Edaravone Diminishes Free Radicals from Circulating Neutrophils in Patients with Ischemic Brain Attack

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Abstract

Objective Treatment with a free radical scavenger could be a new option for ischemic brain attack, however, little is known about the alteration of oxidative stress markers induced by edaravone, a novel free radical scavenger, in human ischemic brain attack.

Methods We investigated the effects of edaravone on the oxidative stress markers in patients with ischemic brain attack. Twenty-one patients with ischemic brain attack and 19 controls were enrolled in this study. Blood samples were obtained just before and soon after the first administration of edaravone (30 mg) or ozagrel (40 mg). Intracellular reactive oxygen species of neutrophils were measured using 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate and a fluorescence-activated cell sorter. Superoxide from neutrophils, induced by phorbol myristate acetate (PMA), was determined by luminol-amplified chemiluminescence assay.

Results Treatment with 30 mg of edaravone significantly decreased the intracellular reactive oxygen species (ROS) of neutrophils (Wilcoxon test, $p=0.0001$) and PMA-induced superoxide produced by neutrophils (Wilcoxon test, $p=0.001$). Ozagrel did not alter the intracellular ROS or superoxide production of neutrophils.

Conclusion Reduction of intracellular ROS and suppression of superoxide production in neutrophils provide a potential explanation for the clinical efficacy of edaravone in patients with ischemic brain attack.

Key words: edaravone, free radical scavenger, reactive oxygen species, neutrophil, ischemic brain attack

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Introduction

Accumulating evidence implicates free radicals in the damage of ischemic brain attack, and free radical scavengers have been proposed as a new clinical strategy. A novel free radical scavenger, edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one), has been shown to prevent vascular endothelial injury *in vitro* (1) and cerebral damage following middle cerebral artery occlusion (2), delayed neuronal death in transient cerebral ischemia (3) and ischemic brain edema in rats (4, 5). Edaravone has been found to inhibit activation of the lipoxygenase pathway in the arachidonic acid cascade

(6) and peroxidation of the phosphatidylcholine liposomal membrane (7), as well as to scavenge hydroxyl radicals.

The clinical efficacy of edaravone on ischemic brain attack has been demonstrated by significant improvement in the functional outcome in a randomized, placebo-controlled, double-blind study (8). However, to date no data is available on the alteration of oxidative stress markers due to edaravone in human ischemic brain attack. We investigated the effects of edaravone on oxidative stress markers of circulating neutrophils in patients with ischemic brain attack.

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Figure 1. Intracellular reactive oxygen species of circulating neutrophils in patients with ischemic brain attack and controls. **A:** Typical flow cytogram of neutrophils (surrounded by an oval). Intracellular reactive oxygen species are presented as relative fluorescence units, i.e., the median channel value of the fluorescing cell population. SS = side scatter dot plot. **B:** Relative fluorescence units of neutrophils before and after treatment with 30mg of edaravone or 40 mg of ozagrel.

Patients and Methods

Twenty-one patients (14 males and 7 females, mean $\pm$ SD; 71 $\pm$ 2 years old, range 52-88) with ischemic brain attack and 19 untreated healthy controls (11 males and 7 females, mean $\pm$ SD; 51 $\pm$ 4 years old, range 23-79) were enrolled in this study under informed consent. Edaravone treatment was started for the patients within 24 hours after the onset of ischemic brain attack. Patients were administered 30 mg of edaravone i.v. over 1 hour twice a day for 3-10 days. Ten patients were treated with 40 mg of ozagrel, a thromboxane A2 synthase inhibitor, over 1 hour before the treatment with edaravone. Blood samples were obtained just before and soon after the first administration of edaravone or ozagrel. Glycerol was used for the patients after the first treatment with edaravone.

To measure intracellular reactive oxygen species of neutrophils, venous peripheral blood was incubated in NH_4Cl (8.26 g/L) KHCO_3 (1.0 g/L) and tetrasodium ethylenediaminetetraacetic acid (0.037 g/L) in Chelex 100-treated water (pH 7.3) for 10 min to lyse erythrocytes, washed with ice-cold 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer and then centrifuged at 1,000 g at 4°C for 5 min. The supernatant was discarded. Neutrophils were resuspended in 1 ml HEPES buffer, and 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (CDCFH-DA; Molecular Probes, Netherlands) (10 μM final concentration) was added to each sample tube; and the tubes were incubated at 37°C

for 30 min before data acquisition by flow cytometry. A fluorescence-activated cell sorter (EPICS XL/XL-MCL System II v3.0, Becton Dickinson) and WinMIDI 28 software were used for acquisition and analysis of data. The stability of instrumental settings including spectral compensations was checked regularly with fluorescent beads (Calibrite, Becton Dickinson). For each 1 ml aliquot of cell suspension, an appropriate electronic gate was generated, and 5,000 events were collected in live mode. Neutrophils were delineated in a forward/side scatter dot plot, and appropriate fluorescence histograms were developed. Fluorescence intensities are reported in relative fluorescence units, i.e., the median channel value of the fluorescing cell population (Fig. 1A).

To measure superoxide produced by neutrophils, neutrophils were resuspended in 1 ml of HEPES buffer by the above method and adjusted to the density of 2×10^5 /ml. Superoxide production by phorbol myristate acetate (PMA) was determined by luminol-amplified chemiluminescence assay using a luminometer (BLR-301, ALOKA, Japan). Neutrophils were stimulated with PMA (0.1 nmol/L), and the resulting light output was continuously recorded on a chart recorder. All results are expressed as kilo-counts per minute (kcpm) using the chemiluminescence emission peak (Fig. 2A).

Results

The controls showed no significant relationship between

Figure 2. Superoxide production of circulating neutrophils in patients with ischemic brain attack and controls. **A:** Typical light output continuously recorded on a chart recorder, reflecting amount of superoxide produced by neutrophils stimulated by phorbol myristate acetate (PMA). **B:** Amount of superoxide produced by neutrophils stimulated by PMA before and after treatment with edaravone or ozagrel. All results are expressed as kilo-counts per minutes (kcpm) using the chemiluminescence emission peak.

age and relative fluorescence units of circulating neutrophils treated with CDCFH (simple regression analysis, $p=0.1832$) or between age and superoxide production by neutrophils (simple regression analysis, $p=0.2406$). The relative fluorescence units of circulating neutrophils in CDCFH in patients with ischemic brain attack before treatment with edaravone or ozagrel (36.8 ± 5.9 , mean $\pm$ SE) was higher than that of controls (19.1 ± 1.8) (Mann-Whitney U test, $p=0.0262$). The relative fluorescence units in CDCFH decreased after treatment with edaravone (26.8 ± 3.8) (Wilcoxon test, $p=0.0001$), indicating that edaravone lowered the intracellular ROS of circulating neutrophils (Fig. 1B). In contrast, the relative fluorescence units in CDCFH showed no significant alteration after the treatment with ozagrel (38.3 ± 7.2).

Amount of superoxide produced by neutrophils in patients with ischemic brain attack tended to be higher than those of controls (Mann-Whitney U test, $p=0.0578$). The superoxide production by neutrophils decreased after treatment with edaravone in patients with ischemic brain attack (Wilcoxon test, $p=0.001$; Fig. 2B). The amount of superoxide from neutrophils showed no significant alteration after the treatment of ozagrel. In one patient, we happened to obtain a blood sample one day before ischemic brain attack. The preliminary data showed that the superoxide produced by neutrophils after ischemic brain attack was increased to 135% of that before ischemic brain attack, indicating an increase in inducible superoxide from neutrophils after ischemic brain attack. No adverse events were observed in patients treated with 30 mg of edaravone.

Discussion

Brain damage in ischemic brain attack is closely related to inflammatory responses, especially infiltration of circulating neutrophils into ischemic tissue (9). Many studies show that inhibition of neutrophil accumulation greatly reduces infarct size (10, 11). Neutrophil accumulation in vessels requires interaction between several adhesion molecules, such as intracellular adhesion molecule (ICAM)-1. Interleukin (IL)-1 β and tumor necrosis factor- α which upregulate ICAM (12) are increased in ischemic brain tissue. Ischemia induces the formation of IL-1, the subsequent expression of ICAM-1, and increased adherence of neutrophils. In ischemic brain tissue, neutrophil, cytokines, and adhesion molecules are closely related to each other.

Neutrophils are a potential source of ROS when activated during inflammatory responses. Free radicals from the neutrophils are produced via activation of NADPH oxidase in the cytoplasmic membrane. An ischemia and reperfusion animal model demonstrated that the major site of superoxide production is the activated leukocytes in the circulating blood (13). Superoxide and H_2O_2 have since been shown to directly trigger leukocyte activation and adhesion to vascular endothelium. Neutrophils might induce damage by causing local vascular occlusion, or they might initiate toxic reactions, including free radical production by NADPH oxidase, production of hypochlorous acid, or protease activation (14). Transgenic mice lacking NADPH oxidase showed reduction

of infarct size by 50%, however, selective elimination of either leukocyte or parenchymal NADPH oxidase did not reduce damage, suggesting both NADPH oxidases need to be eliminated to protect the brain from ischemia (15). A large part of free radical production is dependent on neutrophils, but it could be initiated by NADPH oxidation in parenchymal tissue or in the neutrophils (14). These findings suggest that inhibition of circulating neutrophils in ischemic brain attack could be effective to prevent ischemic brain injury.

We demonstrated here that edaravone diminished free radicals in circulating neutrophils in patients with ischemic

brain attack, which could at least partially explain the clinical efficacy of edaravone on ischemic brain attack (8). We speculate that edaravone eventually protects the ischemic brain by scavenging free radicals from circulating neutrophils as well as from brain tissue.

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