Functional Role of Soluble Receptor for Advanced Glycation End Products in Stroke

Sung-Chun Tang, Yu-Chi Wang, Yu-I Li, Hsiao-Ching Lin, Silvia Manzanero, Yu-Hsuan Hsieh, Simon Phipps, Chaur-Jong Hu, Hung-Yi Chiou, Yi-Shuo Huang, Wei-Shuang Yang, Mark P. Mattson, Thiruma V. Arumugam, Jiann-Shing Jeng

Objective—Little is known about the involvement of the soluble form of receptor for advanced glycation end products (sRAGE) in acute ischemic stroke (IS). Here, we aim to identify the role of plasma sRAGE and high mobility group box 1 (HMGB1) in imaging-confirmed IS patients, as well as mice subjected to focal ischemic stroke.

Methods and Results—IS patients were recruited and plasma samples were collected for the measurement of sRAGE and HMGB1 after stroke. The relation of sRAGE and HMGB1 with acute IS was also investigated in a C57BL/6J mouse model of focal ischemic stroke and primary cortical neurons subjected to oxygen and glucose deprivation. Plasma levels of sRAGE and HMGB1 were both significantly increased within 48 hours after IS, and the sRAGE level was an independent predictor of functional outcome at 3 months poststroke. Immunoprecipitation assays revealed that the binding of plasma HMGB1 to sRAGE increased progressively after IS both in patients and mice. Administration of recombinant sRAGE significantly reduced infiltrating immune cells and improved the outcome of injury in mice, protected cultured neurons against oxygen and glucose deprivation–induced cell death, and ameliorated the detrimental effect of recombinant HMGB1.

Conclusion—Early poststroke plasma sRAGE may play a protective role in IS by capturing HMGB1. Hence, recombinant sRAGE is a potential therapeutic agent in acute IS. (Arterioscler Thromb Vasc Biol. 2013;33:585-594.)

Key Words: animal model ■ HMGB1 ■ inflammation ■ ischemic stroke ■ sRAGE

Stroke is the third major cause of death worldwide and the leading cause of permanent disability. Ischemic stroke (IS) alone accounts for >70% to 80% of all strokes.1–4 Theoretically, acute occlusion of the cerebral arteries results in immediate loss of oxygen and glucose to the core region of the affected brain tissue.5 Hence, a complex of ischemic death and functional deficits.5–8 There is growing evidence implicating the participation of RAGE-ligand interactions in the development and progression of various immune-mediated disorders, including vascular diseases.12,16–18 Previous experimental studies in cultured cells and animal models have demonstrated the central role of mRAGE signaling in the pathogenesis of both chronic atherosclerosis.
and acute vascular insults. Emerging results from human clinical studies also reveal that the plasma levels of sRAGE correlate with the presence or extent of various vascular diseases. Most human studies have shown that the plasma levels of sRAGE are lower in subjects with coronary artery disease, cerebral atherosclerosis, vascular dementia, or individuals at high risk of cardiovascular disease, but contradictory data have also been reported.

Data suggest that HMGB1 is released in large amounts into the extracellular space immediately after an ischemic brain insult and subsequently induces an inflammatory reaction via activation of mRAGE and toll-like receptors in affected brain tissues. However, the role of circulating sRAGE in acute IS is unclear. Only a few studies have addressed the change of serum or plasma sRAGE levels in acute IS patients, and the findings were contradictory to each other. Moreover, clinical applications from these studies remain limited because of the restriction of the study designs. In the present study, we performed a comprehensive investigation involving clinical and basic aspects on the dynamic expression patterns of plasma sRAGE and HMGB1 in imaging-confirmed IS patients, as well as mice subjected to focal IS. We demonstrated an interaction of circulating sRAGE and HMGB1 after acute IS and therapeutic effects of exogenous administration of recombinant sRAGE or HMGB1 in experimental stroke models.

**Materials and Methods**

**Study Population**

This is a multicenter study involving 1 medical center (National Taiwan University Hospital) and 2 regional hospitals (National Taiwan University Hospital, Yun-Lin Branch, and Taipei Medical University-Shuang Ho Hospital). Patients with acute IS who were admitted within 24 hours and received the first blood drawing within 48 hours after onset of the IS were recruited. The diagnosis of all IS patients was confirmed and characterized by head magnetic resonance imaging (diffusion-weighted image) or repeated computed tomography after onset of the IS were recruited. The diagnosis of all IS patients was confirmed and characterized by head magnetic resonance imaging (diffusion-weighted image) or repeated computed tomography exams (performed ≥24 hours after stroke onset to clearly demonstrate the infarct region). Etiologic subtypes of IS were determined based on the Trial of Org 10172 in Acute Stroke Treatment criteria, including large artery atherosclerosis, small vessel occlusion, cardioembolism, stroke of undetermined etiology, and stroke of other determined etiology.

Patients with a known active infection, cancer, renal disease (creatinine >2.0 mg/dL), autoimmune disorder, current steroid treatment, and poor diabetes mellitus control (hemoglobin A1C >8.0%) were excluded. Age- and sex-matched control subjects free of cardiovascular events within 12 months were included for comparison. This study was approved by the ethics committee of the participating hospitals, and all of the patients gave their written informed consent.

**Clinical Protocol**

In acute IS patients, blood samples were drawn at 3 time points, within 48 hours, 2 to 3 days, and 5 to 7 days after the onset of stroke. A single sample of blood was taken from the control subjects. A detailed history of clinical presentation, vascular risk factors, and comorbidity was obtained for each patient. Body mass index was calculated as weight divided by the square of height. Stroke severity at admission was assessed by the National Institutes of Health Stroke Scale (NIHSS). Mortality and functional outcome 3 months after stroke onset were determined. Good outcome was defined as a modified Rankin Scale score of ≤2. Complete blood cell counts and biochemistry were performed at the time of admission.

**Human Plasma Collection and Measurements**

A 10-mL sample of blood was drawn from the controls and IS patients into an EDTA tube, then centrifuged at 300g for 15 minutes, aliquoted into 1.5-mL tubes, and stored at 80°C until use. The plasma levels of total sRAGE and HMGB1 were determined using a commercially available ELISA kit (RAGE, R&D Systems, Minneapolis, MN; HMGB1, IBL, Hamburg, Germany) according to the manufacturer’s protocol. Measurements were performed in duplicate and the results were averaged. Samples with obvious hemolysis, which was visually detected by showing a pink to red tinge inside, were not used for measurements.

**Mouse Focal Cerebral Ischemia/Reperfusion Stroke Model**

Three-month-old C57BL/6j and RAGE knockout (RAGE-KO; generously provided by Dr Ann Marie Schmidt, New York University) male mice were subjected to transient middle cerebral artery ischemia and reperfusion (I/R) injury, as reported previously. Briefly, after making a midline incision in the neck, the left external carotid and pterygopalatine arteries were isolated and ligated with 6-0 silk thread. The internal carotid artery was occluded at the peripheral site of the bifurcation with a small clip, and the common carotid artery was ligated with 6-0 silk thread. The external carotid artery was cut, and a 6-0 nylon monofilament with a tip that was blunted (0.20–0.22 mm) with a coagulator was inserted into the external carotid artery. After the clip at the internal carotid artery was removed, the nylon thread was advanced into the middle cerebral artery until light resistance was felt. The nylon thread and the common carotid artery ligature were removed after 1 hour of occlusion to initiate reperfusion. In the sham group, surgery was performed until the arteries were visualized. Mice were administered 1 mg/kg of sRAGE (1179RG, R&D Systems, Inc), 1 mg/kg of HMGB1 (Hmgbl-2628 mol/L, Creative BioMart), 1 mg/kg of sRAGE + 1 mg/kg of HMGB1, or vehicle (saline) by infusion into the femoral vein (100 μL) 180 minutes after the start of reperfusion. In a separate set of experiments, anesthetized animals from all of the groups (4 to 6 mice per group) underwent cerebral blood flow measurements using a laser Doppler perfusion monitor (PeriFlux System 5000, Järfälla, Sweden). To see whether reduced infarct volume directly related to cerebral blood flow in sRAGE-treated and RAGE-KO animals compared with vehicle-treated or wild-type animals, we measured cerebral blood flow up to 180 minutes after I/R. There were no significant differences between sRAGE or RAGE-KO mice compared with vehicle-treated or wild-type animals (data not shown).

The functional consequences of I/R injury were evaluated using a 5-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously) and were assessed in a blinded fashion. At 72 hours of reperfusion, the mice were killed with a lethal dose of isoflurane. The brains were immediately removed and placed into PBS (4°C) for 15 minutes, and four 2-mm coronal sections were made from the olfactory bulb to the cerebellum. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride in PBS at 37°C for 15 minutes. The stained sections were photographed, and the digitized images were used for analysis. The borders of the infarct in each brain slice were outlined and the area quantified using National Institutes of Health Image J software.

To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. The infarct area was determined by calculating the percentage of infarcted area in each brain slice. Total infarct volume was calculated by integration of infarct areas for all slices of each brain. All of the experimental in vivo procedures described above were approved by the National Taiwan University and the University of Queensland Animal Care and Use Committees.
Mouse Plasma and Brain Tissue Collection and Analysis

A 1.5-mL sample of blood from the inferior vena cava of sham and I/R injury mice was drawn into a tube containing EDTA at 3–24 hr and 72-hour time points (number of mice from each group = 3), centrifuged at 300 g for 15 minutes, aliquoted into 0.5-mL tubes, and stored at −80°C until use. Brain tissues were collected at the same time. Levels of RAGE and HMGB1 in plasma and brain tissues were determined by immunoblot analysis.

Primary Cortical Neuronal Cultures

Dissociated cell neuron-enriched cultures of cerebral cortex were established from 18-D Sprague-Dawley rat embryos, as described.47, 48 Experiments were performed in 7–9-day-old cultures. Approximately 95% of the cells in such cultures were neurons, and the remaining cells were astrocytes. For glucose-deprivation studies, glucose-free Locke’s buffer (Locke without glucose) containing the following: 154 mmol/L NaCl, 5.6 mmol/L KCl, 2.3 mmol/L CaCl2, 1 mmol/L MgCl2, 3.6 mmol/L NaHCO3, and 5 mmol/L HEPES (pH 7.2), supplemented with gentamicin (5 mg/L) was used. The cultured neurons were incubated with glucose-free Locke buffer and incubated for 3 to 24 hours. Controls were incubated in Locke buffer containing 10 mmol/L glucose. For oxygen-glucose-deprivation (OGD) studies, neurons were incubated in glucose-free Locke’s medium in an oxygen-free chamber containing 95% N2 and 5% CO2 atmosphere for 1 hour and then the medium was replaced with Neurobasal medium and the cells were incubated under usual culture conditions for either 12 or 24 hours.

Cell Viability Assay

Cell survival was evaluated with the dye Alamar blue using methods similar to those described previously.49 Dissociated cells were counted and plated in 24-well plates and exposed to treatments for predetermined time periods. The culture medium was removed and replaced with 300 μL/well of 0.5% Alamar blue diluted in Locke solution and incubated for 1 to 2 hours at 37°C in a 5% CO2 incubator. Levels of the Alamar blue reaction product were measured using an HTS 7000 Plus Bio Assay Reader (540-nm excitation and 590-nm emission wavelengths). Values for cultures exposed to experimental treatments were expressed as a percentage of the mean value for untreated control cultures.

Flow Cytometry

Animals were euthanized and perfused with PBS. Ipsilateral (stroke-side) hemispheres were dissected, digested for 30 minutes at 37°C (1.0 mg/mL of collagenase and 0.1 mg/mL of Dnase I in DMEM), and pressed through a cell strainer (40 μm; BD Bioscience). Next, cells were incubated with standard erythrocyte lysis buffer on ice and separated from myelin and debris in a Percoll gradient (GE Healthcare; 1095 g/mL and 1030 g/mL). For surface staining, cells were stained with V450-conjugated Ly6g (BD Bioscience), APC-conjugated CD11c (eBioscience), FITC-conjugated CD11b (BD Biosciences), or PE-Cy7-conjugated CD45 (eBioscience). Flow cytometry was performed using a BD Bioscience LSRII and analyzed using FlowJo software (Tree Star).

Immunoblots

Cell lysates were obtained by washing the cells in ice-cold PBS and resuspending the cell pellets in cell lysis buffer. Brain tissue was extracted using T-PER tissue protein extraction buffer containing a protease inhibitor mixture (Sigma, St. Louis, MO). Protein concentration was determined using a BCA protein assay kit (Fierce, Rockford, IL). Protein in samples (40 μg) was separated by SDS/PAGE (8% to 12%) and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk for 1 hour at room temperature, followed by an overnight incubation at 4°C with primary antibodies. The membrane was then washed and incubated with a secondary antibody for 1 hour at room temperature. Protein bands were visualized by using a chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, United Kingdom).

Immunocytochemistry

Primary neurons were grown on 25-mm microscope coverslips and fixed in 4% paraformaldehyde for 20 minutes. After washing, the coverslips were blocked in PBS containing 10.0% FCS and 0.1% Triton X-100, incubated at room temperature for 1 hour with primary rabbit anti-RAGE antibody (Abcam) and mouse anti-CD45 (Millipore) in blocking buffer, followed by a 1-hour incubation with Alexa Fluor 488 and 568-conjugated specific secondary antibodies (Invitrogen) at room temperature. Images of cells were acquired using an Olympus microscope (Japan).

Immunohistochemistry

Brains were fixed in 4% formaldehyde in PBS (freshly prepared from paraformaldehyde powder) overnight at 4°C before being transferred to sequential 20% and 30% solutions of sucrose (wt/vol) at 4°C until the brains sank to the bottom of the solution. The brains were embedded in TissueTek (Sakura) before sectioning (10-μm sections made using a cryostat) in the coronal anatomic plane. Sections were first exposed for a minimum of 30 minutes to PBS containing 0.1% Triton X-100 (Amresco) and 10.0% normal goat serum (Sigma) to block nonspecific antibody binding, followed by incubation overnight with the relevant primary antibodies (rabbit anti-RAGE antibody from Abcam, and mouse anti-CD45 from Millipore). Sections were then washed and incubated for 1 hour in the presence of appropriate fluorochrome-tagged secondary antibodies. Images were acquired using an Olympus microscope (Japan).

Immunoprecipitation

Protein A agarose beads (Calbiochem, IP06) were washed with 1 mL of radioimmunoprecipitation assay buffer and centrifuged at 4000 rpm for 3 minutes, and this was repeated 3 times. For the precllean step, 10 μL of washed beads were added to each sample and the suspension was shaken gently at 4°C for 1 hour. Afterward, the beads were pelleted by immunoprecipitation and the supernatant was collected. The supernatant was added to 40 μL of washed beads and antibody and then shook at 4°C for 2 hours. The mixture was centrifuged at 4000 rpm for 3 minutes and then washed with 1 mL of radioimmunoprecipitation assay buffer 4 times. Supernatant was discarded and the remaining beads were supplemented with 20 mL of 2X sample buffer and then boiled at 95°C for 3 minutes. Subsequently, the immunoprecipitated materials were subjected to immunoblotting.

Nuclear Fractionation and Nuclear Factor-κB Assay

Cells were scraped and washed with PBS. The cells were then incubated with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.05% NP40, and protease inhibitor [pH 7.9]) on ice for 20 minutes. The supernatant (cytoplasmic fraction) was harvested after centrifugation at 5000 rpm (4°C) for 10 minutes. Meanwhile, the pellet was washed 3 times with PBS and then treated with high salt buffer (5 mM HEPES, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 26% glycerol [vol/vol], and protease inhibitor [pH 7.9]), left on ice for 40 minutes, and vortexed vigorously. The supernatant was harvested after centrifugation at 13000 rpm (4°C) for 15 minutes (nuclear fraction). Both cytoplasmic and nuclear fractions were analyzed by immunoblotting with primary antibodies against phospho-nuclear factor-κB p65 (Cell Signaling), Rb (nuclear marker, Abcam), and [β]-tubulin (cytoplasmic marker, GeneTex).

Statistical Analyses

Statistical analyses were performed using SPSS version 17.0 software (Chicago, IL). Figures were made using GraphPad Prism 4.0 software.
(GraphPad Software, Inc). Experimental results are expressed as mean±SEM. Human plasma levels of sRAGE and HMGB1 were log(10)-transformed for statistical analysis. Comparisons between subgroups were made with Student t test, logistic regression, or ANOVA followed by Newman–Keuls post hoc analysis. Neurological behavior scores were analyzed using a nonparametric Kruskal–Wallis test and Dunn multiple comparison test. Mortality data were analyzed using χ² test.

Results

Plasma Levels of sRAGE and HMGB1 in Acute IS Patients

From July 2008 to July 2010, a total of 84 IS patients were recruited. Twelve patients were excluded because of renal function impairment (n=6) or poor diabetes mellitus control (n=6). Data were acquired from 72 acute IS patients and the same number of control subjects. The basic characteristics of the study participants are shown in Table I in the online-only Data Supplement. IS patients were similar to the controls with respect to age, sex, risk factors, and biochemical data. The etiologic subtypes of IS patients included large artery atherosclerosis in 21 (29.2%); cardioembolism in 27 (37.5%); stroke of other atherosclerosis in 21 (29.2%); small vessel occlusion in 12 (16.7%); and stroke of undetermined etiology in 8 (11.1%); and stroke of undetermined etiology in 8 (11.1%).

As shown in Figure 1A, the initial plasma levels of sRAGE (<48 hours) in the IS patients were elevated significantly when compared with the controls (P<0.01) and then decreased significantly at 5 to 7 days compared with sRAGE (<48 hours; P<0.05). The plasma levels of HMGB1 were significantly higher in the IS patients compared with the controls at all 3 time points after acute stroke (P<0.05; Figure 1B). In addition, the plasma level of log sRAGE (<48 hours) was highly correlated with the NIHSS score at admission (γ=0.36; P=0.002). When the subtypes of IS were considered, the subjects with cardioembolism had significantly higher NIHSS scores, larger infarct volumes, lower levels of glucose and hemoglobin A1C, and higher levels of plasma sRAGE (<48 hours) than other subtypes of IS (Table II in the online-only Data Supplement). Subjects in all of the stroke subgroups exhibited gradual decreases of plasma levels of sRAGE during the 1-week poststroke evaluation period.

Table I documents the basic profiles, stroke severity, and biochemical data in IS patients with good (n=24) or poor (n=48) functional outcome at 3 months after IS. Using univariate analysis the variables correlated with poor functional outcome were older age, female sex, higher NIHSS at admission, larger infarct volume, and higher plasma levels of sRAGE (<48 hours and 2–3 days). Logistic regression analysis shows that older age, female sex, baseline NIHSS, and high plasma sRAGE (<48 hours) independently predicted poor outcome 3 months after IS (Table 2).

Plasma sRAGE and HMGB1 Levels Change in Response to Experimental Stroke in Mice

The relationships among mRAGE, sRAGE, and HMGB1 were further analyzed by measuring the levels of these proteins in brain tissue and plasma samples of I/R injury mice. As shown in Figure 2A through 2C, I/R injury markedly increased levels of mRAGE and HMGB1 in the affected brain tissues as early as 3 hours and were even higher at 24 and 72 hours after I/R, compared with the sham controls. In plasma, sRAGE levels were significantly elevated at the 24-hour poststroke time point, and levels of HMGB1 were significantly elevated at all 3 of the poststroke time points. In summary, the dynamic expression patterns of plasma sRAGE and HMGB1 in acute stages of I/R injury in a mouse stroke model were similar to our clinical data from IS patients. Evidence that HMGB1 binds sRAGE after IS was shown by immunoprecipitation of HMGB1 with a RAGE antibody in plasma samples from mice (Figure 2G) and human IS patients (Figure 2H). The amount of plasma HMGB1 binding to sRAGE increased after stroke both in IS patients and in mice subjected to focal IS.

Administration of sRAGE Protects Mice Against I/R Brain Injury and Protects Cultured Neurons Against OGD and GD

Although we observed that plasma sRAGE does bind HMGB1 after IS, our human data suggest that the amount of endogenous sRAGE generated may be insufficient to inhibit all of the secreted HMGB1. Therefore, we used recombinant sRAGE to determine whether administration of exogenous sRAGE has a beneficial effect in experimental stroke models. First, we performed a dose–response experiment to determine whether sRAGE would protect the brain against ischemic injury. We found that intravenous administration of recombinant sRAGE at concentrations of 0.1 or 0.3 mg/kg, 3 hours after reperfusion, had no significant effect on brain infarct size (Figure 1 in the online-only Data Supplement). However, as shown in Figure 3A and 3B, intravenous administration of recombinant sRAGE at a concentration of 1 mg/kg significantly reduced infarct size (P<0.0001) and improved functional outcome compared with vehicle-treated controls in young 3– to 4-month-old mice subjected to I/R. In contrast, intravenous administration of recombinant HMGB1 (1 mg/kg) resulted in larger infarct volume, and higher plasma levels of sRAGE (<48 hours and 2–3 days). Logistic regression analysis shows that older age, female sex, baseline NIHSS, and high plasma sRAGE (<48 hours) independently predicted poor outcome 3 months after IS (Table 2).

Figure 1. Plasma levels of soluble form of receptors for advanced glycation end products (sRAGE) and high mobility group box 1 (HMGB1) in acute ischemic stroke (IS) patients. Plasma levels of sRAGE increase significantly at 48 hours after stroke, compared with controls, and then decrease at 5 to 7 days (A). Plasma levels of HMGB1 are significantly higher in IS patients than in controls at all 3 time points (B). #P<0.05 vs control. *P<0.05 vs <48-hour samples.
in a larger infarct size and poorer neurological scores compared with vehicle-treated controls and resulted in the worst survival rate among all of the experimental groups (Figure 3C). In addition, premixing sRAGE (1 mg/kg) and HMGB1 (1 mg/kg) effectively reversed the detrimental effect of HMGB1 in the mouse stroke model.

RAGE-KO mice exhibited reduced ischemic brain injury similar to mice treated with sRAGE (Figure 3A and 3B). RAGE-KO mice had significantly reduced neurological deficit score and infarct volume (P < 0.0001) compared with wild-type controls. In addition, all of the RAGE-KO mice survived after I/R injury (Figure 3C). The cerebral blood flow measurements obtained immediately before and after MCAO, and 180 minutes after reperfusion, showed a 90% to 95% reduction in blood flow to the middle cerebral artery during ischemia, which did not significantly differ between groups after reperfusion. In addition, to determine whether sRAGE maintained its protective effect in old animals, we used 12-month-old mice. Intravenous administration of recombinant sRAGE at a concentration of 1 mg/kg significantly reduced infarct size (P < 0.0001) and improved functional outcome compared with vehicle-treated controls in 12-month-old mice subjected to I/R (Figure 3D and 3E).

In the cerebral cortex of sham-operated control mice, little or no immunoreactivity with RAGE antibodies was observed (Figure 3F). At 3 hours after stroke, neurons in the peri-infarct cortex exhibited RAGE immunoreactivity, which remained elevated at 24 and 72 hours after reperfusion (Figure 3H). Cerebral I/R-induced infiltration of CD45 high immune cells into the ipsilateral hemisphere after 1 day (Figure 3I and 3J) was significantly attenuated in mice treated with sRAGE (1 mg/kg, 3 hours after reperfusion). To study the protective mechanisms of sRAGE administration in mice after stroke, we analyzed the levels of phospho-P65, c-Jun amino-terminal kinase, and cleaved caspase 3. sRAGE administration significantly reduced activation and nuclear translocation of p65 protein (Figure 3K and 3L) in the ipsilateral ischemic penumbra at 3- and 24-hour I/R compared with vehicle-treated controls. Administration of sRAGE and premixing sRAGE with HMGB1 ameliorated the aggravating effect of HMGB1 on c-Jun amino-terminal kinase and caspase-3 activities (Figure 3M and 3N) in the ipsilateral ischemic penumbra evaluated at 24-hour I/R compared with vehicle-treated controls.

Using a cell culture model we found that rat primary cortical neurons treated with sRAGE (50 ng/mL) were significantly (P < 0.05) less vulnerable to death caused by GD or combined OGD compared with vehicle-treated control neurons (Figure 4A and 4B). Similar to its action in the mouse stroke model, exogenous HMGB1 (50 ng/mL) significantly (P < 0.05) aggravated GD or OGD-induced neuronal death, and its detrimental effect was reversed effectively by premixing with sRAGE (Figure 4A). To confirm the expression of neuronal RAGE under GD and OGD conditions, we immunostained cultured cortical neurons with RAGE antibody and visualized the immunoreactivity by fluorescence microscopy. In neurons in the control cultures, little or no RAGE immunoreactivity was observed (Figure 4C). At 6 to 24 hours after GD or OGD,

### Table 1. Basic Profiles, Stroke Severity, and Levels of sRAGE and HMGB1 in IS Patients With Good (n=24) or Poor (n=48) Functional Outcomes at 3 Months After IS

<table>
<thead>
<tr>
<th>Variable</th>
<th>mRS ≤2 (n=24)</th>
<th>mRS ≥3 (n=48)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>58.8±15.6</td>
<td>72.0±11.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male, n</td>
<td>20 (83.3)</td>
<td>26 (54.2)</td>
<td>0.019</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.4±4.2</td>
<td>24.6±4.3</td>
<td>0.118</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>10 (41.7)</td>
<td>21 (43.8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Hypertension</td>
<td>16 (66.7)</td>
<td>36 (75.0)</td>
<td>0.578</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>7 (29.2)</td>
<td>12 (25.0)</td>
<td>0.779</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>4 (16.7)</td>
<td>17 (35.4)</td>
<td>0.168</td>
</tr>
<tr>
<td>Smoking habit</td>
<td>13 (54.1)</td>
<td>18 (37.5)</td>
<td>0.212</td>
</tr>
<tr>
<td>History of stroke</td>
<td>4 (16.7)</td>
<td>17 (35.4)</td>
<td>0.170</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>156.8±76.5</td>
<td>134.2±42.5</td>
<td>0.161</td>
</tr>
<tr>
<td>Hemoglobin A₁c, %</td>
<td>7.2±1.2</td>
<td>6.6±1.3</td>
<td>0.170</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>1.3±1.2</td>
<td>1.2±0.3</td>
<td>0.481</td>
</tr>
<tr>
<td>NIHSS, median (25% to 75%)</td>
<td>3 (2–8)</td>
<td>15 (9–18)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infarct volume, mL</td>
<td>19.0±40.9</td>
<td>87.8±123.4</td>
<td>0.010</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>162.2±35.9</td>
<td>160.9±21.0</td>
<td>0.880</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>93.3±21.0</td>
<td>88.0±23.9</td>
<td>0.362</td>
</tr>
<tr>
<td>Body temperature, °C</td>
<td>36.5±0.8</td>
<td>36.4±0.8</td>
<td>0.488</td>
</tr>
<tr>
<td>sRAGE, pg/mL (log transformed)</td>
<td>702.67±526.65</td>
<td>1250.64±1158.5</td>
<td>0.004</td>
</tr>
<tr>
<td>HMGB1, ng/mL (log transformed)</td>
<td>589.39±549.24</td>
<td>1049.34±1390.93</td>
<td>0.014</td>
</tr>
</tbody>
</table>

**Table 2. Factors Leading to Poor Functional Outcome at 3 Months After IS by Logistic Regression Analysis**

<table>
<thead>
<tr>
<th>Covariate</th>
<th>β</th>
<th>P Value</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per y</td>
<td>0.085</td>
<td>0.008</td>
<td>1.09</td>
<td>1.02–1.16</td>
</tr>
<tr>
<td>Female sex</td>
<td>1.977</td>
<td>0.033</td>
<td>7.22</td>
<td>1.18–44.27</td>
</tr>
<tr>
<td>NIHSS, per score</td>
<td>0.269</td>
<td>0.001</td>
<td>1.41</td>
<td>1.11–1.54</td>
</tr>
<tr>
<td>Log sRAGE (&lt;48 h)</td>
<td>2.902</td>
<td>0.037</td>
<td>18.22</td>
<td>11.9–278.15</td>
</tr>
<tr>
<td>Intercept</td>
<td>−18.142</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IS indicates ischemic stroke; NIHSS, National Institute of Health Stroke Scale; and sRAGE, soluble form of receptors for advanced glycation end products.
neurons exhibited robust RAGE immunoreactivity. Furthermore, exogenous administration of sRAGE in culture medium reduced activation of c-Jun amino-terminal kinase, nuclear translocation of p65 protein, and consequent caspase 3 activation in cultured neurons subjected to GD (Figure 4D–4H).

Discussion
There is a growing body of evidence that the receptor for advanced glycation end products and its ligands is involved in the pathogenesis of various disorders, including cardiovascular, neurodegenerative, inflammatory, and autoimmune disorders. Administration of recombinant sRAGE has been shown to block the RAGE signaling pathway in animal models.49–51 Intravenous administration of sRAGE may capture and eliminate circulating RAGE ligands, thus acting as a decoy receptor and protecting against tissue damage. However, there is relatively little knowledge of the regulation and role of sRAGE in human disease. The present study shows that the plasma levels of sRAGE and HMGB1 both were increased within 3 hours of and up to ≥48 hours after stroke onset. Moreover, the level of sRAGE positively correlated with stroke severity and as an independent outcome predictor. Considering the data from the 3 time points examined after acute IS, there was a dynamic pattern of plasma sRAGE from initially high to subsequently lower levels within a short period of time after stroke. These characteristics indicate that plasma sRAGE is not only a surrogate maker for acute IS but may also play some functional roles in the pathogenesis of acute IS.

Previously, other investigators have measured serum or plasma levels of sRAGE in acute IS patients.41–44 In one study, plasma sRAGE was higher in cardioembolic stroke than other IS subtypes, but there were no controls for comparison.41 In another study, low plasma sRAGE was associated with severe leukoaraiosis rather than parameters related to acute IS, although the average plasma sampling was 14 days after IS, and again no controls were included.42 Park et al43 reported that plasma sRAGE levels in acute IS patients were significantly lower than in the control subjects. However, the majority of their strokes were mild (79% of IS patients had NIHSS <5), indicating that the observed sRAGE variation may have reflected the underlying cardiovascular or metabolic diseases rather than acute IS, per se. The study by Montaner et al44 showed that the sRAGE levels in IS patients were significantly higher than those in stroke-mimic patients, but this study did not recruit matched control subjects, and no further association analysis for sRAGE was done. All of the aforementioned studies measured sRAGE levels at only one time point after IS and therefore did not provide information concerning dynamic features of sRAGE levels in acute IS patients.

The present study collected plasma at 3 time points within 1 week after stroke onset, and the first sample was obtained within 48 hours after IS. The diagnoses of acute IS in our recruited patients were confirmed by brain imaging, and most patients had a moderate severity of stroke (79.2% had NIHSS between 4 and 25 points). The same number of age- and sex-matched controls was included for comparison. Therefore, our results may be representative of the specific acute physiological response of circulating sRAGE and HMGB1 after IS rather than other factors. In consideration of different stroke subtypes, patients with cardioembolism had significantly higher levels of sRAGE (<48 hours but not 2- to 3- and 5- to 7-day time points) than the other stroke subtypes, and they

Figure 2. Expression of receptor for advanced glycation end products (RAGE) and high mobility group box 1 (HMGB1) after stroke. Membrane RAGE and HMGB1 levels increase markedly as early as 3 hours and even higher at 24 and 72 hours in ipsilateral brain tissues after ischemia and reperfusion (I/R) vs sham controls (A through C). Plasma expression of soluble form of RAGE (sRAGE) significantly increases at 24 hours after I/R (D and E) and plasma levels of HMGB1 at all 3 time points after I/R (D and F). Immunoprecipitation shows the amount of plasma HMGB1 binding to sRAGE increases after stroke both in mice (G) and IS patients (H). *P<0.05 vs sham levels. Statistical comparisons were made with ANOVA, followed by Newman-Keuls post hoc analysis.
also had greater NIHSS scores and brain infarct volumes than patients with other types of stroke. Thus, the differences of sRAGE levels among different stroke subtypes could be related to the acute stroke severity rather than different etiologies of the stroke subtypes.

The pattern of sRAGE and HMGB1 expression in our clinical study was similarly reflected in our well-controlled mouse I/R injury of stroke model. We showed rapid increase of mRAGE and HMGB1 levels in the affected brain tissue, consistent with previously published studies.21,22 Increased
expression of RAGE after acute IS was further demonstrated by our supplementary data showing RAGE-positive neurons from autopsy brain of a stroke patient and also significant upregulation of RAGE mRNA in buffy coat via quantitative analysis in IS patients compared with the controls (Figures II and III in the online-only Data Supplement), and, theoretically, this may increase the amount of circulating sRAGE. Therefore, we propose that the initial high levels of plasma sRAGE at 48 hours after stroke may reflect the rapid activation of mRAGE after acute IS. Because the physiological concentration of plasma sRAGE was far less than its ligand HMGB1, this may explain why sRAGE levels correlated positively with stroke severity and were a negative outcome predictor.

Our data provide direct evidence that circulating sRAGE has the ability to capture HMGB1 after IS, as determined by immunoprecipitation assay of human and mouse plasma. In addition, we found that sRAGE blocks the neurodegenerative effect of HMGB1 in cultured cortical neurons subjected to GD or OGD, consistent with neutralization of HMGB1 as a mechanism by which sRAGE protects neurons against IS. Our finding that RAGE-KO mice had smaller infarct sizes and better neurological scores after I/R injury compared with wild-type mice is consistent with a detrimental role of mRAGE activation in acute IS. Following this concept, we speculate that higher levels of circulating sRAGE may reduce the activation of mRAGE and improve the outcome of acute IS. The importance of immune cell infiltration during stroke has been demonstrated in several species. Leukocytes are involved in the secondary progression of brain damage after stroke, and there is typically a dramatic accumulation of neutrophils and macrophages in infarcted tissue during reperfusion,46,52 an observation recapitulated in our mouse model. Our results have shown

Figure 4. In vitro study shows exogenous soluble form of receptors for advanced glycation end products (RAGE; sRAGE; 50 ng/mL) significantly protects primary cortical neurons from glucose deprivation (GD) and oxygen GD (OGD) injury, and premixing sRAGE with high mobility group box 1 (HMGB1) can reverse the detrimental effect of HMGB1 (A and B). **P<0.05 vs Locke buffer control cultures. RAGE immunoreactivities in cultured neurons in normal (Neural basal) or following GD or OGD plus reperfusion conditions; cells were counterstained with 4'-6-diamidino-2-phenylindole to label all nuclei and with the neuron-specific marker mitogen-activated protein (MAP) 2 (C). Exogenous sRAGE in culture medium significantly reduces activation of c-Jun amino-terminal kinase (JNK; D and E), consequent caspase 3 activation (D and F), and nuclear translocation of p65 protein (G and H) in cortical neurons following 6- and 24-hour GD condition vs controls. Premixing sRAGE with HMGB1 ameliorates the aggravating effect of HMGB1 on JNK and nuclear factor-κB activities in neurons subjected to GD (D through H). *P<0.05 vs Locke’s buffer control cultures. **P<0.01 vs Locke buffer control cultures. Statistical comparisons were made with ANOVA, followed by Newman–Keuls post hoc analysis.
that treatment with sRAGE after mouse I/R significantly reduced the inflammatory infiltrate to the brain caused by the injury, suggesting that this could be an additional mechanism by which sRAGE minimizes brain damage after IS. However, it is possible that reduced leukocyte infiltration after sRAGE administration may be the result of less inflammation. Further studies are needed to determine whether sRAGE activity in the neural or hematopoietic compartment mediates the effects that we observed.

Our data establish that administration of recombinant sRAGE effectively protects mice from I/R injury and primary neurons from energy deprivation-induced cell death via a mechanism involving reduced activation of c-Jun amino-terminal kinase and NF-κB. There is one other study showing that intraperitoneal injection of sRAGE reduced the infarct size and decreased the number of CD11b-expressing microglial cells in the infarcted hemisphere in a stroke model. The therapeutic potential of recombinant sRAGE has also been suggested from studies of mouse models of acute myocardial infarction and atherosclerosis. Systemic administration of sRAGE may, therefore, be an effective therapeutic approach for all RAGE-mediated vascular disorders.

Study Limitations

Our study did not measure the concentration of endogenous sRAGE that was formed via alternative splicing in IS patients. It therefore remains to be determined which form of sRAGE would be a more representative disease biomarker for acute IS. However, most previous studies, including large prospective cohort studies, have shown good correlations between plasma levels of total sRAGE and the occurrence or severity of targeted diseases. In addition, one recent study revealed that levels of sRAGE and endogenous sRAGE were strongly correlated, and most of the circulating sRAGE seems to be from cleaved wild-type RAGE rather than sRAGE synthesized de novo. Therapeutic effects of exogenous administration of recombinant sRAGE have been established, but whether this results from its action on peripheral or central RAGE ligand-mediated inflammatory process after stroke remains elusive. In addition, we do not have any direct evidence to suggest that intravenous sRAGE crosses the blood-brain barrier. However, because the blood-brain barrier is compromised after stroke, we expect that sRAGE may get into the damaged area. Future studies with labeled recombinant sRAGE may provide valuable information on the issue. Finally, our study showed a wide range of sRAGE concentrations within IS patients. Thus, it is difficult to establish a cutoff point for the lowest level of sRAGE indicating poor functional outcome in IS patients. Nevertheless, although we can use images and clinical symptoms to estimate the stroke severity in stroke patients, early stroke is a dynamic process, and sometimes even clinical symptoms do not reliably reflect the severity of stroke or the degree of brain tissue involvement. We believe that the current work represents a key step toward the application of biochemical markers in clinical stroke management. Further studies with larger sample sizes are necessary to strengthen our findings.

In conclusion, our study supports the pivotal role of sRAGE ligand-mediated signaling in the pathogenesis of IS. Measurement of plasma levels of sRAGE within 48 hours after stroke may be a biomarker for the prediction of the clinical outcome of IS patients. Intravenous administration of recombinant sRAGE is a potential therapy for acute IS.

Sources of Funding

This work was supported by the Taiwan National Science Council grants (97-2314-B-002-127-MY2 and 98-2314-B-002-115-MY3), Australian Research Council Future Fellowship (FT100100427) awarded to T.V.A., and the National Institute on Aging Intramural Research Program.

Disclosures

None.

References


Functional Role of Soluble Receptor for Advanced Glycation End Products in Stroke
Sung-Chun Tang, Yu-Chi Wang, Yu-I Li, Hsiao-Ching Lin, Silvia Manzanero, Yu-Hsuan Hsieh, Simon Phipps, Chaur-Jong Hu, Hung-Yi Chiou, Yi-Shuian Huang, Wei-Shiung Yang, Mark P. Mattson, Thiruma V. Arumugam and Jiann-Shing Jeng

*Arterioscler Thromb Vasc Biol.* 2013;33:585-594; originally published online January 3, 2013; doi: 10.1161/ATVBAHA.112.300523

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/3/585

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/01/03/ATVBAHA.112.300523.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/