Cleaved but not endogenous secretory RAGE is associated with outcome in acute ischemic stroke

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Objective: To investigate the expression patterns of 2 soluble isoforms of receptor for advanced glycation end-product (RAGE), including endogenous secretory RAGE (esRAGE) and cleaved RAGE (cRAGE), and their associations with outcome in acute ischemic stroke (IS).

Methods: Acute IS patients \((n=106)\) and age- and sex-matched controls \((n=150)\) were recruited. Plasma levels of total soluble RAGE (sRAGE) and esRAGE in patients at <48 hours and 48–72 hours after IS and in controls were measured by ELISA. The level of cRAGE was calculated by subtracting the level of sRAGE from that of esRAGE. Poor outcome was defined as modified Rankin Scale score >2 at 3 months after stroke.

Results: The plasma levels of cRAGE were significantly higher and correlated to those of esRAGE \((p<0.001)\). The plasma levels of esRAGE and cRAGE were both significantly higher in IS patients <48 hours and 48–72 hours after onset than in controls, but only level of cRAGE at <48 hours was independently associated with poor outcome after adjusting for clinical variables \((\text{odds ratio } 2.44; 95\% \text{ confidence interval } 1.16–5.16; p=0.019)\).

Conclusion: The plasma level of cRAGE at <48 hours after IS, rather than esRAGE, is a significant predictor of acute IS outcome.

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GLOSSARY

AUC = area under the curve; cRAGE = cleaved receptor for advanced glycation end-products; esRAGE = endogenous secretory receptor for advanced glycation end-products; IS = ischemic stroke; mRS = modified Rankin Scale; NIHSS = NIH Stroke Scale; RAGE = receptor for advanced glycation end-products; sRAGE = soluble isoforms of receptor for advanced glycation end-products.

Many types of cells express the receptor for advanced glycation end-products (RAGE), a cell surface receptor in the immunoglobulin superfamily.\(^1\)\(^2\) A variety of ligands, such as high mobility group box 1, may bind to RAGE and activate intracellular signals including the mitogen-activated protein kinases and nuclear factor kappa-B.\(^2\)\(^3\) There are 2 soluble isoforms of RAGE (sRAGE): endogenous secretory RAGE (esRAGE), formed by alternative splicing of RAGE messenger RNA; and cleaved RAGE (cRAGE), formed by proteolytic cleavage of full-length RAGE protein.\(^4\)\(^5\) esRAGE has and cRAGE lacks the transmembrane domain of RAGE. Acting as a decoy, sRAGE competes with cellular RAGE for ligand binding, and thereby blocks RAGE-associated intracellular signaling.\(^6\)\(^–\)\(^8\)

In our recent study,\(^9\) the plasma level of sRAGE in ischemic stroke (IS) patients increased significantly in the early stage of stroke (<48 hours after onset) and then decreased at later time points (5–7 days). In addition, the plasma level of sRAGE (<48 hours) was independently associated with functional outcome. However, our study did not measure esRAGE concentration and thus did not establish which was the main form of poststroke circulating sRAGE (esRAGE or cRAGE) and which form was the most representative poststroke predictor of outcome.

In the present study, we compared the plasma levels of esRAGE and cRAGE between patients at <48 hours and 48–72 hours after IS onset and age- and sex-matched nonstroke controls. We
also investigated associations of functional outcome with different isoforms of sRAGE at the acute stage of IS.

**METHODS Subjects.** The study included acute IS patients (n = 106) admitted within 24 hours poststroke, and who had blood drawn within 48 hours poststroke, from 2 academic medical centers (National Taiwan University Hospital and Tri-Service General Hospital) and 2 community hospitals (Taipei Medical University Hospital and Taipei Medical University–Shuang Ho Hospital). Head MRI or repeated CT examination was performed to confirm the diagnosis of acute IS. IS patients were further classified into 5 subtypes: large artery atherosclerosis, small vessel occlusion, cardioembolism, other specific etiologies, and undetermined etiology based on the criteria of Trial of Org 10172 in Acute Stroke Treatment. Patients were excluded if they had active infection, autoimmune disease, cancer, impaired renal function (defined as creatinine >2.0 mg/dL), poor sugar control (defined as hemoglobin A1C >8.0%), or steroid therapy. The study also included 150 control subjects matched for sex and age who were free of cerebrovascular disease for more than 12 months.

**Standard protocol approvals, registrations, and patient consents.** This study was approved and performed under the ethical guidelines issued by the institutional ethics committees of the participating hospitals and all patients gave their written informed consent.

**Clinical protocol and plasma collection and measurement.** Blood samples from the IS patients were drawn within 48 hours and 48–72 hours after stroke onset and the interval between the 2 time points was at least 18–24 hours. Control subjects had a single blood sample. Clinical information including stroke presentation, risk factors, comorbidities, laboratory data of complete blood cell counts, and biochemistry at the time of admission were collected from all study subjects. The severity of stroke was assessed by the NIH Stroke Scale (NIHSS). Severe stroke was defined as a score of NIHSS ≥12 based on several previous reported studies. Outcome 3 months after the stroke was represented as the modified Rankin Scale (mRS) score. Poor outcome was defined as mRS score ≥2.

We described methods of standard plasma collection and preparation in our previous study. The plasma levels of sRAGE and esRAGE were assayed using a commercially available kit (RAGE Human ELISA Kit, R&D Systems, Minneapolis, MN; esRAGE Human ELISA Kit, B-Bridge International, Cupertino, CA). The level of cRAGE was calculated by subtracting the level of esRAGE from that of sRAGE based on several previous related studies.

**Statistical analyses.** Continuous data and categorical data were expressed as the mean ± SD and percentage, respectively. NIHSS was expressed as median and interquartile range. In univariate analysis, differences in the clinical data between good and poor outcomes were analyzed by the 2-sample t test, chi-square test, Fisher exact test, and one-way analysis of variance. Then, fitting logistic regression model was conducted for multivariate analysis, employing the possibly related parameters to estimate the individual prognostic effect. Receiver operating characteristic curve was used to explore the usefulness of clinical parameters and cRAGE (<48 hours) in predicting outcome 3 months poststroke. Area under the curve (AUC) estimates were obtained. Two-sided p value ≤0.05 was defined as statistically significant. SPSS software package version 18.0 (SPSS Inc., Chicago, IL) and SAS 9.3 (SAS Institute Inc., Cary, NC) were used for statistical analysis.

**RESULTS** Plasma levels of sRAGE, esRAGE, and cRAGE in IS patients and controls. The study included 106 IS patients and 150 age- and sex-matched nonstroke controls. The basic characteristics of the study subjects are shown in table 1. Compared to controls, IS patients had similar percentage of diabetes mellitus but significantly higher percentage of hypertension and hyperlipidemia. In all study subjects, plasma level of cRAGE was much higher than that of esRAGE, and cRAGE level was significantly correlated with esRAGE level in both controls and IS patients (all p < 0.001; figure, A–C). Importantly, plasma levels of sRAGE, esRAGE, and cRAGE were significantly higher in IS patients (at both <48 hours and 48–72 hours after onset) than in controls (figure, D–F).

**Associations of plasma sRAGE, esRAGE, and cRAGE with 3-month poststroke outcome.** The clinical variables associated with poor outcome were male sex, older age, and higher NIHSS scores (table 2). Of the 3 RAGE-related parameters, higher sRAGE level at <48 hours and cRAGE level at <48 hours and 48–72 hours were significantly associated with poor outcome in univariate analysis. After adjusting for clinical variables possibly associated with outcome, higher plasma cRAGE level at <48 hours was found to be the most significant poor outcome predictor (adjusted odds ratio 2.44, 95% confidence interval 1.16–5.16, p = 0.019) (table 3). Furthermore, there was a trend of increasing the value of AUC for predicting poor functional outcome from 0.822 (0.744–0.900) by using clinical parameters (age, sex, history of diabetes mellitus and atrial fibrillation, NIHSS ≥12) to 0.849 (0.778–0.921) after adding the parameter of level of cRAGE (<48 hours) (p = 0.126).

Between patients subgrouped on the basis of stroke subtype, there were significant differences in
age and glucose levels at admission. Plasma levels of sRAGE, esRAGE, and cRAGE at <48 hours, plasma level of esRAGE at 48–72 hours, NIHSS scores at admission, and percentage of patients with poor outcome were all significantly higher in patients with cardioembolism than in patients with other IS subtypes (table 4).

DISCUSSION

There is increasing evidence demonstrating the crucial role of RAGE–ligand interactions in various immune-mediated disorders, including acute and chronic vascular diseases.20–22 Several previous studies have reported changes in plasma levels of sRAGE in IS patients.23–26 However, whether plasma level of sRAGE is higher or lower in IS patients than controls remains controversial because of various study limitations, such as lack of controls, case selection bias, or measurement not during the acute stage of stroke. In our recent study, plasma was collected from the IS patients at <48 hours, 48–72 hours, and 5–7 days after stroke onset and from nonstroke controls.9 Assays showed that plasma levels of sRAGE increased at <48 hours and decreased at 5–7 days. We also used an experimental mouse stroke model to confirm the dynamic pattern of plasma sRAGE, which increased significantly at the 24-hour poststroke time point and then decreased at later time points.9 Nevertheless, none of the aforementioned studies investigated the individual role of esRAGE or cRAGE in acute stroke.

The results of the present study confirmed that sRAGE level rose within a short period after acute stroke and also demonstrated that both plasma esRAGE and cRAGE levels increased significantly at the same time, implying the rapid activation of both pathways (i.e., alternative splicing of RAGE messenger RNA and proteolytic cleavage of full-length RAGE protein) in acute IS. Importantly, the majority of the circulating sRAGE was cRAGE in our study subjects, which may reflect the significant increase of membrane type of RAGE expression and related intracellular signaling and activation of various proteolytic enzymes such as matrix metallopeptidase 9 in acute IS.27 This hypothesis is supported by statistical evidence showing that plasma levels of sRAGE and cRAGE but not esRAGE at <48 hours are independently associated with outcome in IS patients.

Furthermore, plasma levels of all kinds of sRAGE were significantly higher in patients with cardioembolism than those with other IS subtypes. However, NIHSS scores and percentage of patients with poor outcome were also higher in patients with cardioembolism. Thus differences in sRAGE levels between IS subtypes may be related to the severity of acute

Figure

Associations and expression patterns of soluble isoforms of receptor for advanced glycation end-products (RAGE) in controls and stroke patients

The plasma level of cleaved RAGE (cRAGE) is much higher than that of endogenous secretory RAGE (esRAGE), and cRAGE level is significantly correlated with esRAGE level in all study subjects (all \( p < 0.001 \); A–C). Furthermore, as compared with the controls, the plasma levels of soluble RAGE (sRAGE), esRAGE, and cRAGE are significantly higher in stroke patients (at both <48 hours and 48–72 hours after onset, \( p < 0.05 \)) (D–F). *\( p < 0.05 \), **\( p < 0.01 \). IS = ischemic stroke.
Values are n (%), mean ± SD, or median (interquartile range).

**Table 2** Comparison by functional outcome for patients with ischemic stroke

<table>
<thead>
<tr>
<th></th>
<th>mRS ≤2 (n = 52)</th>
<th>mRS &gt;2 (n = 54)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60.1 ± 13.3</td>
<td>66.5 ± 13.7</td>
<td>0.016</td>
</tr>
<tr>
<td>Male</td>
<td>44 (84.6)</td>
<td>30 (55.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Initial SBP</td>
<td>153.4 ± 22.7</td>
<td>158.9 ± 38.4</td>
<td>0.375</td>
</tr>
<tr>
<td>Initial DBP</td>
<td>92.7 ± 15.7</td>
<td>89.6 ± 25.1</td>
<td>0.452</td>
</tr>
<tr>
<td>Initial HR</td>
<td>78.3 ± 14.1</td>
<td>79.6 ± 17.9</td>
<td>0.683</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>14 (26.9)</td>
<td>19 (35.2)</td>
<td>0.406</td>
</tr>
<tr>
<td>Hypertension</td>
<td>35 (67.3)</td>
<td>43 (79.6)</td>
<td>0.188</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>14 (26.9)</td>
<td>19 (35.2)</td>
<td>0.406</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>11 (21.2)</td>
<td>17 (31.5)</td>
<td>0.274</td>
</tr>
<tr>
<td>History of stroke</td>
<td>9 (17.3)</td>
<td>14 (25.9)</td>
<td>0.349</td>
</tr>
<tr>
<td>Leukocytes, 10^3/μL</td>
<td>8.6 ± 2.5</td>
<td>9.0 ± 3.1</td>
<td>0.441</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>14.1 ± 2.6</td>
<td>14.1 ± 2.1</td>
<td>0.683</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>143.9 ± 54.1</td>
<td>140.6 ± 57.2</td>
<td>0.600</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>0.723</td>
</tr>
<tr>
<td>NIHSS</td>
<td>4 (2–6)</td>
<td>14 (8–20)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NIH Stroke Scale ≥12</td>
<td>7 (13.5)</td>
<td>34 (63.0)</td>
<td>&lt;0.001b</td>
</tr>
</tbody>
</table>

Abbreviations: DBP = diastolic blood pressure; HR = heart rate; mRS = modified Rankin Scale; NIHSS = NIH Stroke Scale; SBP = systolic blood pressure. Values are n (%), mean ± SD, or median (interquartile range).

aFavorable outcome is mRS ≤2 at 3 months after stroke; unfavorable outcome is mRS >2 at 3 months after stroke.
bSignificant.

cerebral injury rather than different etiologies.9 Why the phenomenon that circulating sRAGE is theoretically functionally protective but paradoxically a poor outcome predictor had been discussed in our recent study that the amount of circulating sRAGE is reflecting the degree of cellular RAGE activation but inadequate to counteract the detrimental effect of related ligands in acute IS.9 Interestingly, the expression pattern and the relationship with outcome of cRAGE in acute stroke were similar with homocysteine, an amino acid that inflicts acute damage to the vascular wall and cell.28 Several studies have shown that plasma levels of homocysteine are increased in the acute stage of stroke and positively associated with poor functional outcome at 3 months after stroke onset.29–31

Recently, there have been several studies investigating the impact of cRAGE or esRAGE in different diseases.17–19 One study focusing on patients with severe sepsis showed that serum levels of sRAGE and subtracted sRAGE (cRAGE) but not esRAGE were elevated in severe sepsis vs in healthy controls.17 Importantly, there were significant correlations between the clinical and biochemical parameters related to the severities of sepsis and levels of sRAGE and cRAGE, but not esRAGE. Although the clinical significance of cRAGE was not identical in different studies, all of them showed that levels of cRAGE were higher than esRAGE.

Our study failed to show a significant association between plasma esRAGE level and outcome in acute IS patients, but pattern of plasma esRAGE expression after stroke was similar to that of plasma sRAGE and cRAGE and highly correlated with that of cRAGE. Several previous studies have shown significant association between plasma esRAGE and various metabolic, vascular, and immune disorders.19,32–36 One 4-year follow-up study identified circulating esRAGE level as an independent risk factor for the progression of carotid atherosclerosis in patients with type 1 diabetes mellitus.37 Therefore, further studies are required to identify any independent role of esRAGE and any functional difference between cRAGE and esRAGE in RAGE-mediated poststroke mechanisms.

Our study has several limitations. First, acute stroke can trigger biological reactions in the affected brain region and in circulating blood cells. Thus, it is unclear whether sRAGE and esRAGE (measured in

**Table 3** Relation of RAGE to functional outcome in patients with ischemic stroke

<table>
<thead>
<tr>
<th></th>
<th>mRS ≤2 (n = 52)</th>
<th>mRS &gt;2 (n = 54)</th>
<th>p Value</th>
<th>Adjusted OR (95% CI)a</th>
<th>Adjusted p valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>sRAGE &lt;48 h, μg/dL</td>
<td>1.26 ± 0.75</td>
<td>1.90 ± 1.54</td>
<td>0.009</td>
<td>1.86 (1.05–3.32)</td>
<td>0.034†</td>
</tr>
<tr>
<td>esRAGE &lt;48 h, μg/dL</td>
<td>0.31 ± 0.29</td>
<td>0.39 ± 0.33</td>
<td>0.177</td>
<td>1.36 (0.26–7.17)</td>
<td>0.714</td>
</tr>
<tr>
<td>cRAGE &lt;48 h, μg/dL</td>
<td>0.95 ± 0.62</td>
<td>1.50 ± 1.28</td>
<td>0.006</td>
<td>2.44 (1.16–5.16)</td>
<td>0.019†</td>
</tr>
<tr>
<td>sRAGE 48–72 h, μg/dL</td>
<td>1.17 ± 0.99</td>
<td>1.69 ± 1.66</td>
<td>0.057</td>
<td>1.35 (0.89–2.04)</td>
<td>0.155</td>
</tr>
<tr>
<td>esRAGE 48–72 h, μg/dL</td>
<td>0.24 ± 0.20</td>
<td>0.29 ± 0.22</td>
<td>0.243</td>
<td>1.68 (0.20–14.39)</td>
<td>0.638</td>
</tr>
<tr>
<td>cRAGE 48–72 h, μg/dL</td>
<td>0.93 ± 0.82</td>
<td>1.40 ± 1.48</td>
<td>&lt;0.048</td>
<td>1.50 (0.90–2.49)</td>
<td>0.116</td>
</tr>
</tbody>
</table>

Abbreviations: CI = confidence interval; cRAGE = cleaved receptor for advanced glycation end-products; esRAGE = endogenous secretory receptor for advanced glycation end-products; mRS = modified Rankin Scale; OR = odds ratio; RAGE = receptor for advanced glycation end-products; sRAGE = soluble isoforms of receptor for advanced glycation end-products.

Values are mean ± SD.

†Adjusted by age, sex (male), hypertension, atrial fibrillation, and NIH Stroke Scale ≥12.
plasma) were predominantly from the CNS or circulatory system. Second, the concentration of cRAGE was calculated from levels of sRAGE and esRAGE in each subject rather than measured directly. Whether cRAGE (measured) and cRAGE (calculated) values can differ remains uncertain. However, cRAGE shared the common structure of extracellular domain of RAGE with esRAGE; there is no commercially available method of measuring human plasma cRAGE level, and it will be important to develop a reliable way to directly measure cRAGE. There have been several good examples using the calculated bioparameters as important prognostic factors in different biomedical fields, such as calculated low density lipoprotein cholesterol using the Friedewald formula and estimated glomerular filtration rate.38,39 Third, our sample size was small, though our results were significant. Further studies with a larger sample size or as a second cohort are necessary to confirm our findings. Nevertheless, our current study extends the knowledge and clinical application of various RAGE signaling-related biomarkers in acute stroke patients.

Our study provides evidence that RAGE is synthesized by both the alternative splicing and protease cleavage pathways after the occurrence of acute IS. In addition, plasma sRAGE is mostly plasma cRAGE, and its <48-hour plasma level is the most significant predictor of functional outcome in IS patients.

**AUTHOR CONTRIBUTIONS**


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