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Murine embryonic EGF-responsive ventral mesencephalic neurospheres display distinct regional specification and promote survival of dopaminergic neurons

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Abstract

Similar to embryonic forebrain, the embryonic mesencephalon contains Fibroblast Growth Factor 2 (FGF2)- and Epidermal Growth Factor (EGF)-responsive progenitors that can be isolated as neurospheres. Developmentally, the FGF2-responsive population appears first and is thought to give rise to EGF-responsive neural stem cells. It is not known whether following this developmental switch of growth factor responsiveness ventral mesencephalic (VM)-derived neural stem cells display distinct region-specific properties. We found that murine VM- and dorsal mesencephalic (DM)-derived primary neurospheres isolated with EGF at embryonic day 14.5 differed with respect to neurosphere formation efficacy and size. VM- but not DM-derived spheres expressed *En1*, the molecular marker of isthmic organizer, and contained transcripts of BDNF, FGF2, IGF-I and NT-3. Both VM and DM primary neurospheres were self-renewing and gave rise to astroglial cells, but 20% of VM spheres also generated neurons. According to in vitro properties, DM- and majority of VM-derived EGF-responsive progenitors represent glial precursors. VM- but not DM-derived primary neurospheres enriched their respective conditioned medium with factors that promoted the survival of dopaminergic neurons in vitro, suggesting that ventral mesencephalic EGF-responsive progenitors are endowed with the potential to provide trophic support to nearby nascent dopaminergic neurons. These data may have implications in the treatment of Parkinson's disease.

Keywords: Ventral mesencephalon; Progenitors; Neurospheres; Region specific; Dopaminergic; Glial precursors; FGF2; EGF; Parkinson's disease; *En1*

Introduction

Developmentally, the dorsoventral gradient of the morphogenic molecule sonic hedgehog (SHH) intersects at the embryonic ventral mesencephalon with antero-posterior Fibroblast Growth Factor (FGF) 8 signaling to specify differentiation of embryonic ventral mesencephalic (VM) precursors into dopaminergic cells of substantia nigra (SN) (Ye et al., 1998). Dopaminergic neurons of embryonic SN are generated over the period extending from E10 to E14 in mouse (Lauder and Bloom, 1974). During this period, neural progenitors undergo a developmental switch in growth factor responsiveness resulting in the emergence of Epidermal Growth Factor (EGF)-responsive neural stem cells (Martens et al., 2000; Santa-Olalla and Covarrubias, 1999). It is not known whether resident VM neural stem cells acquire region-specific features following the establishment of the SN.

FGF2 and EGF are the principal mitogens for neural progenitors from many different regions of the developing and adult CNS (for reviews, see Gage, 2000; McKay, 2000; van der Kooy and Weiss, 2000). These mitogens are commonly used in vitro to obtain proliferating progenitors in the neurosphere proliferative and differentiation assay (Reynolds and Weiss, 1996; Reynolds et al., 1992). In the neurosphere assay, proliferation is defined as the ability to generate a floating clonal colony in response to FGF2 and/or EGF, multipotentiality as the ability to generate three main neural cell lineages (neurons, astrocytes and oligodendrocytes) and self-renewal as the ability of cells from dissociated colonies to generate secondary colonies.

FGF2-responsive cells are found much earlier in development than EGF-responsive cells (Ciccolini, 2001; Kalyani et al., 1999; Kilpatrick and Bartlett, 1995; Tropepe et al., 1999).

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Previous studies have suggested a lineage development model whereby FGF2-responsive precursors and progenitors divide symmetrically and give rise to EGF-responsive cells (Tropepe et al., 1999; Martens et al., 2000; Santa-Olalla and Covarrubias, 1999; Ciccolini, 2001; Ciccolini and Svendsen, 1998). When cultured in vitro, forebrain EGF-responsive progenitors exhibit neural stem cell properties: proliferation, multi-potentiality and self-renewal over extended periods of time (Reynolds and Weiss, 1996).

FGF2- and EGF-responsive progenitors are present in the embryonic mesencephalon (Drago et al., 1991a,b; Reynolds and Weiss, 1992). As distinct from FGF2-responsive progenitors in other regions of the embryonic brain, early FGF2-responsive VM progenitors differentiate into dopaminergic neurons (Yan et al., 2001). FGF2-responsive primary VM neurospheres have also been shown to maintain a region-specific gene expression profile representative of their neural region of origin (Hitoshi et al., 2002). It would be tempting to hypothesize that VM progenitors retain region-specific properties following a transition in growth factor responsiveness from FGF2 to EGF. EGF-responsive progenitors derived from the dorsal mesencephalon (DM) would be the most suitable population with which to compare VM progenitors as anatomically the DM belongs to the same brain region as VM but does not normally generate dopaminergic cells. A previous study of mesencephalic FGF2- and EGF-responsive progenitors (Santa-Olalla and Covarrubias, 1995) did not provide data about clonally derived primary neurospheres. Culture conditions in that study were quite different from the classical neurosphere assay (Reynolds and Weiss, 1996), making comparison of results difficult.

In the present work, we report distinct in vitro properties of clonal primary embryonic VM and DM-derived EGF-responsive progenitors. Remarkably, VM- but not DM-derived spheres also demonstrated trophic activity towards dopaminergic cells in vitro, supporting the hypothesis that after the establishment of SN; emerging ventral mesencephalic EGF-responsive progenitors are endowed with the potential to provide trophic support for nearby nascent dopaminergic neurons.

Methods

Neurosphere generation

Time-pregnant C57/BL6 strain mice were used. Embryos were collected in medium consisting of phosphate-buffered saline (PBS), with HEPES 10 mM (Gibco), sodium pyruvate 1 mM (Sigma), 0.6% glucose and penicillin (Gibco, 50 IU/ml) and streptomycin (Gibco, 50 μg/ml). The compartments of interest from individual embryos were pooled and processed as described (Drago et al., 1991a,b), except that serum-free conditions used digestion stopped by soybean inhibitor (0.07%, Sigma). The resultant cell suspension was allowed to settle for at least 30 min, and supernatant was carefully withdrawn. As previously reported (Qian et al., 1997), this method yielded single-cell suspension. Cell viability was typically greater than 95%.

The posterior border of the mesencephalic flexure was defined at the presumptive isthmus. The mesencephalon was divided into dorsal and ventral parts along the presumptive sulcus limitans. The lateral walls of mesencephalic tube were always removed to obtain the ventral and dorsal plates each of which measured about 1 mm in width centered on the midline. The cerebellar anlagen was identified and dissected away while isolating dorsal mesencephalic tissue.

To generate primary neurospheres, cells (at plating density indicated in text) were plated in uncoated 25 cm^2 flasks (Falcon) at a final volume of 5 ml and incubated at 37°C in humidified 5%CO_2/95% atmosphere air. The incubation medium consisted of DMEM/F12/2.5 mM Glutamine (Gibco, Cat. No.: 11320-033), 1% N2 supplement (Gibco), 0.6% glucose, penicillin (Gibco, 50 IU/ml) and streptomycin (Gibco, 50 μg/ml) (referred to as neurosphere medium) supplemented with FGF2 (10 ng/ml, Chemicon) or EGF (20 ng/ml, Chemicon). Alternatively, primary neurospheres were generated by seeding cells in 6-well plates (Nunc) at very low-density, i.e. 5×10^3 cells/well in 3 ml of neurosphere medium supplemented by EGF (20 ng/ml). One milliliter of fresh neurosphere medium containing 70% of the growth factor being tested was added every 48 h. Spheres generated under very low density conditions were used in differentiation assay as stated in the text.

For long-term propagation, neurospheres were collected, trypsinized in bulk and seeded at a density of 1000 cells/ml in the presence of EGF (20 ng/ml) alone.

To assess self-renewal, single neurospheres were individually transferred into 1.5 ml Eppendorf tubes containing 300 μl of neurosphere medium supplemented with 20 ng/ml of EGF. Neurospheres were mechanically triturated and transferred into 96-well plates for 10–14 days.

A neurosphere was defined as a free-floating cellular spherical structure of at least 50 μ in diameter. Sphere size was determined using an eyepiece reticule. The number of neurospheres per flask was determined using a grid device covering the entire field.

Differentiation assay

Intact primary neurospheres were plated onto slide-flasks (Nunc), precoated with poly-L-lysine (PLL, Sigma). Alternatively, primary spheres derived at very low seeding density were plated on 4-well PLL-coated chamber slides (Nunc). Coating was carried out overnight at 37°C; PLL was applied at a final concentration of 0.002% (w/v). Spheres were allowed to attach for 2 h and then were washed three times with mitogen-free neurosphere medium. Attached spheres were then incubated in neurosphere medium for 5–7 days (or as stated in the text), and 30% of the incubation medium was replaced every 3 days.

Primary ventral mesencephalic cultures

The rostral half of the mesencephalic flexure was dissected from 12 to 13 embryos harvested at E14.5, and a single-cell