Neuroscience 162 (2009) 317–327

EDARAVONE ATTENUATES WHITE MATTER LESIONS THROUGH ENDOTHELIAL PROTECTION IN A RAT CHRONIC HYPOPERFUSION MODEL

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Abstract—A multicenter randomized clinical trial demonstrated that acute ischemic stroke patients treated with edaravone, a scavenger of hydroxyl radicals, had significant functional improvement. We tested the hypothesis that edaravone has protective effects against white matter lesions (WML) and endothelial injury, using a rat chronic hypoperfusion model. Adult Wistar rats underwent ligation of bilateral common carotid artery (LBCCA) and were divided into the edaravone group (injected once only immediately after LBCCA [n=39, ED_1]; and injected on three consecutive days [n=39, ED_3]), the vehicle group (n=39), and the sham group (n=15). Cerebral blood flow, Morris water maze performance, footprint test for locomotor function, immunohistochemical analyses and Western blot analysis were performed before and after LBCCA. The ED_3 group upregulated endothelial nitric oxide synthase and attenuated Evans Blue extravasation at day 3 after LBCCA (P<0.05). Edaravone markedly suppressed accumulation of 4-hydroxy-2-nonenal-modified protein and 8-hydroxy-deoxyguanosine (P<0.01), and loss of oligodendrocytes (P<0.05) in the cerebral white matter at days 3, 7, 14, 21 and 28 after LBCCA. These results were more evident in the ED_3 group. Moreover, at day 21 after LBCCA, spatial memory but not motor function, and axonal damage were significantly improved by three-time treatment of edaravone (P<0.05). Our results indicated that 3-day treatment with edaravone provides protection against WML through endothelial protection and free radical scavenging and suggested that edaravone is potentially useful for the treatment of cognitive impairment.

Key words: chronic hypoperfusion, hydroxyl radical scavenger, white matter injury, 4-hydroxy-2-neonenal, endothelial nitric oxide, endothelial dysfunction.

About 50% of the adult brain is composed of cerebral white matter (Miller et al., 1980), which is involved in almost all instances of ischemic stroke. The cerebral white matter consists of abundance of oligodendrocytes, lipid-rich contents of the myelin sheath, and low levels of intrinsic antioxidants. Oligodendrocytes are vulnerable in ischemic insult, and consequently, cerebral white matter can be an enormous source of reactive oxygen and nitrogen species (Sommani et al., 2007). The disorders of small perforating cerebral arteries can cause cerebral white matter lesions (WML), and also explain the progression of WML. In the laboratory, WML can be induced in rat brains under chronic cerebral hypoperfusion by permanent occlusion of both common carotid arteries (Farkas et al., 2007; Watanabe et al., 2006). Such process can also induce endothelial injury in this model (Ueno et al., 2002).

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one) was the first neuroprotective agent approved in 2001 for clinical use in Japan. Recently, a Japanese multicenter randomized clinical trial demonstrated that edaravone significantly reduced brain infarct volume and produced sustained benefits in functional outcomes during a 12-month follow-up (Edaravone Acute Infarction Study Group, 2003). Experimental studies have also documented the brain-protective role of this agent: (1) reduction of brain edema (Abe et al., 1988; Nishi et al., 1989), (2) inhibition of vascular endothelial injury (Watanabe et al., 1988), (3) extension of the therapeutic time window (Zhang et al., 2004), and (4) improvement of neurological deficits (Watanabe et al., 1994). Edaravone can scavenge hydroxyl radicals and peroxynitrite, although it has no major effect on superoxide anion radicals (Banno et al., 2005). Moreover, we have recently reported that edaravone suppressed the accumulation of both 4-hydroxy-2-neonenal (HNE) modified protein and 8-hydroxy-deoxyguanosine (8OHdG) and reduced the sequential inflammatory response in ischemia/reperfusion models (Zhang et al., 2005).

However, little or no information is available on the roles of edaravone in WML and endothelial injury in chronic hypoperfusion. This is important since endothelial injury and blood–brain-barrier (BBB) permeability play important roles in the progression of WML as well as lacunar ischemic stroke involving the occlusion of small perforating cerebral arteries (Wardlaw et al., 2003, 2008). Moreover, recent evidence indicates that edaravone improves functional outcome of patients with lacunar stroke (Mishina et al., 2005). In the present study, we tested the hypothesis that edaravone provides protection against WML and endothelial injury using a rat model of chronic hypoperfusion.

EXPERIMENTAL PROCEDURES

Experimental groups and surgical procedures

All animals used in the present study were acquired and cared for in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Abbreviations: BBB, blood–brain barrier; CBF, cerebral blood flow; eNOS, endothelial nitric oxide synthase; GFAP, glial fibrillary acidic protein; HNE, 4-hydroxy-2-neonenal; LBCCA, ligation of bilateral common carotid arteries; LDL, low-density lipoprotein; PBS, phosphate buffer saline; vWF, von Willebrand factor; WML, white matter lesions; 8OHdG, 8-hydroxy-deoxyguanosine.

0306-4522/09 $ - see front matter
doi:10.1016/j.neuroscience.2009.04.065

All animal procedures were conducted after obtaining approval of the animal care committee of Juntendo University, and efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats (9–11-week-old rats) weighing 280–320 g were purchased from Charles River Institute (Kanagawa Japan) and maintained on a 12-h light/dark cycle with continuous access to food and water. To occlude both common carotid arteries, the rats were anesthetized with 1.0%–2.0% isoflurane in 30% oxygen and then anesthesia was maintained during operation with 70% nitrous oxide. During surgery, a temperature probe was inserted into the rectum, and a heat lamp was applied to maintain body temperature at 37.0–37.5 °C. Through a midline incision, each common carotid artery was carefully separated from the cervical sympathetic and vagal nerves and ligated permanently. At baseline (before ligation of bilateral common carotid arteries [LBCCA]) or at days 3, 7, 14, 21 and 28 after LBCCA, the rats were re-anesthetized with 1% isoflurane and 70% N_2O:30% O_2, and then transcardially perfused with phosphate buffer saline (PBS) and 4% paraformaldehyde. The brain was dissected out immediately, postfixed in 4% paraformaldehyde for 48 h, and stored in 30% sucrose in 0.1 M PBS. For immunohistochemistry, 20-μm-thick free-floating coronal sections of the corpus callosum were prepared for staining.

Drug administration and classification

Rats were divided randomly into the following four groups: (1) the ED_1 group: 55 rats of this group were treated with tail vein injection of edaravone (Mitsubishi, Tanabe Pharma Co., Osaka, Japan) at 3 mg/kg body weight immediately after LBCCA using the method described previously (Yoshida et al., 2005) for 1 day. Sixteen rats died after LBCCA and the injection and the remaining 39 rats were used in the study. (2) The ED_3 group: 52 rats of this group were injected i.v. with 3 mg/kg body weight edaravone immediately after LBCCA once every 24 h for three consecutive days. Thirty-nine rats survived and were used in this study. (3) The vehicle group: 65 rats of this group underwent LBCCA and received i.v. injection of saline solution at a volume similar to edaravone. Thirty-nine rats survived the procedure and were used in the study. (4) The control sham-operated groups: 15 rats underwent the protocol described for the vehicle group, but were spared LBCCA. All 15 rats survived and were used in the experiments. Edaravone was administered while the animal was anesthetized with halothane. To exclude the effect of the anesthesia, saline solution was injected i.v. in the remaining two days in the ED_1 group, and in three consecutive days in the vehicle and sham-operated groups after the procedures.

Measurement of cerebral blood flow (CBF)

CBF was measured in a left temporal window using laser Doppler flowmetry (Laser Tissue Blood Flow Meter FLO-C1; Omega Wave, Inc.). The probe in the shape of straight rectangular sheet (7.5 mm in length and 1.0 mm in-depth) was positioned between the temporal muscle and the lateral aspect of the skull as described previously (Harada et al., 2005). In these experiments, there was no need for craniotomy. CBF was monitored continuously for 3–5 min at each time, before, immediately after, and at days 3, 7, 14, 21 and 28 after LBCCA. Reproducible recorded CBF velocity was obtained.

Water maze task

The water maze task was performed to evaluate any LBCCA-induced learning deficits using the method described previously (Gerlai, 2001). In a 150-cm-diameter circular pool filled with 20-cm-deep water set at 22±1 °C, a circular transparent acrylic platform was prepared, the top surface of which was 3 cm below the water. Water was clouded with milk so as to hide the underwater platform. Rats were released facing the wall from one standard point, and the time taken to escape to the platform was recorded as the escape latency. Each rat performed five trials per day, with a constant inter-trial interval of 30 min, for 3 consecutive days before LBCCA. The escape latency was analyzed before LBCCA and at days 7, 14 and 21 after LBCCA.

Footprint test

For evaluation of locomotor function, footprint analysis was performed as described previously (de Medinaceli et al., 1982). The fore- and hindfeet of the rats were stained with red and blue dye (nontoxic), respectively. Then the animals were trained to walk on a paper-covered narrow runway (1 m long, 7 cm wide). The narrow runway ensured that the animals walked along a straight path. To prevent the rats from pausing while passing the track, a very bright box was placed at the beginning of the runway, and a dark box with food was placed at the end of the track to encourage them to finish the task as fast as possible. To perform the measurements, we excluded the first and the last 15 cm of the footprints. If the rats stopped in the middle of the track, the test was repeated. Hind limb stride and interlimb coordination were measured bilaterally. For analyses, at least four steps from each side per print were measured (n=4, each).

Immunohistochemistry

After incubation in 0.3% H_2O_2 followed by 10% block ace in 0.1% PBS(−), the coronal sections of the corpus callosum were immunostained overnight at 4 °C using a mouse monoclonal antibody against HNE (dilution, 40:1; Japan Institute for the Control of Aging) to assess lipid peroxidation, a mouse monoclonal antibody against 8OHdG (dilution, 100:1; Japan Institute for the Control of Aging) to assess oxidative DNA damage, and a rabbit polyclonal antibody against glutathione-*S*-transferase-pi (GST-pi; dilution, 1000:1; Chemicon International, Inc., Temecula, CA, USA) to assess mature oligodendrocytes. The sections were then treated with secondary antibodies (dilution, 300:1; Vectastain; Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was visualized by the avidin–biotin complex method (Vectastatin, Vector Laboratories) and developed with diaminobenzidine.

Klüver-Barrera staining

The corpus callosum was evaluated for WML by Klüver-Barrera staining. The myelin areas in three sections per animal and both sides of selected areas were stained with Luxol Fast Blue. The severity of WML was graded as 0 (normal), 1 (disarrangement of nerve fibers), 2 (formation of marked vacuoles), and 3 (disappearance of myelinated fibers), according to the previously described grading system (Wakita et al., 2002).

Immunofluorescence staining

Double immunofluorescence staining and confocal laser scanning microscopy (Axiovert 100 mol/L; Carl Zeiss, Microimaging, Tokyo, Japan) were performed. The primary antibodies used were rabbit polyclonal antibody against 3-nitrotyrosine (dilution, 1:50; Upstate Biotechnology, Lake Placid, NY, USA), and mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; dilution, 1:1000; Sigma Aldrich, Inc., St. Louis, MO) as a marker of astrocytes. FITC-conjugated donkey anti-mouse IgG antibody (dilution, 1:300; Amersham, Buckinghamshire, UK) and goat anti-rabbit IgG biotinylated antibody (dilution, 1:300; Vector Laboratories) were used as secondary antibodies. Staining for capillary endothelial cells of the corpus callosum was also performed using mouse monoclonal antibody against endothelial nitric oxide synthase (eNOS; dilution, dilution, 250:1; BD Transduction Laboratories, San Jose, CA, USA) and rabbit anti-human antibody against von Willebrand factor (vWF; dilution, 1:200; Dako, Carpentaria, CA, USA), an endothelial cell marker, as the primary antibodies. FITC-conjugated donkey anti-rabbit IgG antibody (1:300; Amersham)