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Olfactory ensheathing cells promote neurite outgrowth from co-cultured brain stem slice

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ABSTRACT

Cell therapy aiming at the replacement of degenerated neurons is a very attractive approach. By using an established *in vitro* organotypic brain stem (BS) slice culture we screen for candidate donor cells, some of them being further functionally assessed in *in vivo* models of sensorineural hearing loss. Both *in vitro* and *in vivo* systems show that implanted cells face challenges of survival, targeted migration, differentiation and functional integration with the host tissue. Low success rates are possibly due to the lack of necessary neurotrophic factors, adhesion molecules and guiding cues. Olfactory ensheathing cells (OECs) have been shown to express a number of neurotrophic factors and to promote axonal growth through cell to cell interactions. In the present study we co-cultured OECs with organotypic BS slice in order to see if OECs can serve as a facilitator when screening candidate donor cells in an organotypic culture setup. Here we show that OECs when co-cultured with the auditory BS slice not only promote neurite outgrowth from the cochlear nucleus (CN) region of the BS slice but also support cells by having BS slice axons growing along their processes. These findings further suggest that OECs may enhance survival and targeted migration of candidate donor cells suitable for cell therapy *in vitro* and *in vivo*. This article is part of a Special Issue entitled: Understanding olfactory ensheathing glia and their prospect for nervous system repair.

Introduction

Many hearing disorders involve irreversible damage to the inner ear hair cells and components of the hearing pathway such as primary afferent spiral ganglion neurons (SGN), the cochlear nerve and cochlear nucleus (CN) which results in permanent hearing impairment (Nadol et al., 1989; Raveh et al., 2007). Currently, the treatment options for hearing loss include the use of prosthetic devices such as hearing aids and in selected cases cochlear implants (CI) can be used (Khan et al., 2005). It should be noted, however, that the performance of a CI is dependent on the residual SGN including their function. Since regeneration of mature mammalian hair cells and SGN does not occur spontaneously (Roehm and Hansen, 2005) the cell replacement therapy using neural stem cells and progenitor cells presents an attractive treatment option. Different cell types including mouse embryonic stem cells (MESC) (Regala et al., 2005; Boer et al., 2009), embryonic dorsal root ganglion (DRG) neurons (Olivius et al., 2003; Olivius et al., 2004) and mesenchymal stem cells (MSCs) (Matsuoka et al., 2007) have been used for SGN and cochlear nerve replacement.

In vitro and *in vivo* models can be used to assess the feasibility of cell therapy. Candidate donor cells have to survive, migrate, differentiate and get integrated with the host hearing pathway to establish proper functional connections. Since the SGN fibers composing the cochlear nerve send the auditory signal from the hair cells in the inner ear to the CN in the brain stem, any transplanted potential donor cell has to get functionally connected to the CN. An established *in vitro* organotypic culture system (Thonabulsombat et al., 2007; Glavaski-Joksimovic et al., 2009) consisting of an auditory brain stem slice containing CN and a part of the cochlear nerve have been used in our laboratory for the screening of candidate donor cells. A number of cell candidates have already been screened using the co-culture system, including MESC (Glavaski-Joksimovic et al., 2009), embryonic DRG neurons (Thonabulsombat et al., 2007), neural crest cells and human neural progenitor cells (data in prep). The brain stem co-culture system with its controlled microenvironment has turned out to be very useful for evaluating manipulations of the cultured cells. Major issues arising in co-culture systems and when cells are transplanted *in vivo* belong to cell survival, differentiation and growth. These issues are also critical in the *in vivo* transplantation process, presenting technical challenges

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and reducing the positive outcome of the experiments (Olivius et al., 2003, 2004; Regala et al., 2005; Hu et al., 2009).

Biological mechanisms behind the cell survival are mostly undefined and have been shown to largely depend on interactions among cells and the extracellular matrix (Hynes, 2009; Imbeault et al., 2009). Survival-promoting compounds such as brain-derived neurotrophic factor (BDNF) (Lu et al., 2005), glial cell line-derived neurotrophic factor (GDNF) (Lee et al., 2005) and neurotrophins (NTs) (Lu et al., 2008) in the culture medium can significantly enhance stem cell and progenitor cell survival and differentiation. The compound source basically rests on addition of necessary factors to the culture medium or on co-culturing with cells which have been known for producing survival enhancement factors. These types of cells include mesenchymal cells (Crigler et al., 2006; Potapova et al., 2007; Wilkins et al., 2009) and OECs (Boruch et al., 2001; Lipson et al., 2003). In addition to an endogenous production of factors these cells can also serve as guidance cues since they readily respond to the environment (Crigler et al., 2006; Ao et al., 2007).

Cells to be transplanted *in vivo* with the attempt to restore damaged nerves are facing rather more aggressive environment as compared to an *in vitro* setup, including the peripheral nervous system (PNS)/central nervous system (CNS) barrier transitional zone. Transitional zone inhibitory signals make its environment non-permissive for axonal regrowth of damaged neurons as well as for advancement of transplanted cells (Fraher, 2000).

The olfactory nerve is the only cranial nerve in which regeneration can be maintained even following trauma to the olfactory neurons (Zippel, 1993). The latter has the ability of renewal and re-growth across the PNS/CNS barrier transitional zone (Doucette, 1991) throughout the whole mammalian lifetime. It is believed that its regenerative capacity is facilitated by the presence of the OECs (Ramon-Cueto and Valverde, 1995; Raisman and Li, 2007). Due to their unique abilities the OECs have been used as cell candidate for successful axonal re-growth in spinal cord injury models (Ramon-Cueto et al., 2000; Stamegna et al., 2011; Takeoka et al., 2010) as well as in optical nerve injury models (Li et al., 2003).

So far the mechanism by which OECs support axonal re-growth is not fully understood. OECs have been shown to express neurotrophic factors: nerve growth factor (NGF) (Boruch et al., 2001; Lipson et al., 2003), BDNF (Boruch et al., 2001; Lipson et al., 2003) and GDNF (Woodhall et al., 2001), which have neuronal protection probabilities and may promote axon growth.

Grafted OECs may also enhance migration of host Schwann cells into the CNS and stimulate angiogenesis to form a biological environment, which enhances the repair of a spinal cord injury in an *in vivo* model (Ramer et al., 2004). As illustrated *in vitro* the co-cultured CNS neurons send axons directly along the axes of OECs (van den Pol and Santarelli, 2003), which indicates that the interaction between OECs and CNS neurons may be an important factor for the regeneration of nerve fibers. An *in vivo* study also showed that the interaction between OECs and CNS neurons may be an important factor for the reconstruction of the central neural pathway (Vukovic et al., 2007). Some studies suggest that cell to cell interactions may be important for a controlled and induced release of neutrophins from OECs (Lipson et al., 2003).

We hypothesize that the presence of OECs creates a permissive environment for the survival of transplanted cells, their neuronal differentiation and axonal sprouting leading to the establishment of functional connections with neurons originating from the auditory brain stem slice in our *in vitro* model. Before potentially advancing to cultures using candidate donor cells, we presently tested on how OECs derived from adult rat olfactory bulb interact with neurons originating from the cochlear nucleus region of the slice. Our results show that the presence of OECs in the culture significantly promotes axonal growth from the CN region, illustrating that OECs can be used as modulators of axonal growth and guidance in our co-culture system. In future studies the significance of these *in vitro* observations will be further accessed with co-cultured donor cells.

Materials and methods

Animals

Adult Sprague–Dawley rats (SD, 5 weeks old, 150 g, $n=8$) used for primary and purified OEC culture and postnatal pups (P12-P14, $n=10$) used for organotypic BS slice culture were obtained from Harlan (Netherlands). The animals were maintained under standard conditions with food and water available *ad libitum* and sacrificed by decapitation. All animal procedures were conducted in accordance with local ethical guidelines and approved animal care protocol (approval N 296/03 and 464/03).

OECs primary cultures and purification

Adult female SD rat olfactory nerve and glomerular layers of the olfactory bulb were used to obtain an OECs primary culture as previously described (Nash et al., 2001; Johansson et al., 2005). Briefly, the olfactory bulb was carefully dissected out and transferred into Hank's balanced salt solution (HBSS, Gibco) with added antibiotics (Penstrep, 500 U/ml). The olfactory bulb glomerular and nerve layers were collected, minced and trypsinized (0.25% trypsin) for 10 min at 37 °C. After trypsinization the cell suspension was spinned at 450*g* for 3 min and resuspended in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM/Ham's/F-12, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), 1% L-glutamine and penstrep (500 U/ml, Gibco). We adopted the OEC purification method by Nash et al. (2001) to be carried out in three steps. The method employs different sedimentation and attachment abilities of different cell types as a function of time (Polinger, 1970). Initially cells were plated on uncoated culture flask and incubated for 18 h in 10 ml of culture medium (37 °C, 5% CO_2). In the second step this medium with unattached cells suspension was transferred to a new uncoated culture flask and incubated for another 48 h (37 °C, 5% CO_2). In the final step the medium with the remaining cell suspension from the second step was transferred to a poly-D-lysine (PDL) coated 25 cm^2 culture flask to allow attachment of the OECs. The cells were incubated (37 °C, 5% CO_2) in culture medium changed every other day. When confluent the culture was harvested for co-culture with the BS slice. The same procedure was performed using cell culture chamber slides (PDL coated and uncoated; BD, Fisher) to allow OECs phenotypic characterization.

Organotypic brain stem slice cultures

The organotypic BS slice culture protocol has been used previously (Glavaski-Joksimovic et al., 2009). Briefly, SD rat postnatal pups (P12–P14) were sacrificed by decapitation. After decapitation the skulls were opened longitudinally along the midline and excised brains placed in the ice cold dissecting medium (HBSS supplemented with 20% glucose and 1% antibiotic/antimycotic). 300 μm thick transverse sections of the brain encompassing the proximal part of the cochlear nerve and the CN according to local anatomical landmark were obtained using a tissue-chopping device (McIlwain). The BS slices were transferred to sterile Petri dishes containing cold HBSS (pH 7.2) for further separation of the individual slices with a sterile spatula. Slices were propagated as interface cultures on sterile polyester membranes with 0.4 μm pore size and 24 mm in diameter (Corning Inc). The membrane inserts were coated with sterile PDL (5 μg/ml; Sigma) and laminin (10 μg/ml: Gibco). BS slices with cochlear nerve and CN were transferred to coated membrane inserts and placed in a 6-well plate with 1 ml culture medium per well and placed in the incubator (37 °C; 5% CO_2). BS slice culture medium consisted of DMEM supplemented with 30% HBSS, 10% FBS, glucose