Nrf2 and DJ1 are consistently upregulated in inflammatory multiple sclerosis lesions

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Oxidative stress plays a major role in multiple sclerosis (MS), a chronic inflammatory central nervous system (CNS) disease. Invading leukocytes contribute to cell damage and demyelination by producing excessive amounts of cytotoxic mediators, including reactive oxygen species (ROS). To counteract the damaging effects of ROS the CNS is endowed with a repertoire of endogenous antioxidant enzymes, which are regulated by the transcription factor NF-E2-related factor 2 (Nrf2). Upon exposure to ROS, Nrf2 translocates to the nucleus allowing transcriptional activation of various antioxidant enzymes. DJ1 is a protein that is involved in the stabilization of Nrf2 and hence acts as a positive regulator of Nrf2-driven antioxidant protection. Here, we investigate the (sub)cellular localization of Nrf2 and DJ1 in various MS lesion stages and show that Nrf2 is strikingly upregulated in active MS lesions, in both the nucleus and the cytoplasm of infiltrating macrophages and to a lesser extent in reactive astrocytes. Simultaneously, DJ1 protein expression is predominantly increased in astrocytes in both active and chronic inactive MS lesions compared to control brain tissue and normal-appearing white matter. Together, our findings suggest that persistent Nrf2-mediated transcription occurs in active MS lesions, but that this endogenous response is insufficient to prevent ROS-induced cellular damage, which is abundant in inflammatory MS lesions.

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brain tissue and is localized to neurons of distinct neurotransmitter phenotypes and glial cell types [20].

Alterations in DJ1 and Nrf2 expression levels have been observed in various neurodegenerative diseases, including sporadic Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), and other α-synucleinopathies and tauopathies [21–26]. Protective effects of Nrf2 activation have been demonstrated in various animal models for PD [27], AD [28], and ALS [29]. Recent findings point toward important roles for DJ1 and Nrf2 in the pathogenesis of MS, as DJ1 mRNA and protein expression is enhanced in brain tissue of animals suffering from experimental autoimmune encephalomyelitis (EAE), a validated animal model for MS [30], and DJ1 levels are increased in cerebral spinal fluid of patients with relapsing–remitting MS [31]. Further, induction of EAE in Nrf2-knockout mice resulted in a more severe disease, a higher incidence of animals developing EAE, and a more rapid onset. Notably, brain tissue of Nrf2-knockout mice suffering from EAE demonstrated enhanced numbers of infiltrated leukocytes and increased glial cell activation in the spinal cord [32], indicating that Nrf2 is able to modulate immune responses in an experimental model of MS.

Together, these findings implicate the Nrf2 pathway in MS pathogenesis; however, data on the expression of Nrf2 and DJ1 in various MS lesion stages are lacking. To gain insight into the (sub) cellular localization and distribution of Nrf2 and DJ1 we performed a comprehensive immunohistochemical analysis to examine the distribution pattern and cellular localization of DJ1 in various MS lesion stages.

Materials and methods

Autopsy material

For this study, we collected 17 lesions from 11 patients with clinically diagnosed and neuropathologically confirmed MS. Brain tissue was obtained at rapid autopsy and immediately fixed in formalin (in collaboration with The Netherlands Brain Bank, Amsterdam; Dr. I. Huitinga, coordinator). The Netherlands Brain Bank received permission to perform autopsies for the use of tissue and for access to medical records for research purposes from the ethics committee of the VU Medical Center (Amsterdam, The Netherlands). Three cases without neurological disease were selected as controls. Tissue samples from control cases were taken from the subcortical white matter or corpus callosum. MS tissue samples were selected on the basis of postmortem MRI and lesions were classified according to standard histopathological criteria as previously published [33]. Relevant clinical information was retrieved from the medical records and is summarized in Table 1. All patients and controls, or their next of kin, had given informed consent for autopsy and the use of their brain tissue for research purposes.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue was sectioned at 5 μm and stained for proteolipid protein (PLP; clone plpc1; Serotec), major histocompatibility complex (MHC) class II (Dako), DJ1 (Stressgen), and Nrf2 (Santa Cruz). First, sections were deparaffinized in xylene and rehydrated through graded alcohol into distilled water and endogenous peroxidase activity was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol. The slides were rinsed with distilled water and transferred to 10 mM sodium citrate (pH 6.0; DJ1, Nrf2) or formic acid (MHC class II) to achieve heat-induced antigen retrieval. After microwave irradiation for 5 min on the high setting and for 10 min on the medium setting, the slides were cooled to room temperature and rinsed in phosphate-buffered saline (PBS). Serial sections of each tissue sample were incubated overnight with anti-Nrf2 (1:100), anti-DJ1 (1:500), MHC class II (1:100), or PLP (1:500). Then, the slides were incubated with horseradish peroxidase-labeled anti-mouse/rabbit from the Envision kit (Dako) for 30 min at room temperature and finally diaminobenzidine tetrachloride. Between incubation steps, the sections were thoroughly washed with PBS. After a short rinse in tap water the sections were incubated with hematoxylin for 1 min and extensively washed with tap water for 10 min. Finally, the sections were dehydrated with ethanol followed by xylol and mounted with Entellan (Merck, Darmstadt, Germany). All antibodies were diluted in PBS containing 0.1% bovine serum albumin (Boehringer Mannheim).

For immunofluorescence staining, slides of sections were rinsed with distilled water and transferred to formic acid to achieve heat-induced antigen retrieval. After microwave irradiation for 5 min on the high setting and for 10 min on the medium setting, the slides were cooled to room temperature and rinsed in PBS. For cellular localization the sections were incubated overnight with either mouse anti-DJ1 or rabbit anti-Nrf2 followed by incubation with Alexa-488-labeled goat anti-mouse/rabbit (1:400; Molecular Probes). Mouse or rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; Dako), mouse anti-MHC class II (1:100), or mouse anti-Nogo-A (1:20,000; kind gift from Professor Schwab, Department of Neuromorphology, University of Zurich, Switzerland) was subsequently applied for 1 h and followed by incubation with Alexa-594-labeled goat anti-mouse or rabbit (1:400; Molecular Probes). After being washed, the slides were covered with Vectashield (Vector Laboratories) supplemented with 0.4% DAPI to stain nuclei. Microscopic analysis was performed with a Leica TCS SP2 AOBs confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

Results

Lesion classification

Classification of lesion staging was based on standard histopathological staining for inflammatory cells (anti-MHC class II) and myelin (anti-PLP) as described before [12,34]. Based on these findings nine lesions sampled in this study were classified as active with myelin loss (Fig. 1A) and abundant perivascular and parenchymal macrophages (Fig. 1B) and eight lesions as chronic inactive with demyelinated areas (Fig. 1C) containing few MHC class II-positive cells (Fig. 1D).

<table>
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<th>Sex</th>
<th>Postmortem delay (h)</th>
<th>Disease duration (years)</th>
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<td>—</td>
<td>F</td>
<td>8</td>
<td>—</td>
<td>—</td>
<td>Cachexia, uremia</td>
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SP, secondary progressive; PP, primary progressive; F, female; M, male; CVA, cerebrovascular accident.
Nrf2 expression in various MS lesion stages

In normal-appearing white matter we observed a weak Nrf2 staining, which was mainly restricted to the cytoplasm of cells with an astrocyte morphology (Fig. 2A). Nrf2 immunoreactivity in the white matter of nonneurological patients was similar to that observed in normal-appearing white matter (data not shown). In active MS lesions with abundant leukocytes Nrf2 expression was consistently upregulated in infiltrating macrophages and to a lesser extent in reactive astrocytes (Fig. 2B). In chronic inactive lesions Nrf2 staining was observed in astrocytes, albeit less pronounced compared to active lesions (Fig. 2C). We further observed a weak parenchymal Nrf2 staining in chronic lesions (Fig. 2C). In general, there was minimal variety in the expression pattern and intensity of Nrf2 immunostaining between similar MS lesion types of various patients.

Double immunofluorescence staining with MHC class II (Fig. 2D) and GFAP (Fig. 2G) confirmed that Nrf2 (Figs. 2E and H) was localized to MHC class II-positive infiltrating macrophages and reactive astrocytes (Fig. 2I) in inflammatory active lesions. Although Nrf2 immunostaining was predominantly cytoplasmic in both macrophages and astrocytes we observed nuclear staining of Nrf2 in both macrophages (Fig. 2J) and astrocytes (Fig. 2K) in all active lesions. Again, nuclear staining of Nrf2 was more profound in macrophages compared to astrocytes. Interestingly, oligodendrocytes, identified by the expression of Nogo-A, at the border of active lesions lacked evident Nrf2 staining (Fig. 2L).

DJ1 expression in various MS lesion stages

DJ1, a protein that is involved in the stabilization of Nrf2, is weakly expressed throughout the brain parenchyma and in cells with morphological characteristics of astrocytes (Fig. 3A), which is in line with previous findings [26,35]. DJ1 immunoreactivity in white matter of nonneurological patients was similar to that observed in normal-appearing white matter (data not shown). In active inflammatory lesions with abundant MHC class II-immunopositive inflammatory cells numerous astrocyte cell bodies were intensely decorated by anti-DJ1 (Fig. 3B). Hypocellular chronic inactive lesions, characterized by significant loss of myelin and few inflammatory cells (Fig. 3C), contained hypertrophic DJ1-immunoreactive astrocytes throughout the lesion area. Both astrocyte cell bodies and cellular processes were strongly DJ1 immunoreactive in chronic inactive lesions. Importantly, in line with the Nrf2 staining, we did not observe major interindividual differences in DJ1 expression between similar MS lesion types of various patients, suggesting minimal variability in DJ1 distribution and expression levels. To confirm that astrocytes were the main DJ1-immunopositive cell type in MS lesions we performed colocalization studies. Using double immunofluorescence staining we showed that the majority of DJ1-immunoreactive cells are positive for GFAP, indicating that DJ1 is primarily expressed by astrocytes (Figs. 3D–F) in various MS lesion stages.

Discussion

We examined, for the first time, the (sub)cellular localization of DJ1 and Nrf2 proteins in brain specimens of MS patients and observed marked changes in DJ1 and Nrf2 expression in MS lesions compared to surrounding normal-appearing white matter and white matter from nonneurological controls. In active lesions Nrf2 was predominantly expressed in the cytoplasm and nucleus of infiltrating macrophages and to a lesser extent in astrocytes, whereas DJ1 protein expression was restricted to astrocytes. In chronic lesions we found a strong DJ1, not Nrf2, immunostaining in hypertrophic astrocytes.

It has been generally accepted that free radicals and mitochondrial dysfunction contribute significantly to AD pathology. Pioneer work by Smith, Perry, and co-workers demonstrated that oxidative stress and inflammation are early events in AD pathogenesis [36–38]. Inflammation-driven ROS formation induces oxidative stress and damage, which are common pathological features of several other neurological disorders, including MS [6,39]. To control the redox balance cells are equipped with an efficient machinery consisting of multiple antioxidant enzymes that are involved in the detoxification of free radicals.
Nrf2 is a key transcription factor that regulates the production of an array of protective enzymes and it has been reported that DJ1 facilitates activation of the Nrf2 pathway and subsequent production of neuroprotective enzymes [15,16]. However, Johnson and co-workers recently demonstrated that the Nrf2 pathway can still be activated by tBHQ, a potent Nrf2 activator, in DJ1-deficient cells [40].

Notably, recent reports from various research groups showed the involvement of the Nrf2/DJ1 pathway in MS pathology. DJ1 mRNA and protein levels are strikingly upregulated in brain tissue homogenates of an experimental MS animal model and there is a positive correlation between the amount of DJ1 protein and disease severity [30]. DJ1 levels are significantly increased in cerebrospinal fluid samples of relapsing–remitting MS patients and correlate with the Multiple Sclerosis Severity Score, a method for comparing disease progression using single-assessment data [31]. The pivotal protective role of Nrf2 under neuroinflammatory conditions was elegantly demonstrated by Johnson and colleagues, who showed that induction of EAE in Nrf2-knockout mice resulted in a more severe disease and an
increase in infiltrating immune cells [32]. Because ROS are known to mediate transendothelial leukocyte migration [41,42] it is conceivable that the loss of Nrf2 and impaired ROS detoxification might promote leukocyte migration into the CNS. In addition, reduced levels of Nrf2-regulated enzymes in the CNS might aggravate ROS-induced cellular damage and trigger glial activation.

Histopathological studies revealed that reactive astrocytes are the most predominant DJ1-immunopositive cell type in a variety of neurological disorders, such as stroke, Parkinson disease, Alzheimer disease, and several α-synucleinopathies and tauopathies [23–26,35]. Here, we demonstrate that enhanced astrocytic DJ1 expression is not only associated with neurodegenerative diseases but also occurs under inflammatory conditions. Remarkably, even astrocytes in chronic inactive MS lesions expressed high levels of DJ1, indicating that increased astrocytic DJ1 immunoreactivity also occurs in the chronic phase of the disease when inflammation has abated. Conceivably, abundant astrocytic DJ1 expression in chronic inactive lesions might be involved in axonal preservation. In fact, addition of astrocytes to neuronal cultures prevents cell death caused by a number of neurotoxic compounds, and DJ1 knockdown in astrocytes diminishes astrocyte-mediated neuroprotection [43].

Immunohistochemical analysis of Nrf2 protein levels in various neurodegenerative diseases primarily focused on alterations in neuronal expression and localization. Decreased neuronal levels of Nrf2 have been detected in amyotrophic lateral sclerosis [21] and Alzheimer disease brain tissue [22], whereas a strong nuclear Nrf2 staining was observed in nigral neurons of Parkinson disease cases [22]. Here, we show that Nrf2 expression in white matter of nonneurological patients is mainly restricted to the cytoplasm of astrocytes. Interestingly, Nrf2 immunostaining was consistently enhanced in active MS lesions and was predominantly localized to infiltrating macrophages and reactive astrocytes. We observed both cytoplasmic and nuclear staining of Nrf2 in macrophages and astrocytes, indicating that activation of the Nrf2 pathway can occur under inflammatory conditions in macrophages and astrocytes. It has been generally accepted that astrocytes play a dual role in MS pathogenesis as they exert beneficial as well as detrimental roles. The observation that astrocytes in active lesions express enhanced levels of both Nrf2 and DJ1 implies that these cells exhibit a potent transcriptional Nrf2 response and concomitant high levels of Nrf2-regulated proteins. In fact, we and others have shown that various Nrf2-induced enzymes, including NAD(P)H:quinone oxidoreductase-1 and heme oxygenase-1, are upregulated in astrocytes and macrophages in inflammatory MS lesions [12,34,44]. Remarkably, during the chronic phase of the disease Nrf2 levels as well as antioxidant enzymes revert to those observed in control tissue [12]. Taken together, there is ample evidence that astrocytes under pathological conditions express a powerful arsenal of molecules involved in cellular protection, including Nrf2/DJ1. Augmented astrocytic Nrf2/DJ1 expression in MS lesions may protect not only the astrocytes themselves but also surrounding cells and structures, such as axons and oligodendrocytes.

Activation of the Nrf2 pathway together with marked expression of antioxidant enzymes in infiltrated macrophages indicates that macrophages exhibit an efficient system to protect themselves against oxidative damage, which could impair important functions in the defense and repair mechanisms of these cells. Surprisingly, we observed no detectable amounts of Nrf2 in oligodendrocytes in either control white matter or MS brain tissue. In line with these data we previously described a lack of Nrf2-driven antioxidant enzyme expression in oligodendrocyte in MS brain tissue. Oligodendrocyte damage and loss are key features of MS pathology and it has been shown that oligodendrocytes are highly vulnerable to free radicals [45]. Hence, low levels of Nrf2 or impaired Nrf2 activation might underlie the selective vulnerability of oligodendrocytes under neuroinflammatory conditions. Notably, recent data suggest that Nrf2 plays an essential role in oligodendrocyte function and myelination, as Nrf2-knockout mice exhibit severe myelin degeneration and oxidative damage to the myelin sheath [46].
Experimental animal studies have evidently demonstrated the beneficial effects of Nrf2-mediated antioxidant protection in models for neurodegeneration and neuroinflammation cerebral ischemia. Administration of sulforaphane, a potent Nrf2 activator, reduced microglial cell activation and production of inflammatory mediators [47] and improved blood–brain barrier integrity [48] and cognitive function in vivo [49]. Currently, clinical trials with dimethyl fumarate (DMF) are ongoing and show promising results [50]. DMF is able to promote Nrf2-driven antioxidant enzyme production [51], and oral administration of DMF significantly reduced the formation of new MS lesions [50].

In summary, we investigated the (sub)cellular localization of Nrf2 and DJ1 in various MS lesion stages and showed that Nrf2 is strikingly upregulated in active MS lesions, in both the nucleus and the cytoplasm of infiltrating macrophages and to a lesser extent in reactive astrocytes. Similarly, DJ1 protein expression was enhanced in astrocytes in both active and chronic inactive MS lesions compared to control brain tissue and normal-appearing white matter. Increased expression of Nrf2/DJ1 under neuroinflammatory conditions may reflect a protective response to counteract ROS-mediated cellular toxicity. However, although Nrf2-mediated transcription occurs in astrocytes and macrophages in MS lesions, this response is apparently insufficient to prevent ROS-induced cellular damage, which is abundant in MS brain tissue. Importantly, oligodendrocytes at the edge of MS lesions expressed relatively low levels of Nrf2. Hence, we propose that therapeutic strategies aimed at promoting Nrf2-mediated antioxidant protection represent an interesting approach to enhancing levels of antioxidants, thereby interfering with pathological processes underlying MS lesion formation [39].

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