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Research report

Embryonic neural stem cells in a 3D bioassay for trophic stimulation studies

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ABSTRACT

Progenitors were discovered in the corpus striatum several years ago, but little is known about their proliferation and differentiation. The aim of this study was to analyze embryonic progenitor cells from the corpus striatum using a bioassay with trophic stimulation. Primary cells obtained from brains of rat embryos at E13–14 were dissected from striatum niches and cultured in stem cell media. These floating dispersed cells clumped together to forming floating bodies like irregular spheres (spheroids), which were placed in type I collagen gel and cultured under basal conditions or with the addition of NGF, NT-3, or NTN. Optimum growth of neurites was obtained, and after 24 and 48 h, they were measured for number and length. The expression of proliferation markers such as PCNA and Ki67, and of neural progenitor markers such as GFAP, nestin, vimentin, O4, A2B5, Pax6, S100, TubIII, and NeuN, was then analyzed. The initial behavior in cell cultures showed distinguishable spheroids that, when placed in 3D gels and with trophic support, generated neurites. A similar effect was observed in glial cell outgrowth from the spheroids. Our assay showed high reproducibility, short culture time, and high resolution for tracing neuron-neurite outgrowth or visualizing glial outgrowth in a few hours.

1. Introduction

The formation of neurons in the adult brain has contributed to knowledge on brain plasticity. In 1962, Joseph Altman reported the incorporation of tritiated thymidine into neural cell DNA (Altman, 1962, 1969; Altman and Das, 1966), which indicated proliferation in the adult brain. Then, decades later, neuron formation in adulthood was confirmed by Eriksson et al. (1998). There are several regions in the mature brain, such as the olfactory bulb and the hippocampus, where the neuron formation process takes place. New neurons seem to derive from neural stem cells that are first transformed into a progenitor which later forms a neuron. This stem cell resides in the subventricular zone of the lateral ventricles (Corotto et al., 1993) and in the subgranular zone of the dentate gyrus (Gould et al., 1992).

Neural stem cells may increase neuroblast populations by rapid proliferation. Later, they generate a group of progenitors that will remain quiescent in these regions until they replace neurons by migration and differentiation, to finally form part of a network. The same finding has been reported for the subventricular zone in the human brain by Sanai et al. (2004) and for the hippocampus by Eriksson et al. (1998).

Knowledge on brain progenitors is growing exponentially, which may be explained by current interest in brain mechanisms by which different populations of neural cells give rise to cells with a multipotent capacity of differentiation and proliferation. These are stem cells that could give rise to progenitors. This is why the subventricular zone of mammalian brain has become a focus for experimental research but there are also true several aspects that still remain incomplete, among which the potential replacement of neurons in diseases, causing cell loss and presents an interesting challenge. A possible way to induce neurogenesis could be learned from manipulating these pluripotent cells that live inside the brain niches.

The formation of new cells from quiescent stem/progenitors must be regulated precisely to avoid an alteration in circuits where neurogenesis is activated through epigenetic signals. This involves

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activation from quiescent cells to controlled amplification and final differentiation of neurons. Culture systems in collagen gel are specific bioassays that have been intensively used as models to evaluate trophic factors in the survival, differentiation and stimulation of nerve outgrowth *in vitro* (Ebendal et al., 1985; Carri et al., 1998; Morel et al., 2010). However, there are few reports concerning their use for stem and progenitor cells (Cruz Gaitan et al., 2012; Carri et al., 2014).

On the other hand, there is abundant experimental evidence for the adult mammal subventricular zone and hippocampus, where a subgroup of quiescent stem cells is able to generate neurons. The corpus striatum could provide important stem cell *reserves*, but little is yet known about their rate of proliferation and the possible paths of differentiation from the embryonic stage. Recent research studies on neural progenitor cells have provided further knowledge on neural regeneration. A number of chemical factors which mediate these effects have been well characterized and have been studied through different development stages of neuronal and glial cells, though little has been done on progenitors, especially by means of 3D assays. The analysis of this response, neuritogenesis, in spheroids explanted to 3D cultures with type I collagen would facilitate their evaluation since this substrate allows the extension of neuronal cells and their neurites.

Several reports suggest that neuron development in the nervous system may be controlled by similar or analogous mechanisms but they have not been applied to stem cells. In order to study this we prepared trophic factors at 20 ng/ml. These factors derive from families of molecules that may influence survival, differentiation, and outgrowth in length and number (density).

In this paper we first characterize the assay necessary for brain progenitors to be cultured under basal conditions and trophic stimulation with the analysis of survival, differentiation, and neurite outgrowth. In this assay, it was possible to evaluate cells by the effects of trophic molecules, particularly those with clear activity on embryonic cells. We were also able to evaluate the action of other epigenetic signals recently discovered for embryonic stem cells in 3D bioassays of type I collagen. It is essential to identify signals that lead the stem to leave quiescence and express a neuronal or glial phenotype under basal conditions and trophic stimulation. This paper also includes the analysis of the immunoreactive profile of stem cells for the identification of neuronal and glial phenotypic epitopes.

2. Materials and methods

2.1. Donor tissue, stem cells assay

Stem cells were obtained from the corpus striatum of E14.5 fetal Sprague Dawley rats, and kept in sterile Hank's medium (CRL:CD SD BR® Charles River Laboratories, USA). Surgery was performed with a stereo-microscope under sterile air flow in a tissue culture room following a previously described protocol (Rojas Mayorquin et al., 2010). Briefly, an oblique cephalic cut above eye level was performed, and skin and cartilage of the head were removed to expose the brain. The corpus striatum is a clear anatomical region in the developing rat brain and has a niche with considerable neural stem cells. This region has a readily recognizable vascular irrigation, which makes it easy to identify it during explantation. The niche was removed from brain hemispheres, and placed in sterile plastic tubes containing Hank's medium, in a humid chamber at 37 °C for 20 min (see Flow chart phase 1). The explanted brain zones were then mechanically dissociated by gently pipetting resulting in a cell suspension that was centrifuged for 5 min (>1000 rpm) for cell concentration. Pellets were resuspended into mitogenic medium defined for stem cells (75% DMEM, 25% HAM F-12, 1% B27 – all from GIBCO, USA) and a mitogenic factor (bFGF at 10 ng/ml – Invitrogen, USA) (see Table 1).

The first step of cell culture was resuspension, seeding in culture bottles (Corning, USA) and incubation at 37 °C in a 5% CO_2 in a humid atmosphere. After the first 48–72 h of incubation, spheroids of primary cultures were obtained and collected by centrifugation for 5 min. The supernatant was then removed, and the spheroids were mechanically dissociated for resuspension in 1 ml medium defined for stem cells. After this procedure we obtained the spheroids of secondary cultures. The cell suspension was resuspended in a double volume and incubated under the same conditions as those of the primary culture. At 48 h from beginning of the culture floating spheroids contained in an aliquot of 8 μl medium were placed in the center of a 35 mm plastic dish for inclusion in collagen gel for the 3D bioassay. The action of trophic factors on the spheroids of secondary culture was then analyzed using the same protocol as that used to evaluate the primary culture.

2.2. The 3D bioassay and treatments

Our 3D bioassay is based on cell clumps inside collagen gel produced in our lab according to the original procedure described by Elsdale and Bard (1972), Ebendal's protocol (1985) and our group (Carri et al., 1998; Reynaldo et al., 2007; Cruz Gaitan et al., 2012; Carri et al., 2014). Briefly, spheroids were pipetted out from the flask, placed in the center of the 35 mm plastic dish inside collagen gels. The trophic factors like neurturin (NTN), neurotrophin-3 (NT-3) and nerve growth factor (NGF) from Peprotech® were added over the surface of the gel at a final concentration of 20 ng/ml. We also performed cultures without trophic factors, covering them with DMEN to prevent dihydratation (see Table 1). The bioassay was cultured for 48 h and neurite outgrowth was then measured at 24 and 48 h (see Flow chart) with an inverted microscope IM35 from Carl Zeiss with long focal distance lens of 10× or 20× and phase contrast illumination.

2.3. Expression markers in cultured cells.

Cultures grown inside gels were fixed *in toto* with 4% paraformaldehyde at room temperature, and then washed three times each in PBS for 5 min. Permeabilization was performed with 0.02% Triton X-100 for 15 min at room temperature followed by overnight incubation at 4 °C with normally diluted (1:100 in PBS) primary antibody, then washed again three times for 5 min each in PBS, and finally incubated with the secondary antibody diluted in 1:200 PBS for 1 h. DNA nuclei were stained with DAPI (Sigma, USA) at 1 μg/ml concentration for 15 min. After washing three times for 5 min each with PBS, the culture was preserved with Fluoromount-G® (Southern Biotechnology Association, USA) antifading mounting medium. Several primary antibodies were used for detecting antigens, especially two antibodies which show expression of proliferation related identifier proteins (anti-PCNA – from Santa Cruz Biotech SC56 – and anti-Ki67 – from AbCam, USA). Several other antibodies showed neural lineage (anti-GFAP – from SIGMA 63893; anti-nestin from Developmental Studies Hybridoma Bank at the University of Iowa DSHB-RAT401; anti-vimentin – from SIGMA B6630; anti-O4 – anti-oligodendrocyte marker O4 MAB345; anti-A2B5 – prepared by us with DSHB-Clone 05; anti-Pax6 from DSHB-PAX6-Kawakami; anti-S100 from SIGMA, USA; anti-Tub III – from SIGMA USA; anti-NeuN from Chemicon International MAB377B). These primary antibodies were detected by a secondary antibody with red Alexa 555 or green Alexa 488 from Molecular PROBES, USA. Then cultures were washed and mounted with Fluoromount-G. This method provided excellent detection of cellular antigens for visualization of the markers and for morphometric analysis. The immunoprofile analysis was carried out