Changes in Nrf2 Expression in the Spinal Cord in Mouse Model of Experimental Autoimmune Encephalomyelitis

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INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease developed by an autoimmune attack on the central nervous system, characterized by destruction of myelin and multi-centric inflammation. It leads to serious motor disturbance and clinical disorders in MS range from relapsing-remitting to chronic-progressive form, associated with permanent neurological problems such as motor paralysis and ataxia (Tsujimura et al., 2008; Dendrou et al., 2015; Ransohoff et al., 2015). Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is encoded by the NRE2LE gene and is a basic leucine-zipper transcription factor that regulates the expression of cytoprotective proteins including antioxidant enzymes (Kraft et al., 2004). Nrf2 has been reported to mediate neuro-protection in Parkinson’s disease and cerebral ischemia (Shih et al., 2005; Chen et al., 2009) and to direct neuroprotection in the autoimmune neuro-inflammatory response in MS (Kraft et al., 2004; Johnson et al., 2010). Recent findings highlight the importance of Nrf2 in MS pathogenesis; in support of this, Nrf2 enhancement has been reported in the brains of various animal models of MS (Solis et al., 2002; Kraft et al., 2004; Johnson et al., 2010; van Horssen et al., 2010). In addition, Nrf2 was shown to be prominently increased in demyelinated lesions in the spinal cord of human MS patients (Licht-Mayer et al., 2015).

However, previous studies using animal models of MS focused predominantly on Nrf2 expression in the brain. Lately, Nrf2 expression and its association with MS in the spinal cord of MS animal models are beginning to be reported (Linker et al., 2011; Higashi et al., 2017). In the present study, we attempted to investigate the expression of Nrf2 in a cellular subpopulation in the spinal cord using an experimental autoimmune encephalomyelitis (EAE) model.

A B S T R A C T

In this study, we investigated the expression of nuclear factor erythroid-derived 2-related factor 2 (Nrf2) in the spinal cord using an experimental autoimmune encephalomyelitis (EAE) model. MOG35,55 was injected into female mice to induce an autoimmune response. Clinical scoring and a behavioral rotarod test were conducted to assess the successful modeling of EAE. Animals were sacrificed 1, 2, and 3 weeks after immunization. Spinal cords were prepared for morphometry, immunohistochemistry, and western blotting. Multifocal demyelination was observed in the white matter. Nrf2 immuno-reactivity was detected in astrocytes and neurons in spinal cord. Nrf2 immuno-reactive neurons and astrocytes had increased by 2 weeks after immunization, and their up-regulation was maintained until 3 weeks after immunization. Additionally, time-dependent up-regulation of Nrf2 in the spinal cord was investigated by western blotting. The level of NAD(P)H quinone oxidoreductase-1 protein, an Nrf2 target gene, was not changed during week 1, but showed a significant decrease 2 and 3 weeks after immunization. Parallel to up-regulated Nrf2, heme oxygenase-1, another target gene of Nrf2, was significantly augmented until 3 weeks after immunization. We conclude that Nrf2 expression in neurons is involved in an autoimmune attack in the spinal cord in an EAE model.
which serves as an in vivo validation model for MS. Additionally, the EAE model in dogs is also a viable model for canine necrotizing encephalitis (Moon et al., 2015). In the spinal cord, Nrf2 expression was studied using immunohistochemistry and western blotting 1, 2 and 3 weeks after immunization.

MATERIALS AND METHODS

Experimental animals and induction of EAE: Nine-week-old female C57BL/6 mice were achieved from the Hallym University (Chuncheon, South Korea). Animals were housed in a conventional state and allowed free access to food and water. The experimental procedures were adhered to the guidelines (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication). EAE was induced according to the protocol of previous study (Lee et al., 2014). Mice (n=14) were subcutaneously immunized with 200µg of myelin oligodendrocyte glycoprotein (MOG35-55) peptide (AnaSpec; Fremont, CA, USA) which were emulsified in complete Freund’s adjuvant (CFA) (H377Ra; Difco Laboratories, Detroit, MI, USA) supplemented with Mycobacterium tuberculosis (4 mg/ml, Sigma-Aldrich; St. Louis, MO, USA). Mice were intraperitoneally injected with pertussis toxin (200ng, Sigma-Aldrich) on day 0 and 2 after immunization. Only CFA was injected in the control mice.

Clinical scoring and evaluation of behavior: Animals were daily checked for clinical scores with the following criteria - 0, healthy; 1. flaccid tail; 2. hind limb mild paralysis; 3. paralysis of both hind legs with residual mobility; 3.5, complete paralysis of both hind legs; 4, complete paralysis of both hind legs with front limb paralysis; 5, moribund or dead (Pöllinger et al., 2009). The motor performance of EAE mice was assessed using rotarod (Med Associates, Inc., St Albans, VT, USA) by checking the latency time to fall (Tsunoda and Fujinami, 2010).

Histology: Mice (n=7) were deeply anesthetized by isoflurane (3% in oxygen), perfused trans-cardially with PBS and 10% neural buffered formaldehyde (NBF). The spinal cord was dissected and post-fixed with 10% NBF. Tissues were paraffin-embedded and sectioned at 6 µm with microtome. First, sections were stained with Luxol Fast Blue (LFB) (Sigma-Aldrich, St. Louis, MO, USA) to assess demyelination.

For immunohistochemistry (IHC) and double immunofluorescence staining, sections were processed according to the previous study (Chang et al., 2012). Briefly, sections were incubated (overnight at 4°C) with rabbit anti-Nrf2 monoclonal antibody (1:200, NOVUS Biologicals, Littleton, CO, USA), and biotinylated-conjugated anti-rabbit antibody, horseradish peroxidase-conjugated streptavidin (1 hr at room temperature). IHC sections were visualized by diaminobenzidine tetrachloride (Vector Lab, Burlingame, CA, USA) and contrasted with hematoxylin counterstain. For double staining, sections were incubated with anti-Nrf2 monoclonal antibody (1:100), mouse anti-glial fibrillary acidic protein (GFAP) (1:200, Chemicon International, Temecula, CA), and mixture of both FITC-conjugated goat anti-mouse IgG (1:200, Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch). Countersait was conducted with 4,6-diamidino 2-phenylindole (DAPI; Vector Lab) for nucleus. Images were taken using digital image analyzer (MCID; Inter-Focus Imaging Ltd., Cambridge, UK) and confocal microscope (LSM510 META NLO, Carl Zeiss, Germany). Numbers of Nrf2 immuno-reactivity positive (+) cells were counted in the spinal cord.

Western blotting: Mice (n=7) in each group were sacriﬁced and spinal cords were dissected and stored at -80°C until analysis. Tissues were processed according to the previous study (Chang et al., 2012). After blotting, membranes were blocked with skim milk and incubated with Nrf-2 antibody and other antibodies (NQO1, HO1, Actin and secondary HRP-conjugated anti-IgG). Immuno-reactive proteins were detected using ECLplus Western Blot Detection Kit (GE Healthcare, Piscataway, NJ, USA). Bands density was quantitated using a density-metric Multi Gauge program (Fujifilm Corporation, Tokyo, Japan).

Statistical analysis: Data was analyzed using ANOVA followed by Duncan’s multiple range as post hoc comparison test with SAS (SAS Institute Inc., version 9.2, Cary, NC, USA). A value of significant threshold was set as P<0.01. The analysis of motor performance of behavioral test adopted a Kruskal-Wallis nonparametric one-way analysis of variance and followed by a two-tailed Mann-Whitney U test. Statistically, a value of P<0.05 was significant.

RESULTS

Clinical and behavior scores in EAE model: Clinical scores of EAE induction were initially recorded at day 11 after MOG35-55 immunization, and clinical signs progressed rapidly. In most mice in the experimental group, clinical scores were graded “5” 3 weeks after MOG35-55 immunization (Fig. 1A). The staying period on the rotarod in the MOG35-55 immunization group was significantly shorter than that of the control group. The latency time of immunized mice declined significantly beginning 1 week after MOG35-55 immunization. After 3 weeks, none of the mice in the MOG35-55 immunization group were able to remain on the rotating drum (Fig. 1B).

LFB staining and measurement of demyelination: Areas of delamination appeared in a multifocal fashion in the white matter of spinal cords of animals in the MOG35-55 immunization group, while this phenomenon was not observed in the control group (Figs. 2A-F). The delamination areas showed significant time-dependent increases after MOG35-55 immunization (Fig. 2G).

Nrf2 IHC in spinal cord: In the control group, Nrf2 immunoreactivity was detected in the grey and white matter of the spinal cord. The numbers of observed Nrf2+ cells in the spinal cord were similar to the control and 1 week after MOG35-55 injection. Thereafter, the number of Nrf2+ cells in the spinal cord increased significantly compared to the controls 2 and 3 weeks after MOG35-55 injection. Subsequently, 3 weeks after MOG35-55 injection, the number of Nrf2+ cells in grey matter had decreased compared to that at 2 weeks (Fig. 3).
Fig. 1: Clinical score (A) and behavioral test (B). Clinical score was significantly increased on 11th day, after the induced immunization (MOG_{35-55} peptide injection), most of the mice clinical score were “4” on 3rd week (A), results shown the daily mean clinical scores (n=21). Immunized mice latency time (second) was significantly declined after 1 week on rotarod test, the mice could not maintain on rotating drum after 3 weeks (B). Data are expressed as the mean ± S.E.M., and representative of experiments repeated three times.

Fig. 2: Detection of demyelination on the spinal cord with LFB staining (A-F). Demyelination has not been shown by the control group (A; ×100; B and C; ×200). However, MOG_{35-55} peptide treatment group’s demyelination was significantly increased with time dependently at 3 week (D; ×100; E and F; ×200). Demyelination area in the spinal cord is outlined by the dashed black line. Demyelination data was present with graph (G). Data are expressed as the mean±S.E.M. and representative, ANOVA, P<0.01, Duncan test. Meanwhile, demyelination was not detected in the gray matter of spinal cord.

**Double immunofluorescence staining:** Double staining was performed to differentiate the type of cells in the spinal cord expressing Nrf2 (Fig. 3). Large numbers of astrocyte in the white matter expressed Nrf2, while some neurons in gray matter showed Nrf2 positivity. We confirmed that, in the spinal cord, the Nrf2 expressing cells in white matter were astrocytes, while in the gray matter, Nrf2 was expressed in neurons (Fig. 3).

**Western blot analysis:** The results of western blotting revealed a pattern of Nrf2, HO1, and NQO1 protein expression in the spinal cord at 1, 2 and 3 weeks after MOG_{35-55} immunization. As EAE progressed, the level of Nrf2 was significantly upregulated compared to the control at 2 and 3 weeks after immunization and peaking at 2 weeks. We also detected time-dependent changes in the Nrf2 target gene HO1, with increases during weeks 2 and 3 after immunization. In contrast, another Nrf2 target gene, NQO1, remained unchanged 1 week after immunization and significantly decreased 2 and 3 weeks after MOG_{35-55} immunization (Fig. 4).

**DISCUSSION**

This study was designed to investigate the morphology of demyelination and the expression of Nrf2 in the EAE mouse spinal cord. LFB staining demonstrated multifocal time-dependent demyelination in the white matter of the spinal cord. Nrf2 upregulation was detected in astrocytes in the white matter and in neurons in the gray matter. The numbers of Nrf2+ astrocytes and neurons increased 2 weeks after MOG_{35-55} immunization, and then decreased at 3 weeks. The protein expression levels of Nrf2 measured by western blotting were similar to the immunohistochemistry results, showing a peak at 2 weeks and a subsequent decrease 3 weeks post-MOG_{35-55} immunization.
Fig. 3: Immunohistochemistry of Nrf2 in the spinal cord (A-F) and the positive cell number in gray matter (G) and white matter (H). Control (A and D), 2 weeks (B and E) and 3 weeks (C and F) after MOG<sub>35-55</sub> peptide treatment. Data are expressed as the mean±S.E.M. and representative. ANOVA, P<0.01, Duncan’s test. Arrows are marked neurons of Nrf2 immuno-reactivity. A-F: x200. Double immunofluorescence staining of Nrf2 (red), astrocytes (GFAP, green) and neurons (blue) 3 week after immunizations (I: x100, J: x200). Nrf2 and astrocyte co-location was detected in white matter, and co-location with neuron was detected in grey matter of spinal cord. Nrf2 expressed cell is astrocyte in white matter and is neuron in grey matter of spinal cord. Arrow indicated Nrf2 expressed neurons in the grey matter, arrow head indicated Nrf2 expressed astrocytes in white matter.

Experimentally, MOG<sub>35-55</sub> immunization is a convenient animal model of EAE, which may correspond to human MS (Lee et al., 2014). In this study, clinical grading scores increased 2 weeks after MOG<sub>35-55</sub> immunization, and behavioral disorders were recorded after 1 week. In the spinal cord, demyelinated white matter was observed 1 week after MOG<sub>35-55</sub> immunization, and demyelination progress paralleled the clinical symptoms and behavioral disorders. These phenomena were reported in our previous study and are known to be typical characteristics of MS (Lee et al., 2014).

An inflammatory response and oxidative stress are detected in MS, and Nrf2 has been reported as being closely related to these inflammatory and oxidative stresses in the brain of MS patients (Gilgun-Sherki et al., 2004; Pöllinger et al., 2009; Johnson et al., 2010; Rosenbaum et al., 2012). In our EAE-modeled spinal cords, increased Nrf2 protein levels were again confirmed. In double-stained tissue, the Nrf2<sup>+</sup> cells were astrocytes in the white matter and neurons in the grey matter. Nrf2<sup>+</sup> neurons have been reported in the brains of EAE animal model (Linker et al., 2011).
Additionally, the activation of Nrf2-ARE pathway was detected in the spinal cord of amyotrophic lateral sclerosis (ALS) model mice (Kraft et al., 2007). Furthermore, Vargas et al., reported that the astrocytes had strong Nrf2 expression and enhanced immune response in spinal cord (Vargas et al., 2005).

The present results confirmed that, within 3 weeks of immunization, expression of Nrf2 is detectable in the neurons and astrocytes in the grey and white matter, respectively, of the spinal cord of EAE mice. Neurons also showed expression of Nrf2 in the grey matter of spinal cord, where the number of Nrf2+ cells was augmented at 2 and 3 weeks after immunizations in this study. Recently, Linker et al. reported that Nrf2 was expressed in the neurons and astrocytes of the spinal cord 41 and 74 days after EAE modeling, respectively. (Linker et al., 2011). Accordingly, Nrf2 was reported to be expressed in the astrocytes and macrophages of spinal cord lesions in MS patients (van Horssen et al., 2010). However, Nrf2 was also detected in the oligodendrocytes at the initial demyelination sites in MS patients (Licht-Mayer et al., 2015). Interestingly, the number of Nrf2+ neurons paralleled the level of Nrf2 protein determined by western analysis, and grey matter of the spinal cord was unaffected by the inflammatory and oxidative attack of MOG35-55 immunizations. This suggests that Nrf2 expression in neurons is more closely related to the impact on the protein level of Nrf2 in the spinal cord after EAE. Additionally, Johnson et al. reported that Nrf2 knockout exacerbated the severity of EAE, indicating that Nrf2 may participate in delaying the development of autoimmune disease (Johnson et al., 2010).

To clearly define the meaning of Nrf2 upregulation in the previous and present studies, we focused on the fact that Nrf2 is an oxidative stress response mechanism initiating the expression of cytoprotective proteins. For instance, targets of Nrf2 include NQO1, glutamate-cysteine ligase catalytic (Gclc), HO1, glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), and multi drug resistance-associated proteins (Mrps) (Solis et al., 2002; Maher et al., 2007; Wang and Dore, 2007; Yamamoto et al., 2008; Jarmi and Agarwal, 2009; Zhao et al., 2009).

The protein level of Nrf2 was upregulated in spinal cord tissue in the EAE model of MS and paralleled enhanced expression of the HO1 protein in the present study. Similarly, ginsenoside Rb1-induced protection of neural progenitors from oxidative stresses was mediated by upregulation of Nrf2 and its target HO1 (Ni et al., 2014). The protective effects of HO1 are associated with attenuation of excessive nitric oxide production and cellular toxicity. Several earlier reports revealed HO1 involvement in acute lung injury sepsis, atherosclerosis, hypertension, and kidney injury (Yamamoto et al., 2008; Jarmi and Agarwal, 2009). Our finding that HO1 is induced in the spinal cord in the EAE model indicates concomitant induction of HO1 in the Nrf2-dependent cytoprotective mechanism. NQO1 is another target gene of Nrf2 (Wang et al., 2007) and catalyzes the reduction of quinones while preventing their participation in redox cycling. NQO1 is a broad-spectrum antioxidant that maintains antioxidative alpha-tocopherol and coenzyme Q10 in their active states by scavenging superoxide (Kraft et al., 2004; Wang et al., 2007). During autoimmune demyelination, NQO1 undergoes time-dependent changes different from Nrf2 and HO1 expression. In the present study, the NQO1 level in the spinal cord was significantly decreased 2 and 3 weeks after immunization. However, NQO1 expression was increased in the brain in the EAE animal model (Li et al., 2013). Along with Nrf2-HO1, NQO1 was increased in the brain as a protective mechanism against lead toxicity (Ye et al., 2016). To the contrary, Agúndez et al. reported that the NQO1 gene does not show a significant correlation in comparison between MS patients and healthy controls (Agúndez et al., 2014). These inconsistent NQO1 results suggest the possibility of site-dependent differences in NQO1 expression or Nrf2-independent changes in NQO1 expression in the spinal cord. However, clarifying the correlation of NQO1 with Nrf2 in the spinal cord in this model will require additional intensive studies.
Conclusions: The present results indicate the involvement of Nrf2 signaling in regulation of cytoprotective genes in response to inflammatory mediators in MS pathology in spinal cords. Along with the induction of Nrf2, there are multidirectional changes in the expression of potent target genes including HO1 and NQO1. Expressional change of Nrf2 in the spinal cord of the EAE model suggests that future investigations in multiple sclerosis patients focusing on this molecule will open a window to increase understanding of the pathology of this disease.

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Authors contribution: HJ Tae, SM Nam, JH Lee and JH Lee conceived and conducted experiments, and collected the data. HJ Tae, SM Nam, JH Lee and JH Lee analyzed the data and wrote the manuscript. BJ Chang participated in designing and discussing the study. All authors approved the final version of the manuscript.

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