High Plasma Cyst(e)ine Level May Indicate Poor Clinical Outcome in Patients With Acute Stroke: Possible Involvement of Hydrogen Sulfide

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INTRODUCTION

Excitotoxic amino acids—glutamate in particular—have long been thought to be central in the pathophysiology of neuronal death in acute ischemic stroke (1, 2). For example, Castillo et al (3) showed that elevated plasma levels of glutamate and glycine correlated with larger infarct size and worse neurologic deficit at 48 hours. Skvortsova et al showed acute increases in cerebrospinal fluid levels of glutamate and aspartate, which correlated with severity (4). However, there are no clinical data on the correlation of plasma levels of these and other amino acids with long-term outcome at 3 months, which is the usual outcome measure in clinical trials assessing the treatment of acute ischemic stroke.

Strong evidence links elevated plasma homocysteine with increased risk of acute stroke (5), recurrent stroke within 15 months (6), and secondary vascular events in patients with stroke within 24 months (7). Homocysteine may be converted to cysteine (Cys) through an intermediate cystathionine, thus suggesting a possible association of the actions of the 2 amino acids in stroke. Plasma Cys has been reported to be as important a risk factor as homocysteine in coronary heart disease (8). Unlike homocysteine, which acts as an agonist at the glutamate-binding site as well as a partial agonist at the glycine-binding site of the NMDA receptors (9), Cys is not known to be an NMDA receptor agonist. However, it causes neuronal cell death that can be prevented by NMDA antagonists (10). It may exert an indirect action on the glutamate receptors through the excitatory amino acids or Cys derivatives such as S-nitrosocysteine or cysteine sulfinate (11). Cys has been reported to be elevated in brain ischemia in experimental animals (12).

It may be hypothesized that high Cys would increase the ischemic damage after a stroke, and this may have clinical relevance. To study this hypothesis, we investigated whether plasma levels of cyst(e)ine obtained from patients within 12 hours of stroke onset bore a significant relationship with long-term clinical outcome at 3 months. We also investigated in a rat stroke model whether Cys loading increased the infarct volume by exacerbation of the ischemic damage after a stroke.
MATERIALS AND METHODS

Clinical Studies in Patients With Acute Stroke Participants

Thirty-six patients with a clinical diagnosis of acute stroke were recruited over a period of 7 months for this study. Patients had to be previously independent (Rankin 0–1) and presenting within 12 hours of stroke onset with significant limb weakness ≥1 on the National Institute of Health Stroke Scale (NIHSS). Patients who awoke with symptoms were assumed to have a stroke onset at the time they went to sleep or when they were last seen to be well. Exclusion criteria included renal impairment (defined as a serum creatinine of over 200 mmol/L), coma, nonavailability for reassessment at 3 months after stroke (e.g., nonresidents), and anticipated life expectancy of less than 6 months as a result of other medical conditions. This study was approved by the hospital ethics committee.

Procedures

After clinical assessment by a stroke neurologist, informed consent was obtained and blood samples were withdrawn from patients. All patients were admitted to the Neurology Department Stroke Service and had standard investigations (including brain imaging) and management. Demographic details and risk factors were routinely entered into the Singapore General Hospital Stroke Database. Stroke subtype was classified according to the Oxfordshire Community Stroke Project classification.

Patients were assessed at 24 to 48 hours for early deterioration from stroke progression or complications (secondary outcome measure) and at 90 days after stroke onset using the modified Rankin scale (primary outcome measure). The clinical assessors were blinded to the neurochemical results.

Amino Acid Analysis

Cys and its dimer cystine are nonessential amino acids obtained from the diet and also synthesized in the liver from methionine (13). They are both present in the plasma, but the sulfhydryl group of Cys is easily oxidized to cystine on standing at neutral pH when exposed to air (14). Plasma levels of cystine may thus be used to represent the combined cystine and Cys levels.

A minimum of 2.5 mL of blood was withdrawn by venipuncture into tubes containing lithium heparin as an anticoagulant and immediately centrifuged at 3000 rpm for 10 minutes to separate plasma from red blood cells. The supernatant was stored at −27°C for a maximum of 2 days. Thawed samples were then placed in a Millipore ultrafree-MC filter unit and centrifuged at 4°C for 2 hours at 10,000 rpm (8,832 g) to obtain the ultrafiltrate that was free of plasma proteins.

Amino acid analysis was performed by ion exchange chromatography through a cation exchange resin column (Beckman 6300 analyzer, Fullerton, CA). Samples were eluted with acidic lithium citrate buffer solutions (1% for 32 minutes followed by 2% for 54.5 minutes and then 1% for 67.5 minutes) at varying pH (pH 2.8, 3.3, 3.7, 3.9 and 2.8 for 32, 46, 8.5, 52.5, and 15 minutes, respectively) and temperature (30°C for 11 minutes, raised to 61°C in 21.6 minutes and held for 50.4 minutes, raised to 70°C in 6.3 minutes and held for 52.7 minutes, and finally decreased to 30°C in 12 minutes). Amino acids in the eluate were reacted with ninhydrin in a heated coil and then through a flow cell where the absorbance was read at 570 nm. Data collection and analysis were performed using “system gold” software dedicated to this system. External reference standards provided by the manufacturer were run together with each batch of samples. An internal standard was used to check for extraction in each sample, and a known normal control sample was run with each batch. For comparison, methionine and the 3 amino acids related to the glutamate receptor systems, namely glutamate, aspartate, and glycine, were also measured.

Animal Studies

Middle Cerebral Artery Occlusion

Cerebral ischemia was induced by permanent occlusion of the left middle cerebral artery (MCA) using a subtemporal approach (15). Male Wistar rats (200–250 g) were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally). MCA was exposed through a subtemporal craniectomy and cauterized from the point proximal to its origin to the point where it intersects the inferior cerebral vein. Rectal temperature was maintained at 37 ± 0.5°C using an electric blanket with a thermocouple probe. All procedures were performed in accordance with the guidelines set by the Laboratory Animal Center, National University of Singapore adapted from Howard-Jones (16), and all efforts were made to minimize suffering and the number of rats used. Cys was administered by intraperitoneal or intracerebroventricular (lateral ventricle: AP –0.8, ML 1.5, DV –3.8 mm from Bregma) injection one hour before middle cerebral artery occlusion (MCAO); sham-operated control rats received saline (n = 5–9) per dose level given by either route of administration. Aminooxyacetic acid (AOAA) was administered intraperitoneally 10 minutes before Cys.

Quantitative Measurement of Infarct Volume

After MCAO or sham operation (24 hours), rats were killed and the entire forebrain was sectioned into 12 coronal sections (1 mm) and placed into multiwell plates containing 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated for one hour. The unstained infarct areas were analyzed by image processing software (Scion Image for Windows version 4.0.2). True infarct volumes were calculated by correcting for edema as previously described (17).

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed 24 hours after MCAO for saline-pretreated and Cys-pretreated rats (2 per group) to demonstrate in vivo the change in infarct volume after Cys treatment in support of the quantitative measurement after death. A 2.0-Tesla whole-body system (Bruker MEDSPEC S200 Avance, Ettlingen, Germany) with gradients capable of 30 mT/m magnitude was used. A home-built 1H solenoid with an inner diameter of 45 mm was used for excitation and reception of the radiofrequency signals (18). T2-weighted fast-spin-echo sequence was performed with
parameters of 4000/100/4 (TR/TE/eff/NEX). The axial in-plane resolution was 115 × 230 μm. The slice thickness was 1.5 mm.

Histology
Rats pretreated with saline or Cys (3 per group) were killed 24 hours after MCAO and intracardiac perfusion with 10% formalin was performed. The brains were then removed and processed for conventional histologic analysis using the standard hematoxylin & eosin staining.

Statistical Analysis
All statistical analyses, including one-way analysis of variance, independent sample t-test, and Fisher’s exact test, were performed by SPSS for Windows (version 13).

RESULTS

Patients
The clinical characteristics of the patients are listed in Table 1. The gender, age, and ethnic distribution of the patients were in keeping with the profile of stroke patients in Singapore. Follow up was complete for all patients. At 24 to 48 hours, 9 patients had early deterioration (8 with motor progression and one with deterioration of conscious level) as a result of the acute stroke. At 3 months, 11 of the patients had good outcome (Rankin 0–1), 20 had poor outcome (Rankin 2–5), and 5 (Rankin 6) were dead. Causes of death were stroke progression (2 patients), acute myocardial infarction (2 patients), and septicemia (one patient).

Amino Acid Measurements
A significant association was found between plasma cyst(e)ine levels and patient outcome, with lowest levels in patients who had good outcome (Rankin 0–1), intermediate levels in patients with poor outcome but were alive (Rankin 2–5), and highest levels in deceased patients (Table 2). Using a dichotomized Rankin measure of outcome, patients with a poor outcome (Rankin >1) had a significantly higher plasma cyst(e)ine level at baseline (p < 0.05) than patients with a good outcome (Rankin 0–1). This finding remains significant even when patients with primary intracerebral hemorrhage are excluded from the analysis. Cyst(e)ine was also significantly elevated in patients who had early stroke deterioration (Table 3) but did not correlate with baseline NIHSS scores. Methionine, glutamate, aspartate, and glycine levels did not show any such association (Tables 2 and 3). There was no relationship between amino acid measurements and stroke subtype (Table 4).

Univariate analysis also showed that stroke subtype, baseline NIHSS score, and early deterioration were significantly different in the outcome groups. Stepwise linear regression with these and age and atrial fibrillation (conventional stroke prognostic factors) showed that only cyst(e)ine levels (β = 0.48, p = 0.001) and the NIHSS score (β = 0.44, p = 0.001) were independent predictors of poor outcome at 3 months, accounting for 51% of the variance. Thus, cyst(e)ine may provide additional prognostic information to physical examination.

Effects of Middle Cerebral Artery Occlusion After Cysteine Pretreatment
Dose-dependent administration of Cys (intraperitoneal or intracerebroventricularly) increased the infarct volume

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of Patients with Stroke</th>
<th>Good Outcome (n = 11)</th>
<th>Poor Outcome (n = 20)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>11</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Age (years), mean (range)</td>
<td>64.6 (37–77)</td>
<td>67.3 (38–84)</td>
<td>NS*</td>
</tr>
<tr>
<td>Gender, male:female</td>
<td>5:6</td>
<td>13:12</td>
<td>NS†</td>
</tr>
<tr>
<td>Race, Chinese:Malay/Indian</td>
<td>8:3</td>
<td>20:5</td>
<td>NS†</td>
</tr>
<tr>
<td>Atrial fibrillation, present:absent</td>
<td>1:10</td>
<td>8:17</td>
<td>NS†</td>
</tr>
<tr>
<td>Type of stroke, total anterior circulation infarct/partial anterior circulation infarct/hemorrhage: lacunar infarct</td>
<td>1:10</td>
<td>20:5</td>
<td>p &lt; 0.001†</td>
</tr>
<tr>
<td>Early deterioration, present:absent</td>
<td>0:11</td>
<td>9:16</td>
<td>p &lt; 0.05†</td>
</tr>
<tr>
<td>Mean baseline NIHSS, standard deviation</td>
<td>4.0, 2.3</td>
<td>11.2, 7.0</td>
<td>p &lt; 0.001*</td>
</tr>
</tbody>
</table>

* Statistical analysis by t-test.
† Statistical analysis by 2-tailed Fisher’s exact test.

<table>
<thead>
<tr>
<th>TABLE 2. Amino Acid Levels (mean ± standard deviation, μmol/L) and Patient Outcome at 3 Months</th>
<th>Good Outcome (n = 11)</th>
<th>Poor Outcome (n = 20)</th>
<th>Dead (n = 5)</th>
<th>Significance (one-way analysis of variance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acid</td>
<td>(Rankin 0–1)</td>
<td>(Rankin 2–5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyst(e)ine</td>
<td>61 ± 12</td>
<td>67 ± 9</td>
<td>82 ± 14</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Methionine</td>
<td>22 ± 5</td>
<td>21 ± 6</td>
<td>29 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Glutamate</td>
<td>46 ± 19</td>
<td>52 ± 22</td>
<td>30 ± 14</td>
<td>NS</td>
</tr>
<tr>
<td>Aspartate</td>
<td>12 ± 2</td>
<td>12 ± 2</td>
<td>13 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>Glycine</td>
<td>205 ± 54</td>
<td>182 ± 43</td>
<td>216 ± 62</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.

<table>
<thead>
<tr>
<th>TABLE 3. Amino Acid Levels (mean ± standard deviation, μmol/L) and Early Deterioration at 24 to 48 Hours</th>
<th>Early Deterioration</th>
<th>No Early Deterioration</th>
<th>Significance (independent sample t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acid</td>
<td>At 24–48 Hours Poststroke (n = 9)</td>
<td>At 24–48 Hours Poststroke (n = 27)</td>
<td></td>
</tr>
<tr>
<td>Cyst(e)ine</td>
<td>77 ± 15</td>
<td>65 ± 11</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Methionine</td>
<td>22 ± 6</td>
<td>22 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>Glutamate</td>
<td>44 ± 19</td>
<td>48 ± 22</td>
<td>NS</td>
</tr>
<tr>
<td>Aspartate</td>
<td>13 ± 3</td>
<td>12 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Glycine</td>
<td>184 ± 38</td>
<td>197 ± 54</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.
TABLE 4. Amino Acid Levels (mean ± standard deviation, μmol/L) Against Type of Stroke

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Total Anterior Circulation Infarct (n = 12)</th>
<th>Partial Anterior Circulation Infarct (n = 5)</th>
<th>Lacunar Infarct (n = 15)</th>
<th>Primary Intracranial Hemorrhage (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyst(e)ine</td>
<td>69 ± 14</td>
<td>67 ± 13</td>
<td>65 ± 12</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>Methionine</td>
<td>25 ± 9</td>
<td>23 ± 3</td>
<td>21 ± 6</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Glutamate</td>
<td>41 ± 15</td>
<td>51 ± 28</td>
<td>47 ± 16</td>
<td>54 ± 29</td>
</tr>
<tr>
<td>Aspartate</td>
<td>12 ± 2</td>
<td>13 ± 3</td>
<td>12 ± 1</td>
<td>12.0 ± 0.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>192 ± 54</td>
<td>182 ± 37</td>
<td>208 ± 55</td>
<td>177 ± 60</td>
</tr>
</tbody>
</table>

FIGURE 1. Dose-dependent increases in infarct volume in rats after intraperitoneal (IP) or intracerebroventricular (ICV) injections of cysteine one hour before middle cerebral artery occlusion. Control rats (C) received saline and the mean infarct volumes (193 ± 9 or 220 ± 9 mm³, respectively) were taken as 100%. One-way analysis of variance: for IP, $F_{3, 27} = 10.815$, $p < 0.001$, $n = 6–9$; for ICV, $F_{3, 16} = 18.888$, $p < 0.001$, $n = 5$ per group. Bars represent standard error of mean. *, $p < 0.005$ by post hoc analysis (Scheffe) against the control group.

FIGURE 2. MRI (Fig. 2). Consistently, histologic studies demonstrated extensive primary infarct and edema (note the partial obliteration of the lateral ventricles) in the ipsilateral cerebrum of Cys-pretreated rats (Fig. 3B). The ischemic penumbra extended further dorsally in the cortex to include the primary somatosensory cortex (S1) (Fig. 3B). Damage in the septodiencephalic region appeared to be most severe, where much of the ipsilateral caudate–putamen nucleus was affected (Fig. 3B, D). In the saline-pretreated control rats, the ischemic penumbra extended rostrocaudally from the septodiencephalic to the caudal diencephalic regions of the cerebrum, similar to that observed in the Cys-pretreated group, but the depth of damage and edema were much less, i.e. the caudate–putamen was only marginally involved (Fig. 3A). Under high magnification, it could be clearly observed that neurons appeared normal, retaining most of the typical histological features (Fig. 3C), in contrast to the shrunken and hyperchromatic neurons observed in the Cys-pretreated caudate putamen (Fig. 3D).

To test the hypothesis that the observed neurotoxicity of Cys was mediated by its conversion to hydrogen sulfide (H₂S), AOAA, an inhibitor of cystathionine β-synthase (CBS, EC 4.2.1.22) that is responsible for the conversion of Cys to H₂S in the brain (19), was coadministered with Cys. It was observed that AOAA completely abolished the Cys-induced increase in infarct volume (Fig. 4). This strongly suggests that H₂S is the mediator of the observed Cys effect.

**DISCUSSION**

Despite the well-established neurotoxic properties of the excitatory amino acids, there is no direct proof that glutamate excitotoxicity is an important mechanism affecting or determining clinical outcome in stroke. Clinical trials of a variety of drugs that are NMDA receptor antagonists or modulators of the NMDA receptor channel have not been successful to date (20, 21). Consistently, we found no association between the plasma levels of glutamate, aspartate, and glycine and the long-term clinical outcome in patients with acute stroke. In contrast, long-term clinical outcome appeared to be related to the plasma levels of cyst(e)ine. It is important to note the observed elevations in cyst(e)ine levels do not appear to be an epiphenomenon of stroke severity as they did not correlate with baseline NIHSS scores or with stroke subtype. Hence, although the poor outcome group had more severe stroke subtypes than the good outcome group, multiple regression analysis showed that cyst(e)ine remained an independent predictor of outcome.

The observed increase in cyst(e)ine levels in patients who had poorer outcome might be the result of increased release after the stroke. If so, the present findings raise the possibility that these amino acids are involved in the pathophysiology of acute stroke. On the other hand, it is entirely possible that the cyst(e)ine levels were raised because of other comorbidities that may be present even before the stroke. Although raised homocyst(e)ine levels may in principle lead to increased cyst(e)ine levels, it has been reported that plasma Cys remained unchanged in some stroke patients with hyperhomocyst(e)inemia (22). Clarification on this point may be forthcoming with further investigations.

Whatever the reason for the raised cyst(e)ine levels, its presence seems to exacerbate the detrimental effects of an ischemic insult both in acute stroke patients and in experimental stroke in rats. In the animal study, the plasma concentration of Cys achieved at the Cys loading dose used (10 mmol/kg) was likely to be much higher than that obtained in patient plasma, approximately 60 to 80 μmol/L. However, cross-species extrapolation is not meaningful. For many drugs, it is known that human requires a much lower dose (on a unit body weight basis) than rats for the same effect.

Cys is known to be toxic to neurons. It causes neuronal death when given orally to infant mice (23) and has also been shown to be important in the pathology of brain injury in immature animals after hypoxic–ischemic brain injury (24).
Using a rat hippocampal slice preparation, Cys was shown to be innocuous under normal conditions but causes toxicity to neurons deprived of glucose, oxygen, or both (25). Extracellular levels of Cys have also been found to be markedly elevated after ischemic brain injury caused by carotid artery ligation in Mongolian gerbils (12). Thus, elevation in extracellular Cys may occur during brain ischemia and contribute to the pathophysiology of ischemic brain injury. Our animal model data support this view and suggest that elevated plasma cyst(e)ine may be responsible for worse outcome in clinical stroke.

The toxicity of Cys has been shown to be mediated through the NMDA receptor and can be blocked by various NMDA antagonists (11, 26), although Cys is not an agonist on the NMDA receptors. Interestingly, Cys is the precursor of H$_2$S, a novel neuromodulator (27) that can enhance NMDA receptor function (28). It is, therefore, possible that high Cys may be translated into increased production of H$_2$S, which mediates tissue injuries through the NMDA receptors. This is strongly supported by the present observation that the proinfarct effect of Cys was completely abolished by inhibition of the conversion from Cys to H$_2$S (Fig. 4).

Elevated plasma homocysteine is strongly linked with increased risk of acute stroke (5–7). Homocysteine may be converted by the action of cystathionine $\beta$-synthase (CBS, EC 4.2.1.22) to cystathionine, which, in turn, is acted on by

**FIGURE 2.** Example of magnetic resonance T2-weighted image of control (left panel, [A–H]) and L-cysteine-pretreated (1.0 mmol/kg intracerebroventricular, right panel, [A–H]) rats 24 hours after middle cerebral artery occlusion. Note the increased infarct caused by cysteine particularly in the striatum (C–F).
Cystathionine γ-lyase (CSE, EC 4.4.1.1) to form Cys. Thus, increased homocysteine may lead to increased Cys and H2S production. Moreover, it has been reported that CBS may condense Cys and homocysteine to form cystathionine and H2S (29). These strongly suggest a possible association and interaction of the actions of Cys and homocysteine in stroke through the production of H2S.

In conclusion, this is the first study on the plasma cyst(e)ine levels in patients with acute stroke. Our results suggest that cyst(e)ine may play an important role in the clinical outcome of these patients. Therefore, understanding the involvement of H2S in ischemic brain damage may give rise to new therapeutic approaches in acute stroke.

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REFERENCES


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