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High serum levels of BMP-2 correlate with BMP-4 and BMP-5 levels and induce reduced neuronal phenotype in patients with relapsing-remitting multiple sclerosis☆

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ABSTRACT

Blockage of bone morphogenetic protein (BMP) signaling is required for differentiation of neurons and oligodendrocytes from neural stem cells (NSCs). Sera of untreated relapsing-remitting multiple sclerosis (RR-MS) patients expressed significantly higher levels of BMP-2 compared to sera of healthy controls. BMP-2 levels correlated with BMP-4 and -5 levels only in sera of untreated MS patients. Furthermore, sera of untreated patients inhibited the neuronal differentiation of RA-treated P19 cells, which was associated with induction of phospho-SMAD signaling pathway. These results suggest that BMP-2 sera levels may play a role in the failure of remyelination and neuro-regeneration in RR-MS.

1. Introduction

Multiple sclerosis (MS) is a chronic disease with multiple foci of inflammatory lesions in the central nervous system (CNS) that include demyelination, oligodendrocyte death, axonal loss and neuronal degeneration (Barnett and Prineas, 2004; F. et al., 2011; Stadelmann et al., 2008). CNS regeneration and healing are dependent upon effective neurogenesis of neural stem cells (NSCs) that can differentiate into mature neurons and glia (Alvarez-Buylla and Garc\ía-Verdugo, 2002). Neuronal precursor cells (NPCs) and oligodendrocytes (OPCs) are recruited into MS lesions, however, they are unable to differentiate into mature functioning oligodendrocytes or neurons cells (Kotter et al., 2006). The damaged lesions are populated by reactive astrocytes in the process of astrogliosis that leads to the formation of nonfunctional glial scars (Dutta and Trapp, 2007; Prineas, 2001). Although compensation mechanisms may be activated by the generation of new neurons in early stages of neuronal loss (Chang et al., 2008) and the remyelination of the MS lesions (Franklin et al., 1997), the loss of neurons was shown to lead to irreversible neurological disability (Dutta and Trapp, 2011).

Many intrinsic factors were found to influence axonal/neuronal repair and remyelination. Among them LINGO-1 (leucine-rich repeat- and Ig domain-containing nogo receptor-interacting protein 1) and polysialylated-neural cell adhesion molecule (PSA-NCAM) were found to act as axonal inhibitory signals that prevent myelination (Lee et al., 2007; Charles et al., 2002). Difference to epigenetic changes linked to the recruitment of histone deacetylases (HDACs) were found to affect the remyelinating potentials of oligodendrocytes (Ye et al., 2009).

Bone morphogenetic proteins (BMPs) are secreted proteins that belong to the transforming growth factor-β (TGF-β) superfamily. They have an important role in bone formation, as well as in the formation of the heart and cartilage, and in neural development (Rivera-Feliciano and Tabin, 2006; Shu et al., 2011; Urist, 1965). BMPs and many of their antagonists affect neurogenesis in the adult brain. It has been demonstrated that the fate specification of the NSCs is partially dependent upon the balance between BMPs and their antagonists. BMPs are intrinsic factors that were found to inhibit neuronal fate of NSC differentiation and to promote astroglial lineage fate (Gross et al., 1996; Lim et al., 2000; Zimmerman et al., 1996). While, it was shown that noggin, a BMPs antagonist, can expand hippocampal progenitors in the subgranular zone (SGZ) and in the subventricular zone (SVZ) (Bonaguidi et al., 2008). It promotes neurogenesis and decreases gliogenesis. Shh, a BMPs regulator, was shown to be necessary for the proliferation and long term maintenance of the SVZ and SGZ stem cells (Lai et al., 2003; Palma et al., 2004).

Pathological studied of multiple sclerosis demyelinating lesions found an expression of BMPs protein and mRNA in the lesions. It was also shown that leukocytes express BMP-4 and BMP-5, suggesting that lesion-infiltrating leukocytes contribute, at least partially, to the

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presence of BMP-4 and BMP5 in the MS lesions (Deininger et al., 1995a).

We had reported that peripheral blood mononuclear cells (PBMCs) from relapsing-remitting multiple sclerosis (RR-MS) patients express and secrete higher levels of BMP-2, BMP-4 and BMP-5 than healthy individuals (Mausner-Fainberg et al., 2013).

The pluripotent P19 cells are mice embryonic carcinoma cells, which can be differentiate into neurons by exposure to retinoic acid (RA) (Jones-Villeneuve et al., 1982). P19 cells are widely used as a model system for analyzing neural differentiation and activities ((Martins et al., 2005; Monzo et al., 2012; Yuahasi et al., 2012).

It has been previously demonstrated that P19 cells that were treated with BMP-2 or BMP-4 lost their capacity to differentiate into neurons, but not to astrocytes (Bani-Yaghoub et al., 2000).

We now examined BMP-2, BMP-4 and BMP-5 levels in both the sera and CSF of RR-MS patients, and investigated their biological significance in the neurogenesis process using an in vitro model of P19 neural cells.

2. Methods

2.1. Subjects

The institutional ethics committee approved all experiments and informed consent was obtained from all participants. Patients with confirmed RR-MS who were attending the Neuroimmunology Clinic at the Tel Aviv Sourasky Medical Center were included in the study. Blood samples were drawn from (i) 102 patients with RR-MS who were in clinical remission for at least 3 months (60 untreated patients and 42 patients treated with interferon-β1a [IFN-β]) (ii) 44 age-matched healthy controls and (iii) 20 patients with other neurological diseases (OND), that included: patients with early onset dementia, headaches, epilepsy, peripheral neuropathy, systemic. No patient had any other chronic inflammatory condition, and none of the participants was being treated with other immune/inflammatory-related medication (e.g., non-steroidal anti-inflammatory drugs, steroids or other immunosuppressive agents).

CSF was drawn from 18 untreated RR-MS patients and from 10 age-matched donors who were undergoing diagnostic testing for non-infectious or inflammatory medical conditions, e.g., headache and eye disease (controls). Tables 1 and 2 describe the clinical characteristics of the study participants whose sera and CSF samples were collected, respectively.

2.2. Sandwich ELISA for BMP-2, BMP-4 and BMP-5 protein detection

BMP-2, BMP-4 and BMP-5 were measured using Duoset ELISA development system kits (R & D Systems) according to the manufacturer's instructions. Briefly, flat-bottom 96-well plates (Thermo Fisher Scientific) were coated with monoclonal mouse anti-human BMP-2, anti-human BMP-4 or anti-human BMP-5. Samples were added, and the captured BMP-2, BMP-4 and BMP-5 were detected using biotinylated mouse anti-human BMP-2, BMP-4, and BMP-5, respectively, followed by the addition of streptavidin conjugated to horseradish peroxidase (HRP). Tetramethylbenzidine reagent was used as a substrate solution, and the reaction was terminated by the addition of 2N H_2SO_4. A Termo Max ELISA reader (Molecular Devices microplate reader, USA) was used for the quantifications. BMP-2, BMP-4 and BMP-5 levels could be detected within a range of 46.8–3000 pg/ml, 15.6–1000 pg/ml and 187.5–12,000 pg/ml, respectively.

Table 2
Clinical characteristics of the CSF donors.

	Controls	Untreated RR-MS patients
Number	10	18
Male	6	4
Female	4	14
Age (mean ± SD)	36.8 ± 14.7	31.6 ± 7.7
Age range, years	16–62	20–46
EDSS (mean ± SD)	–	1.8 ± 1.4
EDSS range	–	0–5
Disease duration (mean ± SD)	–	1.2 ± 2.3
Disease duration range, years	–	0–7

2.3. P19 cell culture

P19 cells (Sigma Aldrich) were grown in α-minimum essential medium (Gibco) containing 7.5% calf serum (Biological Industries), 2.5% fetal calf serum (Biological Industries), and 1.2% penicillin–streptomycin (Biological Industries) at 37 °C, 5% CO_2. The cells were replenished with fresh medium every 48 h. The differentiation protocol that was followed was previously described elsewhere (Bani-Yaghoub et al., 2000). Briefly, a concentration of 2×10^5 P19 cells/60 mm bacterial grade plate was treated with medium containing 5×10^{-7} M all-*trans*-retinoic acid (RA) (Sigma Aldrich, R2625), 5 ng/ml rhBMP-2 (R & D Systems), and 500 ng/ml neutralizing anti-BMP-2/4 (R & D Systems), and the different sera were diluted 30-fold with the medium. The medium with the different stimulations was replenished once after 48 h. Following 4 days of stimulations, the aggregates which had formed during RA treatment were dispersed enzymatically (0.05% v/v trypsin–0.02% v/v EDTA) and mechanically, and the cells were plated in tissue culture grade dishes.

2.4. Immunofluorescent staining for MAP-2

At day 7 (3 days post-stimulations), the cells were re-cultured in 24-well plates with coverslips (De-Groot Laboratories) at a final concentration of 5×10^4 cells/well. On the following day, the cells were washed with PBS, fixed with 4% paraformaldehyde (Electrone Microscopy Sciences) for 15 min and permeabilized with 0.5% Triton Tx (Sigma Aldrich) for 3 min. A blocking buffer (10% fetal bovine serum, 0.1% BSA, 0.05% Tween) was added for 30 min. The cells were incubated with a primary antibody (rabbit MAP-2 monoclonal antibodies [mAbs]) (1:100, D5G1, Cell Signaling) for 30 min. After several

Table 1
Clinical characteristics of the sera donors.

	Healthy controls	Untreated RR-MS patients	IFN-β treated RR-MS patients	Other neurological diseases
Number	44	60	42	20
Male	24	16	13	3
Female	20	44	29	17
Age (mean ± SD)	35.7 ± 10.6	37 ± 10.3	45.2 ± 11.4	42.8 ± 14
Age range, years	18–77	20–62	25–69	19–67
EDSS (mean ± SD)	–	1.2 ± 1.2	1.2 ± 1.3	–
EDSS range	–	0–5.5	0–4	–
Disease duration (mean ± SD)	–	5.5 ± 7	11.6 ± 9	–
Disease duration range, years	–	0–27	1–36	–