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Research Report

Diffusible, membrane-bound, and extracellular matrix factors from olfactory ensheathing cells have different effects on the self-renewing and differentiating properties of neural stem cells

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ABSTRACT

Transplantation of olfactory ensheathing cells (OECs) has been a promising strategy in enhancing central nervous system (CNS) regeneration. However, little is known about the effects of transplanted OECs on the self-renewal, neurogenesis, and oligodendrogenesis of neural stem cells (NSCs), which are known to play a very important role in the repair of damaged CNS tissue. In this study, we investigated the influence of diffusible, membrane-bound, and extracellular matrix factors from OECs on the self-renewal and differentiation properties of NSCs. We found that diffusible factors from cultured OECs promoted self-renewal, whereas the extracellular matrix molecules from OECs increased neurogenesis and oligodendrogenesis of NSCs. Furthermore, we demonstrated that directly coculturing OECs and NSCs inhibited not only self-renewal but also neurogenesis and oligodendrogenesis of NSCs. We propose three models for the interaction between transplanted OECs and endogenous NSCs. Our findings provide new insight into the ability of OECs to promote CNS repair and also indicate potential targets for manipulation of these cells to enhance their restorative ability.

1. Introduction

Neural stem cells (NSCs) are defined by their ability to self-renew over many generations while retaining the potential to generate both neurons and glia (Gage, 2000). NSCs were believed to have an extraordinary capacity to repair the damaged nervous system. In the last decade, NSCs were extensively studied with the aim of using endogenous and/or donor NSCs to replace neurons and restore circuitry in a neurodegenerative microenvironment (Gage et al., 1995; Nunes et al., 2003; Cummings et al., 2006; Ogawa et al., 2002). However, most of this work has only demonstrated the limited contribution of NSCs to brain repair after injury (Magavi et al., 2000; Arvidsson et al., 2002; Nakatomi et al., 2002). One of the

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Abbreviations: OEC, olfactory ensheathing cell; NSC, neural stem cell; CNS, central nervous system
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most challenging aspects of NSCs is that their differentiation potential in vivo tends to be biased toward glial lineages, particularly during degeneration or injury (Cao et al., 2001, 2002; Vroemen et al., 2003). In addition to their limited differentiation potential, adult NSCs renew far less frequently than their embryonic counterparts and therefore may be more difficult to expand into the large cultures required for clinical applications (Doetsch et al., 1999; Morshead et al., 1998).

Many researchers have focused on promoting CNS repair using NSCs by searching for conditions that will overcome these limitations. The strategies developed include transplantation of NSCs with direct administration of growth factors (Ohori et al., 2006), genetic engineering of NSCs by expressing bioactive molecules (Setoguchi et al., 2004), or combined transplantation of NSCs with other cell types, such as Schwann cells and olfactory ensheathing cells (OECs) (Zhang et al., 2007; Srivastava et al., 2009). Some of these efforts, such as cotransplantation of OECs and NSCs, were able to partially overcome these restrictions and enhance the restorative ability of NSCs (Srivastava et al., 2009).

The OECs are unique glial cells with axonal growth-promoting properties. During development, OECs are thought to play a critical role in promoting axonal growth as they accompany the olfactory axons from their initial peripheral site in the olfactory plate to the CNS (Li et al., 1997; Ramón-Cueto and Avila, 1998). Currently, OEC transplantation has emerged as a very promising experimental therapy to treat CNS injuries and demyelinating diseases (Raisman, 2001). Many transplantation studies showed that OECs could promote axonal regeneration and functional recovery (Li et al., 1997; Ramón-Cueto et al., 1998; Imaizumi et al., 2000; Nash et al., 2002; Cao et al., 2004; Shyu et al., 2008). The mechanism of this reparative effect may involve many factors, such as overcoming local inhibitory influences (Filbin n 2003), reinstating intrinsic growth programs in the neurons (Goméz et al., 2003), and cooperating with the host astrocytes to make the interface penetrable by axons (Li et al., 2004). Self-renewal, neurogenesis, and oligodendrogenesis of endogenous NSCs are considered to play important roles in the repair of CNS tissue after injury. However, the effects of transplanted OECs on these functions are not well understood.

In the present study, we systematically explored the effects of diffusible, membrane-bound, and extracellular matrix factors from OECs on the self-renewal, neurogenesis, and oligodendrogenesis of NSCs using various coculture experiments to determine how OECs affect these functions.

2. Results

2.1. *OECs stimulate the proliferation of NSCs*

We first used nestin immunostaining to determine the purity of NSCs. We dissociated the primary and tertiary neurospheres respectively, plated them on laminin-coated coverslips and stained the cells with nestin. Nearly all the cells expressed the NSC-marker nestin. Furthermore, when the dissociated tertiary spheres were plated in serum-free defined medium (DM) for 5 days, they could differentiate into neuronal progenitors (DCX^+), mature neurons ($MAP2^+$), astrocytes ($GFAP^+$), and oligodendrocytes ($O4^+$; Supplementary Fig. 1). These data indicated that the tertiary neurospheres primarily contained NSCs, and these cells still maintained their pluripotency.

To measure the effect of OECs on the proliferation of NSCs, GFP^+ neurospheres were mechanically dissociated and planted directly on the OEC monolayer. Three days later, BrdU was introduced into the medium. As shown in Figs. 1A and B, BrdU incorporation by NSCs cultured directly on OEC monolayer (OEC-co) increased significantly compared with the control NSCs cultured on PLL in DM ($P<0.01$). These results revealed that direct contact with OECs significantly promoted the proliferation of NSCs.

The ability of the OEC monolayer to promote NSC proliferation could be due to secreted molecules, factors associated with the extracellular matrix, or cell membrane-associated molecules. To study the potential effect of OEC extracellular matrix molecules on NSC proliferation, we plated NSCs onto water-lysed OECs (OECs-lys) and compared their proliferation to negative-control NSCs cultured in DM only and positive-control NSCs cultured on a whole OEC monolayer. Our previous study revealed that lysed OECs leave behind a matrix footprint, and this remaining extracellular matrix contained considerable amounts of laminin (Cao et al., 2007). In this experiment, we found that NSCs proliferated more intensely on OECs-lys than in control DM ($P<0.01$, Figs. 1A and B).

We then examined whether the effects of OECs on NSC proliferation depended on membrane-bound factors. We plated NSCs directly onto lightly fixed OECs (OECs-fix). BrdU incorporation by NSCs on OECs-fix increased significantly compared to NSCs in control DM ($P<0.01$; Figs. 1A and B). Finally, we explored the effect of OEC diffusible factors by culturing NSCs in conditioned medium from OECs (OECs-CM). A significant difference was found between NSCs cultured in OECs-CM and that in control DM ($P<0.01$; Figs. 1A and B). These results indicate that the matrix molecules, membrane-bound factors, and diffusible factors of OECs are all responsible for promoting NSC proliferation.

2.2. *Diffusible factors from OECs promote NSC self-renewal*

We tested NSCs cultured in different conditions for the expression of nestin protein. As shown in Figs. 1A and C, NSCs cultured in OECs-CM, but not with OECs-lys or OECs-fix, showed significantly increased nestin-positive cell proportions. Since nestin is an immature cell marker, and self-renewal involves both proliferation and the maintenance of an undifferentiated state, our observation that OECs-CM promoted NSC proliferation and generated more $nestin^+$ progeny suggests that diffusible factors from OECs may promote NSC self-renewal.

To further explore this possibility, we established a secondary neurosphere-forming assay to examine whether diffusible factors from OECs could promote the development of secondary neurospheres (Fig. 2A). Increased secondary neurosphere formation reflects a greater probability of self-renewing symmetric divisions (Ramírez-Castillejo et al., 2006). As shown in Figs. 2B and C, significantly higher