E-selectin Deficiency Attenuates Brain Ischemia in Mice

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SUMMARY

Aims: To determine whether E-selectin deficiency can attenuate brain ischemia in a mouse model of focal cerebral ischemia. **Methods:** E-selectin was determined in spontaneously hypertensive rats (SHRs) and stroke-prone spontaneously hypertensive rats (SHR-SPs). E-selectin knockout ($Es^{-/-}$) mice and wild-type control (WT) mice underwent permanent distal middle cerebral artery occlusion (MCAO). Behavioral analyses were performed followed by the measurement of infarct areas. Myeloperoxidase (MPO) protein was determined by Western blot. IL-6, IL-1 β , and TNF- α were detected by ELISA. *In situ* detection of apoptotic cells was performed by TUNEL staining. **Results:** The brain and serum E-selectin levels were higher in SHR-SPs than in SHRs ($P < 0.05$) after salt intake. E-selectin deficiency improved neurological function and reduced infarct area in cerebral ischemic mice. MPO and IL-1 β were lower in $Es^{-/-}$ mice than in WT mice. In addition, the number of apoptotic cells in $Es^{-/-}$ mice was significantly less than in WT mice after MCAO. **Conclusions:** E-selectin deficiency presents protective effect on cerebral ischemia. This protective effect is likely achieved by the inhibition of inflammation and apoptosis.

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The first three authors contributed equally to this work.

Introduction

Stroke has become the leading cause of mortality and morbidity in China, in both urban and suburb population, with 1,500,000–2,000,000 new strokes per year. There are over 7,000,000 stroke survivors and an estimated two-thirds of them require lifelong assistance [1]. In addition to physical disabilities, the detrimental effects of stroke also extend to significant emotional burden, and further the substantial economic costs.

Inflammation plays an important role in a variety of vascular diseases, including stroke [2–4]. The biomarkers of inflammation can predict risk of primary ischemic stroke [5]. A clinical study indicated that several inflammatory factors, including E-selectin, are significantly higher in stroke patients compared with controls until 72 h after onset of symptoms, suggesting a possible diagnostic role of these markers [6, 7]. During the progression of ischemic stroke, leukocyte recruitment occurs and then results in local tissue damage. Central to this process is the vascular endothelium expression of P- and E-selectin in the first few minutes to hours after cerebral ischemia, leading to leukocyte or neutrophil migration into brain tissue, cytokine release, and free radical-mediated damage [8]. E-selectin is a glycoprotein adhesion molecule specifically expressed on activated endothelial cells. In stroke-prone spontaneously hypertensive rats (SHR-SP), mucosal tolerance to

E-selectin prevents spontaneous ischemic and hemorrhagic strokes [9] and protects against ischemic brain damage after permanent middle cerebral artery occlusion (MCAO) [10]. All of these results suggested the important role of E-selectin in ischemic stroke.

In this study, we investigated further the protective effect of E-selectin deficiency on ischemic brain injury in E-selectin knockout mice. It might provide a new way for the prevention and treatment of ischemic stroke.

Materials and Methods

Animals

Male spontaneously hypertensive rats (SHR) and SHR-SP (4–5 months old) were provided by the Animal Center of the Second Military Medical University. E-selectin knockout ($Es^{-/-}$) mice and their wild-type control (WT) mice were introduced from Jackson Laboratory (Bar Harbor, ME, USA). All animals were housed at 23–25°C, with 12/12 h lighting schedule (8:00–20:00 light, 20:00–8:00 dark) and free access to standard food and drinking water. All animal subjects used in this experiment received humane care in compliance with institutional guidelines for health and care of experimental animals.

Middle Cerebral Artery Occlusion

Mice were anesthetized by using 1.5% isoflurane in 70%/30% NO/O₂ and subjected to permanent focal ischemia [11, 12]. The left middle cerebral artery was occluded by electric coagulation just proximal to the pyramidal branch. Body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ by using a heating pad. Sham-operated mice (WT mice) underwent the same surgical procedure just without electric coagulation of the middle cerebral artery.

Behavioral Tests

Two behavioral tests were performed by an observer blinded to the treatment groups 24 h after middle cerebral artery occlusion (MCAO) operation. These two tests had been used previously to evaluate the neurologic function: (1) body asymmetry test, to measure motor asymmetry, mice were examined for head swings while being suspended by their tails, as previously described [12, 13] and (2) beam test, to assess motor coordination and balance after stroke injury [12, 14]. Mice were placed on one end of a narrow round beam (5 mm diameter and 900 mm in length) and the latency to traverse the central 80% of the beam toward the enclosed escape platform at the other end was recorded. Data are expressed as mean latency to cross the beam of 3 trials.

Evaluation of Cerebral Infarction

Mice were euthanized and their brains were removed for the examination of infarct area after behavioral tests. Serial coronal sections (1 mm apart) were stained with 2,3,5-triphenyltetrazolium chloride (TTC) for assessing infarct area. Sections were digitized, and the area of infarct and noninfarct tissues was outlined using Image J (National Institutes of Health, Bethesda, MD, USA). The ischemic lesion area was calculated as the difference between the area of the nonischemic hemisphere and the normal area of the ischemic hemisphere [15].

Histological Examination

At 24 h after MCAO operation, mice were anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed and embedded in paraffin. Histological sections were cut for hematoxylin–eosin (HE) stain-

ing or terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining, respectively. The histopathologic changes of brain tissue after MCAO were detected by microscope.

Apoptosis Detection

Eight WT mice and four *Es*^{-/-} mice were used in this experiment. Four WT mice received sham operation and the other mice received MCAO operation. For the detection of DNA fragmentation, TUNEL staining was performed by using an *in situ* apoptosis detection kit (Roche, Basel, Switzerland). The number of TUNEL-positive cells per square millimeter of tissue at the margin of the infarct area was counted under high-power magnification ($\times 400$) [16].

Western Blotting

Western blot was performed to detect expression of brain MPO protein. WT mice received sham operation or MCAO operation, and *Es*^{-/-} mice received MCAO operation ($n = 3$ for each group). Twenty-four hours later, brain tissue of infarct side was homogenized in lysis buffer. Total protein was extracted and protein concentration was determined by a protein assay kit. Protein samples were electrophoresed on gradient sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and subsequently electrotransferred to PVDF membranes. Membranes were treated with blocking buffer (5% skimmed milk in TBST buffer) for 3 h at room temperature, followed by incubation with primary antibodies for anti-MPO (1:500 dilution) for 3 h at room temperature. The membranes were washed with TBST and then incubated with anti- β -actin (1:5000; Sigma, St Louis, MO, USA) and secondary antibodies (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at room temperature and then developed with an enhanced chemiluminescence system (ECL kit, Pierce Biotechnology Inc., Rockford, IL, USA) [17, 18].

Statistical Analysis

Data are expressed as mean $\pm$ SD. Differences were evaluated by two-tailed Student's *t*-test (two groups) or one-way ANOVA (three groups). Statistical significance was set at $P < 0.05$.

Figure 1 E-selectin level in SHR and SHR-SP fed on normal rat chow or high salt rat chow (containing 4% NaCl). (A) brain tissue. (B) serum. Data are represented as mean $\pm$ SD, $n = 8$ in each group. * $P < 0.05$ versus SHR.

Figure 2 E-selectin deficiency improved sensorimotor functions and decreased infarct area after MCAO. (A) body asymmetry test. (B) beam test. (C) infarct area (% of hemisphere). Data are expressed as mean $\pm$ SD, $n = 7$ in each group. * $P < 0.05$, ** $P < 0.01$ versus WT mice.

Results

E-selectin Level in SHR and SHR-SP

Difference comparison in E-selectin level was performed between male SHR and SHR-SP. The animals were divided into four groups: SHR normal control group, SHR-SP normal control group, SHRs high salt group, and SHR-SPs high salt group. Normal control rats receive normal rat chow and high salt group rats receive rat chow containing 4% NaCl for 1 month. As shown in Figure 1, there was no significant difference in E-selectin level between SHR and SHR-SP receiving normal rat chow (Figure 1A and B). But E-selectin level in brain tissue (Figure 1C) and serum (Figure 1D) in SHR-SP was significantly higher than in SHR after rats received high salt rat chow for 1 month ($P < 0.05$).

E-selectin Deficiency Improved Neurological Behavior and Reduced Infarction Area after MCAO

We investigated whether E-selectin deficiency would improve neurological functions in mice 24 h after MCAO. In the body asymmetry test, WT mice displayed more frequent turn toward contralateral side after MCAO. Es^{-/-} mice showed significantly decreased biased swing activity compared with WT mice ($P < 0.05$, Figure 2A). In the beam test, shorter time was needed for Es^{-/-} mice to cross the beam compared with WT mice ($P < 0.05$, Figure 2B).

Figure 3 E-selectin deficiency decreased IL-1 β level and MPO expression in mice after MCAO. TNF α (A) and IL-6 (B) were increased 8 h after MCAO, but not affected by the E-selectin deficiency. (C) IL-1 β level was lower in Es^{-/-} mice than in WT mice 8 h after MCAO. $n = 7$ in each group of A–C. (D) MPO expression was decreased by the E-selectin deficiency. Blots are representative of 3 separate rats. * $P < 0.05$, ** $P < 0.01$ versus sham-operated mice; # $P < 0.05$ versus WT mice with MCAO.

The infarct areas in the $Es^{-/-}$ mice and WT mice 24 h after MCAO are shown in Figure 2C. The infarct areas in the $Es^{-/-}$ mice were significantly smaller compared with those in the WT mice ($P < 0.01$).

Effect of E-selectin Deficiency on Cytokines and Neutrophil Infiltration in Brain after MCAO

Compared with sham-operated mice, TNF α , IL-6, and IL-1 β levels were significantly increased in mice underwent MCAO. E-selectin deficiency did not affect the levels of TNF α and IL-6 in ischemic mice, but IL-1 β level was significantly decreased in $Es^{-/-}$ mice than in WT mice 8 h after MCAO (Figure 3A–C).

Myeloperoxidase level in brain tissues was measured using Western blot method to investigate neutrophil infiltration 24 h after MCAO. As shown in Figure 3D, the little expression of MPO protein was seen in sham-operated WT mice, while in WT mice subjected to MCAO operation, there was a significant increase in MPO level. In $Es^{-/-}$ mice subjected to MCAO, expression of MPO was significantly decreased compared with that in WT mice with MCAO.

E-selectin Deficiency Attenuated Apoptosis in Brain after MCAO

HE staining (Figure 4A) revealed the marked cerebral damage both in WT mice and $Es^{-/-}$ mice 24 h after MCAO. Surviving cells in ischemic area were less than in normal tissue. But the surviving cells were more in ischemic cerebrum of $Es^{-/-}$ mice than in that of WT mice (low-power magnification, $\times 100$). Under high-power magnification ($\times 400$), the condensed, irregularly shaped nuclei in ischemic area can be seen.

TUNEL-positive cells displayed brown staining within the nucleus of apoptotic cells. The sham-operated mice displayed almost no positive TUNEL reactivity, whereas the ischemic mice had strikingly positive TUNEL reactivity in ischemic cerebral tissue. The quantitative results showed that the TUNEL-positive cells were significantly more in $Es^{-/-}$ mice compared with WT mice (Figure 4B).

Discussion

Increasing evidence implicates inflammation in the pathogenesis of brain ischemia [19, 20]. Focal cerebral ischemia elicits an inflammatory response characterized by the infiltration and accumulation of leukocytes and monocytes/macrophages and the secretion of inflammatory mediators. Adhesion molecules are involved in leukocyte rolling, firm adhesion, and transmigration across endothelial cells. During this process, E-selectin is needed to play as an initiator. E-selectin belongs to the selectin family of adhesion molecules and is observed only on activated endothelium. It can facilitate the firm attachment of leukocytes into the arterial wall.

In this study, we compared the expression level of E-selectin in SHR with that in SHR-SP. SHR-SP is a substrain of SHR with much more vulnerability to stroke [21, 22]. It is now a commonly used animal model for studying the pathogenesis of stroke. These rats develop a stroke at a mean age of 10 (males) to 14 months

Figure 4 E-selectin deficiency reduced cell apoptosis in ischemic brain tissue. (A) Hematoxylin- and eosin-stained sections of mouse brain 24 h after MCAO. Ischemic cerebral cortex displayed marked tissue damage. Condensed, irregularly shaped nuclei can be seen (arrows). a, ischemic area. (B) *In situ* detection of apoptotic cells by the TUNEL method was performed 24 h after MCAO. TUNEL-positive cells are indicated by arrows. a, ischemic area. Quantitative presentation of the TUNEL analysis (TUNEL-positive cells per square centimeter) is shown in column diagram. Data are expressed as mean $\pm$ SD ($n = 4$). * $P < 0.05$, ** $P < 0.01$ versus sham-operated mice; ### $P < 0.01$ versus WT mice with MCAO.

(females) [23, 24]. As both strains (SHR and SHR-SP) are hypertensive, a comparison of SHR-SP with SHR would provide new insights into the pathogenesis and development of stroke beyond hypertension. Our results indicated the increasing tendency of E-selectin level in SHR-SP than in SHR, although there was no statistical significance. Because SHR-SP develops a stroke at a mean age of 10 months, while the animals used in this study are very young (4–5 months old), the E-selectin expression difference between SHR and SHR-SP may have not appear. So we treated the rats with high salt, an accelerator for stroke development [25, 26], for 1 month. The significant higher E-selectin level was then found in SHR-SP than in SHR, suggesting that E-selectin may be related with the onset of spontaneous stroke in SHR-SP.

To investigate the role of E-selectin in cerebral ischemia, MCAO was performed in $Es^{-/-}$ mice and their wild-type control mice. Our data demonstrated that the infarct area was reduced in $Es^{-/-}$ mice compared with WT mice 24 h after MCAO. This was consistent with previous studies [9, 10] that mucosal tolerance to E-selectin exhibited protective effect against spontaneous stroke and permanent MCAO. In addition, our finding also demonstrated

that E-selectin deficiency improved neurological function 24 h after MCAO.

Several pro-inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β , have been reported to be upregulated in response to brain ischemia [27–30]. Although similar results were obtained in this study that TNF- α , IL-6, and IL-1 β were all significantly elevated after MCAO, only IL-1 β was decreased in Es^{-/-} mice, that is, only IL-1 β was associated with the protective effect induced by E-selectin deficiency. There is evidence that IL-6 in the stroke-lesioned rodent brain is produced mainly by neurons, and TNF- α and IL-1 β are produced mainly by microglia and macrophages [31–34], but the subsets of microglia and macrophages producing IL-1 β are apparently different from those producing TNF- α as demonstrated by flow cytometry studies [35]. We speculated that E-selectin deficiency may affect those IL-1 β -producing microglia or macrophages, and then cause reduced IL-1 β production, but further studies were needed for this hypothesis.

In the current study, we also found that E-selectin deficiency alleviated pathological injury and cell apoptosis after ischemic stroke in mice. At the early phase of inflammation, neutrophils are the mainly implicated cells that infiltrate into the brain [36]. These neutrophils produce a number of potentially harmful substances, including toxic oxygen metabolites and destructive

enzymes with neurotoxic properties [37]. On the other hand, IL-1 β also exerts neurotoxic properties in ischemic stroke and blocking its effect has been shown to reduce ischemic brain damage [38, 39]. So, E-selectin deficiency can reduce the production of neurotoxic substances, including IL-1 β , and then protect cells from apoptosis after cerebral ischemia.

In conclusion, our results revealed the highly attractive therapeutic target of decreasing E-selectin in ameliorating ischemic cerebral injury. And for the first time, we proved that this protective effect is likely achieved by the inhibition of inflammation and apoptosis.

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Conflict of Interest

The authors declare no conflict of interest.

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