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Edaravone, a free radical scavenger, ameliorates experimental autoimmune encephalomyelitis

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ABSTRACT

Reactive oxygen species (ROS) are implicated in the pathogenesis of multiple sclerosis (MS) and its murine model experimental autoimmune encephalomyelitis (EAE). The effect of edaravone, a free radical scavenger, on EAE was investigated in this study. Treatment with edaravone significantly ameliorated the clinical severity of EAE, and a reduced infiltration of lymphocytes was observed based on a histological analysis. The expression of inducible NO synthase (iNOS) in the spinal cords appeared to be reduced by the treatment with edaravone and this effect was confirmed *in vitro*. A reduction of both the cellular infiltration and the expression of iNOS may therefore underlie the mechanisms of the beneficial effect of edaravone on EAE.

Experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease of the central nervous system (CNS) provoked by myelin antigens, is widely used as an animal model of multiple sclerosis (MS) [26]. Evidence has been accumulating suggesting the association of reactive oxygen species (ROS) with the pathogenesis of EAE and MS [7,8,14,15]. ROS themselves directly cause demyelination, and they also activate nuclear transcription factor kappa B (NF-κB), thus resulting in the up-regulation of tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS). Compounds scavenging ROS have been reported to ameliorate the severity of EAE in several studies [9,10,16,19–21]. α-Lipoic acid (LA) reduces the expression of adhesion molecules and inhibits migration of T cells and monocytes [4,17,21]. Uric acid, another antioxidant, reduces the expression of inflammation-related molecules in the CNS such as iNOS and nitrotyrosine, a marker of peroxynitrite reactivity [24].

Edaravone is a free radical scavenger which potently reduces hydroxyl radicals (OH^-). There is significant evidence of its beneficial effect on experimental models of ischemia [1,11,13,22,27,28]. It has been administered to a large number of patients with cerebral infarction since it was approved by the Japanese Ministry of Health, Labor and Welfare in 2001. It has shown an inhibitory effect on the expression of iNOS in astrocytes and the production of nitric oxide (NO) by microglia [3,27]. Therefore, the effects of edaravone on EAE were examined and its underlying mechanisms were also investigated.

Female SJL mice of 8 weeks old (Nippon SLC, Hamamatsu, Japan) were immunized with 150 μg proteolipid protein $(PLP)_{139–151}$ peptide (HSLGKWLGHPDKF, Biologica, Nagoya, Japan) in complete Freund's adjuvant (CFA) supplemented with 200 μg of H37Ra mycobacterium tuberculosis (Difco, Detroit, MI, USA) as an actively induced EAE model. Edaravone was kindly provided by the Mitsubishi Wellphama Co. and was administered intraperitoneally twice a day from 5 days post-immunization (dpi) to 19 dpi (6 mg/kg/day, $n = 16$). The same volume of physiological saline was used as control ($n = 15$). The severity of EAE was evaluated in a blinded fashion using following scale as previously described [18]. 0, normal; 1, limp tail; 2, mild paraparesis of the hind limbs with unsteady gait; 3, moderate paraparesis with preservation of voluntary movement; 4, paraplegia; 5, moribund. All experimental procedures were approved by the institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine.

Three mice from each group were randomly selected and sacrificed on 12 dpi and their lumbar spinal cords were prepared for histological and immunohistochemical analyses. Their

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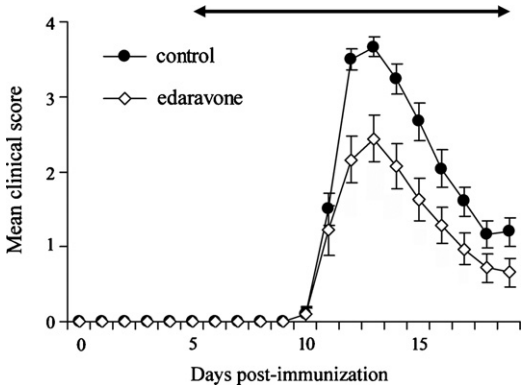

Fig. 1. Clinical severity of EAE. The mean maximum clinical score (2.6 ± 0.32) of mice treated with edaravone (open diamonds, $n = 16$) was significantly reduced in comparison to that (3.8 ± 0.08) of control mice (black circles, $n = 15$). $p < 0.01$. An arrow above the graph indicates duration of the treatment. Data are expressed as the mean ± S.E.M.

mean clinical scores were 2.0 in the edaravone-treated group and 3.3 in the vehicle-treated group. Five paraffin sections (4 μm thick) were cut from each tissue specimen at 50 μm intervals and thirty sections in total were stained with hematoxylin and eosin (H&E). The number of inflammatory foci per section was counted in a blinded fashion. For immunohistochemical analysis, cryosections (10 μm thick) were incubated with hamster anti-CD3 antibody (1:40, BD Biosciences, Tokyo, Japan), rabbit anti-Iba-1 antibody (1:200, Wako Pure Chemical Industries, Osaka, Japan), or rabbit anti-iNOS antibody (1:50, BD Biosciences) in PBS containing 0.1% bovine serum albumin at 4 °C overnight. The sections were then incubated with biotin-conjugated secondary antibodies for 2 h and then were incubated with streptavidin-conjugated Alexa Fluor 488 (1:200, Molecular Probes, Eugene, OR, USA) for 30 min.

The microglial cell line, GMI-M6-3 was maintained as previously described [12]. The cells were incubated with edaravone for 24 h, followed by incubation with recombinant mouse IFN-γ (10 U/ml, R&D Systems) and agonistic anti-CD40 antibody (1 μg/ml, HM40-3, BD Bioscience) in the presence of edaravone. The cells were collected 24 h later and their protein extracts were subjected to a Western blot analysis. The blots were incubated with rabbit anti-iNOS (1:200) or mouse anti-β-actin (1:20000, Sigma) antibodies at 4 °C overnight, and then were subsequently incubated with appropriate secondary antibodies conjugated with horseradish peroxidase, then visualized by enhanced chemiluminescence (ECL) reagents (GE Healthcare Bio-sciences, Uppsala, Sweden). The image of the film was captured and analyzed using Fluorchem IS 8000 (Alpha Innotech, San Leandro, CA, USA). The relative intensity of each band was determined and normalized to the intensity of β-actin. The Mann–Whitney *U* test was used for the statistical analysis for clinical scores. $p < 0.05$ was considered to be significant.

The treatment with edaravone reduced the mean clinical score of EAE (Fig. 1). The mean maximum clinical score [2.6 ± 0.32 (mean ± S.E.M.)] in the edaravone-treated group was significantly lower than that (3.8 ± 0.08) of the vehicle-treated group ($p < 0.01$). Since the beneficial effect of edaravone on the severity of EAE was recognized, we next evaluated its effect on inflammatory cellular infiltration in the spinal cord. A marked reduction in the inflammatory cellular infiltration in the spinal cords was observed in the edaravone-treated mice in contrast to the control mice with EAE (Fig. 2A and B), and the number of inflammatory foci per section tended to be reduced in the edaravone-treated group (Fig. 2C). An immunohistochemical analysis revealed that CD3-immunoreactive (IR) T lymphocytes, which were markedly infiltrated into the spinal cords of the control mice with EAE, were reduced by the treatment with edaravone (Fig. 2D and E). The effect of edaravone was investigated on the morphology of microglia and iNOS expression in the spinal cord of EAE because edaravone has an inhibitory effect on the activation of microglia and the expression of iNOS [3,27]. Many of the Iba-1-IR cells in the spinal cords of control mice with EAE exhibited morphological change consistent with activated microglia, while many of those in edaravone-treated mice looked less activated (Fig. 2F and G). The expression of iNOS was seen especially in the perimeningeal region of the spinal cords of control mice with EAE, but these iNOS expressing cells, presumably macrophages, were scarcely seen in the edaravone-treated mice (Fig. 2H and I). The expression of iNOS in microglia was induced by co-stimulation with agonistic anti-CD40 antibody and IFN-γ, and this expression was inhibited by the incubation with edaravone in a dose-dependent manner (Fig. 3).

The reduction of cellular infiltration in the spinal cords of edaravone-treated mice may suggest that edaravone influences the trafficking of immune cells through the BBB. α-Lipoic acid (LA) inhibits transmigration of lymphocytes by the inhibition of very late activation antigen-4 (VLA-4) expression and matrix metalloproteinase-9 (MMP-9) synthesis [17]. This inhibitory effect is explained by its inhibitory effect on the NF-κB activation [16,17,25]. Schreibelt et al. reported that LA inhibits transendothelial migration of monocytes by the reduction of cytoskeletal rearrangement of endothelial cells [21]. Given these facts, the inhibitory effect of edaravone on the infiltration of immune cells observed in the current experiment may be associated with the reduction of adhesion molecules or MMPs or with the regulation of cytoskeletal changes in endothelial cells.

In the pathogenesis of EAE, activated lymphocytes infiltrating into the CNS produce pro-inflammatory cytokines, which is augmented by interaction with non-professional APC such as microglia. Microglia are also activated in these circumstances and increase the production of inflammatory mediators such as TNF-α, iNOS and a large quantities of ROS including superoxide (O_2^-), OH^-, hydrogen peroxide (H_2O_2) and NO. ROS themselves can activate NF-κB, which thus leads to a further up-regulation of inflammatory molecules [7]. iNOS catalyzes large amount of NO synthesis in pathological conditions including EAE and MS, and the pathogenic role of iNOS in EAE has been shown by the fact that knockdown of iNOS and an inhibitor of iNOS ameliorate EAE [5,6]. It is also suggested that NO may cause mitochondrial dysfunction which leads to axonal degeneration [23]. In addition, a recent study revealed that the iNOS expression in macrophages is associated with functional disturbances of axons in adoptive-transferred EAE [2]. In the present study, the expression of iNOS in macrophages/microglia appears to be reduced in the spinal cords of edaravone-treated mice with EAE and the microglial production of iNOS is inhibited by the addition of edaravone *in vitro* (Figs. 2H and I and 3). These results are consistent with those of previous reports [3,27]. This inhibitory effect of edaravone on iNOS expression may therefore contribute, at least in part, to its beneficial effect on the severity of EAE.

In summary, the current study demonstrated that edaravone ameliorates the severity of EAE. Edaravone attenuates inflammatory cellular infiltration into the CNS and seems to suppress the activation of macrophages/microglia and the expression of iNOS. These mechanisms may together contribute to its beneficial effect on EAE. The dose of edaravone administered in this study (6 mg/kg/day) is equal to that applied to a model of ischemia in previous reports [1,22,27] and no adverse effects were observed. Because edaravone has been administered to patients experiencing a stroke for 6 years without any severe adverse effects, this drug may therefore also be applicable to the treatment of MS.