neurons but glial cells that the cultured rats NSCs differentiated into in adult human CSF.

Methods

Isolation and differentiation of fetal rat NSCs

Mesencephalic NSCs from E14 rat were prepared as previously described [11]. In brief, a pregnant female SD rat (Center of experimental animals, medical college of Wuhan university, China) was killed according to NIH guidelines and eight fetal rats were obtained. The fetal brains were dissected under a stereomicroscope and washed with PBS for three times. Single cell suspensions of cortex and hippocampus were obtained using trypsin, DNase, and mechanical trituration. Cells were centrifuged at 1,000 rpm for 5 min, discarding the supernatant. The single cells were resuspended and primarily plated at a density of 1×10^5 cells/ml in culture bottle (4 ml/bottle) and grown in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10 % fetal bovine serum (FBS, Sigma, USA), penicillin and streptomycin. Cultures were incubated at 37 °C, 5 % CO₂ in a humidified atmosphere.

Collection of adult human CSF

The CSF samples were taken for diagnostic purpose from adult patients in the Neurological Clinic of Renmin hospital, Wuhan University by a lumbar puncture. Scientific use of CSF samples was approved by the local Ethics Committee in accordance with the Declaration of Helsinki. All patients gave a written informed consent for the diagnostic procedure. Lumbar puncture was performed by standard protocols. CSF was only used if all standard parameters were normal (cell count, glucose, lactate and albumin content, immunoglobulin quotient). Surplus CSF from diagnostic samples of all patients was pooled, and then aliquoted and frozen at -80°C until use.

Group and immunocytochemistry

Growth of the cultured cells were observed daily using inverted microscope, which provided the timing for immunocytochemistry. Five days following culture in vitro, the cultured cells began to show spindle shape with

short extensions, when immunohistochemistry staining to nestin (1:100; Chemicon International, Temecula CA, USA) was carried out to clarify the presence of NSCs.

NSCs were seeded on poly-D-lysine-coated coverslips in triplicate at the density of 2×10^4 cells/well. All cells were allocated into two groups randomly. NSCs in control group were cultured continuously in DMEM/F12 supplemented with 10 % FBS; NSCs in experiment group were cultured in 1 ml of CSF sample.

For examination of NSCs differentiation, immunohistochemistry was carried out. Briefly, the cells were fixed in a PBS solution containing 4 % paraformaldehyde for 1 h, and washed 3 times with PBS. The cells were blocked with a PBS solution containing 1 % bovine serum albumin (BSA) and 0.3 % Triton for 30 min at room temperature. After removing this blocking reagent, cells were incubated in a humidity chamber at 4 °C overnight with primary antibodies composed of mouse anti-NSE (1:500, Sigma, USA) and rabbit anti-GFAP (1:2,000, Millipore, USA). Then cells were washed 3 times with PBS and incubated for 2 h in the dark at room temperature in the presence of the fluorescent secondary antibodies (Alexa fluor 594 donkey anti-mouse IgG, 1:800, and Alexa fluor 488 donkey anti-rabbit IgG, 1:400, Invitrogen, USA). Finally, the cells were incubated with DAPI for 20 min at room temperature to stain the cellular nuclei. The preparations were analyzed under a fluorescent Olympus BX-51 microscope.

Results

Cells derived from fetal brain showed NSCs identity in the differentiating medium

Proliferating ability of stem cells, demonstrated as neurosphere formation in other reports simulated by EGF and/or FGF, was not studied here. The cells of fetal brain adhere rapidly following being added into the differentiating medium containing FBS. Then these cells gradually showed shapes of spindle or star with minor sprouts. Immunocytochemistry demonstrated positive staining to nestin at fifth day post-culture (Fig. 1).

No neuronal differentiation from NSCs cultured in CSF

The extensions of NSCs in the control group, cultured at the presence of DMEM/F12 containing FBS, grew longer and more as time went on. About 10 days later most of these NSCs showed spindle shapes, multipolar and had longer sprouts, which were of good refraction, and even interweaved into nets. Immunocytochemistry demonstrated positive staining to NSE (Fig. 2). In contrast, intense cells

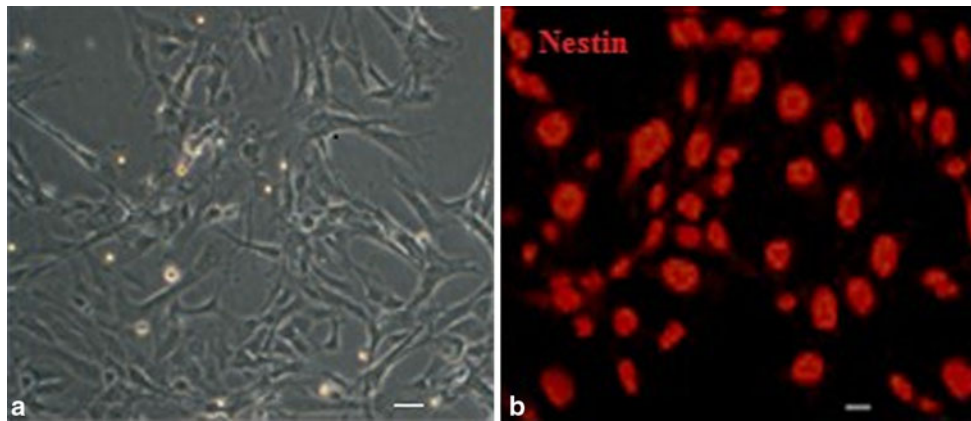


Fig. 1 Culture of cells derived from fetal brain. **a** Cells showed shapes of spindle or star, with minor sprouts under an inverted microscope at fifth day post-culture. **b** Immunocytochemistry demonstrated positive staining to nestin at fifth day post-culture. Scale bar 50 μ m

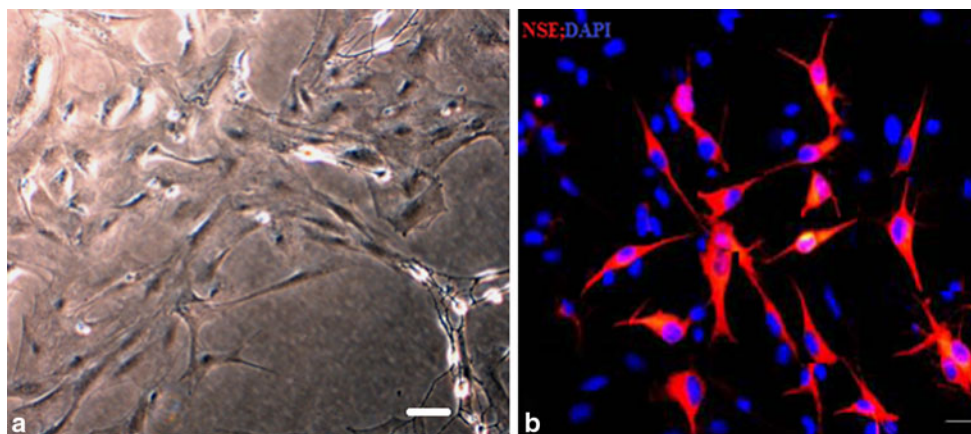


Fig. 2 Growth and differentiation of NSCs in cultured medium. **a** Multipolar NSCs showed spindle shapes with longer sprouts interweaving into nets on tenth day post-culture. **b** For immunostainings, NSCs were stained with NSE. Scale bar 50 μ m

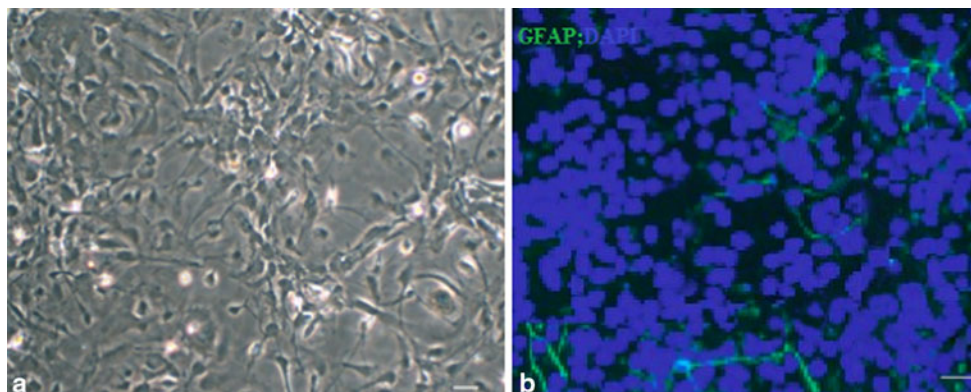


Fig. 3 Growth and differentiation of NSCs cultured in CSF. **a** Intense cells showed smaller size, circle or irregular shape in the inverted microscope on third day post-culture. There was no apparent spindle,

with smaller size, circle or irregular shape were found in the experiment group on third day post-culture. There was no apparent spindle, multipolar cells. These cells in the experiment group were GFAP staining (+) (Fig. 3).

multipolar cells. **b** For immunostainings, NSCs were stained with GFAP. Scale bar 50 μ m

CSF cannot support newborn neurons to survive

NSCs of the control group, which were proved as NSE (+), were transferred into CSF for further culture. The previous

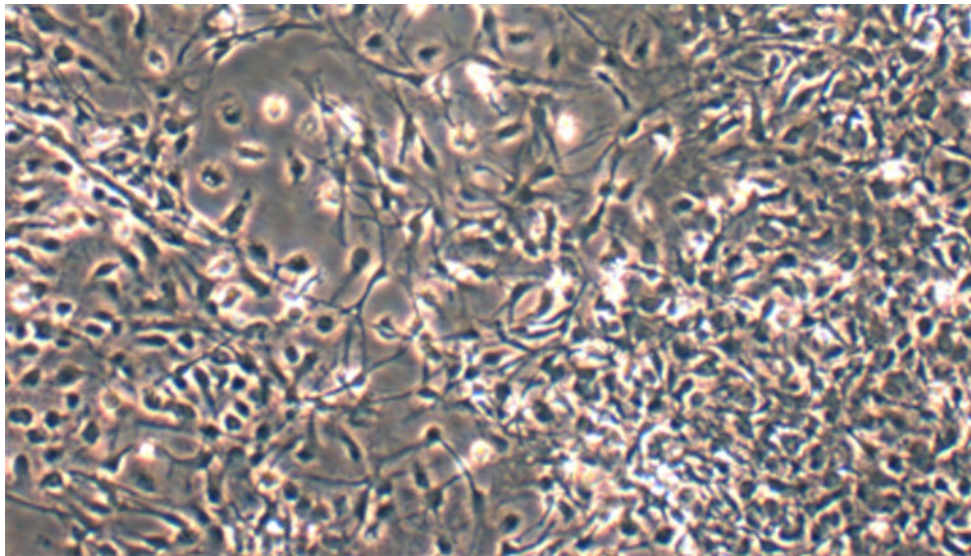


Fig. 4 Growth of the cultured neurons in CSF. The whole visual field was full of newborn glial cells on the third day under an inverted microscope

neurons with larger size were found dissolving gradually 1 day later. There were a lot of newborn cells with smaller size and irregular shape. All neurons disappeared on the third day, and the whole visual field was full of newborn glial cells (Fig. 4). It was obvious that there were no neurons morphologically in the CSF, so immunostaining was not done.

Discussion

Stem cell transplantation has shown partial recovery in rodents *in vivo*. Intrastratially transplanted cultured rodent and human NSCs showed long-term survival, extensive distribution, and morphological maturation in rats. It has also been reported that transplantation of NSCs leads to a reduction of functional impairment in animal models of different neurodegenerative disorders and SCI. These effects, however, were mainly obtained by local transplantation directly into the spinal cord parenchyma. In contrast, intrathecal transplantation seems a more feasible approach to clinical patients. Thus, operation risks could be minimized, and a widespread distribution of NSCs into the lesioned CNS could be achieved. At the same time, cell distribution could be restricted to the primarily affected organ, and side effects arising from peripherally applied cells could be avoided [12]. Interestingly, injected NSCs tend to attach primarily to the lesion site, from which they invade the CNS tissue [13].

An ideal candidate for clinical transplantation to patients with SCI should at least meet requirement as follows: (1) proved effect on neural recovery *in vivo* by animal experiments; (2) as few manipulation as possible to the

candidate cells *in vitro*; (3) an effective and save approach for transplantation. Most of current studies aim to evaluate the proliferating ability of stem cells, and so stem cells are cultured *in vitro* in a serum-free culture medium supplemented with the mitogens epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2). NSCs cultured with these mitogens will form multicellular aggregates, so-called neurospheres. However, this condition is apparently inappropriate to patients for the probability that mitogens may give rise to gene mutation. Cells of the fetal brain in our study were cultured in DMEM/F12 supplemented with FBS, without mitogens, and were found to adhere rapidly. These cells were proved to be NSCs with positive nestin staining on the fifth day. Most of the NSCs further differentiated into neurons with positive NSE staining after another 5 days. However, they differentiated only into astroglia when cultured in adult human CSF, which is similar to the report of Buddensiek [14]. The presumed reasons included: (1) proliferation and differentiation of NSCs were regulated not only by endogenous gene, but also by exogenous signals. It is probable that there are certain factors in CSF which inhibits neurogenesis but facilitates gliogenesis from NSCs. It is reported, both *in vitro* and *in vivo*, that the FGF2 from the E-CSF has an effect on the regulation of neuroepithelial cell behavior, including cell proliferation and neurogenesis [15]. (2) Compared with glial cells, neuronal differentiation and survival depend much on the microenvironment. DMEM/F12 supplemented with FBS, but not CSF, can satisfy the conditions that neuronal differentiation requires. Thus, most of the NSCs transplanted through lumbar puncture will differentiate into astroglia in CSF rather than neuron or oligodendrocytes. The main role that the transplanted

NSCs in CSF played in neural recovery was not substitution of the lost neurons but providing neurotrophic factor merely. In a very recent study in China, cord blood stem cells were transplanted into 22 patients with SCI intrathecally [16]. As a result, nine cases did not respond clinically to transplantation at all; among them six cases suffered from complete SCI. Other 13 cases showed partial clinical recovery. Given the fact that NSCs themselves promote neural recovery in vivo in rodents, it indicated that the approach of transplantation weakens the effect of NSCs.

Implanted NSCs cannot differentiate into neuron in CSF. Transplanting differentiated neurons from NSCs in vitro into CSF seems to be an alternative method. CSF acts merely as a medium for transport of the differentiated neurons, rather than promoting NSCs to differentiation. Unfortunately on adding the pre-differentiated neurons into adult CSF in our study, they began to dissolve 1 day post-culture and disappeared 4 days after. The visual field was full of newborn glial cells. It is concluded that neurons cannot survive for a longer period in CSF. The implanted neurons would die soon unless they were recruited to the injury site rapidly.

In fact, there is no general agreement on the fate of the NSCs in CSF [9, 17]. Influence of CSF on the proliferation of NSCs was not taken into account in the present study. Our research explored preliminarily the influence of 1 ml of CSF to NSCs differentiation in vitro, and the results demonstrated that adult human CSF cannot promote NSCs to differentiation toward neuron, nor support newborn neurons survival. But the differentiation of stem cells is regulated by multiple factors [18]. Further studies are required to confirm the clinical feasibility of transplantation via CSF, including issues as follows: the effect of circulating CSF (such as renewal of the CSF daily) on NCSs instead of 1 ml, the effect of human CSF on cord blood-derived stem cells besides rat NSCs, and the effect of culturing conditions on NSCs differentiation.

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