Polyamine Oxidase and Acrolein as Novel Biochemical Markers for Diagnosis of Cerebral Stroke

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Background and Purpose—We found previously that plasma levels of acrolein (CH2=CHCHO) and spermine oxidase (SMO) were well correlated with the degree of severity of chronic renal failure. The aim of this study was to test whether the levels of these 2 markers and of acetylpolyamine oxidase (AcPAO) were increased in the plasma of stroke patients.

Methods—The activity of AcPAO and SMO and the level of protein-conjugated acrolein in plasma of the stroke patients and normal subjects were measured by high-performance liquid chromatography and ELISA, respectively. Focal infarcts were estimated by MRI or computed tomography (CT).

Results—The levels of AcPAO, SMO, and acrolein were significantly increased in the plasma of stroke patients. The size of stroke was nearly parallel with the multiplied value of acrolein and total polyamine oxidase (AcPAO plus SMO). After the onset of stroke, an increase in AcPAO first occurred, followed by increased levels of SMO and finally acrolein. In 1 case, an increase in AcPAO and SMO preceded focal damage as detected by MRI or CT. Furthermore, stroke was confirmed by MRI in a number of mildly symptomatic patients (11 cases) who had increased levels of total polyamine oxidase and acrolein. Among apparently normal subjects (8 cases) who had high values of acrolein×total polyamine oxidase, stroke was found in 4 cases by MRI.

Conclusions—The results indicate that increased levels of AcPAO, SMO, and acrolein are good markers of stroke. (Stroke. 2005;36:2609-2613.)

Key Words: acrolein ■ polyamines ■ stroke

Polymines (putrescine, spermidine, and spermine) are essential for normal cell growth.1,2 Polymines exist mostly as polyamine-RNA complexes and thus affect various steps of translation.3,4 However, 1 of the oxidative products of polymines, acrolein, which is mainly generated from spermine by spermine oxidase (SMO), is strongly cytotoxic. In a cell culture system, acrolein was found to be cytotoxic at micromolar concentrations.5 Recently, we found that the level of acrolein in plasma is well correlated with the degree of severity of chronic renal failure.6 In the plasma of patients with renal failure, there was a decreased level of spermine and increased levels of putrescine, SMO, and free and protein-conjugated acrolein.6 Our results suggested that acrolein would increase in the plasma of patients having other diseases that involve cell damage. Stroke is a sudden focal neurological deficit caused by vascular insult, accompanied by cell damage in the central nervous system. At present, there is no simple biochemical marker for diagnosis of stroke. In this report, we examined whether protein-conjugated acrolein and polyamine oxidase (SMO and acetylpolyamine oxidase [AcPAO]) are suitable biochemical markers for stroke.

Materials and Methods

Patients
Plasma samples were collected from 35 control subjects without stroke (20 males, 15 females; 67.8±2.3 years of age) and 62 patients with stroke (33 males, 29 females; 70.7±1.7 years of age). Stroke patients were defined as having focal infarcts detected by MRI or computed tomography (CT; 41 patients were lacunar and 21 patients were large artery) and managed according to Japanese Guideline for the Management of Stroke (2004). In brief, edaravone, ozagrel, or argatroban was medicated to the patients within the first 10 to 14 days, and ticlopidine hydrochloride or aspirin was medicated after a few days to weeks of onset of stroke. Patients with chronic renal failure were excluded from the study, including the control group. Human blood was collected with procedures approved by the ethics committees of Chiba University. Clinical investigations were conducted in accordance with the Declaration of Helsinki principles. Blood containing 3 U/mL heparin was centrifuged at 1500g for 10 minutes at 4°C. The supernatant (plasma) was carefully collected to avoid contamination by erythrocytes.

Measurement of Polymines and Free Acrolein in Plasma
Amino acids in plasma were removed by cellulose phosphate column chromatography before polyamine analysis, and polyamine contents...
were measured by high-performance liquid chromatography (HPLC) as described previously.6,7 Free acrolein formed from 3-aminopropanal or 3-acetamidopropanal was measured as described previously.6

Assay for SMO and AcPAO in Plasma
The reaction mixture (0.075 mL) containing 10 mmol/L Tris-HCl, pH 7.5, 0.2 mmol/L spermine or N-acetylspermine, and 0.065 mL plasma was incubated at 37°C for 48 hours. To 0.02 mL of the reaction mixture, 0.55 mL of 5% trichloroacetic acid was added and centrifuged at 12,000 g for 10 minutes. A 10-μL aliquot of the supernatant was used for the polyamine measurement by HPLC.7 The activity of SMO and AcPAO was expressed as nanomole spermidine increase per milliliter plasma.

Measurement of Protein-Conjugated Acrolein
Protein-conjugated acrolein (N-(3-formyl-3,4-dehydropiperidino)-lysine [FDP-lysine]) was determined by the method of Uchida et al8 using ACR-LYSINE ADDUCT ELISA SYSTEM (NOF Corporation) and 0.05 mL plasma. After the reaction was terminated, absorbance at 450 nm was measured by a microplate reader Bio-Rad Model 550.

Imaging
All patients underwent T1- and T2-weighted MRI, and some patients underwent fluid-attenuated inversion recovery (FLAIR) and CT. All MRI was performed in 5- to 8-mm thickness with 1- to 2-mm slice gap with a 1.5-T MRI unit (Signa; GE Medical Systems). A standard head coil with a receive–transmit birdcage design was used. The maximum size of focal infarcts was measured using 5 or 10 mm length calibration accompanied in each image.

Statistics
Values are indicated as median±interquartile deviation or means±SE. Groups were compared using Wilcoxon ranking sum test. Regression curves were drawn by the least square method at the secondary dimension.

Results
Increase in AcPAO, SMO, and FDP-Lysine in Plasma of Patients With Stroke
Spermine is metabolized via 2 pathways: 1 involves conversion to spermidine and 3-aminopropanal by SMO, and the other involves metabolism to spermidine and 3-acetamidopropanal by spermidine/spermine N-acetyltransferase (SSAT) and AcPAO.9,10 We reported that acrolein produced from 3-aminopropanal is toxic in FM3A cells.5 We also found that acrolein was produced from 3-acetamidopropanal, although its production was low (data not shown). Accordingly, the activities of SMO and AcPAO were measured together with the level of protein-conjugated acrolein (FDP-lysine) in plasma of the patients with stroke. Blood samples were collected during medical examination, and AcPAO, SMO, and FDP-lysine were measured together with various biochemical markers. As shown in Figure 1, the levels of AcPAO, SMO, total polyamine oxidase (total PAO; AcPAO plus SMO), and FDP-lysine were significantly higher in the plasma of the patients with stroke. Blood samples were collected during medical examination, and AcPAO, SMO, and FDP-lysine were measured together with various biochemical markers. As shown in Figure 1, the levels of AcPAO, SMO, total polyamine oxidase (total PAO; AcPAO plus SMO), and FDP-lysine were significantly higher in the plasma of the patients with stroke. The median levels of AcPAO, SMO, total PAO, and FDP-lysine in patients with stroke compared with control subjects increased from 0.9 to 3.1, from 3.2 to 4.7, from 4.5 to 8.0, and from 14.4 to 21.3 nmol/mL plasma, respectively. When we analyzed the level of polyamines in plasma from 12 patients from day 1 to 20 after the onset of stroke, there was a tendency for putrescine levels to be increased, whereas levels of spermine and spermidine were significantly decreased (data not shown).

These results support the idea that AcPAO and SMO are released from nerve, glia, or other cells during the early period of stroke, leading to reduced levels of spermidine and spermine and increased levels of acrolein (FDP-lysine).

The data for 25 patients were analyzed with regard to time after onset of the stroke. As shown in Figure 2A, an increase in AcPAO occurred first, followed by increases in SMO and then FDP-lysine. For 3 patients whose plasma was collected at 2 time points after onset of stroke, an earlier increase in SMO compared with FDP-lysine was confirmed (Figure 2B). This is probably because of the fact that it takes time to produce acrolein from spermine by total PAO, especially by SMO.

We then examined whether the increase in total PAO and FDP-lysine is correlated with the size of stroke from day 1 to 20 after onset. We did this with 16 patients in this time window. Because the maximal increases in total PAO and FDP-lysine occurred at different times (Figure 2), and the
increase in FDP-lysine is dependent on changes in PAO, the multiplied value of FDP-lysine by total PAO was compared with the size of stroke. Statistical significance was greater in the multiplied value ($P = 9.3 \times 10^{-7}$) than FDP-lysine ($P = 6.6 \times 10^{-5}$) or total PAO ($P = 7.0 \times 10^{-5}$; Figure 1; Table 1).

When the cutoff value (93.2) was set up at the third quartile of no stroke, the multiplied value showed high true positive value: 75.2%. The size of stroke was nearly paralleled with the multiplied value of FDP-lysine by total PAO (Table 1).

There was also a patient who came to our hospital with a suspected stroke (Figure 3). On day 1 (within 6 hours after onset of stroke), the levels of AcPAO and SMO were elevated (25.8- and 2.05-fold above controls), together with a small increase in FDP-lysine (1.17-fold). At that time, focal infarcts were not observed by either MRI (T2-weighted MRI) or CT. On day 2, a large infarct was clearly observed at the left temporal lobe by MRI (T2-weighted MRI). On day 7, the levels of AcPAO, SMO, and FDP-lysine were still elevated, and infarction was clearly observed by CT. Thus, the increase in AcPAO and SMO in plasma was the very early diagnostic marker to confirm stroke in this patient.

Levels of other biochemical markers were measured together with AcPAO, SMO, and FDP-lysine. Except for AcPAO, SMO, and FDP-lysine, the values were within the normal range or only slightly higher than normal (Table 2).

Prediction of Stroke by Total PAO and FDP-Lysine
There were 11 patients suspected to have a stroke but who had only mild symptoms such as numbness of the limbs or headache. The levels of total PAO and FDP-lysine in the plasma were elevated in these patients (Figure 4A), and MRI confirmed infarction on the brain of all 11 patients; an example is shown in Figure 4A.

Among 39 apparently normal subjects, 8 subjects were beyond the cutoff value (93.2) of the multiplied value of total PAO and FDP-lysine. Thus, MRI studies were done in these 8 subjects, and evidence for stroke was found in 4 of them.
Table 2. Biochemical Markers in Blood of the Patients With Stroke

<table>
<thead>
<tr>
<th>Biochemical Marker</th>
<th>Values of Normal Subjects</th>
<th>Values of Patients</th>
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<tbody>
<tr>
<td>AcPAO, nmol SPD/mL plasma</td>
<td>0.9 ± 1.0^c</td>
<td>3.1 ± 2.7^c</td>
</tr>
<tr>
<td>SMO, nmol SPD/mL plasma</td>
<td>3.2 ± 0.9^c</td>
<td>4.7 ± 1.7^c</td>
</tr>
<tr>
<td>Total PAO, nmol SPD/mL plasma</td>
<td>4.5 ± 0.9^c</td>
<td>8.0 ± 3.8^c</td>
</tr>
<tr>
<td>FDP-lysine, nmol/mL plasma</td>
<td>14.4 ± 2.5^c</td>
<td>21.3 ± 4.0^c</td>
</tr>
<tr>
<td>AST; GOT, IU/L</td>
<td>22.3 ± 0.8</td>
<td>25.8 ± 4.1</td>
</tr>
<tr>
<td>ALT; GPT, IU/L</td>
<td>18.6 ± 1.4</td>
<td>30.4 ± 7.4</td>
</tr>
<tr>
<td>LDH, IU/L</td>
<td>190.0 ± 5.0</td>
<td>199.0 ± 9.0</td>
</tr>
<tr>
<td>ALP, IU/L</td>
<td>206.0 ± 11</td>
<td>229.9 ± 6</td>
</tr>
<tr>
<td>Total protein, g/dL</td>
<td>7.2 ± 0.1</td>
<td>6.7 ± 0.1</td>
</tr>
<tr>
<td>UA, mg/dL</td>
<td>5.3 ± 0.3</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>15.0 ± 0.6</td>
<td>15.9 ± 2.1</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>T-CHO, mg/dL</td>
<td>190.0 ± 5.7</td>
<td>187.8 ± 7.2</td>
</tr>
<tr>
<td>γ-GTP, IU/L</td>
<td>31.4 ± 4.3</td>
<td>38.6 ± 10</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>149.9 ± 15</td>
<td>116.2 ± 9.9</td>
</tr>
<tr>
<td>CPK, IU/L</td>
<td>101.6 ± 11</td>
<td>571 ± 7</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>101.9 ± 2.7</td>
<td>120.8 ± 11</td>
</tr>
<tr>
<td>WBC, 10^3 cells/μL</td>
<td>6.2 ± 0.3</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>RBC, 10^6 cells/μL</td>
<td>4.5 ± 0.1</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>HGB, g/dL</td>
<td>14.0 ± 0.2</td>
<td>13.4 ± 0.4</td>
</tr>
<tr>
<td>PLT, 10^3 cells/μL</td>
<td>250.0 ± 10</td>
<td>210.0 ± 11</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
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Discussion

The data in this study indicate that total PAO and FDP-lysine (protein-conjugated acrolein) are good markers for stroke. It has been reported that the property of the blood-brain barrier changes during cerebral ischemia so that various proteins are released into blood. This is consistent with the idea that spermine, spermidine, and polyamine oxidase are derived from damaged cells in the central nervous system. Although AcPAO does not produce much acrolein, it increased first before SMO and FDP-lysine. Actually, increase in AcPAO was faster than focal infarcts observed by MRI (T2-weighted MRI). Thus, the timing of the increase in AcPAO is probably as early as the detection of focal infarct observed by diffusion-weighted MRI. However, it takes >24 hours to measure AcPAO activity. Experiments are now in progress to develop rapid immunoassay system of AcPAO. It is also noted that the value derived from FDP-lysine × total PAO was correlated with the size of stroke. Such information is probably helpful for diagnosis and treatment of patients with stroke. It is also possible to predict stroke in asymptomatic subjects if total PAO and FDP-lysine in the plasma of such subjects were routinely measured.

Figure 4. Levels of total PAO and FDP-lysine in the plasma of mildly symptomatic patients and MRI of 1 of the patients (A) and detection of infarction from apparently normal subjects (B). A. The levels of total PAO and FDP-lysine in the plasma of 11 mildly symptomatic patients (4 males, 7 females; 71.0 ± 3.7 years of age) were shown in medium ± interquartile deviation. The median values of these markers in plasma of the mildly symptomatic patients were 7.0 nmol spermidine increase/mL plasma and 24.3 nmol/mL plasma, respectively. MRI of 1 of the mildly symptomatic patients was constructed by the method of FLAIR pulse sequence. As control, MRI of a normal subject and a patient with stroke was shown. B. MRI was made in 8 apparently normal subjects whose multiplied value of total PAO and FDP-lysine was beyond the cutoff value. Diagnosis of those 8 apparently normal subjects and MRI of typical 4 examples were shown. Percentage of detection of stroke from apparently normal subjects with higher multiplied value was 50%.
infarct, and that the multiplied value is only correlated with the size of infarct. To decrease false negatives of lacunar stroke, it is important to develop a more sensitive method to measure total PAO and FDP-lysine.

When spermine and acetylspermine were used as substrates, enzymes to degrade these substrates were termed as SMO and AcPAO, respectively. However, it is possible that other amine oxidase(s) may be involved in these 2 enzymatic activities as in the case of chronic renal failure, in which diamine oxidase is also slightly involved in SMO activity of some individual patients.6

When spermine is metabolized by SMO, H2O2 is produced together with acrolein. The concentration of H2O2 necessary to cause cell toxicity was much higher than that of acrolein.5 Our results suggest that acrolein is a more sensitive marker than H2O2 for stroke.

There are reports that 3-aminopropanal, which automatically produces acrolein, is generated from spermine and is strongly involved in cell damage during ischemia in rats.12,13 It has been reported that acrolein can also be produced from membrane phospholipids, although the major aldehydes produced during lipid peroxidation are 4-hydroxy-2-nonenal and malondialdehyde.14 We measured acrolein produced from arachidonic acid under the same conditions in which acrolein has been reported to be produced from membrane phospholipids.8 However, acrolein production was very low (data not shown). Thus, our results suggest that acrolein is mainly produced from spermine.

There are also reports that SSAT and SMO are induced during kidney ischemia-reperfusion injury in rats,15 and spermine and spermidine decreased after transient focal cerebral ischemia in spontaneously hypertensive rats.16 Although induction of AcPAO was not examined, this may also occur together with SSAT and SMO. So these metabolizing enzymes are released together with spermine and spermidine into blood and significant level of acrolein is formed in blood during ischemia. It has been recently reported that matrix metalloprotease-9 and S100B, a specific protein in astroglial during ischemia. It has been recently reported that matrix metalloprotease-9 and S100B, a specific protein in astroglial during ischemia. It has been recently reported that matrix metalloprotease-9 and S100B, a specific protein in astroglial during ischemia. It has been recently reported that matrix metalloprotease-9 and S100B, a specific protein in astroglial during ischemia. 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