Establishing Conditions for the Enrichment of Oligodendrocytes from Cultures of Neurospheres Derived from Embryonic Rat and Mouse Brains

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Abstract

Mammalian embryonic neural stem cells (NSC) form a unique population of cells that exhibit stem cell functions including self- renewal and multi- lineage differentiation. When cultured in a defined serum-free medium containing epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF), NSC proliferate forming free floating clonally derived clusters of cells referred to as neurospheres. When exposed to serum, the stem cell progeny differentiate into the three primary cell types (neurons, astrocytes and oligodendrocytes) found in the CNS. While large numbers of neurons and astrocytes can be generated, the relative numbers of oligodendrocytes generated is low. The aim of this study was to develop culture conditions to increase the numbers of oligodendrocytes in cultures of embryonic mouse and rat neurospheres. Early passage embryonic mouse and rat neuroshpheres were differentiated as whole neurospheres or single cells in several media formulations with or without the inclusion of serum. Oligodendrocytes were identified by immunostaining with anti-O4 and MBP antibodies. The addition of various cytokines affected the frequency and morphology of oligodendrocytes and this effect was modulated by the absence or presence of serum. In differentiated cultures of mouse neurospheres, the frequency of oligodendrocytes was consistently higher in serum alone compared to serum plus Sonic Hedgehog (SHH) or serum plus PDGF-AA. Similarly, the frequency of oligodendrocytes detected in cultures of rat neurospheres exposed to serum was higher compared to serum plus EGF or FGF only. Since serum was efficient at inducing oligodendrocyte differentiation, we screened sera from different animal species derived from fetal or adult sources. In mouse cultures we found that adult sera from different animal species induced a 2-fold increase in the frequency of oligodendrocytes compared to fetal bovine sera. This result however, was not observed in cultures of rat neurospheres. The results suggest there may be an inducing factor in adult animal sera that is effective at promoting oligodendrocyte differentiation in embryonic mouse derived neurospheres. In conclusion, the addition of adult sera was more effective in increasing the relative number of oligodendrocytes in differentiated embryonic mouse neurosphere cultures. Methods for enriching particular phenotypes derived from NSC may be useful for *in vitro* studies of cellular function and tissue transplantation.

Introduction

Growth factor responsive cells from the embryonic and adult mammalian CNS that exhibit stem cell features have been isolated *in vitro*. These neural stem cells (NSC), which grow as clusters of cells called neurospheres, are capable of self-renewal and can be expanded *ex vivo* in a defined serum-free medium containing NSC mitogens such as epidermal growth factor (EGF) and fibroblast growth factor (FGF). When exposed to serum, the NSC progeny differentiate into the three primary cell types found in the CNS, neurons, astrocytes and oligodendrocytes. Under these conditions, large numbers of neurons and astrocytes can be generated, however numbers of oligodendrocytes generated is typically low.

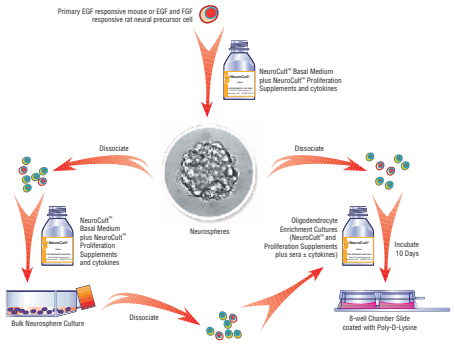
This study aimed to establish culture conditions using cytokines to increase the numbers of oligodendrocytes generated from neurospheres cultures of embryonic rat and mouse CNS tissue.

Methods

Primary Cell Isolation. Striata, Cortex and Ventral Mesencephalon (VM) were dissected from Embryonic Day 14 CD1 albino mouse embryos (Charles River) using standard microdissection techniques. All tissues were collected in PBS containing 2% glucose. Dissected cortical region from E18 Sprague/Dawley rat were obtained from BrainBits Inc (Illinois, USA).

Neurospheres Cultures. Primary rat and mouse CNS tissue was mechanically dissociated with a fire-polished Pasteur pipette in the appropriate growth culture medium (NeuroCult™-Mouse Basal Medium; StemCell Technologies Inc, Catalog #05700 or NeuroCult™-Rat Basal Medium; StemCell Technologies Inc, Catalog #05770 supplemented with NeuroCult™ Proliferation Supplements; StemCell Technologies Inc, Catalog #05701 and 20 ng/mL human EGF for mouse or 20 ng/mL human EGF, 10 ng/ml basic FGF and 20 ng/mL heparin for rat cells respectively). Mouse cells were cultured for seven days, while rat cells were cultured for four days. At the end of the culture period, rat and mouse neurospheres were harvested, dissociated into a single cell suspension and total viable cells were counted based on Trypan blue exclusion. Cells were passaged into secondary cultures and also used in oligodendrocyte enrichment cultures (Figure 1).

Figure 1. Culture and differentiation of mouse and rat neurospheres



Oligodendrocyte Enrichment Cultures. Passage 1 or 2 neurospheres from rat and mouse cell cultures were harvested, centrifuged at 400 rpm and dissociated into single cells in growth factor-free medium. Cells were counted based on Trypan blue exclusion and 10^5 cells were plated per well in 8-well cultureware chamber slides coated with Poly-D-Lysine (BioCoat BD #35-4688) in the various oligodendrocyte enrichment media (Table 1). In all cases, the base media consisted of the NeuroCult™-Mouse Basal Medium or NeuroCult™-Rat Basal Medium for mouse and rat cells respectively, supplemented with NeuroCult™ Proliferation Supplements. The various sera and cytokines listed in Table 1 were added separately to the base media.

Table 1. Sera and cytokines tested in the oligodendrocyte enrichment cultures

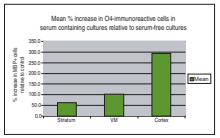
Rat	Mouse
Serum and Cytokines	
1% FBS A	1% FBS A
1% FBS A + EGF	1% FBS A + Sonic Hedgehog (SHH)
1% FBS A + FGF	1% FBS A + PDGF*
1% FBS A + EGF + FGF	
Sera Screening	
1% FBS B	1% FBS B
1% FBS C	1% FBS C
1% Human Serum	1% Human Serum
1% Horse Serum	1% Horse Serum
§Control - No Serum or Cytokines	§Control - No Serum or Cytokines

*PDGF = platelet-derived growth factor AA
§Controls also included media containing growth factors alone without serum (data not shown)

Results

We first determined the numbers of oligodendrocytes produced from three regions of the embryonic mouse neuroaxis (striata, cortex and ventral mesencephalon (VM)). Passaged 1 or 2 cells (10^5) generated from striata, cortex and VM were differentiated in base medium (NeuroCult™-Mouse Basal Medium supplemented with NeuroCult™ Proliferation Supplements) alone or with the addition of 1% FBS A.

Figure 2. Comparison of the % increase in the number of O4-immunoreactive cells in serum or serum-free, cultures (controls), of differentiated neurospheres derived from three mouse brain regions



Values are mean (n=3)

Summary. The percentage increase in O4-positive cells in serum containing cultures compared to serum-free controls was highest in the cortex relative to the striatum and VM. Based on this result we used cortical tissue in the remaining experiments as this region had the largest increase in oligodendrocytes relative to control conditions (serum-free).

In vivo, the initial induction of NSC to differentiated into oligodendrocyte precursor cells (OPC) in the ventricular ventral zone requires sonic hedgehog (SHH). Once OPC are committed to the oligodendrocyte lineage, PDGF-AA and FGF-b play a role in the outward migration of OPC from ventral ventricular zone. We measured the enrichment of oligodendrocytes in cultures containing SHH and PDGF in the presence or absence of serum relative to serum alone.

Figure 3. The mean % of MBP-immunoreactive cells relative to total cells generated in mouse oligodendrocyte enrichment cultures containing serum ± cytokines

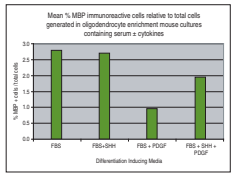
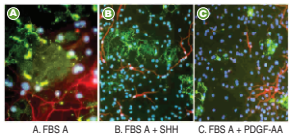


Figure 4: Immunostaining of MBP+ cells from mouse oligodendrocyte enrichment cultures containing serum and cytokine combinations



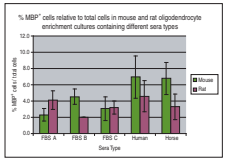
A. FBS A B. FBS A + SHH C. FBS A + PDGF-AA

Staining of oligodendrocytes with anti-MBP is shown in green while neurons stained with B-Tubulin antibody are shown in red and DAP1 counterstain in blue.

Summary. In mouse oligodendrocyte enrichment cultures, no significant difference was seen by the addition of SHH, PDGF, or a combination of both cytokines.

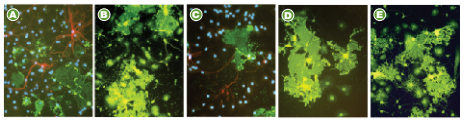
Our data suggest that PDGF and SHH, in combination with 1% serum, do not increase the number of oligodendrocytes in oligodendrocyte enrichment cultures derived from mouse cortical neurospheres. Since serum is an efficient inducing factor for generating oligodendrocytes in this culture system we screened a number of sera obtained from various animal species either from fetal or adult animals.

Figure 5: The mean % of MBP-reactive oligodendrocytes relative to total cells (DAP1+) generated from mouse (n=4) and rat (n=2) cortical neurospheres in oligodendrocyte enrichment cultures containing different sera



Values are mean ± SE (n=4 mouse; n=2 rat)

Figure 6: Immunostaining of oligodendrocytes with anti-MBP from mouse oligodendrocyte enrichment cultures containing various sera types



A. FBS A B. FBS B C. FBS C D. Human Serum E. Horse Serum

Staining of oligodendrocytes with anti-MBP is shown in green while neurons stained with B-Tubulin antibody are shown in red and DAP1 counterstain in blue.

Summary. The mean % oligodendrocytes (determined by MBP positive staining), was approximately 2-fold higher in mouse cultures containing adult human or horse sera, relative to mouse cultures containing the three tested fetal sera. There was no difference in the mean % oligodendrocytes between cultures containing the sera obtained from the different adult animal species. An increase in the % of oligodendrocytes was not observed in oligodendrocyte enrichment cultures of embryonic rat neurospheres supplemented with the adult or fetal sera. While these results indicate that adult sera is better than fetal sera at increasing the number of oligodendrocytes, this effect may be species specific.

Conclusion

Our results suggest the presence of an oligodendrocyte proliferation/differentiation/survival factor in adult sera. This is consistent with the possible presence of an inducing agent during the post-natal period that promotes glial cell development.

Future Studies

- Determine whether the enriching sera is increasing oligodendrocyte number by proliferation, differentiation or enhanced survival.
- Screen a wider variety of sera.

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