Evaluation of Therapeutic Efficacy of Free Radical Scavenger in Patients with Ischemic Stroke
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Abstract
To explore if there is free radical damage to DNA in stroke patients and to evaluate the efficacy of a free radical scavenger, edaravone, we evaluated 23 patients with acute ischemic stroke and compared with 8 non-stroke patients.

Patients were divided into an edaravone-treated group (n = 16) and an edaravone non-treated group (n = 7). We evaluated efficacy of edaravone by calculating urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) and serum S100β per unit volume of cerebral infarction. Urine was collected for 24 h for 8-OHdG measurement and serum was sampled for S100β analysis at the 3 rd, 4 th, 5 th, 7 th, and 14 th day after onset. All patients were treated similarly except for edaravone use. Total urinary 8-OHdG content during the 3 rd to 5 th day was significantly higher in stroke patients than in non-stroke patients (p<0.01). The total urinary 8-OHdG contents showed significant correlation to volume of infarction (p<0.05), modified Rankin Scale (p<0.05), and serum S100β values (p<0.05). Edaravone treatment did not show significant effects on delta 8-OHdG values divided by volume of infarction.

These findings suggest the damage in acute ischemic stroke patients involved free radical induced DNA damage; however, the origin of 8-OHdG may come from a whole body condition. In contrast, S100β index (individual S100β values minus value at 14 th day divided by volume of infarction) showed significant reduction in the edaravone-treated group (p<0.05). Edaravone treatment may be protective reducing S100β release per unit volume of infarction, that is, edaravone may have rescued some cells or areas inside the cerebral infarction.

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Key words: S100beta, 8-OH-dG, ischemic stroke patients, edaravone, free radical

Introduction
Reactive oxygen radicals have been implicated in the pathogenesis of ischemic cerebral damage. These oxygen radicals induce lipid peroxidation, protein oxidation, and DNA damage, causing both acute and chronic cerebral damage. Evidence suggests that reactive oxygen species, most likely nitric oxide, superoxide ions, and hydroxy radicals mediate oxidative DNA damage. Damage to nuclear DNA is a consistent feature of ischemic brain injury and may be both a cause and consequence of cell death processes.

Various markers of oxidative damage have been implicated in cerebral ischemic damage; however, few markers have been confirmed with human stroke patients, except oxidized LDL which was designed for lipid peroxidation. The detection of a new carbonyl group, dityrosine and oxidized histidine has been measured to indicate protein oxidation; however, markers for DNA oxidation were few.
Only in recent years 8-hydroxy-2′-deoxyguanosine (8-OHdG) has emerged as a marker of oxidative stress and involvement of 8-OHdG in ischemic stroke was suggested in an animal model. As a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis, and diabetics, urinary 8-OHdG is measured because it is noninvasive and technically less involved.

To clarify if stroke patients produce larger amounts of 8-OHdG is important since several free radical trapping agents are now under clinical trial, and one drug, edaravone, is already clinically used in Japan. Therefore, we examined the levels of urinary 8-OHdG in patients with an acute phase of relatively severe ischemic stroke, and compared this to levels in non-stroke inpatients. The urinary 8-OHdG values were evaluated for correlation to serum S100β value, NIH stroke scale, and modified Rankin scale so that it could be determined whether 8-OHdG is useful as a clinical indicator of prognosis of acute ischemic stroke.

To assess the efficacy of drugs, clinical symptoms are usually used. However, if an easy, objective method of evaluation is available, drugs can be used with more confidence in the clinical setting. We evaluated the efficacy of edaravone treatments as determined by urinary 8-OHdG and serum S100β measurements.

Patients and Methods

Twenty-three patients (10 females and 13 males, mean age 72 years) with acute ischemic stroke of relatively severe grade admitted to the hospital of Nippon Medical School and the affiliated hospitals were included in this study. The patients were diagnos ed according to present history (sudden onset), existence of atrial fibrillation by electrocardiogram, and hypercoagulability with measurements of thrombin-antithrombin complex, plasminogen-plasmin inhibitor complex, and D-dimer. Patients with middle cerebral artery or internal carotid artery occlusion were retrospectively selected with magnetic resonance angiography. All patients were diagnosed as cardioembolic stroke, except for two patients diagnosed with paradoxical embolic stroke. Non-stroke inpatients without fever or severe conditions were selected as the control patients (3 females and 6 males, mean age 65 years). Patients with serum cre-a tin values of more than 1.5 mg/dl were excluded. The control group included patients with Parkinson disease, multiple system atrophy, third nerve palsy, epilepsy, transient ischemic attack, and Amiotrophic Lateral Sclerosis (ALS). Informed consent was obtained from all patients or families if the patients were unconscious. All stroke patients were treated with heparin and glycerol as normal therapy. The free radical scavenger, edaravone, could be used for patients who arrived at the hospital within 24 hours after onset of symptoms. These patients were assigned to the edaravone-treated group. If the patient arrived after 24-48 hours after onset, edaravone could not be used because of the limitation of use of edaravone by the health insurance system in Japan. Those patients were assigned to the edaravone non-treated group.

In the morning (7 AM), serum was collected from patients and frozen until measured. Urine was collected for 24 hours through intrabladder catheter for the stroke patients. Control patients collected their urine by themselves. The next morning at 7 AM, the accumulated urinary volume was measured and 5 ml was collected and frozen until measured. Urine and serum samples were measured every day from the day of admission to the 14 th day after onset. For the control patients, three consecutive days without significant changes in vital signs were selected for the sample collection. Total urinary content of 8-OHdG was the sum of the three days excretion (from the 3 rd to 5 th day) calculated after multiplying the measured value with the urine volume. S100β value peaked between the 3 rd to 5 th day and then went down to near control value at 14 th day after onset.

Measurement of volume and total surface area of infarction

From 1 to 7 days after onset of infarction, most of the patients underwent MRI measurement. One patient underwent CT scanning because of a pacemaker implantation. MRI (or CT) films were scanned and the area of infarction with diffusion-weighted image was measured with NIH image software. The infarct volume was calculated by integrating the
area multiplied by the thickness of slices. Circumferences of the infarct area in the scanned film were also measured and multiplied by the slice thickness and integrated to mimic total surface area of the infarct.

**Evaluation of Neurological Symptoms**

On admission, neurological symptoms were assessed with NIH stroke scale and at discharge (76 ± 66 days after onset of infarction) with modified Rankin (mRankin) scale.

**S-100β and 8-OHdG measurement**

SRL Inc. (Tokyo, Japan) laboratory determined 8-OHdG and S100β without knowledge of the study design or subject group. Urine samples were centrifuged at 5,000 g for 10 min and the supernatant was used for the determination of 8-OHdG with an enzyme-linked immunosorbent assay (ELISA) (New 8-OHdG check ; Japan Institute for the Control of Aging, Shizuka, Japan). S100β was also measured with ELISA methods with specific monoclonal antibodies (HyTest Ltd, Turku, Finland) against human S100 protein.

**Statistics**

All values are reported as means ± S.D. The S100β values were collected at 3, 4, 5, 7, and 14 days after onset; however, the highest values were picked up for correlation study (usually the value during 3 rd to 5 th days). Total urinary 8-OHdG content was the sum of the total release into the urine during the 3 rd to 5 th days. Regression analyses were performed and correlation was calculated by the Spearman rank method for comparison involving NIH stroke scale or mRankin scale. Other correlations of continuous variables were analyzed with Pearson’s correlation coefficient. For comparing the effects of edaravone, 2 factor analysis of variance (ANOVA) was performed.

**Results**

1. *Urinary 8-OHdG in stroke patients*

Characteristics of enrolled stroke patients and control patients were not significantly different, thus mean age was 73.3 ± 7.5 versus 65.3 ± 12, number of patients with history of diabetes mellitus was 7 versus 4, number of patients with history of smoking was 3 versus 2, respectively. Total urinary 8-OHdG content during the 3 rd to 5 th day after onset showed significant elevation in stroke patients compared to non-stroke control patients (p<0.01, t-test, Fig. 1A). The changes of urinary 8-OHdG contents are shown in Fig. 1B. Urinary 8-OHdG content was elevated from the 3 rd day, and reached a peak...
value during the 3rd and 5th day and then gradually decreased; however, the values showed higher tendencies on the 14th day after onset.

2. Relationship between urinary 8-OHdG and other parameters

The relationship between 8-OHdG and the volume of infarction was significant \( (p<0.05, \text{Fig. 2A}) \). The correlation between urinary 8-OHdG and serum S100β was also significant as shown in Fig. 2B \( (p<0.05) \). Urinary 8-OHdG content showed significant correlation to mRankin scale \( (p<0.05) \) at discharge \( (76 \pm 66 \text{ days after onset}) \); however, the correlation to NIH stroke scale on admission was not significant \( (p=0.052) \) (Fig. 3).

3. Evaluation of efficacy of edaravone treatments

Characteristics of edaravone treated patients and non treated patients were not significantly different, thus mean age was 71.1 ± 14 versus 72.2 ± 8.2, number of patients with history of diabetes mellitus was 5 versus 2, number of patients with history of smok-
The effects of edaravone treatment were evaluated for S100β (A) or 8-OHdG (B). Delta serum S100β values were calculated as individual values minus the data of 14th day. Delta urinary 8-OHdG values were calculated as individual values minus the lowest value during the 14 days. Open and closed circles denote edaravone non-treated and treated groups, respectively. There was a significant difference between edaravone-treated and non-treated groups for S100β, however, no significant difference was found for 8-OHdG.

Fig. 4. The effects of edaravone treatment were evaluated for S100β (A) or 8-OHdG (B). Delta serum S100β values were calculated as individual values minus the data of 14th day. Delta urinary 8-OHdG values were calculated as individual values minus the lowest value during the 14 days. Open and closed circles denote edaravone non-treated and treated groups, respectively. There was a significant difference between edaravone-treated and non-treated groups for S100β, however, no significant difference was found for 8-OHdG.

Fig. 5. (A) There was a significant correlation between S100β and total surface area of infarction (p < 0.01). Delta S100β values (see Fig. 4) divided by total surface area of infarction were calculated and the effect of edaravone was compared (B). Edaravone treatment significantly reduced the value (p < 0.001).

There was a significant correlation between serum S100β concentration and volume of infarction was reported in the literature\textsuperscript{31}. Our data also showed the significant relationship between serum S100β and volume of infarction (r = 0.699, p < 0.01).

To evaluate the effect of edaravone on the volume of infarction, we calculated the delta S100β (individual data minus the data of 14th day) divided by the volume of infarction (named S100β index). The results are shown in Fig. 4A. The edaravone-treated groups showed higher values compared to the edaravone non-treated group (2 factor ANOVA, p < 0.05). Delta 8-OHdG was also calculated as the individual data minus the lowest values during the 14 days. We calculated the delta urinary 8-OHdG divided by the volume of infarction; however, the edaravone treatment did not yield significant effects (Fig. 4B).

4. Comparison using total surface area of infarction

There was a significant correlation between se-

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rum S100β and total surface area of infarction (p<0.01, Fig. 5). If the delta S100β value (see above) was divided by the total surface area of the infarction instead of the volume of infarction, the statistical significance between the edaravone treated group and the edaravone non-treated group became stronger (p<0.001).

Discussion

This study yielded novel findings on the status of acute cerebral ischemia, such that there was participation of DNA damage in patients with acute ischemic stroke. Similar to our previous findings with smaller numbers of patients[23], the total content released in urine during the 3 rd to 5 th days after onset of infarction was significantly higher in stroke patients (Fig. 1B).

Nuclear DNA from tissue is usually the site of oxidation damage[22] and among all purine and pyridine bases, guanine is most prone to oxidation. Upon oxidation, a hydroxyl group is added to the 8 th position of the guanine molecule and an oxidatively modified product, 8-OHdG, is produced as one of the predominant forms of free radical-induced lesions of DNA. Oxidized nucleosides and bases are fairly water-soluble and are excreted in the urine without being further metabolized, and urinary 8-OHdG is considered to be an important biomarker of generalized, cellular oxidative stress and a DNA repair product[23]. There are reports showing increase of 8-OHdG with aging[20], during carcinogenesis[25], during radiotherapy[20], in smokers[27], and in patients with diabetes[28]; however, there are no reports showing the increase of 8-OHdG in stroke patients, except in our preliminary study[15]. Since our non-stroke control patients include patients with diabetes, the value of urinary 8-OHdG content with normal control could be lower. Because there are many reports showing DNA damage in cerebral ischemic tissue in animal experiments[23], the present results of increased 8-OHdG values in stroke patients could partly reflect ischemic damage of brain. However, as shown in Fig. 4B, if we adjust the increased 8-OHdG values according to the infarct volume, there was no significant effect of the free radical scavenger, edaravone. Furthermore, the levels of 8-OHdG in the edaravone non-treated group tended to be lower than those of the edaravone-treated group, probably because of the infarct volume, which was larger in the edaravone non-treated groups. These results may imply that the released 8-OHdG is not mainly due to cerebral ischemic tissue. If it were mainly due to cerebral ischemic tissue like S100β, the value might have been reduced with a free radical scavenger (Fig. 4A). However, urinary 8-OHdG values showed significant correlation to stroke patients’ prognosis as evaluated with mRankin scale at discharge (average 76 days). We believe that urinary 8-OHdG is a marker of whole physical condition, including severity of acute cerebral ischemia.

Diagnostic tests based on blood markers are commonly used for cardiogenic disorders; however, there are few markers specific for cerebral disorders[25]. Recently S100β was reported to be useful to evaluate brain cell damage[22]. It was also reported that S100β was correlated to infarct volume and prognosis[25]. In this study, we could also confirm the significant correlation between serum S100β concentration and volume of infarction (p<0.01) or the patients’ prognosis (p<0.05). Furthermore, we could show the S 100 β index (value of delta S100β divided by volume of infarction) significantly decreased with edaravone treatment (Fig. 4A), that is, edaravone reduced the release of S100β per unit volume of cerebral infarction. Since infarct volume was evaluated mostly with diffusion-weighted magnetic resonance imaging, these results suggest that there may be surviving cells or areas which can be saved inside the high intensity with diffusion weighted image. To explore edaravone’s effects on penumbra (peri-infarct) area, we measured the total surface area of infarction. If the delta S100β values were divided by total surface area of infarction instead of the volume of infarction, the effects of edaravone became more significant (p<0.001, Fig. 5), suggesting that the site of action of edaravone could be the border zone of infarction as reported in animal experiments[22]. Although the origins of S100β may involve extracerebral tissue, including schwann cells and others[22,23], those factors would contribute equally to both groups. We suggest that edaravone is clinically effective in terms of protecting astrocytes, as seen in reduced S100β release. Our evaluation methods to de-
termine the efficacy of therapeutic drugs may be valuable for clinical treatment of stroke.

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**References**

脳梗塞患者へのフリーラジカルスカベンジャー投与の治療効果の評価

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脳梗塞患者のフリーラジカルによる DNA 傷害の有無、およびフリーラジカルスカベンジャーであるエダラボンの効果の評価のために、脳梗塞急性期患者 23 人の尿中 8-hydroxy-2′-deoxyguanosine (8OHdG) および血中 S100β を測定し検討した。対照として非脳梗塞患者 8 人を用い、16 名のエダラボン使用群と 7 名の非使用群の二群に分類した。8OHdG 総量は、第 3 から第 4 病日までの間、対照群と比較して脳梗塞群で有意に高値を示した (p<0.01)。8OHdG の総量は、脳梗塞体積 (p<0.05)、modified Rankin Scale (p<0.05)、血中 S100β 濃度 (p<0.05) と有意な相関を認め、脳梗塞急性期の病態にはフリーラジカルによる DNA 傷害が関与していることが示唆された。一方、第 14 病日を基準にした第 3、4、5 病日の S100β index 値（脳梗塞単位体積あたりの S100β 値）はエダラボン使用群で有意に低値であった (p<0.05)。エダラボンは脳梗塞体積あたりの S100β 値を低下させたが、これはエダラボンが脳梗塞部位の組織あるいは病巣を保護したことによると考えられた。

キーワード：S100beta、8-OH-dG、脳梗塞患者の予後、エダラボン、フリーラジカル