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Research Report

Novel effects of edaravone on human brain microvascular endothelial cells revealed by a proteomic approach

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ABSTRACT

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenger used for acute ischemic stroke. However, it is not known whether edaravone works only as a free radical scavenger or possess other pharmacological actions. Therefore, we elucidated the effects of edaravone on human brain microvascular endothelial cells (HBMECs) by 2 dimensional fluorescence difference gel electrophoresis (2D-DIGE). We found 38 protein spots the intensity of which was significantly altered 1.3 fold on average ($p < 0.05$) by the edaravone treatment and successfully identified 17 proteins of those.

Four of those 17 proteins were cytoskeleton proteins or cytoskeleton-regulating proteins. Therefore, we subsequently investigated the change of size and shape of the cells, the actin network, and the tight junction of HBMEC by immunocytochemistry. As a result, most edaravone-treated HBMECs became larger and rounder compared with those that were not treated. Furthermore, edaravone-treated HBMECs formed gathering zona occludens (ZO)-1, a tight junction protein, along the junction of the cells. In addition, we found that edaravone suppressed interleukin (IL)-1β-induced secretion of monocyte chemoattractant protein-1 (MCP-1), which was reported to increase cell permeability.

We found a novel function of edaravone is the promotion of tight junction formations of vascular endothelial cells partly via the down-regulation of MCP-1 secretion. These data provide fundamental and useful information in the clinical use of edaravone in patients with cerebral vascular diseases.

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1. Introduction

The neurovascular unit, formed mainly by neuronal cells, microvascular endothelial cells (MECs), and astrocytes, is an integrated functional structure connecting the brain circulation with the functions of neurons (del Zoppo, 2009; del Zoppo, 2010; Iadecola, 2004; Lo et al., 2003; Lo et al., 2004). Recently, protection of the neurovascular unit is highlighted as a strategy for treatment of acute ischemic stroke, as STAIR VII (Stroke Treatment Academic Industry Roundtable) recommends (Lo and Rosenberg, 2009). Among the neurovascular unit components, MECs are the only cell types to contact with blood flow directly and, thus, are the responsible part of the blood–brain barrier. In the early phase of acute ischemic stroke, MECs are impared by oxidative stress and inflammation (Iadecola et al., 2006). Therefore, the protection of MECs is essential to that of the neurovascular unit.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is widely used for acute ischemic stroke in Japan (Abe et al., 1988; Albers et al., 2011). Edaravone is reported to work as a free radical scavenger to inhibit lipid peroxidation and other oxidation (Lukic-Panin et al., 2010; Mizuno et al., 1998; Watanabe et al., 1994). However, it remains to be discovered whether or not edaravone possesses functions other than antioxidative functions. A recent study showed that edaravone reduced the risk of bleeding after treatment with tissue plasminogen activator (tPA) treatment by decreasing MMP-9 (matrix metallopeptidase 9) (Yagi et al., 2009). This may indicate multifunctional aspects of edaravone. Therefore, we attempted to elucidate novel actions of edaravone on human brain (HB)MECs, one of the essential components of the neurovascular unit, by proteomics.

2. Results

2.1. *Comprehensive detection of HBMEC proteins altered by edaravone using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)*

We attempted to identify HBMEC proteins affected by edaravone using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). Specifically, we treated HBMEC with 10 mM edaravone, for 4 h. This concentration of edaravone did not affect the survival of HBMEC (data not shown). Then we extracted proteins from the treated HBMEC and also from those not treated. We then labeled each protein sample with Cy5 and separated the samples by 2D-DIGE, together with Cy3-labeled mixture of all the samples as an internal control. We conducted this experiment in triplicate, as shown in Fig. 1A.

We then compared the intensity of each of the detected protein spots between the edaravone-treated and non-treated HBMEC samples. As a result, we detected a total of 1003 protein spots which were matched among the 6 panels shown in Fig. 1A. Among those 1003 protein spots, the intensity of 179 protein spots were altered 1.3 fold on average, among which the changes of 38 spots were statistically significant ($p<0.05$, Table 1).

2.2. *Identification of proteins by mass spectrometric analysis*

We subsequently identified the proteins of the 38 spots that were altered by the edaravone treatment.

Peptides were recovered from the protein spots on the 2DE gels after in-gel digestion and were subjected to MALDI-TOF/TOF mass spectrometric analysis. As a result, 17 protein spots were identified (Table 2). The identified proteins included an antioxidant of nicotinamide adenine dinucleotide phosphate (NADPH): adrenodoxin oxidoreductase. However, interestingly, most of the identified proteins did not appear to be related to the anti-oxidative functions of edaravone.

Four of the 17 identified proteins were cytoskeleton-related proteins. The intensity of actin and vimentin (Spot nos. 703, 673, and 729) were increased by the edaravone treatment. Also, Rho H (Spot no. 1206), which was reported to antagonize classical Rho proteins that suppress formation of actin filaments (Li et al., 2002; Tapon and Hall, 1997), was decreased by the edaravone treatment. These data indicate that the cytoskeleton of HBMECs are up-regulated by the treatment of edaravone. The other 4 protein spots were carbohydrate metabolism-related (Spot nos. 916, 715, 562, and 641).

2.3. *Effects of edaravone on the cytoskelton of HBMECs*

We, therefore, subsequently investigated the cytoskeleton of HBMECs by fluorescently-labeled phalloidin, which bound to actin filaments. As a result, most edaravone-treated HBMECs became larger and rounder compared with those not treated (Fig. 2A). This change of the cell size and shape brought about by edaravone was also observed in a different cell type of Hela cells (Fig. 2B). Thereby, edaravone most likely has the potential to promote cytoskeleton formation of cells in general.

2.4. *Effects of edaravone on the tight junction of HBMECs*

The increase of the cell size and rounder shape caused by edaravone may also promote the tight junction of HBMECs, which is an essential structure for controlling permeability of vascular epithelium. We, therefore, investigated the effects of edaravone on the tight junction of MECs, focusing on ZO-1, one of the major tight junction proteins, which contributes to the junction formation and epithelial polarization (Umeda et al., 2004; Umeda et al., 2006). As shown in Fig. 3A, when half-confluent HBMECs were stimulated by edaravone, HBMECs showed a gathering of ZO-1 along the junction of the cells. A similar experiment using human umbilical vein endothelial cells (HUVECs) showed similar results. Taken together with the up-regulation of the cell size and round shape formation, this suggests that edaravone would promote a tight junction formation between MECs. This may lead to the down-regulation of the cell permeability and protect the endothelial barrier.

Following ischemic stroke, interleukin (IL)-1β is produced in brain endothelial cells (Buttini et al., 1994; Davies et al., 1999; Sairanen et al., 1997; Touzani et al., 1999) IL-1β is known to damage the tight junction through the up-regulation of