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A comparison of epithelial and neural properties in progenitor cells derived from the adult human ciliary body and brain☆

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ABSTRACT

Cells isolated from the ciliary body (CB) of the adult human eye possess properties of retinal stem/progenitor cells and can be propagated as spheres in culture. As these cells are isolated from a non-neural epithelium which has neuroepithelial origin, they may have both epithelial and neural lineages. Since it is the properties of neural progenitor cells that are sought after in a future scenario of auto-transplantation, we wanted to directly compare human CB spheres with neurospheres derived from the human subventricular zone (SVZ), which is the best characterized neural stem cell niche in the CNS of adults. The CB epithelium was dissected from donor eyes ($n = 8$). Biopsies from the ventricular wall were harvested during neurosurgery due to epilepsy ($n = 7$). CB and SVZ tissue were also isolated from Brown Norwegian rats. Dissociated single cells were cultivated in a sphere-promoting medium and passaged every 10–30 days. Fixed spheres were studied by immunohistochemistry, quantitative RT-PCR and scanning/transmission electron microscopy. We found that both CB and SVZ spheres contained a mixed population of cells embedded in extracellular matrix. CB spheres, in contrast to SVZ neurospheres, contained pigmented cells with epithelial morphology that stained for cytokeratins (3/12 + 19), were connected through desmosomes and tight-junctions and produced PEDF. Markers of neural progenitors (nestin, Sox-2, GFAP) were significantly lower expressed in human CB compared to SVZ spheres, and nestin positive cells in the CB spheres also contained pigment. There was higher expression of EGF and TGF-β receptors in human CB spheres, and a comparative greater activation of the canonical Wnt pathway. These results indicate that adult human CB spheres contain progenitor cells with epithelial properties and limited expression of neural progenitor markers compared to CNS neurospheres. Further studies mapping the regulation between epithelial and neural properties in the adult human CB spheres are vital to fully utilize them as a clinical source of retinal progenitor cells in the future.

1. Introduction

During embryogenesis, the multipotent neuroectoderm of the optic cup develops into the central retina and a peripheral region called the ciliary margin (CM) (Klassen et al., 2004a). While the central retina becomes an integrated part of the central nervous system (CNS), the CM gives rise to two non-neural structures: the double-layered epithelium of the ciliary body (CB) and the distal iris (Perron and Harris, 2000; Perron et al., 1998). However, the CM contains multipotential retinal progenitor cells even at a late stage of development, and in certain lower vertebrates it is a source of progenitor cells for retinal regeneration throughout life (Wetts et al., 1989).

In adult humans, the retina is considered to have limited regenerative potential, and severe injuries will lead to permanent damage. Interestingly, current studies have revealed that the CB (Coles et al., 2004; Tropepe et al., 2000; Xu et al., 2007), in addition to the retina itself (Carter et al., 2007; Klassen et al., 2004b; Lawrence et al., 2007; Mayer et al., 2005), even in adulthood harbors cells with the characteristics of neural stem cells (NSCs); (1) they are undifferentiated and have the capability of cell division and self-renewal (2) they are multipotent, i.e. they have the ability to differentiate into both neurons and glia found in the retina (Boulton and Albon, 2004; Gage, 2000).

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In vitro, NSCs grow in suspension as characteristic spherical aggregates (neurospheres) containing a mixture of multipotent neural stem cells normally present in very small numbers as well as progenitor cells that are more restricted in their proliferative and phenotypic potential (Arsenijevic et al., 2001; Kukekov et al., 1999). Previous studies of CB spheres from the adult eye have mainly focused on the differentiation of cells along various retinal phenotypic pathways after attached colony formation or after grating *in vivo* (Coles et al., 2004; Tropepe et al., 2000). Only recently, studies have focused on the characterization of the sphere composition during *in vitro* cultivation (Kohno et al., 2006; Xu et al., 2007). As CB spheres are isolated from a non-neural epithelium of neuroepithelial origin, it is vital to map their regulation of epithelial and neural properties to fully utilize them as a clinical source of retinal progenitor cells in the future.

In the present paper, we have for the first time combined immunohistochemistry, molecular biology and electron microscopy to compare CB spheres isolated from the adult human eye with neurospheres derived from the adult human subventricular zone (SVZ), which is considered the largest and best characterized stem cell niche in the CNS (Gage, 2002; Liu et al., 2003). We have also compared the two sphere-forming cell populations from adult rats. Our data indicate that adult human ciliary body spheres contain epithelial-like cells with decreased expression of neural stem cells' markers compared to CNS neurospheres.

2. Material and methods

The research was conducted in accordance with the Declaration of Helsinki and all tissue harvesting was approved by the Norwegian National Committee for Medical Research Ethics. All animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were enucleated from cadavers ($n = 8$) as previously described (Slettedal et al., 2007). The CB epithelium was carefully dissected under an operating microscope and placed in Leibowitz-15 medium (L15) (Invitrogen, Carlsbad, CA). Only donors with no history of known eye diseases were chosen. The mean donor age was 52 ± 22 years (mean ± SD, $n = 8$) and the mean time from death to preservation was 16.3 ± 7.0 h. Biopsies from the ventricular wall ($n = 7$) were harvested from temporal lobe specimens obtained during neurosurgery due to medical intractable epilepsy (Westerlund et al., 2003). The mean patient age was 27 ± 19 years. The CB epithelium and ventricular wall tissue were also isolated from 3 to 4 week old female Brown Norwegian rats after decapitation ($n = 8$).

2.1. Cell culture

The CB and SVZ tissue were mechanically separated and placed in a medium containing Dispase (1.2 U/ml, Roche Diagnostics, Basel, Switzerland) for 10 min followed by extensive pipette trituration. The dissociated suspension was passed through a 70 μm strainer (BD Biosciences, San Jose, CA), and re-suspended as single cells at a final density of 150 000 cells/ml. Cells were cultured in a DMEM/F12 (Invitrogen) based sphere-promoting optimized for propagation of adult human NSCs (Moe et al., 2005) containing Hepes buffer (1 M, 0.8%, Invitrogen), B27 supplement (2%, Gibco Life Tech), EGF (20 ng/ml, R&D Systems, Minneapolis, MN), bFGF (10 ng/ml, R&D Systems), Heparin (2.5 μg/ml, LEO Pharma, Denmark) and Penicillin/Streptomycin (100 U/ml, Sigma, St.Louis, MO). To optimize propagation of adult CB progenitors, Sonic hedgehog (Shh) (Moshiri et al., 2005) (50 ng/ml, human specific, R&D Systems) and fetal calf serum (FCS, 1%, PAA Laboratories, Pasching, Austria) were added. In experiments to directly compare sphere-size of CB and SVZ spheres, Shh and FCS were also added to SVZ spheres. Cells were cultured in 6 well plates (Ultralow cluster plate, Corning Life Sciences, Mass) at +37 °C in 6% CO_2 and 20% O_2. The cultures were supplemented with bFGF and EGF twice a week. The spheres were passaged every 10–30 days before the spheres reached 100 μm in diameter and their center became necrotic. To facilitate visual control during fixation for electron microscopic studies some spheres were grown for longer period of times. The spheres were studied just before passage 2–3 (P1 + P2), which we consider to be the earliest time-point for future clinical transplantation.

2.2. Light microscopy and immunostaining

Spheres were fixed in neutral buffered 4% paraformaldehyde, embedded in OCT (Tissue-TEK, Sakura Finetek, CA), cut into 10 μm sections on a freezing microtome, thawed onto Super Frost/Plus object glasses (Menzel-Gläser, Braunschweig, Germany) and stored at −20 °C. Immunostaining was performed as previously described (Olstorn et al., 2007). The following primary antibodies and dilutions were used (rb: rabbit, ms: mouse, gp: guinea pig, gt: goat): glial fibrillary acidic protein (GFAP, rb, 1:1000; Dako, Carpinteria, CA), β-III-tubulin (ms, 1:1000; Sigma), HuN (ms, 1:200; Chemicon, Temecula, CA) Ki-67 (rb, 1:100; Dako), human specific nestin (HuNest, ms, 1:1000; R&D Systems), laminin (rb, 1:200; Sigma), Sox-2 (rb, 1:500; Chemicon), pigment epithelium-derived factor (PEDF, ms, 1:200; Chemicon), Cytokeratin 19 (ms, 1:100; DAKO), Cytokeratin 3/12 (ms, 1:500; Acris-ab), Connexin-43 (ms, 1.50; Sigma), E-cadherin (ms, 1:50; Dako), N-cadherin (ms, 1:50; Dako), Vimentin (rb, 1:200; NeoMarkers), Oct $^3/_4$ (gt, 1:1000; R&D Systems), olig-2 (gt, 10 μg/ml; R&D Systems), Tenascin C (gt, 1:20; R&D Systems). Hoechst (1:500; Invitrogen) was used for nuclear staining. As secondary antibodies the fluorescent markers Cy3 (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488 (1:500; Molecular Probes, Eugene, OR) were used.

For peroxidase staining, we used a standard peroxidase technique (DAB detection kit) with an automated immunostaining system (Autostainer 360, Lab Vision, Fremont, CA). Sections were analyzed using an Olympus IX 81/MT20 microscope equipped with Cell R software or an Olympus BV 61 FluoView confocal microscope (Olympus, Hamburg, Germany). The expression pattern was evaluated by two independent investigators and semiquantified as previously described (Raeder et al., 2007).

2.3. Transmission electron microscopy

The spheres were fixed for 30–60 min at room temperature by immersion in freshly prepared mixed aldehyde-fixation containing 0.1 M sodium cacodylate buffer, 2% glutaraldehyde, 2% paraformaldehyde and 0.025% $CaCl_2$, pH 7.4 (Nour et al., 2004). Fixation was continued overnight at 4 °C, postfixed in 1% osmium tetroxide, and dehydrated through a graded series of ethanol up to 100%. The spheres were then immersed in propylene oxide for 20 min and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA). Ultra-thin sections (60–70 nm thick) were cut on a Leica Ultracut Ultramicrotome UCT (Leica, Wetzlar, Germany) and examined using a CM120 transmission electron microscope (Phillips, Amsterdam, The Netherlands).

2.4. Scanning electron microscopy

Mixed aldehyde-fixated spheres were dehydrated in increasing ethanol concentrations, packed in filter paper and dried according to the critical point method (Polaron E3100 Critical Point Dryer, Polaron Ecq. Ltd., Watford, UK) using CO_2 as the transitional fluid. The spheres were gently transferred to carbon stubs and coated with a 30 nm thick layer of platinum in a Polaron E5100 sputter coater (Polaron) before being examined and photographed with an