Elevation of CRABP-I in the Cerebrospinal Fluid of Patients With Moyamoya Disease

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Background and Purpose—The etiology of moyamoya disease (MMD) remains obscure. This study was undertaken to identify specific proteins associated with the pathogenesis of MMD.

Methods—We studied cerebrospinal fluid (CSF) from 20 patients with angiographically confirmed MMD (4 boys and 16 girls; age range, 3 to 13 years; mean, 7.5 years) and 4 control patients with cerebral palsy who underwent selective dorsal rhizotomy (2 boys and 2 girls; age range, 5 to 10 years; mean, 7.3 years). CSF proteins were analyzed by 2-dimensional polyacrylamide gel electrophoresis, and protein identification was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The presence of specific CSF protein in patients with MMD was confirmed by Western blotting. In addition, cerebral CSF was also tested in 7 patients who had other brain diseases but no MMD (2 boys and 5 girls; age range, 1 to 12 years; mean, 6.9 years).

Results—We identified 1 polypeptide spot (M, of 13 to 15 kDa and isoelectric point of 5 to 5.5) that was differentially expressed in the CSF samples of MMD patients (mean optical density intensity, 0.36±0.24; range, 0.05 to 0.92) and control spinal CSF samples (mean, 0.03±0.04; range, 0 to 0.08; P=0.002). This polypeptide was identified as cellular retinoic acid–binding protein (CRABP)-I. High levels of expression of CRABP-I in the CSF from 17 MMD children were confirmed by Western blotting.

Conclusions—The analysis of the CSF of MMD patients reveals high CRABP-I expression. The present study suggests that the elevation of CRABP-I in CSF may be a candidate for pathogenesis of MMD. (Stroke. 2003;34:2835-2841.)

Key Words: cerebrospinal fluid ■ electrophoresis, gel, two-dimensional ■ moyamoya disease ■ receptors, retinoic acid ■ spectrometry, mass, matrix-assisted laser desorption-ionization

Moyamoya disease (MMD) is the most common pediatric cerebrovascular disease in East Asia and is characterized by a progressive occlusion of the internal carotid artery or of its terminal branches, accompanied by the formation of extensive collateral vessels (“moyamoya” vessels) at the base of the brain.

The occlusion of major intracranial arteries is attributed to eccentric fibrocellular thickening of the intima due to proliferation of smooth muscle cells and fibrosis.1-3 Pathological findings suggest the presence of growth factors that promote the migration and proliferation of smooth muscle cells from the media to the intima.4 Basic fibroblast growth factor (bFGF),1,5-8 transforming growth factor-β (TGF-β),1,5-8 and platelet-derived growth factor (PDGF)10-12 have been reported to be involved in the pathogenesis of MMD. The increased expression of bFGF in the major intracranial cerebral arteries, superficial temporal arteries, and cerebrospinal fluid (CSF) has been previously reported in MMD.1,5-8 Moreover, the expression levels of TGF-β1 in smooth muscle cells, cultured from superficial temporal arteries, and the serum level of TGF-β1 were reported to be elevated in MMD.9 Additionally, smooth muscle cells cultured from arteries with MMD involvement have been known to express fewer PDGF receptors and to show a diminished response to serum mitogens, especially PDGF.10-12 However, the pathogenesis of MMD remains unknown because it is not clear whether these factors are causative or are simply associated with the pathogenesis, for example, the angiogenesis associated with this disease.4

The low mortality rate, limited availability of surgical specimens from affected internal carotid artery, and nonexistence of an animal model have been obstacles to basic research involving pediatric MMD. These limitations suggest that the analysis of CSF proteins offers one of the most effective means of investigating the pathogenesis of this disease.13 Proteomics, the large-scale study of proteins, has
been associated traditionally with the separation of a large number of proteins from a given cell line or organism by 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE). 2-D PAGE separates crude protein mixtures in a single gel according to their relative molecular masses (M_r) and isoelectric points (pI). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) is an analytical tool for the characterization of gel-isolated proteins. With the use of this technique, gel-separated proteins can be excised and digested enzymatically, their mass spectra can be obtained, and, finally, these spectra can be automatically searched for with the use of databases.

In this study we analyzed the CSF proteins of MMD patients by 2-D PAGE and identified a specific protein using MALDI TOF MS.

### Subjects and Methods

#### Patient Population

For 2-D PAGE and MALDI TOF MS, we obtained the CSF of 20 patients with angiographically confirmed MMD (4 boys and 16 girls; age range, 3 to 13 years; mean, 7.5 years) and the CSF of 4 patients with cerebral palsy who underwent selective dorsal rhizotomy (2 boys and 2 girls; age range, 5 to 10 years; mean, 7.3 years) (Table). For Western blotting, we added 7 patients with other brain diseases (5 during brain tumor surgery and 2 during surgery for epilepsy surgery) but with no evidence of MMD (2 boys and 5 girls; age range, 1 to 12 years; mean, 6.9 years).

All patients with MMD suffered sustained repetitive transient ischemic attacks, and 12 of the 20 patients showed evidence of infarction on MRI. Angiography showed several Suzuki stages in the group, but most of the hemispheres were in stages 2 and 3. We have operated on pediatric MMD patients with encephalo-duro-arterio-synangiosis (EDAS) and bifrontal encephalo-galeo-(periosteal)-synangiosis (EGS), with the aim of improving the blood flow.
supply not only to middle cerebral artery territory but also to the anterior cerebral artery territory.

**Sample Collection and Protein Preparation**

The CSF of moyamoya patients was collected from the cerebral subarachnoid space (the frontal cortex or the superficial sylvian fissure) during bypass surgery, which was performed at least 4 weeks after the last stroke attributed to MMD. In the 4 patients with cerebral palsy, CSF was obtained from the spinal subarachnoid space during selective dorsal rhizotomy. In the 7 patients with other neurological disorders, CSF was obtained from the cerebral subarachnoid space during operation.

CSF samples were stored at −80°C before use. For protein preparation, 1 mL of the CSF was mixed with 250 μL of 50% trichloroacetic acid (Merck Co) and centrifuged at 16 000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was washed with ethyl ether (Merck Co). After it was washed, the protein pellet was dissolved in a solution containing 8 mol/L urea, 4% CHAPS, 40 mmol/L Tris base, 100 mmol/L dithiothreitol. Protein concentrations were determined by the Bradford method (Bio-Rad).

**Two-Dimensional Polyacrylamide Gel Electrophoresis**

All of the reagents used for 2-D PAGE were obtained from Amersham Pharmacia Biotech, Korea Ltd. 2-D PAGE was performed by vertical electrophoresis (Bio-Rad), IPGPhor was used to perform the first-dimensional isoelectric focusing with the use of an immobiline DryStrip (18 cm, pH 3 to 10, Amersham Pharmacia Biotech), and the PROTEAN 2, 2-DE system (Bio-Rad) was used for the second-dimensional SDS-PAGE (7% to 17% gradient gel). A total amount of 75 μg of protein was loaded to the IPG (immobilized, pH 3 to 10, nonlinear gradient) strips by in-gel rehydration with the use of IPGPhor (18 cm, Amersham Pharmacia Biotech).

Isoelectric focusing was performed successively at 500 V for 1 hour, 1000 V for 1 hour, and 8000 V for 5 hours. After isoelectric focusing, strips were incubated with equilibration buffer 1 (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, bromophenol blue (BPB), 2% dithiothreitol) and buffer 2 (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, BPB, 2% dithiothreitol, 2.5% iodoacetamide) for 15 minutes each. Equilibrated strips were placed on polyacrylamide gradient slab gels (7% to 17% gradient). Separation was continued at 10 mA per gel in running buffer (25 mmol/L Tris, pH 8.8, 198 mmol/L glycine, 0.1% SDS) until the BPB reached the bottom of the gel. After electrophoresis, the protein spots were visualized by using a modified silver-staining protocol compatible with MALDI TOF MS.

The silver-stained gel was scanned with the use of a densitometer 800 (Bio-Rad), and the digitalized image was analyzed with the use of PDQUEST software (version 6.1, Bio-Rad). The optical density (OD) of specific proteins in MMD patients and in controls was analyzed statistically with the Mann-Whitney test. In MMD patients, the associations between clinical features and the ODs of specific proteins were also determined. Values of P<0.05 were considered significant.

**Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry**

Proteins were identified by peptide mass fingerprinting methods. Selected protein spots were cut from gels with a spot cutter (Bio-Rad). The excised gel spots were destained in 100 μL of destaining solution (30 mmol/L potassium ferricyanide, Sigma Chemical Co), in 100 mmol/L of sodium thiosulfate (Merck Co), with shaking for 5 minutes. After the solution was removed, the gel spots were incubated with 200 mmol/L of ammonium bicarbonate (Sigma Chemical Co) for 20 minutes. The gel pieces were then dried in a speed vacuum concentrator for 5 minutes and then rehydrated with 20 μL of 50 mmol/L ammonium bicarbonate containing 0.2 μg of modified trypsin (Promega) for 45 minutes on ice. After the solution was removed, 30 μL of 50 mmol/L ammonium bicarbonate was added, and the digestion was performed overnight at 37°C. After residual trypsin was removed, the peptides were desalted with the use of a C18 nanoscale (pore size 18 cm) column (homemade). The peptides were then eluted with 0.8 μL of matrix solution (70% acetonitrile [Merck Co], 0.1% trifluoroacetic acid [Merck Co], and 10 mg/mL of α-cyano-4-hydroxycinnamic acid [Sigma Chemical Co]). The eluted peptides were then spotted onto a stainless steel target plate, and peptide masses were determined with a MALDI TOF MS (Micromass). The unit was calibrated with the use of a trypsin auto digestion product (m/z 2211.105). Peptide masses were matched with the theoretical peptide masses of all human proteins in the NCBI database with the use of Mascot software and Profound software.

**Western Blot Analysis**

The presence of cellular retinoic acid–binding protein (CRABP)-1 in the cerebral subarachnoid CSF of 17 MMD patients, the spinal subarachnoid CSF of 4 cerebral palsy patients, and the cerebral subarachnoid CSF of 7 patients with other neurological disorders was confirmed by Western blotting. We were unable to perform Western blotting in 3 MMD patients because of the shortage of CSF samples.

CSF samples were concentrated by trichloroacetic acid precipitation, and 50 μg of protein was denatured with SDS loading buffer for 10 minutes at 70°C and then separated on 16% SDS-polyacrylamide denaturing gel and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech). After it was blocked in Tris-buffered saline containing 5% nonfat dry milk, the membrane was incubated with the primary antibody, goat anti-human CRABP-1 (1:500) (Santa Cruz Biotechnologies) in TBS containing 0.1% Tween-20 for overnight at 4°C. After it was washed, the blot was incubated with horseradish peroxidase–conjugated mouse anti-goat secondary antibody (1:1000) for 30 minutes at room temperature. Horseradish peroxidase–conjugated anti-human albumin antibody (1:2000) (KOMA Biotech Inc) was used as a loading control. Detection was performed with the use of an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

**Results**

**Analysis of CSF Protein by 2-D PAGE**

We performed comparative proteome analysis of the CSF from MMD patients and 4 patients with cerebral palsy (Figure 1). Approximately 500 spots were consistently observed on each gel by silver staining, and the patterns of the silver-stained gels were compatible with those of the master gels of CSF reported in the SWISS-2DPAGE database. One polypeptide spot (M r=13 to 15 kDa, pl=5 to 5.5) was observed to be differentially expressed in the CSF samples of MMD patients and cerebral palsy patients. In patients with MMD, the mean OD of this spot was 0.36, ranging from 0.05 to 0.92; however, the mean OD of this spot in control patients was 0.03, ranging from 0 to 0.08, and this difference was significant (P<0.002). However, no significant relationship was found between the OD of this spot and the presence of infarction on MRI or Suzuki angiographic grade.

**Identification of MMD-Associated Protein by MALDI TOF MS**

The protein was identified by peptide mass fingerprinting by MALDI TOF MS. Individual peptide peaks in the mass spectrum were utilized in a Mascot Search, and the MMD-associated protein in the CSF from MMD patients was identified as CRABP-1 (Figure 2). Sixteen mass values were searched, and 7 were matched, giving a sequence coverage of 48%. This analysis was performed with a mass tolerance of 70 ppm.
Confirmation of MMD-Specific Protein by Western Blotting
Consistent with the 2-D gel result, the high expression of CRABP-I (15 kDa) in MMD CSF was confirmed by Western blotting (Figure 3). CRABP-I was not detected in the spinal and cerebral subarachnoid CSF of the control patients, except for 1 with a cavernous malformation.

Discussion
In 1999, Hojo et al. separated CSF proteins by 2-D PAGE and detected a polypeptide spot ($M_r=12$ kDa, $pI=5.35$) in the CSF samples of 3 MMD patients. However, they were unable to characterize the polypeptide. Using 2-D PAGE in a large number of CSF samples, we also detected a polypeptide spot ($M_r=13$ to 15 kDa, $pI=5$ to 5.5), which we assume was the same spot found by the Japanese group. We identified this polypeptide by MALDI TOF MS as CRABP-I, and the high expression of CRABP-I in the CSF of MMD patients was confirmed by Western blotting.

The relationship between elevated CRABP-I and the presence of cerebral infarction is believed to be negligible, because in the present study we examined chronic-stage MMD patients (ie, the CSF was taken at least 4 weeks after the previous ischemic event), and the OD of CRABP-I was not related to the presence of infarction. To rule out the possibility of sampling variation between the subarachnoid CSF from the cerebral cortex in MMD patients and the subarachnoid CSF from the spinal space in controls, we performed Western blotting for CRABP-I in another control subarachnoid CSF, ie, from the cerebral cortex of patients with other brain diseases but with no MMD. With the exception of 1 case with cavernous malformation, the control

Figure 1. Analysis of CSF protein by 2-D PAGE. By comparative proteome analysis of the CSF from 20 patients with MMD and 4 patients with cerebral palsy, approximately 500 spots were consistently observed on each gel by silver staining. We identified 1 polypeptide spot ($M_r=13$ to 15 kDa, $pI=5$ to 5.5) that was differentially expressed in the CSF samples of cerebral palsy patients (left) and the CSF of MMD patients (right). Enlarged maps of the CSF proteins showed that the spot, indicated by a circle, was present in all patients with MMD but not in the patients with cerebral palsy.

Figure 2. Identification of protein associated with MMD by MALDI TOF MS. The analysis of individual peptide peaks produced by trypsin digestion showed that the overexpressed protein was CRABP-I. Sixteen mass values were searched, and 7 were matched; therefore, sequence coverage was 48%. In this analysis, the mass tolerance was 70 ppm.
subarachnoid CSF from the cerebral cortex showed no CRABP-I expression. The elevation of CRABP-I in the CSF of cavernous malformation might suggest that CRABP-I is more generally related to cerebrovascular disease. This is not a unique concern of CRABP-I. For example, Malek et al. found that CSF bFGF, a well-known angiogenic substance in MMD, is significantly elevated in patients with MMD as well as in patients with arteriovenous malformation and brain tumor. The addition of new control groups including infarct and ischemia might be able to answer more clearly whether CRABP-I is a general marker for cerebrovascular disease (ie, infarct or ischemia) or a specific one for MMD. However, we were not able to reflect this point in our study for the following reasons: First, it was difficult to recruit a new control group because of the rarity of pediatric stroke patients with no evidence of MMD. Next, CRABP-I was detected in MMD patients without infarcts as well as with infarcts on MRI, and the OD of CRABP-I showed no difference according to the presence of infarction.

Retinoids are natural and synthetic derivatives of vitamin A and have recently been identified as critical regulators of vascular smooth muscle cell differentiation and growth. Several studies have shown that retinoids attenuate growth factor–stimulated smooth muscle cell proliferation and neointimal formation. In addition, the inhibitory effects of retinoids on neointimal formation were suggested to be modulated by the expression of genes involved with smooth muscle cell proliferation and migration.

The multiple biological activities of retinoic acid (RA), a hormonally active metabolite of vitamin A, are mediated by 2 classes of proteins: the retinoic acid receptors (RARs) and the cellular RA-binding proteins (CRABP-I and CRABP-II). The RARs encompass ligand-inducible transcription factors that belong to the superfamily of nuclear hormone receptors and that are specifically activated by RA. CRABPs bind retinoids and may modulate their intracellular steady state concentration. Two isoforms (CRABP-I and CRABP-II) have been characterized. Both are expressed in the embryo, and CRABP-1 is ubiquitously expressed in various adult tissues. Although the exact function of CRABP-I is not completely understood, it has been proposed that CRABP-I negatively regulates the activity of RA by enhancing the production of RA-metabolizing enzymes and by increasing the rate at which RA is degraded. Boylan et al. showed that stably transfected F9 cells, which overexpress CRABP-I, require a higher concentration of RA to initiate differentiation, whereas F9 cells, which have a reduced CRABP-I level as a result of the stable transfection of a CRABP-I anti-sense expression vector, require a lower concentration of RA to initiate differentiation. Neuville et al. reported that CRABP-I is selectively expressed by a subpopulation of rat smooth muscle cells prone to migrate, proliferate, and give rise to intimal hyperplasia.

Although the pathogenesis of MMD remains obscure, pathological findings strongly suggest that the disease process occurs mainly in the intima. Moreover, after the proliferation and migration of smooth muscle cells were identified as mechanisms of intimal thickening and moyamoya vessel formation, many investigators turned to the study of growth factors, cytokines, and their receptors. Retinoids have been shown to significantly modulate the expressions of growth factors, which may induce and promote the development of intimal thickening in MMD (Figure 4). Boyle et al. demonstrated that the combination of an RAR ligand and a retinoid X receptor (RXR) agonist can maximally repress the angiogenic FGF-binding protein gene, which facilitates the actions of FGF1 and FGF2. TGF-β1, a potent activator of differentiation, is also known to be susceptible to the antagonistic effects of all-trans retinoic acid (tRA). RARs and RXR-α downregulate TGF-β1 promoter by antagonizing AP-1 activity, which is required for TGF-β1 gene expression. In addition, an early report showed that tRA suppressed PDGF-BB–stimulated smooth muscle cell proliferation in human intimal smooth muscle cells. Similarly, tRA and 9-cis RA blocked PDGF-BB–induced rat aortic smooth muscle cell proliferation. These observations suggest that retinoid signaling plays an important role in the pathogenesis of MMD by controlling growth factor expres-
Retinoids

inhibition

CRABP-I

inhibition

Growth factors & cytokines
(bFGF, TGF-β, PDGF etc.)

Smooth muscle cell
proliferation & migration

Intimal thickening

Moyamoya disease

Figure 4. Potential mechanism linking CRABP-I to pathogenesis of MMD. Retinoids attenuate growth factor–stimulated smooth muscle cell migration and proliferation. In this way, retinoids inhibit neointimal thickening, which is the histological hallmark of MMD. CRABP-I negatively regulates the retinoid activity by enhancing the production of RA, metabolizing enzymes, and increasing the rate at which RA is degraded.

In conclusion, the analysis of the CSF of MMD patients revealed that CRABP-I was highly expressed. Because CRABP-I is known to attenuate the inhibitory effect of RA on growth factor–stimulated smooth muscle cell proliferation, the high expression of CRABP-I in cerebral CSF may be associated with intimal thickening in MMD. It remains to be determined how the retinoid pathway is involved in intimal thickening in MMD and whether retinoids may be efficacious in the treatment of MMD.

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