Neuroscience 280 (2014) 142–155

HUMAN DENTAL PULP STEM CELLS RESPOND TO CUES FROM THE RAT RETINA AND DIFFERENTIATE TO EXPRESS THE RETINAL NEURONAL MARKER RHODOPSIN

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Abstract—Human adult dental pulp stem cells (DPSCs) are self-renewing stem cells that originate from the neural crest during development and remain within the dental pulp niche through adulthood. Due to their multi-lineage differentiation potential and their relative ease of access they represent an exciting alternative for autologous stem cell-based therapies in neurodegenerative diseases. In animal models, DPSCs transplanted into the brain differentiate into functional neurons or astrocytes in response to local environmental cues that appear to influence the fate of the surviving cells. Here we tested the hypothesis that DPSCs might be able to respond to factors present in the retina enabling the regenerative potential of these cells. We evaluated the response of DPSCs to conditioned media from organotypic explants from control and chemically damaged rat retinas. To evaluate cell differentiation, we analyzed the expression of glial fibrillary acidic protein (GFAP), early neuronal and retinal markers (polysialic acid-neural cell adhesion molecule (PSA-NCAM); Pax6; Ascl1; NeuroD1) and the late photoreceptor marker rhodopsin, by immunofluorescence and reverse transcription polymerase chain reaction (RT-PCR). Exposure of DPSC cultures to conditioned media from control retinas induced a 39% reduction on the number of DPSCs that expressed GFAP; the expression of Pax6, Ascl1, PSA-NCAM or NeuroD1 was undetectable or did not change significantly. Expression of rhodopsin was not detectable in control or after exposure of the cultures with retinal conditioned media. By contrast, 44% of DPSCs exposed to conditioned media from damaged retinas were immunopositive to this protein. This response could not be reproduced when conditioned media from Müller-enriched primary cultures was used. Finally, quantitative RT-PCR was performed to compare the relative expression of glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) in DPSC co-cultured with retinal organotypic explants, where BDNF mRNA expression was significantly upregulated in retinal-exposed cultures. Our data demonstrate that DPSC cultures respond to cues from the rat retina and differentiate to express retinal neuronal markers.

Key words: dental pulp stem cells, retina, Müller glia, neuronal differentiation.

INTRODUCTION

Human postnatal dental pulp stem cells (DPSCs) constitute a heterogeneous population of progenitor cells with self-renewal capacity, a large degree of variability in differentiation potential and distinct regenerative capabilities (Gronthos et al., 2000, 2002; Nosrat et al., 2004; d'Aquino et al., 2007; Aänismaa et al., 2012; Sakai et al., 2012). In addition to their osteogenic, dentinogenic, adipogenic, myogenic, chondrogenic and melanocytic differentiation potential (Batouli et al., 2003; Takeyasu et al., 2004; d'Aquino et al., 2007; Paino et al., 2010; Nozaki and Ohura, 2011), DPSCs respond to inductive and environmental cues to differentiate into mature neuronal cells both in culture and *in vivo* (Arthur et al., 2008; Kadar et al., 2009; Király et al., 2009; Osathanon et al., 2011; Kanafi et al., 2014).

DPSCs exert an intriguing regenerative potential in the damaged central nervous system of the rodent; this has been attributed in part, to their ability to replace lost neurons through differentiation, owing to their neural crest developmental origin, (Agnes et al., 2008; Leong et al., 2012) but mainly to the production of neurotrophic factors (NTFs) that promote neuron survival and axon guidance (Nosrat et al., 2004; Agnieszka et al., 2009; Leong et al., 2012; Mead et al., 2013).

As the most accessible part of the vertebrate CNS for visualization and drug/cell delivery, the retina constitutes an optimal experimental model to evaluate cell-based regenerative therapies that could eventually be clinically relevant, as treatment of a number of irreversible retinopathies that progress with significant destruction of retinal architecture. The vertebrate retina, developed

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Abbreviations: BDNF, brain-derived neurotrophic factor; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DPSCs, dental pulp stem cells; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; GDNF, glial cell-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HFFs, human foreskin fibroblasts; NGF, nerve growth factor; NTFs, neurotrophic factors; ONL, outer nuclear layer; PBS, phosphate-buffered saline; PSA-NCAM, polysialic acid-neural cell adhesion molecule; PCR, polymerase chain reaction; RGCs, retinal ganglion cells; RT-PCR, reverse transcription polymerase chain reaction.

http://dx.doi.org/10.1016/j.neuroscience.2014.09.023
0306-4522/

from the neuroepithelium of the optic vesicle and cup, contains six types of neurons (photoreceptors: cones and rods, retinal ganglion cells (RGCs), bipolar, horizontal and amacrine cells) and three types of glia: astrocytes, microglia and Müller cells. All these cell types are arranged in three nuclear layers separated by two plexiform layers that contain the synapses between neurons (Livesey and Cepko, 2001).

DPSCs were shown to promote cell survival and neuritogenesis in an *in vitro* co-culture assay with retinal primary cultures from adult rats, and RGC survival and axon regeneration in an *in vivo* model of optic nerve crush (ONC) (Mead et al., 2013). These neuroprotective and proregenerative effects were mainly attributed to their ability to release of the neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). However, these results also suggested that retinal glia might have a role in the induction of DPSC-mediated effects (Mead et al., 2013). This observation would be in agreement with the well-established role of extrinsic signals as modulators of the phenotypic potential of neural stem cells and with the notion that local tissue-specific environmental cues might be critical in maintaining a favorable or adverse regenerative-environment (Suh et al., 2009; Sequerra et al., 2013).

Although the adult mammalian retina has long being considered a ''non-neurogenic'' environment, Müller glial cells have been shown to be a possible source of retinal progenitors with neurogenic capacity (Ooto et al., 2004; Osakada et al., 2007; Karl et al., 2008; Takeda et al., 2008; Zhao et al., 2014). Mammalian differentiated Müller glia acquire the ability to dedifferentiate, proliferate and differentiate into retinal neuronal lineages in response to tissue damage or extrinsic signals (Ooto et al., 2004; Lawrence et al., 2007; Karl et al., 2008; Bhatia et al., 2011; Ramirez et al., 2012; Reyes-Aguirre et al., 2013). Moreover they also instruct residing retinal progenitors to differentiate toward cells that express early neuronal markers (Simón et al., 2012).

This work was designed to test the hypothesis that DPSCs might be able to express a regenerative potential on their exposure to retinal environmental cues. If this were to be the case, Müller glia would be a likely candidate providing these instructions. We found that conditioned media obtained from the rat retina organotypic cultures induce morphological changes, upregulation of early and late retinal neuronal markers and an increase in BDNF expression in DPSC cultures. However, the same response was not elicited when conditioned medium from differentiated and dedifferentiated Müller cell-enriched primary cultures was evaluated.

EXPERIMENTAL PROCEDURES

Cell culture

DPSCs. Human third molars were collected from healthy adults (19–35 years of age) undergoing routine extractions with informed consent and under approved guidelines set by the Federal Commission for Protection against Sanitary Risks (12 TR 09 011 003). Teeth were cleaned with commercial mouthwash and cracked open using a vice to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root, minced with scalp and digested in a solution of 3 mg/ml collagenase type I for 30 min at 37 °C. After collagenase inactivation with 20% fetal bovine serum (FBS), cell suspensions were centrifuged at 300*g* for 10 min. Cultures were established by seeding dental pulp cell suspensions at a density of 1.5×10^5 of cells per 25 cm^2 in OPTIMEM medium (Invitrogen, Eugene, OR, USA) supplemented with 2% fetal bovine serum, 100 U/ml penicillin, 100-μg/ml streptomycin, 1× ITS, 10-μg/ml epidermal growth factor (EGF) and 10-μg/ml fibroblast growth factor (FGF) (growth medium for dental pulp cells; control medium) and then incubated at 37 °C in 5% CO_2.

Human foreskin fibroblasts (HFFs). HFFs were purchased from ATCC Primary Cells Solutions, catalog number PCS-201-010 batch 59532382.

Müller cells. Laboratory animals were treated and handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines of the internal animal care committee (CICUAL-CINVESTAV). Long Evans Rats (14 days of age) were lethally anesthetized with an IP injection of sodium pentobarbital before enucleating both eyes. A single cut was made carefully severing the retina from the optic nerve. The eyeballs were immersed in ice-cold Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen) and then rinsed in cold OPTIMEM containing 100 U/ml penicillin and 100-μg/ml streptomycin. Eyes were kept overnight at room temperature in OPTIMEM medium supplemented with 4% FBS and 100 U/ml penicillin and 100-μg/ml streptomycin. After that, the eyes were digested in a solution of 0.1% trypsin and 3-mg/ml collagenase type I for 30 min at 37 °C. After enzyme inactivation with FBS 4% the retinal pigment epithelium, the lens and the vitreous body were displaced and each retina was minced with scissors. Cultures were established by seeding the tissue into T-75 flasks in OPTIMEM medium supplemented with 4% fetal bovine serum, 100 U/ml penicillin, 100-μg/ml streptomycin. The medium was changed 24 h after seeding to eliminate suspended cells.

Müller cell cultures were dedifferentiated with 50 μM Glutamate for either 4 or 24 h before harvesting the conditioned media (Reyes-Aguirre et al., 2013). The conditioned media collected from normal or dedifferentiated Müller cells were filtered through a 0.2-μm membrane filter and stored at −20 °C until use.

Flow cytometry analysis of DPSCs

Expression of the cell surface markers CD14, CD31, CD44, CD45, CD73, CD90, CD105 and HLA-DR on DPSC was analyzed by flow cytometry in a FACSCalibur cytometer (Becton Dickinson, Franklin