Expression of Thrombomodulin in Patients With Spontaneous Occlusion of the Circle of Willis

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**Background and Purpose:** We examined the expression of thrombomodulin, a recently isolated anticoagulant protein, in endothelial cells from patients with spontaneous occlusion of the circle of Willis (cerebrovascular moyamoya disease) to determine whether lack of the expression of thrombomodulin might lead to the thrombogenicity in patients with this disease.

**Methods:** The intracranial internal carotid arteries, the external carotid arteries, and the vertebral or basilar arteries from 12 autopsied patients who had this disease and eight control autopsied patients were examined immunohistochemically by using the antisera against human thrombomodulin.

**Results:** All of the endothelial cells from the patients with this disease and from the control patients were positive for thrombomodulin. Immunoelectron microscopy also disclosed normal localization of thrombomodulin on the luminal plasma membrane. Immunohistochemically, we could find no significant differences in the expression of thrombomodulin among the arteries examined in this study.

**Conclusions:** We conclude that as far as we investigated immunohistochemically, the thrombogenicity in this disease is almost unlikely to depend on the abnormal expression of thrombomodulin. 

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**Key Words** • endothelium • moyamoya disease • thrombomodulin

Spontaneous occlusion of the circle of Willis (cerebrovascular moyamoya disease) is characterized by stenotic or occlusive lesions around the terminal portions of the internal carotid arteries and abnormal vascular networks at the base of the brain.1-6 The obstructive vascular lesions of the internal carotid arteries are due to eccentric fibrous thickening of the intima.5,6 Although the etiology of this intimal lesion has not been clarified, some authors emphasize the importance of thrombogenesis as a causative factor.6-8 Nevertheless, there are few reports concerning the mechanism through which the patients with this disease tend to form thrombi.

Thrombomodulin, a thrombin receptor on endothelial cells, acts as a cofactor for the thrombin-catalyzed activation of protein C.9 Activated protein C acts as an anticoagulant during blood coagulation by inactivating the coagulation cofactors, factor V and VIII.10,11 Furthermore, thrombomodulin has been found to block the procoagulant activities of thrombin; thrombomodulin directly blocks the thrombin-catalyzed fibrinogen clotting and factor V activation and the platelet activation.9 Thus, thrombomodulin acts as an inhibitor of intravascular coagulation. Thrombomodulin is thought to have important roles in thrombogenesis in vivo.12 Recently the purification of human thrombomodulin and sequencing of its cDNA has been achieved.13,14 and its distribution has been studied in various human organs.15,16 The intracranial extracerebral arteries are known to contain thrombomodulin, although the intracerebral arteries in humans do not contain thrombomodulin.15,16

We set out to ascertain whether the expression of thrombomodulin in the endothelial cells may lead to the tendency to form thrombi in patients with spontaneous occlusion of the circle of Willis. Although one case report studying the expression of thrombomodulin in the endothelial cells of a superficial temporal artery from a patient suffering from both this disease and von Willebrand disease has been published,17 no studies have been performed on the intracranial arteries. In the present study, we performed an immunohistochemical study on the expression of thrombomodulin in endothelial cells of the terminal portions of the internal carotid arteries, the external carotid arteries, and the vertebral or basilar arteries from 12 autopsied patients with this disease.

**Materials and Methods**

We examined endothelial cells taken from the autopsy materials of 12 patients diagnosed as having had spontaneous occlusion of the circle of Willis according to the criteria proposed by the Research Committee on Spontaneous Occlusion of the Circle of Willis (Moyamoya Disease) of the Ministry of Health and Welfare of Japan. There were three males and nine females whose ages ranged from 14 to 55 (average, 40.8) years at the time of autopsy. Endothelial cells from eight control patients (five men and three women) were also examined. The ages of the control patients ranged from 19 to

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Autopsy cases of spontaneous occlusion of the circle of Willis and the arteries whose endothelial cell linings were identified and examined for the expression of thrombomodulin. L, left; R, right; ICA, internal carotid artery; VA, vertebral artery; BA, basilar artery; ECA, external carotid artery.

*Stenotic portions showing characteristic intimal lesions for spontaneous occlusion of the circle of Willis.

87 (average, 69.5) years at the time of autopsy. Only one patient, a 19-year-old man with an arteriovenous malformation in the vertebrobasilar system, had significant cerebrovascular complications. Neither the patients nor the control patients had associated conditions that were thought to influence the expression of thrombomodulin.

We examined the endothelial cells of arteries from the internal carotid, vertebrobasilar, and external carotid systems. In this study, we identified endothelial cell linings immunohistochemically with positive reactivity for rabbit anti-human von Willebrand factor antibody (DAKOPATTS, Denmark).

The circle of Willis and its major branches were embedded in paraffin after immersed fixation in 10% formalin. Histological sections 3 μm thick were prepared and processed for indirect immunoperoxidase staining for thrombomodulin and von Willebrand factor. After treatment with normal swine serum (1/20 dilution; DAKOPATTS), the histological sections were incubated in either anti-human thrombomodulin polyclonal antibody (10 μg/mL) or anti-human von Willebrand factor polyclonal antibody (1/100 dilution; DAKOPATTS) for 1 hour at room temperature in a humidified chamber. After being washed with 0.01 M phosphate buffered saline (PBS), the sections were similarly incubated in peroxidase-conjugated affinity-purified swine immunoglobulins to rabbit immunoglobulins (1/100 dilution; DAKOPATTS) for 30 minutes at room temperature. The sections were washed again with PBS and reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojin Chemical, Kumamoto, Japan) and hydrogen peroxide (0.2 mg/mL DAB and 0.005% hydrogen peroxide in 0.5 M Tris-HCl, pH 7.4). They were then counterstained with hematoxylin and observed under light microscopy.

The histological sections (3 μm) of 10% formalin-fixed tissue embedded in paraffin were also processed for immunoelectron microscopy. The histological sections on slides were immunostained for human thrombomodulin according to the indirect immunoperoxidase method as described above, then postfixed in 1% osmium tetroxide (E. Merck, Darmstadt, FRG) for 1 hour, dehydrated in a graded ethanol series, and flat
embedded on slides in epoxy resin (Epok 812, Oken, Tokyo, Japan). Ultrathin sections were made, stained with lead nitrate (Wako Pure Chemical, Osaka, Japan), and examined with an electron microscope (JEM 1200EX, Tokyo, Japan).

Specificity of the anti-human thrombomodulin polyclonal antibody that was used in this study had already been demonstrated by absorption with purified human thrombomodulin. Negative controls in this study were prepared by replacing the first antibodies by PBS.

**Results**

The arteries whose endothelial cells were identified are listed in Table 1. In four patients (4, 6, 7, and 12), we were able to examine the endothelial cells located in the terminal portions of the internal carotid arteries showing the typical stenotic lesions of this disease (Figure 1, panel A). Significant disruption of the internal elastic lamina was also undetectable. Expression of thrombomodulin in the endothelial cells of these arterial lesions was confirmed immunohistochemically by the indirect immunoperoxidase method (Figure 1, panel B).

In two patients (3 and 10), we examined the endothelial cells from not only the internal carotid but also the vertebrobasilar and external carotid systems. All of these endothelial cells showed positive reactivity for thrombomodulin. Among the three systems, no significant differences in the expression of thrombomodulin could be detected immunohistochemically (Figure 2). Furthermore, immunoelectron microscopy revealed that thrombomodulin was asymmetrically distributed on the luminal but not the abluminal plasma membrane of the endothelial cells (Figure 3).

The percentage of the endothelial cells positive for thrombomodulin in the remaining cells in the section was 100% in every artery of both the patients with this disease and the control patients. Immunohistochemically, we could find no significant differences in the expression of thrombomodulin in either the patients or the control patients, regardless of the patient's age and the time after death.

**Discussion**

The obstructive vascular lesion at the terminal portions of the internal carotid arteries is thought to be the primary and essential lesion in spontaneous occlusion of the circle of Willis. We examined the expression of thrombomodulin, a recently recognized anticoagulant cofactor, in the intracranial internal carotid arteries, as well as the external carotid arteries and the vertebral or basilar arteries, of 12 patients with this disease. Although the vascular lesion of this disease is thought to involve the extracranial vessels including the external carotid artery, the focal etiologic factors around the terminal portion of the internal carotid artery might exist to form the characteristic lesion of this disease.

In our present study, we confirmed the expression of thrombomodulin in the endothelial cells from all of the intracranial internal carotid arteries studied, including four in which characteristic stenotic lesions of this disease were observed.

We also confirmed the localization of thrombomodulin on the luminal plasma membrane by immunoelectron
microscopy, although the cytoplasmic organelles could not be analyzed in detail because of postmortem changes. This result is consistent with a previous report describing the ultrastructural localization of thrombomodulin.18

As far as we examined by the immunohistochemical methods as described above, there were no abnormalities in the expression of thrombomodulin in endothelial cells from the patients with spontaneous occlusion of the circle of Willis. This result supports the idea that the thrombogenicity in this disease is unlikely to depend on the abnormal expression of thrombomodulin. However, the possibility remains that either functional abnormalities of the thrombomodulin molecule or transient downregulation of the expression of thrombomodulin in a certain stage of the patient's history, which could not be detected in our present study, might contribute to the tendency to form thrombi in this disease.

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