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Long-distance effects of inflammation on differentiation of adult spinal cord neural stem/progenitor cells

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ABSTRACT

Studies in multiple sclerosis have demonstrated that normal-appearing white matter can harbor pathological changes. Here we investigated the effects of neuroinflammation, modeled by experimental autoimmune encephalomyelitis (EAE) on neural stem/progenitor cells (NPCs) located distally to inflammatory foci. We observed that EAE-derived NPCs had a lower capacity to differentiate into oligodendrocytes and an increased neuronal differentiation than control NPCs. This finding was corroborated with changes in gene expression of early differentiation genes. We conclude that inflammation has a long range effect on the NPCs in the diseased central nervous system, reaching NPC populations outside the lesion sites.

1. Introduction

Neural stem/progenitor cells (NPCs) are located at different sites in the adult central nervous system (CNS) i.e. the subventricular zone (SVZ) the subgranular zone, as well as along the central canal of the spinal cord (Weiss et al., 1996; Doetsch et al., 1999). The NPCs can be isolated, expanded and in vitro differentiated into neurons, oligodendrocytes and astroglia (Reynolds and Weiss, 1996; Shihabuddin et al., 1997). Furthermore, these cells are triggered to proliferate, migrate and differentiate following various CNS injuries, such as spinal cord injury (Johansson et al., 1999; Meletis et al., 2008), traumatic brain injury and experimental autoimmune encephalomyelitis (EAE) (Danilov et al., 2006; Picard-Riera et al., 2004; Pluchino et al., 2008). In EAE, we and others have demonstrated that NPCs from the central canal of the spinal cord are recruited to CNS lesions, where they differentiate. In a recent publication, we have demonstrated that EAE affects the transcriptome of the NPCs, inducing the expression of inflammation and neurodegeneration-related genes, while decreasing the expression a core gliogenic signature. Functionally, the NPCs were rendered more neurogenic, differentiating into more neurons and less oligodendrocytes than corresponding controls (Covacu et al., 2014). In the present study, our aim was to elucidate whether NPCs located outside inflammatory sites also are transcriptionally and functionally affected in EAE. The rationale of the study stems from investigations done in multiple sclerosis (MS), the human disease modeled by EAE. It was reported already in 1978 that unaffected areas of the CNS in MS patients appear to have normal-appearing white matter (NAWM) (Allen and McKeown, 1979) which actually may contain pre-active lesions (De Groot et al., 2001). A recent study suggests that these pre-active lesions are not associated with blood–brain barrier disruption but a yet unknown intrinsic trigger of innate immune activation which induces clustering of microglia.

In this study we have focused on "normal appearing" parts of the spinal cord (NASC) in EAE animals and investigated potential distant inflammatory effects on spinal NPCs. By immunizing rats with myelin oligodendrocyte glycoprotein (MOG) we induced a relapsing-remitting type of EAE characterized by inflammatory and demyelinated lesions in the CNS, mainly in the spinal cord (Storch et al., 1998). NPCs were isolated and propagated in vitro and their differentiation pattern and expression of relevant genes were investigated. To discriminate between NPCs isolated from lesion (or inflamed spinal cord, ISC) versus ex-lesion sites (or normal-appearing spinal cord, NASC), we measured the levels of the nitric oxide ($NO^{\bullet}$) oxidation product, nitrite, in the culture supernatant. Nitrite levels are commonly used to identify ongoing $NO^{\bullet}$ production during inflammatory activity within fluid compartments of the CNS as well as well as elsewhere in the body (Kornelisse

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et al., 1996; Brundin et al., 1998, 1999; Clark et al., 1996; Johnson et al., 1995; Danilov et al., 2003).

In this study, we demonstrate that NPCs residing distally to inflamed areas in the spinal cord, i.e. in NASC regions, are affected by inflammation. NPCs derived from NASC regions display an increase in neuronal differentiation and a decreased ability to differentiate into oligodendrocytes in vitro. These findings shed light into the pathological mechanisms in MS-affected CNS, where the regenerative capacity might not only be affected in direct proximity to the inflammatory sites, but more generally, even affecting areas distant to ongoing inflammation.

2. Methods

2.1. Experimental animals and EAE induction

DA female rats 7–8 weeks old (Scanbur B&K, Sollentuna, Sweden, http://www.scanbur.eu/) were kept at the animal facility at Karolinska University Hospital. The rats were anesthetized with isoflurane, chosen due to its low influence on intracranial pressure (Forane; Abbott Laboratories, Abbot Park, IL) and immunized subcutaneously with an injection at the dorsal tail base with 200 μl inoculum containing 20 μg recombinant myelin oligodendrocyte glycoprotein (rMOG) produced in house (Amor et al., 1994) and a 1:1 ratio of incomplete Freunds adjuvant (IFA) and PBS (Sigma-Aldrich, St. Louis, MO). The rats were clinically assessed daily for signs of EAE from day 9 until day 42 post-immunization (p.i.). The clinical score was as follows: 0, no clinical signs of EAE; 1, tail weakness or tail paralysis; 2, hind-limb paraparesis or hemiparesis; 3, hind-limb paralysis or hemiparalysis; 4, tetraplegia; and 5, death. The experimental procedures were approved by Stockholm county ethical committee. Animal care was in accordance to the recommendations at Karolinska Institute. The experiments were performed in accordance with the ARRIVE criteria (Kilkenny et al., 2010).

2.2. Cell culture

Animals were sacrificed using carbon dioxide. Spinal cords were harvested from animals with clinical score 1–3 sacrificed beginning from day 16 p.i. up to day 42 p.i. NPCs from spinal cord were isolated from different levels (cervical, above Th2; thoracic Th2-Th12; and caudal, below Th12). After extracting the spinal cord the tissue was divided and the connective tissue was peeled off. This procedure was visualized by using a microscope. The spinal cord was mechanically dissociated with scalpels and scissors according to a modification of a previously described protocol by Johansson et al. (1999). The tissue was placed in a dissociation medium, consisting of 200 U/ml DNAse (Sigma-Aldrich) and 10 U/ml papain (Worthington) and incubated at 37 °C for 15 min with intermittent trituration. To remove the myelin debris the cells were resuspended in 0.9 M sucrose in Hanks' balanced salt solution (HBSS) (Invitrogen) and pelleted at 750 g for 10 min followed by additional washing with L15. The cells were seeded at a density of 150,000 cells per dish on 10 cm Ø petri dishes in culture medium, contained of Dulbecco's modified Eagle's medium/F-12 containing B27 supplement (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies, Invitrogen AB, Stockholm, Sweden, http://www.invitrogen.com) as well as 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich, Stockholm, Sweden, http://www.sigmaaldrich.com) and 20 ng/ml basic fibroblast growth factor (bFGF, R&D systems). Neurospheres from the spinal cord-derived NPCs were propagated and passaged twice and used in experiments as single cells after the last passage. For differentiation, single cell suspensions were seeded on poly(D-lysine)-coated plates (Sigma-Aldrich), and cultured in medium lacking EGF but supplemented with 1% fetal calf serum (FCS) (Life Technologies). Cells for Sox2 immunostaining were seeded on the plates 1 day before fixation to avoid differentiation.

2.3. Quantitative real-time PCR

RNA was purified using an RNeasy kit (Qiagen) and cDNA was subsequently prepared using the iScript kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR (qPCR) was performed using a BioRad iQ5 iCycler Detection System with a three-step PCR protocol (95 °C for 10 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s), using SYBR Green (Bio-Rad). Expression levels corrected for amplification efficiency and normalized to housekeeping gene expression, were analyzed using iQ5 v2.0 software (BioRad). The primers used for SYBR Green reactions were designed using Express software (Applied Biosystems). *Gapdh*, *Hprt*, and *β-actin* expression were used as reference genes (see Table 1).

2.4. Colorimetric measurement of nitrite

After first passage supernatants were collected into Eppendorf tubes previously washed with nitrite-free water and autoclaved and stored in −20 until use. Concentration of nitrite in supernatants was measured by Griess reaction using sodium nitrite ($NaNO_2$) as standard. Supernatants were mixed with Griess Reagent (Sigma-Aldrich) 1:1 in 96-wells ELISA plates and incubated for 15 min. The absorbance was read at 562 nm using an ELISA reader (EMax Precision Microplate Reader, Molecular Devices). The standard curve was constructed of a nine-fold dilution series of sodium nitrite dissolved in stem cell media.

2.5. Immunohistochemistry

Cells were differentiated on poly-D-lysine coated plates (Sigma-Aldrich), fixed with 4% parafolmaldehyde in phosphate-buffer saline (PBS) (Bie&Berntsen A-S) blocked in PBS/0.1% saponin/10% goat serum and incubated with the primary antibody over night (o.n.). After washing the secondary antibody was applied for 1 h at room temperature (r.t.). Concentration and antibodies used were rabbit anti-glial fibrillary acidic protein (GFAP) 1:1000 (Dako), mouse anti-galactocerebroside (Gal C) 1:100 (Millipore), mouse anti-β-III-tubulin, 1:100 (Millipore), mouse anti-CD11b 1:200 (Millipore), rabbit anti-Sox2 1:100 (Millipore), mouse anti-Sox2 1:100 (Millipore), mouse anti-nestin 1:100 (Millipore), mouse anti-BrdU (dilution according to supplier, Amersham), rat anti-BrdU 1:50 (AbD Serotec), goat anti-rat Alexa 488 1:00 (Invitrogen) Cy3 donkey anti-mouse 1:1000 (Jackson ImmunoReseach), Alexa 488 donkey anti-rabbit 1:500 (Invitrogen) and Alexa 594 goat anti-mouse IgG 1:100 (Invitrogen). For the Sox2 immunostaining the cell membrane was permeabilized with a buffer containing hepes, saccharose, NaCl, MgCl and Triton X.100 before adding the antibody. For visualizing all cells the nuclei were counterstained with DAPI (Invitrogen). Glasses were mounted in Mowiol (Cabiochem). Sections were incubated o.n. at +4 °C with one or combinations of primary antibody. Antibodies were diluted in PBS containing 1% BSA and 0.3% Triton X-100 and incubated for 24 to 72 h at +4 °C. Secondary antibodies diluted in PBS were used. For BrdU labeling sections were first rinsed in PBS followed by incubation with 0.1 M NaOH for 2 min and then in PBS pH 8.5 for 30 s. Sections were then incubated in primary

Table 1
PCR primers.

Target	Forward primer	Reverse primer
gapdh	TCAACTACATGGTCTACATGTTCCAG	TCCCATTCTCAGCCTTGACTG
β-actin	CGTGAAAAGATGACCCAGATCA	AGAGGCATACAGGGACAACACA
β-actin	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC
β-III-tubulin	GGGCCTTTGGACACCTATTCA	GCCCTCTGTATAGTGC CCTTTG
gfap	ACAGACTTTCTCCAACCTCCAGAT	TCTTTAC CACGATGTTCCTCTTGA
neurogenin-2	CCAACTCCACGTCCCATAC	GAGGTGCATAACGGTGCTTCTC
mash-1	AGCGCAACCGGGTCAA	CGCGCGGATGTATTCGA
hes-1	CAGAAAGTCATCAAAGCCTATCATG	TCAGTGTTTTCAGTTGGCTCAAAC
notch-1	GGGCAAACCATGCAGGAAT	AGCGCAAACTGCCACAAGT