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Research article

Neurogenic potential of spinal cord organotypic culture

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HIGHLIGHTS

- Long-term culturing of spinal cord slices stimulate proliferation of NSC.
- In organotypic culture NSC express Oct3/4, *Dppa1* and nestin.
- NSC differentiate not only into astrocytes but in motor neurons and interneurons.

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ABSTRACT

There are several neurogenic niches in the adult mammalian central nervous system. In the central nervous system, neural stem cells (NSC) localize not only to the periventricular area, but are also diffusely distributed in the parenchyma. Here, we assessed neurogenic potential of organotypic cultures prepared from adult mouse spinal cord. Slices were placed on Millipore inserts for organotypic culture and incubated in neurobasal media supplemented with B27 and N2 for up to 9 weeks. After 3–4 weeks, the cell's aggregates formed in the slices. The aggregate's cells were BrdU-uptake, nestin and alkaline phosphatase positive. At the later stage of incubation, we observed Oct3/4 in the inner mass of the neurospheres as well as expression of *Dppa1*, which is an Oct-4 downstream target gene and a marker for pluripotency. To check differentiation, the formed neurospheres were isolated and cultured for several days in differentiation media. The obtained data demonstrated the cells from isolated neurospheres differentiate into astrocytes and MAP2-positive neurons. Immunostaining for HB9 and Lim2 revealed subsequent differentiation of MAP2-positive cells into motor neurons and interneurons, respectively. We hypothesized neuronal loss and/or long-term culturing of spinal cord slices may trigger a reset of the internal cell program and promote proliferation and further differentiation of NSC.

1. Introduction

The last decade demonstrated stemness potential in almost all adult mammalian tissues, including the central nervous system. In the brain, stem cells localized not only to the periventricular area but also diffusely distributed in the parenchyma [9,13,31,35]. Spinal cord injuries were shown to stimulate the formation of neural stem cells (NSC) in the injured zone. These cells have potential to differentiate into the neurons and glia *in vivo* as well as *in vitro* [22,35,36]. Spinal cord organotypic culture is a common *ex vivo* tissue model for studying neurological diseases, injury [11,17,30], or neurogenic potential of transplanted stem cells [4,14,16]. In this study, we investigated the spontaneous formation of NSC neurospheres, in spinal cord organotypic culture, which further gave rise to mature neurons and astrocytes. The induction of NSC in spinal cord organotypic culture can provide a new tool for generation of spinal cord stem cells for research purposes. Potentially, these spheres can be obtained in sufficient quantities for future therapeutic applications.

2. Methods

Eight-weeks old male mice (CD-1, Harlan, Indianapolis, IN) were used in this study. All procedures and tissue collection methods were approved by the East Carolina University Animal Care and Use Committee.

2.1. Culturing of spinal cord slices

Organotypic spinal cord cultures were prepared from lumbar and/or thoracic spinal cords. The spinal cords were collected from

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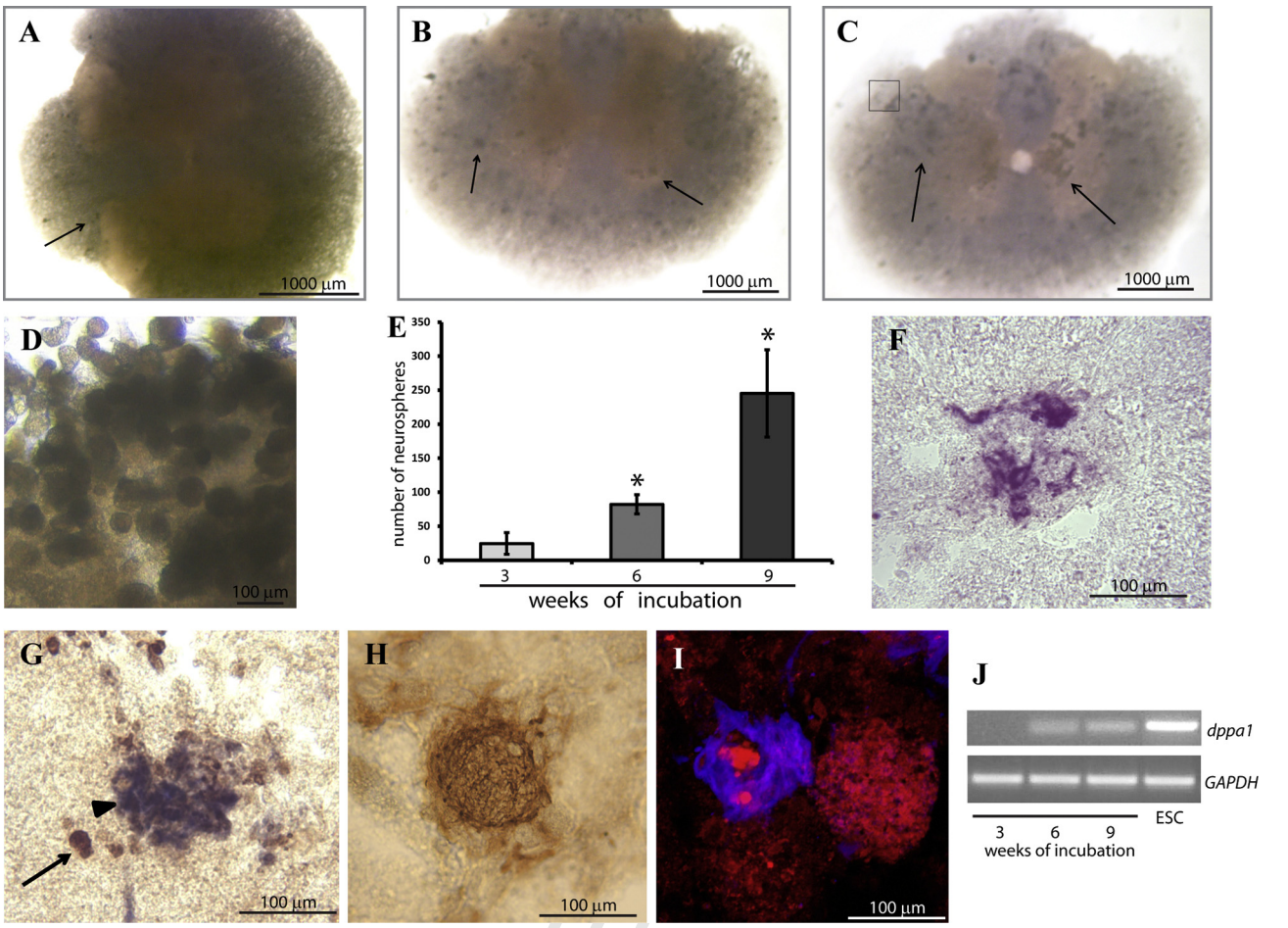

Q6 **Fig. 1.** Analysis of the cell aggregates in the organotypic culture of mouse spinal cord.
The photographs of organotypic culture of mouse spinal cord after 3 (A), 6 (B) and 9 weeks (C). (D) Higher magnification of 9-weeks organotypic culture boxed in C. After 3 weeks of culturing the neurospheres (arrows) appear inside and on the surface of the spinal cord slices (A–C). (E) The number of neurospheres continued to grow until the experiment terminated. Axis *y*: the number of neurospheres per slice ($n = 15$). Data are shown as means ± SE. $^*p < 0.05$ *versus* 3 weeks incubation period.
The cryostat sections (F–I – 6 μm) of a slice cultured for 9 weeks. (F) Histochemical detection of alkaline phosphatase; (G) double staining for BrdU by immunohistochemical method (brawn) and histochemical detection of alkaline phosphatase (violet), arrow indicates BrdU positive cells and arrowhead points on double positive cells; (H) immunohistochemical staining for nestin using ABC elite kit (Vector) and DAB (3,3′-diaminobenzidine tetrahydrochloride) for visualization. (I) Confocal image of the tissue stained for GFAP (blue) and Oct3/4 (red); (J) RT–PCR product demonstrated *Dppa1* expression appeared after 6 weeks and continued to be detected after the 9 weeks incubation period. ESC – undifferentiated, pluripotent mice embryonic stem cells (D3 cell line, American Type Culture Collection Manassas, VA) were used as a positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3 adult mice (CD-1; Harlan, Indianapolis, IN) under sterile conditions and sectioned transversely into 500-mm slices with a tissue chopper. Fifteen slices were cut from each animal. The tissue was divided into 3 groups: 3 weeks incubation; 6 weeks incubation; and 9 weeks incubation. Each group contained 5 slices from each animals ($n = 15$ per group). Slices were cultured in Millicell CM semipermeable culture inserts (Millipore, Billerica, MA) at a density of 5 slices/well in Neurobasal medium supplemented with B27, N2, L-glutamine (2 mM), 100 units/ml penicillin, 100 μg/ml streptomycin. The medium was changed twice weekly. After 3 weeks, the culture media was additionally supplemented with 10% horse serum. To analyze proliferation, the culture was treated with BrdU (5 mg/ml) twice in 24 and 12 h before fixation. After 3, 6 and 9 weeks, 10 slices from each group were fixed in 4% paraformaldehyde, and the rest 5 from each group slices were collected in TIZOL reagent (Invitrogene) for RNA analysis.

2.2. The neurosphere's differentiation

The neurospheres were collected from 9 weeks old cultures prepared from lumbar and/or thoracic spinal cords of 3 adult mice per 3 slices from each animal. The neurospheres were isolated manually using 200 μl pipets under microscopic control. The neurospheres were seeded on collagen-coated round coverslips (12 mm diameter) placed in 24 well plates at 20–30 neurospheres per well for further culturing. To induce differentiation, the medium was exchanged for DMEM/F12 medium supplemented with B27, L-glutamine (2 mM), 20 ng/ml EGF, 20 ng/ml b-FGF, heparin (5 mg/ml), 100 units/ml penicillin, 100 μg/ml streptomycin. After 12 h, 6 coverslips were fixed for analysis of nestin and MAP2 expression to check for an early differentiation stage. The neurospheres on 18 coverslips were cultured further. After 3 days, growth factors (EGF and b-FGF) were removed from the media, and 1% of fetal calf serum was added. The neurospheres were cultured for another 4 days and then fixed in 4% paraformaldehyde for further immunofluorescent staining.

2.3. Antibodies used for immunostaining

Anti-BrdU (1:500; Roche Molecular Biochemicals), anti-Oct-3/4 (1:100, R and D systems), anti-microtubule-associated protein 2 (1:100, MAP2, Chemicon), anti-MNR2/HB9 and anti-Lim2 (1:100,

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