Protein Z in Ischemic Stroke and its Etiologic Subtypes

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Background and Purpose—Protein Z is a vitamin K–dependent plasma protein whose significance in arterial thrombosis remains uncertain. The objectives of this study were to determine the association between protein Z, ischemic stroke, and etiologic subtypes of ischemic stroke.

Methods—We conducted a case-control study of 173 hospital cases of first-ever ischemic stroke and 186 randomly selected community controls. Using established criteria, we classified cases of stroke by etiologic subtype. Protein Z concentrations were measured during the first 7 days and at 3 to 6 months after the acute stroke event.

Results—Blood levels of protein Z measured within 7 days of acute stroke were significantly higher in cases than in controls (geometric mean, 1.46 versus 1.16 μg/mL; P<0.0001). Compared with the lowest tertile, the upper 2 tertiles of protein Z were associated with an adjusted odds ratio (OR) of ischemic stroke of 1.75 (95% CI, 1.00 to 3.07) for the second tertile and 3.07 (95% CI, 1.73 to 5.45) for the upper tertile. The adjusted odds of ischemic stroke caused by large-artery atherothrombosis was nearly 8-fold greater for those with protein Z concentrations in the upper tertile compared with the lower tertile (OR, 7.91; 95% CI, 3.11 to 20.14). The adjusted odds of ischemic stroke due to small-artery disease (OR, 1.79; 95% CI, 0.83 to 3.87) and cardioembolism (OR, 1.80; 95% CI, 0.58 to 5.64) was also increased among individuals with protein Z concentrations in the upper tertile compared with the lower tertile, but not significantly so. There was no significant difference between mean protein Z concentrations among cases in the convalescent phase (3 months) after stroke and age- and sex-matched controls.

Conclusions—There is a strong, independent relationship between elevated blood levels of protein Z and ischemic stroke during the acute phase, particularly ischemic stroke due to large-artery atherothromboembolism, which is no longer evident during the convalescent phase. These results are consistent with the notion that protein Z is either an important factor in the pathogenesis of ischemic stroke due to large-artery atherothromboembolism or an acute phase reactant. Further studies are required to elucidate whether protein Z has a causative or prognostic role in acute arterial thrombosis.

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Key Words: blood proteins ▪ cerebrovascular accident ▪ intracranial embolism ▪ intracranial thrombosis ▪ protein Z

Protein Z is a vitamin K–dependent plasma glycoprotein, synthesized by the liver, which was identified in bovine plasma in 19771 and in human plasma in 1984.2 Unlike the human form, bovine protein Z contains a 36–amino acid C-terminus extension that enhances the binding of thrombin to phospholipid and promotes thrombus formation.3,4 Deficiency of bovine protein Z is therefore associated with an increased risk of bleeding.5 By contrast, the human form of protein Z is believed to suppress thrombus formation. At phospholipid surfaces, human protein Z forms a calcium-dependent complex with activated coagulation factor X, which serves as a cofactor to enhance the action of a protein Z–dependent protease inhibitor (ZPI) by >1000-fold.6–8 The end result is an inhibition of activated factor X and suppression of thrombus formation. Deficiency of human protein Z has been reported to be prothrombotic.9–12

In young adults, an association between protein Z deficiency and an increased risk of ischemic stroke has been reported,13,14 However, these results conflict with those of others who either found no association between protein Z and ischemic stroke or found an increased risk of stroke among patients with elevated concentrations of protein Z in the blood.15 To further clarify the relationship between protein Z and ischemic stroke, we performed a case-control study of consecutive patients admitted to the hospital with ischemic stroke and a similar number of age- and sex-matched population controls.

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2415
Protein Z plasma concentrations were measured in the first few days after stroke and during the convalescent phase at 3 to 6 months.

### Subjects and Methods

The study was approved by the institutional review board of Royal Perth Hospital, and informed consent was provided by all study participants.

### Cases

Consecutive patients presenting to Royal Perth Hospital between March 1996 and June 1998 with first-ever ischemic stroke were approached for consent to participate in our study. Stroke was defined as a clinical syndrome characterized by rapidly developing clinical symptoms and/or signs of focal, and at times global, loss of brain function, with symptoms lasting >24 hours or leading to earlier death and with no apparent cause other than that of vascular origin.17 Ischemic stroke was defined as a stroke with either a normal CT brain scan, CT or MRI brain scan evidence of a recent infarct in the clinically relevant area of the brain (performed within 3 weeks of the event), or at autopsy. Patients with cerebral hemorrhage, cerebral venous thrombosis, or liver disease were not included. Baseline demographic data (age, sex), history of conventional vascular risk factors (hypertension, diabetes, hypercholesterolemia, current smoker), and history of previous vascular events (myocardial infarction, angina, claudication, amputation for peripheral vascular disease) were obtained. All patients underwent a CT brain scan. Echocardiography and extracranial duplex ultrasound were performed at the discretion of the clinician. Within 7 days of the acute stroke event, a blood sample was obtained for protein Z measurement. Survivors were requested to return for review at 3 to 6 months after the acute event, at which time a second blood sample was taken to measure protein Z levels in the convalescent state. Patients taking oral anticoagulants at the time of stroke or subsequently treated with oral anticoagulants were excluded from the study.

On the basis of clinical evaluation and results of imaging studies, the study neurologist (G.J.H.) (who remained blinded to the results of laboratory assays) classified all strokes into 4 major etiologic subtypes according to the following predefined criteria:18; (1) large-artery disease: ischemic stroke with (a) evidence of extracranial or intracranial occlusive large-artery disease (eg, Doppler, angiographic), and (b) no major cardioembolic source (atrial fibrillation, intracardiac thrombus, prosthetic heart valve), and (c) clinical opinion that the most likely cause of brain infarction was atherothrombosis involving the aortic arch, carotid arteries or major branches (main stem middle cerebral artery), or vertebral, basilic, and posterior cerebral arteries; (2) small-artery disease: ischemic stroke with (a) consciousness and higher cerebral function maintained, plus (b) 1 of the classic lacunar syndromes (ie, pure motor hemiparesis, pure hemisensory loss, pure hemisensory-motor motor loss, ataxic hemiparesis) or nonlacunar small-artery critical syndromes (eg, basilar branch artery syndromes), and (c) CT or MRI brain scan, performed within 3 weeks of symptom onset, that is either normal or shows a small deep infarct in the basal ganglia, internal capsule, or brain stem; (3) cardioembolic disease: ischemic stroke with (a) a major cardioembolic source, plus (b) no definite evidence of occlusive large-artery disease, and (c) clinical opinion that the most likely cause of brain infarction was embolism from the heart; and (4) other: ischemic stroke that did not meet the criteria for 1 of the categories outlined above (eg, periprocedural, hypoperfusion, dissection, procoagulant state), for which there was >1 likely explanation (eg, concurrent large-artery occlusive disease and major cardioembolic source) or for which the etiology of stroke could not be determined.

### Controls

Control subjects were randomly selected from the Western Australian electoral roll, stratified by 5-year age group, sex, and postal code. A letter of invitation to participate, together with a stamped and self-addressed envelope, was sent to potential controls. Nonresponders were contacted by telephone. Controls who agreed to participate in the study were required to fast for a minimum of 8 hours before their appointment and were given the option of attending the hospital outpatient clinic or being visited at home by the study nurse. Baseline demographic data (age, sex), history of conventional vascular risk factors, and history of previous vascular events were obtained for each control. A blood sample was obtained for protein Z measurement. Controls were excluded if they were taking oral anticoagulants.

### Laboratory Analysis

Blood samples were collected and processed with the use of a standardized protocol and were analyzed in the central core laboratory. Blood samples were collected into a 1:10 volume of 3.8% sodium citrate (Greiner) and centrifuged at 1300g for 10 minutes. The plasma was separated, centrifuged a second time at 1300g for 10 minutes, and stored at −80°C until assayed. The samples were thawed for the first time to perform these assays.

Blood concentrations of protein Z were determined with a commercially available enzyme-linked immunosorbent assay manufactured by Diagnostica Stago. The reference range for protein Z is 0.26 to 3.76 μg/mL; the intra-assay coefficient of variation is 8%, and the interassay coefficient of variation is 5%. Reference protein Z was used to prepare assay calibrators. Samples from cases and controls were assayed at random and in duplicate.

### Statistical Analysis

Means or proportions for baseline demographics and vascular risk factors were calculated for cases and controls. The significance of any difference between cases and controls was tested with Student’s t test for means and a χ² test for proportions. Because protein Z levels were skewed (ie, not normally distributed), geometric means were calculated after log transformation of the raw data, and the significance of any difference in geometric mean between cases and controls was tested with Student’s t test.

In cases of stroke, protein Z concentrations measured during the acute phase and at 3 to 6 months of follow-up were compared with a paired Student’s t test. Follow-up levels also were compared in controls with an unpaired Student’s t test. The significance of any relationship between blood levels of protein Z and the timing of blood sampling during long-term follow-up was examined by Pearson correlation coefficient.

Logistic regression was used to examine the association between protein Z levels during the acute phase (independent variable) and ischemic stroke (dependent variable) after the protein Z level was categorized into tertiles for all the cases and controls. Adjusted estimates were obtained with the use of a separate model that controlled for age, sex, conventional vascular risk factors, and history of previous vascular events. Separate logistic regression models were used to examine the association between protein Z and etiologic subtypes of ischemic stroke after adjustment for age, sex, conventional vascular risk factors, and history of previous vascular events. Linear trend of the association was examined by fitting a logistic model with the tertile level treated as a continuous variable. Results are expressed as odds ratios (ORs) and 95% CIs. Statistical significance for all analyses was taken as a 2-sided P < 0.05.

A linear regression model was used to explore baseline predictors of protein Z levels in the blood, including age, sex, conventional vascular risk factors, and past history of vascular disease.

### Results

One hundred seventy-three consecutive patients with ischemic stroke (112 men, 61 women; mean age, 65.8 years [SD 12.6]) and 186 controls (114 men, 72 women; mean age, 67.1 years [SD 11.8]) were studied.

### Baseline Characteristics of Cases and Controls

The age and sex distribution of cases and controls was similar. With the exception of hypercholesterolemia, a greater
proportion of cases featured conventional risk factors for vascular disease (hypertension, diabetes, current smoker, and previous arterial vascular event) (Table 1).

**Protein Z Concentrations in Ischemic Stroke Cases and Controls**

Geometric mean concentrations of protein Z measured within 7 days of acute stroke were significantly higher in cases than in controls (1.46 versus 1.16 µg/mL; P < 0.0001) (Table 2). In the 79 patients in whom protein Z concentrations were measured at 3 to 6 months after the acute stroke event, mean protein Z concentrations were significantly lower during the convalescent phase compared with baseline (1.14 versus 1.36 µg/mL; P = 0.001) and were not significantly different compared with controls (1.14 versus 1.16 µg/mL; P = 0.77). There was no significant correlation between protein Z concentrations and the timing of the follow-up sample taken after (90 to 100, 100 to 149, 150 to 199, >200 days) the acute stroke (P = 0.4).

**Association Between Protein Z Concentration and Acute Ischemic Stroke**

A significant, graded association between ischemic stroke and increasing concentrations of protein Z was seen for all stroke as well as stroke due to large-artery atherothrombosis (Table 3). This association was independent of baseline differences between cases and controls, including age, sex, conventional vascular risk factors (hypertension, diabetes, hypercholesterolemia, smoking), and past history of vascular events. Among cases and controls with protein Z concentrations >1.70 µg/mL (the upper tertile), the adjusted odds of ischemic stroke was 3.07 (95% CI, 1.73 to 5.45) compared with protein Z concentrations in the lowest tertile. The adjusted odds of ischemic stroke caused by large-artery atherothrombosis was nearly 8-fold greater in those with protein Z concentrations in the upper tertile compared with the lower tertile (OR, 7.91; 95% CI, 3.11 to 20.14). The adjusted odds of ischemic stroke due to small-artery disease (OR, 1.79; 95% CI, 0.83 to 3.87) and cardioembolism (OR, 1.80; 95% CI, 0.58 to 5.64) was also increased among individuals with protein Z concentrations in the upper tertile compared with the lower tertile, but not significantly so.

**Baseline Predictors of Protein Z**

None of the baseline patients’ characteristics, including age, sex, conventional vascular risk factors (or combinations of risk factors), and past history of vascular disease, were found to be independently predictive of protein Z concentrations in the blood.

**Discussion**

This is one of the largest studies to date that has examined the association between protein Z levels and ischemic stroke and is the only study to use a broad range of patients and community-based controls. Our results demonstrate that protein Z concentrations are elevated in the acute phase of all etiologic subtypes of ischemic stroke compared with community controls matched for age and sex. We have also demonstrated a strong graded relationship between elevated protein Z levels in the acute phase and ischemic stroke due to large-artery atherothrombosis with a similar, but not statisti-

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**Table 1. Baseline Demographics, Conventional Risk Factors, and History of Previous Vascular Events in Stroke Cases and Controls**

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=173)</th>
<th>Controls (n=186)</th>
<th>OR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean±SD</td>
<td>65.8±12.6</td>
<td>67.1±11.8</td>
<td>0.99</td>
<td>0.98–1.01</td>
<td>0.33</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>112 (64.7)</td>
<td>114 (61.3)</td>
<td>1.16</td>
<td>0.76–1.76</td>
<td>0.50</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>94 (54.3)</td>
<td>62 (33.3)</td>
<td>2.38</td>
<td>1.55–3.65</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>45 (26.0)</td>
<td>21 (11.3)</td>
<td>2.76</td>
<td>1.57–4.87</td>
<td>0.0003</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>38 (22.0)</td>
<td>41 (22.0)</td>
<td>1.00</td>
<td>0.60–1.64</td>
<td>0.96</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>63 (36.4)</td>
<td>33 (17.9)</td>
<td>2.62</td>
<td>1.61–4.27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Previous vascular event, n (%)</td>
<td>46 (26.6)</td>
<td>23 (12.4)</td>
<td>2.57</td>
<td>1.48–4.46</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

*χ² test for categorical data, unpaired Student’s t test for continuous data.

**Table 2. Protein Z During the First 7 Days and 3 to 6 Months After Stroke in Cases and Controls*  

<table>
<thead>
<tr>
<th>Protein Z</th>
<th>Cases (n=173)</th>
<th>Controls (n=186)</th>
<th>P Value†</th>
</tr>
</thead>
</table>
| Geometric mean, µg/mL | 1.46 (1.35, 1.58) | 1.16 (1.07, 1.26) | Acute vs control: P<0.0001†  
| (95% CI)   | 1.14 (0.97, 1.33)  | Follow-up vs control: P=0.001‡ |

*Geometric means presented.
†Paired t test was used for the comparison between protein Z levels during the first 7 days after stroke and follow-up (3 to 6 months); unpaired t test for others.
‡In 79 cases, protein Z levels were measured both during the first 7 days and at 3 to 6 months after stroke (1.36 µg/mL vs 1.14 µg/mL; P=0.001).
The majority of previous studies have measured protein Z only in the convalescent phase, at least 2 months after acute stroke.\textsuperscript{13–16} While our findings of elevated levels during the first 7 days after stroke could be explained by an acute phase response, others have suggested that protein Z is a negative acute phase reactant.\textsuperscript{19,20} C-reactive protein (a well-recognized acute phase protein) is also markedly elevated during the first 7 days after acute ischemic stroke, although, unlike protein Z, it remains persistently elevated for at least 3 to 6 months, most likely as a result of persistent inflammation.\textsuperscript{21} Whether protein Z levels are elevated as a consequence of the acute stroke (eg, protein Z has been identified in atherosclerotic plaques and may be released into the blood during endothelial cell activation or after acute plaque rupture\textsuperscript{22}) or whether elevated levels cause stroke or may be important in the pathogenesis of atherothrombosis remains to be clarified.

In the convalescent phase (3 to 6 months after the acute stroke event), there was no significant difference in protein Z concentrations between cases and controls. This finding cannot be explained by the use of oral anticoagulant therapy because we excluded persons taking warfarin from our study, and it contrasts with the findings of 3 earlier case-control studies.\textsuperscript{13,14,16} The first of these measured protein Z plasma concentrations at least 3 months after ischemic stroke and found that 20\% of patients had levels below the normal range compared with 5\% of controls.\textsuperscript{13} The second study measured protein Z plasma concentrations on 2 occasions, 4 days and 2 months after stroke, and found that levels were approximately 20\% lower than those of controls at both time points.\textsuperscript{14} The third study measured protein Z plasma levels at least 2 months after the acute stroke event and found a 4-fold increased risk of ischemic stroke in patients with high (>160\%) concentrations of protein Z.\textsuperscript{16} The results of a fourth study by Lopaciuk et al.,\textsuperscript{15} however, are consistent with ours in that they found no difference in protein Z plasma levels between stroke cases and controls when measured at least 4 months after the acute stroke event.

There are several possible explanations for the divergent findings of previous studies. First, there were considerable differences in case mix and controls among the studies. Three of the 4 previous studies included stroke patients with a mean age of <50 years,\textsuperscript{13,15,16} while the fourth study included predominantly Hispanic patients with a mean age of 57 years.\textsuperscript{14} Our patients were considerably older. We included consecutive, predominantly white, patients with a mean age of 65 years, which is more likely representative of the mean age of stroke in the general community.\textsuperscript{23} Genetic factors may play a disproportionately greater role in the etiology and pathogenesis of stroke in selected younger patients and in certain ethnic populations and may also be an important determinant of protein Z levels.\textsuperscript{24,25} Apart from our study, only 1 study\textsuperscript{15} randomly selected controls from the general community. This is also the only study whose findings were identical to ours.

Second, the time between the acute stroke event and measurement of protein Z levels varies among studies.\textsuperscript{13–16} This may be an important cause of differences between studies because we have demonstrated that protein Z plasma levels vary after an acute stroke event. Third, some studies have reported that patients with untreated hypertension, hyperlipidemia, or diabetes have higher protein Z levels than patients without these risk factors.\textsuperscript{14,19} Therefore, it is possible that by altering the level of these risk factors, differences in use of antihypertensive or lipid-lowering therapy after
acute stroke may have given rise to variations in protein Z plasma concentrations between studies. However, the effects of these or other therapeutic interventions on protein Z concentrations have not been elucidated and require further study.

Our study has several potential limitations. First, although cases were classified prospectively and recruited consecutively and controls were randomly selected from the community, the potential for confounding can never be eliminated in an observational study. Second, there was incomplete follow-up of stroke patients at 3 to 6 months after the acute event. However, baseline characteristics and protein Z levels during the first 7 days after the acute event were similar in patients who returned for follow-up compared with those who did not return for follow-up. Furthermore, to overcome any bias from incomplete follow-up, we restricted our comparison of protein Z levels during the first 7 days and at 3 to 6 months to those patients who were tested at both time points. Third, mean protein Z levels in our study were lower than reported in previous studies. The explanation for this is not clear, although it may relate to differences in the duration of storage of samples before protein Z levels were measured. However, this does not affect the validity of the comparison between cases and controls in our study because both groups will have been similarly affected.

In conclusion, our study has demonstrated an independent association between increasing blood levels of protein Z and increased risk of ischemic stroke, particularly stroke due to large-vessel atherothrombosis. Whether elevated protein Z concentrations are a cause or consequence of ischemic stroke remains unclear. Additional prospective studies should be performed to further elucidate the possible causal nature of this association.

References