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Experimental Encephalomyelitis Induces Changes in DJ-1: Implications for Oxidative Stress in Multiple Sclerosis

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ABSTRACT

DJ-1 plays an important role in oxidative stress, and is involved in various neurodegenerative diseases. Accumulating evidence suggests a central role for oxidative stress in multiple sclerosis (MS). The aim of this study was to examine whether changes occur in DJ-1 expression in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). We found upregulation of DJ-1 mRNA and protein expression levels in EAE and a correlation between disease severity and increased DJ-1 levels. Although DJ-1 isoforms were more alkaline in controls, in EAE, a shift was noted toward acidic isoforms. ROS induced by SIN-I exposure led to an increase in DJ-1 mRNA and protein levels in human glioma U-87 cells. Immunocytochemical staining demonstrated that DJ-1 is present both in the cytoplasm and the nuclei of these cells. This is the first report of modulation of DJ-1 expression in EAE. Upregulation of DJ-1 was noted in EAE, and similar results were observed in glioma cells exposed to ROS. In view of the accumulating evidence on the central role of oxidative stress in MS, and the importance of DJ-1 in oxidative stress management by the CNS, we believe that DJ-1 will be found to have a central role in MS. *Antioxid. Redox Signal.* 8, 1987–1995.

INTRODUCTION

MULTIPLE SCLEROSIS (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS), characterized by various symptoms of neurologic dysfunction. MS and its animal model, experimental autoimmune encephalomyelitis (EAE), are believed to result from autoimmune-mediated activated immune cells such as T and B lymphocytes, as well as macrophages and microglia (22). Pathologically, MS is characterized by perivenous infiltration of lymphocytes and macrophages into the CNS parenchyma, resulting in demyelinative lesions termed plaques. These plaques, which are the hallmark of MS, are associated with oligodendrocyte death, axonal damage, and neuronal loss. The view that MS can be considered an inflammatory neurodegenerative disease is supported by studies demonstrating neuronal and axonal injury in regions remote from acute plaques, as well as imaging studies that demonstrated changes in normal-appearing white and grey matter (10, 11, 16).

The etiology of MS has not yet been fully elucidated, and it is attributed to both genetic and environmental causes. Accumulating data indicate that oxidative stress plays a major role in the pathogenesis of MS. Reactive oxygen species (ROS), leading to oxidative stress, generated in excess primarily by activated microglia, have been implicated as mediators of demyelination and axonal damage in both MS and EAE (13, 18). The neurotransmitter glutamate is one of the sources of oxidative stress in MS, primarily through activation of its ionotropic receptors (3, 17, 41).

Oligodendrocytes, the myelin-producing cells of the CNS, are also highly vulnerable to glutamate excitotoxicity, mainly via the AMPA/kainate receptors (31, 47). ROS cause damage to cardinal cellular components such as lipids, proteins, and nucleic acids, resulting in cell death. Weakened cellular antioxidant defense systems in the CNS of MS patients, result-

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ing in increased vulnerability to ROS effects, may increase CNS damage (19).

DJ-1, a small 189-amino-acid protein that is ubiquitously expressed and highly conserved throughout diverse species, was discovered as a novel oncogen in 1997 (34). Accumulating data revealed its involvement in various cellular processes, especially in oxidative stress (9, 24, 25, 33, 48). DJ-1 is known to have several isoforms with isoelectric points between 5.5 and 7, with dominance of alkaline isoforms in normal conditions (2, 9). On ROS exposure, accumulation of more-acidic isoforms of DJ-1 occurs, mediated through oxidation of cysteine residues (2, 9, 25, 33).

DJ-1 is widely distributed and highly expressed in the brain, and is not confined to a single functional system or anatomic location (1, 40). DJ-1 is expressed in neurons of different neurotransmitter phenotypes and in all glial cell types, such as astrocytes, microglia, and oligodendrocytes (1). Recently DJ-1 mutations were discovered to cause familial Parkinson disease (PD) (7). A postmortem study of brain samples from sporadic PD brains versus control found that acidic isoforms of DJ-1 are more abundant in PD brains (2). DJ-1 immunoreactivity was detected in other neurodegenerative diseases including multisystem atrophy, Alzheimer disease, progressive supranuclear palsy, frontotemporal dementia with parkinsonism linked to chromosome 17, and Pick disease (2, 36, 37). These data support the notion that different neurodegenerative diseases, characterized by ongoing oxidative stress, may share a common mechanism, in which DJ-1 might play a key role.

The central role of oxidative stress in MS and the importance of DJ-1 in CNS handling of ROS increased the possible importance of DJ-1 in MS. Therefore, we examined whether changes in DJ-1 expression occur in its animal model, EAE. In this study, we determined changes in DJ-1 mRNA and protein expression levels in various disease severities and compared them with controls. Modulation of DJ-1 expression in EAE might imply a possible role for DJ-1 in MS.

MATERIALS AND METHODS

Materials

Reagents used were as follows: Tri-reagent (Sigma, St. Louis, MO, U.S.A.); rabbit anti-DJ-1 (Chemicon, Temecula, CA, U.S.A.); mouse anti-actin (Sigma); Alexa 568-conjugated goat anti-rabbit (Molecular Probes, Invitrogen, Eugene, OR, U.S.A.); horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit (Sigma); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma); 3-(4-morpholinyl)-sydnonimine (SIN-1) (Sigma); Super Signal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, U.S.A.); BCA protein assay kit (Pierce Biotechnology); myelin oligodendrocyte glycoprotein (MOG; Weizmann Institute, Rehovot, Israel); random primer (Invitrogen, Carlsbad, CA, U.S.A.); Sybr green PCR master mix (Applied Biosystems, Warrington, U.K.); RNase inhibitor (RNAguard; Amersham Pharmacia Biotech); Super Script II RNase H-reverse transcriptase (Invitrogen); Ready gel for polyacrylamide electrophoresis IEF pH 5–8 (Bio-Rad Laboratories, Hercules, CA, U.S.A.); IEF 10x anode buffer (Bio-Rad Laboratories); IEF 10x cathode buffer (Bio-Rad Laboratories); Dulbecco's modified Eagle's medium (DMEM) (Biological Industries Israel Beit Haemek, Kibbutz Beit Haemek, Israel); fetal calf serum (FCS) (Biological Industries Israel Beit Haemek); diethyl pyrocarbonate (DEPC) water (Biological Industries Israel Beit Haemek); and Complete protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany).

Other chemicals were purchased from Sigma Chemicals Co.

Animals

Six- to 8-week-old C3H.SW female mice weighing 20 g were obtained from Harlan Laboratories, Rehovot, Israel. The animals were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 40%), and a 12-h light/dark cycle, and were allowed free access to food and water. The animals and protocol procedures were approved and supervised by the Animal Care Committee at the Rabin Medical Center.

Induction of chronic EAE

Chronic EAE was induced according to previously described procedures (19). In brief, female C3H.SW mice (6–8 weeks old) were immunized twice, at day 1 and day 8, by subcutaneous injection with an emulsion containing myelin oligodendrocyte glycoprotein (pMOG35–55), in complete Freund's adjuvant (CFA) containing 200 μg heat-activated *Mycobacterium tuberculosis* in a total volume of 0.2 ml.

Mice were followed up for clinical disease severity daily, by using a score scale: 0, no disease signs; 1, loss of tail tonicity; 2, mild hindlimb weakness; 3, complete hindlimb paralysis; 4, paralysis of four limbs; 5, moribund; and 6, death (32). Mild disease severity was defined as clinical scores ≤2; moderate disease severity was defined as clinical score of 3; and severe disease was defined as scores ≥4.

Thirty control and MOG-induced EAE mice were killed at different clinical disease severities. Tissues were frozen in liquid nitrogen and stored at −80°C until protein and RNA extractions.

Cells

Human glioma U-87 MG cells were obtained from the ATCC (an established cell line from a 44-year-old white woman). Cells were grown under sterile conditions as a monolayer in DMEM medium supplemented with fetal calf serum (FCS) (10%), gentamicin (50 mg/ml), and glutamine (5 m*M*) in a 5% CO_2 humidified atmosphere at 37°C. The medium was routinely changed, and the cells were passaged every 5 days. All experiments were performed on cells near confluence in 10-cm plates.

Glioma cells were exposed to 3-(4-morpholinyl)-sydnonimine (SIN-I) (0–5 m*M*; Sigma), which is a peroxynitrite free radical donor, to produce ROS formation.

Protein extraction and Western blotting

Proteins were extracted from brain tissue by grinding in lysis buffer containing 250 m*M* sucrose, 10 m*M* KCl, 1.5 m*M*

$MgCl_2$, 2 m*M* EDTA, 20 m*M* Hepes, and protease inhibitors cocktail (Roche). To prepare whole-cell lysate from the glioma U-87 cells, cells were trypsynized, centrifuged, and resuspended in lysis buffer. Cell debris was removed by centrifugation at 20,000 *g* for 15 min at 4°C. Protein concentration was determined by the BCA method (Pierce). Twenty-five micrograms of total protein from brain samples or glioma cells lysate was separated by 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membranes were probed with rabbit anti-DJ-1 (1:5,000; Chemicon Laboratories), and mouse anti-actin antibodies (1:10,000; Sigma), followed by horseradish peroxidase–conjugated secondary antibody (1:10,000; Sigma), and developed with the Super Signal West Pico Chemiluminescent substrate (Pierce).

Isoelectric focusing (IEF)

Proteins from EAE brain extracts were separated in pH 5–8 ranges of isoelectric focusing phoresis gel (Bio-Rad), transferred onto nitrocellulose membranes, and blotted with rabbit anti-DJ-1 antibodies (Chemicon).

RNA isolation

Total RNA was isolated from cultured glioma U-87 cells and mice brain tissues by using a commercial reagent TriReagent (Sigma) and the manufacturer's recommended procedure. The amount of RNA was determined spectrophotometrically by using the ND-1000 spectrophotometer (Nano-drop). RNA quality was verified by measuring the OD260:OD280 ratio. RNA was stored at −80°C until used.

cDNA synthesis

First-strand cDNA synthesis was carried out in a final reaction volume of 20 μl containing 1 μg of the total RNA, random primer (1.3 μ*M*, Invitrogen UK) in diethyl pyrocarbonate (DEPC) water at a total volume of 10 μl. After incubation in 70°C for 10 min and cooling to 4°C for 10 min, the following reagents were added to a final concentration of 1x Buffer supplied by the manufacturer, 10 m*M* DTT, 20 μ*M* dNTPs, 20 U of RNase inhibitor (RNAguard, Amersham Pharmacia Biotech), and 10 U of the enzyme Super Script II RNase H-reverse transcriptase (Invitrogen). RT reaction was performed at 25°C for 10 min, 42°C for 2 h, followed by 70°C for 15 min and 95°C for 15 min. Samples were stored at −20°C until used.

Real-time quantitative reverse transcription polymerase chain reaction (PCR)

Real-time quantitative PCR of the desired genes was performed in an ABI Prism 7700 sequence detection system (Applied Biosystems) by using Sybr green PCR master mix (Applied Biosystems) and the following primers: GAPDH sense CGA CAG TCA GCC GCA TCT T, GAPDH antisense CCA ATA CGA CCA AAT CCG TTG; MOG sense CCT GGT TGC CTT GAT CAT CTG CTA C, MOG antisense TCT ACT CGG TAT CCA GAA TGT GTC TG; DJ-1 sense CAT GAG GCG AGC TGG GAT TA, and DJ-1 antisense GCT GGC ATC AGG ACA AAT GAC. The GAPDH gene, which served as an internal control, is a valid reference "housekeeping" gene for transcription profiling, which was also used for real-time quantitative PCR experiments in previous studies. EAE induction and clinical severity did not affect the constitutive expression of the reference gene, GAPDH, nor did SIN-I treatment of glioma U-87 cells.

For quantification of DJ-1, MOG, and GAPDH mRNA, real-time quantitative PCR was performed in duplicates. The target gene (*i.e.,* DJ-1 or MOG), and the reference gene, GAPDH, PCR amplification was performed for each sample in separate wells of the same PCR reaction plate, which also contained a standard curve for each gene amplified and no template controls (NTC). Optimal experimental parameters (hybridization temperature, elongation time, and primer concentrations) were determined for each primer pair. For each gene, verifying a single peak in melting curve analysis assessed the specificity of the PCR product.

The PCR was performed in a total volume of 20 μl containing 1 μl of the previously described cDNA, 1 μl each of the 3′ and 5′ primers (final concentration of 500 n*M* each), 10 μl of Sybr Green Mix, and 8 μl of DEPC water.

The amplification protocol was 40 cycles of 95°C for 15 sec followed by 60°C for 1 min each. Quantitative calculations of the gene of interest (DJ-1 or MOG) versus GAPDH was done using the ΔΔCT method, as instructed in the user bulletin 2 ABI prism 7700 sequence detection system (updated 10/2001).

Immunocytochemistry

For immunocytochemistry, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then incubated in a blocking solution followed by 1-h incubation at room temperature with rabbit anti-DJ-1 antibodies (1:1,000; Chemicon). After washing with PBS, the cells were incubated with fluorescent Alexa 568-conjugated goat anti-rabbit antibodies (1:4,000; Molecular probes) for 1 h at room temperature.

Cell viability

Cell viability was determined by the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay. Cells were plated in 96-well plates, and viability after SIN-I treatment was analyzed by adding MTT solution to each well (reaching a final concentration of 0.5 mg/ml) followed by incubation at 37°C for 3 h. The medium was then removed, and the formazan crystals were dissolved in DMSO. Absorbance was determined at 564 nm in a microplate reader. Cell viability was evaluated in triplicate for each treatment. All experiments were repeated at least 3 times.

Statistical analysis

Statistical analysis was done by using the SPSS software. We used Student's *t* test or one way ANOVA for comparisons between groups (as appropriate). Correlation was calculated by using Pearson's rho. Results are presented as mean ± standard deviation. Statistical significance is defined as a p value of ≤ 0.05.

RESULTS

To examine possible involvement of DJ-1 in EAE, we induced chronic EAE by injection of MOG into female C3H.SW mice (6–8 weeks old). MOG-induced EAE led to various disease severities ranging from no apparent disease to moribund (clinical scores of 0–5). Brains of EAE mice, killed at various disease-severity scores, were compared with brains of control mice, because DJ-1 is reported to be present in the brain.

We found decreased MOG mRNA levels in EAE brains. Moreover, an inverse relation was noted between MOG mRNA levels and the clinical disease severity (*i.e.*, as the clinical score of EAE increased, the levels of MOG mRNA declined (Fig. 1). Pearson's rho coefficient for MOG mRNA levels versus clinical severity score was -0.53 ($p = 0.024$).

To evaluate whether changes in DJ-1 were induced by EAE, we extracted RNA from brains of EAE mice, at various disease-severity scores, and compared it with RNA extracted from brains of healthy littermates that served as controls. We discovered that EAE induced upregulation of DJ-1 mRNA (Fig. 2). Furthermore, the increase in DJ-1 mRNA was augmented as the disease severity increased, up to terminal stages, where a decline in DJ-1 mRNA levels was observed (Fig. 2). The changes in DJ-1 mRNA levels with EAE disease severity were significant ($p = 0.01$).

For further evaluation, DJ-1 protein levels, extracted from brains of EAE mice killed at various disease-severity scores, and brains of control mice, were assessed by using Western blot. We found that with increased disease severity, an increase of DJ-1 protein levels occurs, whereas in terminal stages of the disease, a decline in DJ-1 protein levels is noted (Figs. 3A and B). The pattern of the changes in DJ-1 protein

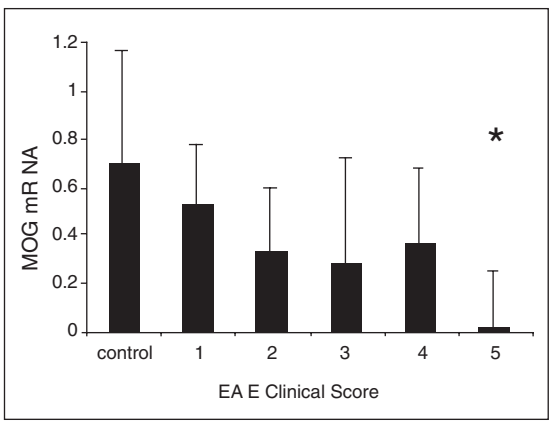

FIG. 1. Myelin oligodendrocyte glycoprotein (MOG) mRNA levels in brains of EAE mice declined as disease severity increased. Female C3H.SW mice (6–8 weeks old) were immunized twice by subcutaneous injection with MOG. Induced EAE mice were killed at different clinical disease severities. Total RNA was isolated from mice brain tissues, and real-time quantitative PCR of MOG was performed as described in Materials and Methods. Quantitative calculations of MOG versus GAPDH were done by using the ΔΔCT method. *$p < 0.05$ versus control.

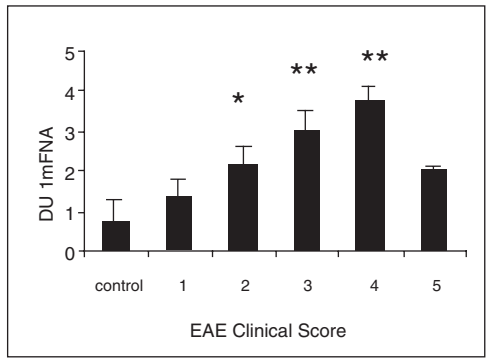

FIG. 2. mRNA levels of DJ-1 in brains of EAE mice. Female C3HSW mice (6–8 weeks old) were immunized twice by subcutaneous injection with MOG. Induced EAE mice were killed at different clinical disease severities. Total RNA was isolated from mice brain tissues, and real-time quantitative PCR of DJ1 was performed as described in Materials and Methods. Increased disease severity upregulated DJ1 mRNA levels, whereas in terminal stages, mRNA levels were downregulated. *$p < 0.05$, **$p < 0.01$ versus control.

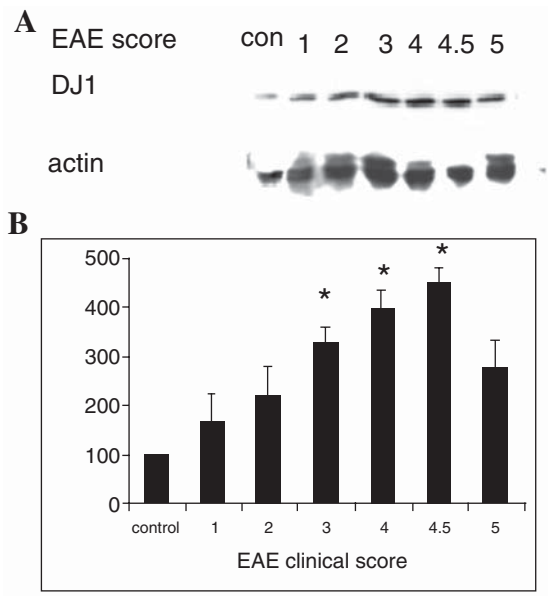

FIG. 3. DJ-1 protein expression levels in brains of EAE mice. Induced EAE mice were killed at different clinical disease severities. Proteins were extracted from brain tissues, and DJ-1 expression was determined by Western blot analysis, as described in Materials and Methods. (**A**) The membranes of Western blot were probed with rabbit anti-DJ-1 (1:5,000) and mouse anti actin (1:10,000) antibodies. (**B**) Quantification of DJ-1 levels per actin levels. Actin was used as an internal protein control. The data presented here are from a representative experiment repeated 3 times with similar results. Increased disease severity upregulated DJ-1 expression levels, whereas in terminal stages, DJ-1 levels were downregulated. *$p < 0.05$.

levels with increased EAE clinical severity is similar to the changes demonstrated in DJ-1 mRNA levels.

By using isoelectric focusing (IEF) for DJ-1, we found that in control brains, alkaline DJ-1 isoforms are the dominant DJ-1 form. EAE induced a shift toward the acidic isoforms (Fig. 4). Whereas mild disease caused a nonsignificant change in the partition of DJ-1 isoforms, as the disease severity increased to moderate and severe clinical disease, an increase in the acidic isoforms of DJ-1 existed at the expense of the alkaline ones (Fig. 4).

Next we analyzed the effect of SIN-I exposure on DJ-1 mRNA and protein levels *in vitro*, by using human glioma U-87 cells as a cellular model. SIN-I is a peroxynitrite free radical donor implicated in oligodendrocyte damage and MS pathology (29, 39, 43). SIN-I induced glial cell death with increasing concentrations, as evaluated by the MTT viability assay (Fig. 5). SIN-I exposure (0.5 m*M*) led to an increase in DJ-1 mRNA levels within 1 h ($p = 0.002$; Fig. 6). Exposure to 0.5 m*M* SIN-I also led to elevation of DJ-1 protein levels in glioma cells (Fig. 7A and B). These changes are in agreement with the DJ-1 changes seen in the brains of EAE mice described earlier.

To evaluate the distribution of DJ-1 in glial cells, we immunocytochemically stained U-87 glioma cells for DJ-1. The immunocytochemical staining demonstrated that DJ-1 is present both in the cytoplasm and the nuclei of these cells (Fig. 7C).

DISCUSSION

This study demonstrated changes in DJ-1 mRNA and protein expression levels in EAE. To our knowledge, this is the first report about the involvement of DJ-1 in EAE or MS. We observed that EAE caused the induction of DJ-1 mRNA as well as the upregulation of DJ-1 protein levels. Moreover, we found a correlation between DJ-1 mRNA and protein levels and clinical severity of the disease. In addition, EAE induces changes in the isoelectric point of DJ-1.

Evidence has suggested that DJ-1 plays an important role in the oxidative stress response and has a protective role against ROS-induced cell death. By short interfering RNA, DJ-1 knockdown rendered neuroblastoma cells susceptible to ROS-induced cell death (42, 48), whereas overexpression of DJ-1 dramatically reduced hydrogen peroxide treatment–induced death (24, 48). DJ-1 dosage effect is suggested because exposure of neurons derived from brains of DJ-$1^{+/+}$, DJ-$1^{+/-}$, and DJ-$1^{-/-}$ mice embryos to H_2O_2 led to a striking increase in DJ-1–deficient neuron death, whereas an intermediate amount of cell death was observed in DJ-$1^{+/-}$ neurons (24). Initial ROS accumulation (15 min after H_2O_2 treatment) was unaltered in DJ-1–deficient cells (30). However, 6 h after H_2O_2 exposure, DJ-1 knockout cells displayed increased apoptosis and robustly augmented protein carbonyl accumulation (30). These studies imply that DJ-1 may have a key role in the cellular defense mechanisms against oxidative stress.

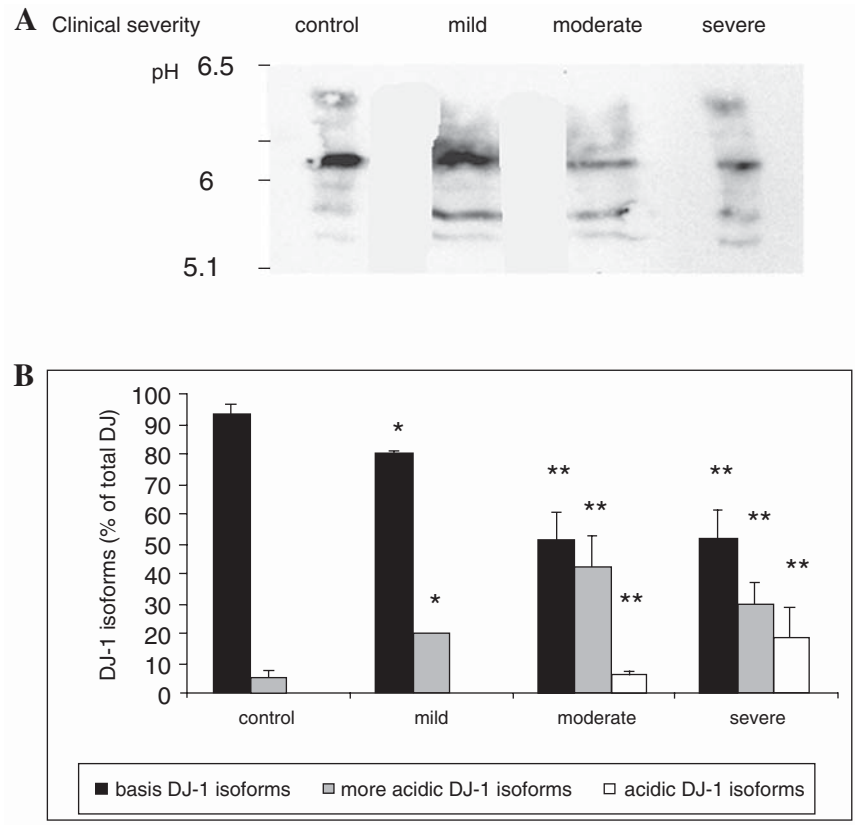

FIG. 4. Multiple isoforms of DJ-1 in brains of EAE mice. Induced EAE mice were killed at different clinical disease severities. Proteins were extracted from brain tissues, and DJ-1 pI was determined by isoelectric focusing (IEF), as described in Materials and Methods. IEF demonstrated a shift of DJ-1 from alkaline isoforms in control toward acidic isoforms in severe disease. (**A**) Blot of IEF gel. (**B**) Quantification of DJ-1 isoforms. *$p <$ 0.05, **$p < 0.01$ versus the control levels of the isoforms.

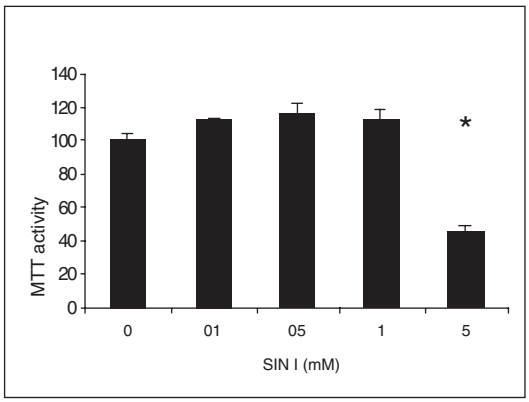

FIG. 5. Effect of SIN-I on glioma U-87 cell viability. Glioma U-87 cells were treated for 24 h with SIN-I at various concentrations (0.5–5 m*M*). Cell viability was evaluated by MTT assay, as described in Materials and Methods. *$p < 0.05$.

Oxidative stress is implicated in the pathophysiology of MS. Numerous studies of MS patients have shown increased free radical activity and deficiencies in important antioxidant enzymes compared with those in healthy controls (5, 20, 23, 27, 35, 46). Accumulation of protein carbonyls, lipid peroxidation products, and oxidative damaged DNA was detected in brains, CSF, and plasma of MS patients (5, 20, 23, 35). Increased reactive oxygen and nitrogen species production by leukocytes, mononuclear cells, and activated microglia in MS patients has also been demonstrated (15, 28, 45). Oxygen and nitrogen free radicals generated by activated microglia have been implicated as mediators of demyelination, oligodendroglial death, and axonal injury in both EAE and MS (6, 28, 44).

Peroxynitrite and nitric oxide, which was shown to be produced by reactive astrocytes via cytokine-mediated induction of nitric oxide synthase, are important mediators of oxidative stress in MS and EAE (12, 14, 29). As exposure to SIN-I led to ROS via peroxynitrite formation, we treated glioma U-87 cells with increasing concentrations of SIN-I. Elevated DJ-1 mRNA (Fig. 6) and protein levels (Fig. 7A and B) were observed in glioma cells exposed to SIN-I, similar to the increased DJ-1 levels observed in the brains of EAE mice (Figs. 2 and 3). These changes signify that upregulation of DJ-1 was indeed induced by oxidative stress.

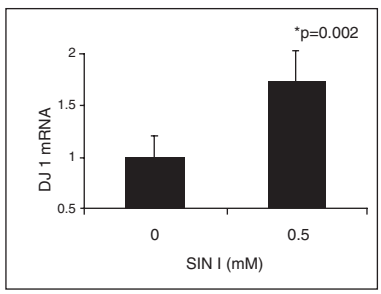

FIG. 6. SIN-I upregulated DJ-1 mRNA levels in U-87 glioma cells. U-87 glioma cells were treated for 1 h with SIN-I (0.5 m*M*). Total RNA was isolated from the cells and real-time quantitative PCR of DJ-1 was performed, as described in Materials and Methods. Quantitative calculations of DJ-1 versus GAPDH were done by using the ΔΔCT method. SIN-I exposure induced increased DJ-1 mRNA levels. *$p < 0.01$.

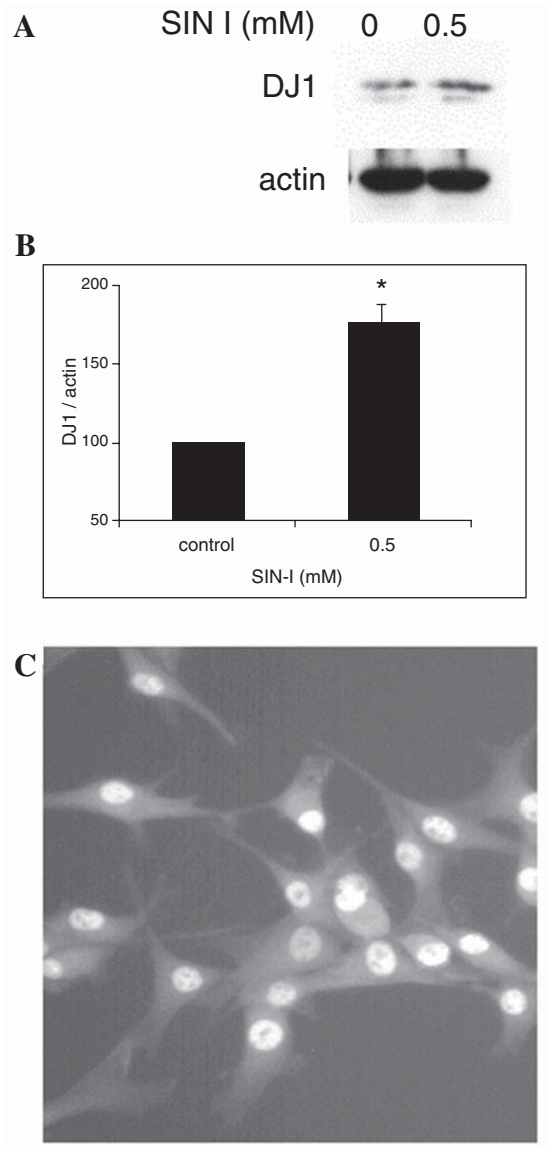

FIG. 7. SIN-I induced upregulation of DJ-1 expression in U-87 glioma cells. Proteins were extracted from cells treated with 0.5 m*M* SIN-I for 24 h. (**A, B**) DJ-1 expression levels were determined by Western blot analysis, as described in Materials and Methods. (**A**) The membranes of Western blot were probed with rabbit anti-DJ-1 (1:5,000) and mouse anti-actin (1:10,000) antibodies. (**B**) Quantification of DJ-1 levels per actin levels. Actin was used as an internal protein control. The data presented here are from a representative experiment repeated 3 times with similar results. *$p < 0.05$. (**C**) DJ-1 is present both in the cytoplasm and the nuclei of U-87 glioma cells, as shown by immunocytochemistry. In control cells, in which anti-DJ-1 antibody was withdrawn from the samples, no staining occurred. Original magnification, ×20.

Interleukin (IL)-1α/β and tumor necrosis factor (TNF)-α/β play a predominant role in the inflammatory reaction in MS and EAE (4, 8, 38). These cytokines were found to inhibit the expression of myelin genes, MOG, CNPase, and PLP, in human primary oligodendrocytes. These effects were blocked by antioxidants, indicating that they are mediated through alteration of cellular redox (21). Gilgun-Sherki *et al*. (19) also reported that MOG mRNA levels in EAE are lower than those in controls. The reduced MOG mRNA levels in EAE brains demonstrated by us (Fig. 1) support these studies. Moreover, we found that MOG mRNA levels progressively decrease with increased disease severity in the brains of EAE mice, thus implying increased oxidative stress with elevated disease-severity score.

Similar to the changes in DJ-1 isoforms described in brains of parkinsonian patients (2), by using isoelectric focusing, we found an increase in the more acidic DJ-1 isoforms in EAE brains (Fig. 4). As the clinical severity of EAE increased, we observed a shift in the distribution of DJ-1 isoforms toward an augmentation of the more acidic isoforms. *In vitro* studies demonstrated that the shift toward more acidic isoforms results from oxidation of cysteine residues on DJ-1 protein, and is dependent on the severity of the oxidative stress (2, 9, 33). It has been suggested that the shift in the pI of DJ-1 can serve as a useful indicator of oxidative stress status both *in vivo* and *in vitro* (2, 33). The changes in DJ-1 pI isoforms found in our study accompanied disease severity; therefore, we believe that they correspond to increased levels of oxidative stress in the brains with the progression of EAE.

Koch *et al.* (26) demonstrated that increased ROS formation occurs in all subgroups of MS. However, significant positive correlations have been established between spontaneous and PMA-induced production of ROS in leukocytes isolated from MS patients and clinical parameters calculating disease disability such as the expanded disability status scale (EDSS), as well as brain lesions load evaluated by MRI and visual evoked potentials (15). Increased ROS with augmented disease activity supports our findings of enhanced DJ-1 mRNA and protein levels as well as accumulation of acidic DJ-1 isoforms, with the progression of EAE.

In moribund EAE mice, we found a decline in DJ-1 mRNA (Fig. 2) and protein levels (Fig. 3), although the shift toward acidic isoforms is retained (Fig. 4). A possible explanation for these results is that DJ-1 is part of the cellular defense systems coping with oxidative stress. The oxidative stress inflicted on the CNS increases as the clinical severity of EAE increases, upregulating DJ-1 expression. However, in terminal disease stages, the defense mechanisms fail, and DJ-1 levels also decrease. Nevertheless, oxidative stress is still elevated, even in terminal disease stages, leading to a shift in the partition of DJ-1 isoforms, increasing the relative amount of acidic isoforms. This observation can serve as a diagnostic tool, helping to determine disease activity and oxidative stress levels. The immunostaining for DJ-1, both in the cytoplasm and in the nucleus, may indicate that DJ-1 has multiple functions in the cells, yet to be discovered.

In conclusion, our study demonstrated changes in DJ-1 levels and pI isoforms in the brains of EAE mice, as well as in glioma cells exposed to SIN-I–induced ROS. We found that with increased disease severity, DJ-1 mRNA and protein levels increase, whereas in terminal stages of the disease, they are reduced. These results, combined with the previously cited evidence on the role of DJ-1 in oxidative stress, imply that EAE induction leads to the accumulation of oxidative stress, which is augmented with disease severity and which produces the upregulation of DJ-1 as part of the defense mechanisms. The decreased DJ-1 levels in terminal stages of the disease may result from overwhelming damage and the collapse of the cellular protective mechanisms, rendering them incapable of coping with the damage.

To the best of our knowledge, the involvement of DJ-1 in EAE is reported for the first time in this study. In view of the accumulating evidence concerning the central role of oxidative stress in MS, and the importance in the CNS of DJ-1 in oxidative stress management, we believe that DJ-1 will be found to have a central role in MS. Further studies on the involvement of DJ-1 in MS, and possible ways to assess DJ-1 levels and pI isoforms changes in MS patients are desired. Detection of DJ-1 changes with altered disease activity in MS patients may lead to the development of a method to assist in the early and accurate diagnosis of the increase in disease activity and might be a basis for therapeutic intervention.

ABBREVIATIONS

CNS, Central nervous system; CSF, cerebrospinal fluid; CFA, complete Freund's adjuvant; EDSS, expanded disability status scale; EAE, experimental autoimmune encephalomyelitis; FCS, fetal calf serum; IL, interleukin; IEF, isoelectric focusing; MDA, malondialdehyde; MS, multiple sclerosis; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; MOG, myelin oligodendrocyte glycoprotein; PD, Parkinson disease; PMA, phorbol-12-myristate-13-acetate; PCR, polymerase chain reaction; ROS**,** **r**eactive oxygen species; SIN-I, 3-(4-morpholinyl)-sydnonimine; TNF, tumor necrosis factor.

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