Experimental Eye Research 98 (2012) 28–36

Contents lists available at SciVerse ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Activation of neural progenitor cells in human eyes with proliferative vitreoretinopathy☆

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ARTICLE INFO

Article history:
Received 15 December 2011
Accepted in revised form 14 March 2012
Available online 21 March 2012

Keywords:
neural
stem cell
progenitor cell
retina
ciliary body epithelium
Müller glia
iris pigmented epithelium
proliferative vitreoretinopathy

ABSTRACT

In addition to the ability for self-renewal and functional differentiation, neural stem/progenitor cells (NSCs) can respond to CNS injuries by targeted migration. In lower vertebrates, retinal injury is known to activate NSCs in the ciliary marginal zone (CMZ). Cells expressing markers of NSCs are also present in the ciliary body epithelium (CE) and in Müller glia in the peripheral retina (PR) of the adult human eye. However, these cells seem to be quiescent in the adult human eye and recent reports have shown that CE cells have limited properties of NSCs. In order to further clarify whether NSCs exist in the adult human eye, we tested whether NSC-like cells could be activated in eyes with proliferative vitreoretinopathy (PVR). The PR and CE were studied for NSC-associated markers in human enucleated control eyes and eyes with confirmed PVR, as well as in a mouse model of PVR. Furthermore, cells isolated from vitreous samples obtained during vitrectomies for retinal detachment were directly fixed or cultured in a stem cell-promoting medium and compared to cells cultured from the post-mortem retina and CE. *In situ* characterization of the normal eyes revealed robust expression of markers present in NSCs (Nestin, Sox2, Pax6) only around peripheral cysts of the proximal pars plana region and the PR, the latter population also staining for the glial marker GFAP. Although there were higher numbers of dividing cells in the CE of PVR eyes than in controls, we did not detect NSC-associated markers in the CE except around the proximal pars plana cysts. In the mice PVR eyes, Nestin activation was also found in the CE. In human PVR eyes, proliferation of both non-glial and glial cells co-staining NSC-associated markers was evident around the ora serrata region. Spheres formed in 7/10 vitreous samples from patients with PVR compared to 2/15 samples from patients with no known PVR, and expressed glial − and NSC-associated markers both after direct fixation and repetitive passages. In conclusion, the adult human eye may harbor two different populations of neuroepithelial stem/progenitor cells; a non-glial population located in the proximal pars plana around peripheral cysts in addition to a population with Müller glia characteristics. Yet, we only found that the glial population was able to respond to retinal injury by targeted migration into the vitreous.

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1. Introduction

During development of the human retina, neuroepithelial stem/progenitor cells (NSCs) from the inner layer of the optic cup give rise to both distinct cell types of the neuroretina that becomes an integrated part of the central nervous system (CNS), as well as two non-neural structures; the double-layered ciliary body epithelium (CE) and the iris pigmented epithelium (IPE) (Perron and Harris, 2000). In adults, the retina is considered to have limited regenerative potential, and severe injuries lead to permanent damage (Klassen et al., 2004). However, in cold-blooded vertebrates such as fish and amphibians NSCs located in a circumferential zone of cells known as the ciliary

☆ Grant information: Supported by the Research Council of Norway, Norwegian Center for Stem Cell Research, the Norwegian Association of the Blind and Partially Sighted, Blindemisionen IL, the Faculty of Medicine, University of Oslo and Oslo University Hospital. Furthermore, supported by grants the TÁMOP 4.2.1./B-09/1/KONV-2010-0007 project implemented through the New Hungary Development Plan, co-financed by the European Social Fund.

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doi:10.1016/j.exer.2012.03.008

marginal zone (CMZ), situated between the retina and the CE, can regenerate new retinal neurons throughout life (Perron and Harris, 2000). In addition, new retinal neurons are generated at the peripheral edge of the postnatal chick retina up to one month after hatching (Fischer and Reh, 2000). There is also evidence of a similar CMZ-like region in monkeys (Fischer et al., 2001) and humans (Martinez-Navarrete et al., 2008) (Bhatia et al., 2009), although these cells seems to be in a quiescent state (Bhatia et al., 2010).

Indications of an analogous stem cell-like population in the CE of the human retina (Coles et al., 2004; Tropepe et al., 2000; Xu et al., 2007) has prompted a number of investigations of their proliferative and differentiation potential *in vitro* and *in vivo* after transplantation to the mouse eye (Inoue et al., 2010). Since both the CE and IPE are derived from the neuroepithelium during embryonic development, but are much more surgically accessible than the neuroretina, huge expectations have emerged for these locations as candidate sources for stem cell therapies. However, recent investigations have provided evidence that the mammalian CE does not have the abilities of NSCs as previously thought. We have recently shown that sphere-forming cells isolated from the adult human CE and IPE have more epithelial properties and limited expression of NSC-associated markers compared to progenitor cells isolated from the human brain (Froen et al., 2011; Moe et al., 2009). Furthermore, other groups have shown that although cells isolated from the CE could be induced to express low levels of neuronal markers, they retained their epithelial morphology and failed to differentiate into retinal neurons (Bhatia et al., 2011; Cicero et al., 2009; Gualdoni et al., 2010). In addition, Bhatia et al. has also shown that the normal adult human CE lack crucial markers of NSCs such as Nestin *in situ* (Bhatia et al., 2009).

A somatic stem cell is commonly defined as a cell with the ability to self-renew and give rise to all the functional cell types of the organ from which they originate (Gage, 2000; Moe et al., 2005; Reh, 2002). In suspension culture, NSCs have the ability to form spheres with a uniform well-defined spherical contour that are mainly formed through cellular divisions (Gage, 2000; Reynolds and Weiss, 1992; Westerlund et al., 2003). Although the sphere-forming process is not specific for stem cells, their three dimensional structure is known to consist of a hierarchical organization with both undifferentiated cells and more differentiated progeny (Louis et al., 2008; Singec et al., 2006). Another key property of NSCs is detection and targeted migration into CNS lesions (Aboody et al., 2000; Imitola et al., 2004; Olstorn et al., 2007). One relatively common CNS lesion in ophthalmology is the development of proliferative vitreoretinopathy (PVR) after retinal detachment (RD) surgery. If NSCs are present in adult mammalian eyes, they might detect retinal injury and respond upon PVR formation by activation and targeted migration into the lesion area. In order to further clarify whether NSCs exist in the adult human eye, we carefully investigated the CE and PR for NSC-associated markers in human enucleated control eyes and eyes with confirmed PVR, as well as in a mice model of PVR. Finally, we looked for signs of targeted migration of NSC-like cells in the vitreous of patients operated with vitrectomy for RD and PVR formation.

2. Materials and methods

2.1. Dissection procedure

All experiments were conducted in accordance with the Declaration of Helsinki and all tissue harvesting was approved by the Local Committees for Medical Research Ethics.

2.1.1. In situ analysis of PR and CE

Control eyes (with no known PVR) were enucleated from human cadavers 24–48 h post-mortem as previously described (Slettedal et al., 2007). Samples were fixed in 4% fresh paraformaldehyde (PFA). The anterior segment was removed and axial sections were made from the iris to the mid-peripheral retina (Fig. 1A) and the specimens were then embedded in paraffin prior to sectioning. One enucleated cadaveric eye with known chronic RD and PVR formation (Fig. 2A), as well as two eyes enucleated due to extensive PVR and phthisis development from a collection of PFA- and paraffin-embedded ophthalmic pathology specimens were also included in the study.

2.1.2. Vitreous samples

After written informed consent, vitreous samples were obtained during vitrectomies for RD with or without confirmed PVR based on evaluation of wide angle images (Optomap P200Tx, Optos, Dunfermline, UK) (Fig. 4A–C). Cases where retinotomies, retinectomies or cutting of the retinal tear was performed got excluded from the study. The vitreous samples were centrifuged at 15 000 rpm for 5 min and the resulting pellets were either fixed in 4% PFA (direct fixation) or cultivated *in vitro*.

2.1.3. Retinal and ciliary epithelial tissue

Retinal tissue was carefully isolated from cadaveric eyes. The CE was isolated as previously described (Moe et al., 2009). No attempt was made to separate the pigmented from the non-pigmented CE in the present study.

2.2. In vitro cultures

The tissues were rinsed in Leibowitz-15 medium (L15, Invitrogen, Carlsbad, CA) and incubated with trypsin-EDTA (0.05%, Invitrogen) for 5 + 5 min followed by careful trituration. The cell suspension was passed through a 70 μm strainer (BD Biosciences, San Diego, CA). The cells were cultured in DMEM/F12 containing B27 supplement (2%, Invitrogen), EGF (20 ng/ml, R&D Systems), bFGF (10 ng/ml, R&D Systems, MN), 1% fetal calf serum (FCS, Sigma–Aldrich), Heparin (2.5 μg/ml, LEO Pharma, Denmark) and Penicillin/Streptomycin (100 U/ml, Sigma–Aldrich, St. Louis, MO) at 37 °C in 5% CO_2 and 20% O_2. Cultures were supplemented with bFGF and EGF twice a week and passaged every two to three weeks by incubation in trypsin-EDTA (0.05%, Invitrogen) for 2 × 4 min.

2.3. Mouse model of proliferative vitreoretinopathy induced by dispase

In order to reproduce the pathological environment of PVR formation in a controlled animal study, we utilized a mouse model of PVR induced by intravitreal injection of the proteolytic enzyme dispase. This model is known to induce glial activation as well as both epi – and subretinal membrane formation (Canto Soler et al., 2002; Frenzel et al., 1998). All animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Study protocols were approved by the Animal Care Committee of the University of Debrecen. Female 4–6 months old wildtype mice (C57/BL6, $n = 6$) were anesthetized with pentobarbital (90 mg kg^{-1}, i.p.), received one drop of 1% procaine hydrochloride (Novocaine) for local anesthesia and one drop of tropicamide (Mydrum) for iris dilatation. 4 μl of dispase (Sigma; 0.4 U μl^{-1}, dissolved in sterile physiological saline) was injected intravitreally in the right eyes under microscopically control using an automatic pipette fitted with 30G 1/6 needle, as previously described (Canto Soler et al., 2002). Control animals received 4 μl of sterile physiological saline solution. Stratus Optical Coherence Tomography images (OCT, Zarl Zeiss Meditec, DublinCA) were taken following injections to monitor disease progression (Fig. 3). Control and dispase treated mice were sacrificed between 7

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