Increased Expression of Hepatocyte Growth Factor in Cerebrospinal Fluid and Intracranial Artery in Moyamoya Disease

Rina Nanba, MD, PhD; Satoshi Kuroda, MD, PhD; Tatsuya Ishikawa, MD, PhD; Kiyohiro Houkin, MD, PhD; Yoshinobu Iwasaki, MD, PhD

Background and Purpose—The etiology of moyamoya disease still remains unknown. This study was aimed to explore the role of hepatocyte growth factor (HGF), a strong inducer of angiogenesis, in development of moyamoya disease.

Methods—We studied cerebrospinal fluid (CSF) from 39 patients with moyamoya disease (24 children and 15 adults), 6 control patients with cervical spondylosis, and 7 control patients with internal carotid artery occlusion. CSF level of HGF was determined by enzyme-linked immunosorbent assay technique. We also evaluated the distribution of HGF and its cellular receptor c-Met in the carotid fork obtained from 2 patients with moyamoya disease and 2 control patients.

Results—CSF level of HGF was 408.2 ± 201.6 pg/mL and 443.2 ± 193.5 pg/mL in patients with cervical spondylosis and internal carotid artery occlusion, respectively (mean ± SD). On the other hand, CSF level of HGF was 820.3 ± 319.0 pg/mL in patients with moyamoya disease, being significantly higher than those in 2 control groups (P < 0.01). Both HGF and c-Met were widely distributed in the media and thickened intima of the carotid fork in patients with moyamoya disease but not in control patients.

Conclusions—This study revealed that HGF is densely found in the carotid fork, and its CSF level is markedly elevated in moyamoya disease, suggesting that HGF may be a key protein for pathogenesis of moyamoya disease. (Stroke. 2004; 35:2837-2842.)

Key Words: angiogenesis ● hepatocyte growth factor ● moyamoya disease ● protooncogene protein c-met

Moyamoya disease is a specific cerebrovascular disease that features stenosis or occlusion of the terminal portion of the bilateral internal carotid arteries (ICAs) associated with abnormal vascular network.1 The histopathological findings in the circle of Willis are fibrocellular thickening of the intima, an irregular undulation (waving) of the internal elastic lamina, and the attenuation of the media, suggesting that the disease process occurs mainly in the intima.2 The “moyamoya” vessels in the basal ganglia and thalamus show either dilated thin-walled arteries, or obstruction from recent thrombi, or mural thickening with or without elastosis or fibrosis. Moreover, similar lesions can be found in the vessels of the other organs, such as heart and kidney.3

Etiology of the disease is still unknown; however, several epidemiological studies suggest the infection in head and neck regions may be related to moyamoya disease, although a certain infectious pathogen has not been determined.4 Some genetic factors may also play an important role in pathogenesis of moyamoya disease. The hypothesis is based on the fact that familial occurrence is recognized in ≈10% to 15% of patients5 and that the incidence of moyamoya disease is much higher in Far Eastern than in Western countries.6

Alternatively, previous studies have shown that certain growth factors or cytokines are elevated in the cerebrospinal fluid (CSF) of patients with moyamoya disease. Expression of basic fibroblast growth factor (bFGF) is reported to increase in the CSF and in the intra- and extracranial arteries.7–11 Soriano et al recently reported that CSF levels of soluble vascular cell adhesion molecule type 1, intercellular adhesion molecule type 1, and E-selectin are elevated in moyamoya disease and suggested ongoing inflammatory processes in the central nervous system.12 Recently, Kim et al characterized a specific protein, cellular retinoic acid-binding protein-I, from the CSF of patients with moyamoya disease.13

On the other hand, hepatocyte growth factor (HGF) was discovered as a growth factor of hepatocytes and was subsequently shown to have numerous functions in every tissue. Currently, HGF is also known as a strong inducer of angiogenesis and is more potent than vascular endothelial cell growth factor or bFGF.14 All biological responses to HGF are mediated through a tyrosine kinase receptor encoded by the c-Met protooncogene.15 A local HGF...
system (HGF and c-Met) is expressed in vascular cells,\textsuperscript{14} and elevated serum level of HGF has been recognized as an independent indicator of systemic atherosclerosis\textsuperscript{16} or hypertension.\textsuperscript{17}

This study, therefore, was aimed to assess the role of HGF in development of moyamoya disease. For this purpose, we measured CSF level of HGF and evaluated the distribution of HGF and c-Met in the carotid fork specimens obtained from patients with moyamoya disease.

Materials and Methods

Quantitative Analysis of HGF in CSF

We studied CSF from 39 patients with moyamoya disease. All patients were diagnosed as moyamoya disease on cerebral angiography. There were 15 males and 24 females. They included 24 pediatric and 15 adult patients. Their clinical diagnosis included transient ischemic attack (TIA) in 27 patients, cerebral infarction in 9 and intracranial bleeding in 3. MRI was performed to evaluate cerebral infarction in all patients. Clinical features of the patients are summarized in Table. We also studied CSF from 7 patients with TIA caused by unilateral ICA occlusion (aged 55 to 71 years) and from 6 patients with cervical spondylosis (aged 26 to 70 years) as the controls.

CSF samples were collected from patients with moyamoya disease during bypass surgery. All patients underwent bypass surgery 1 to 3 months after their onset. After small opening of arachnoid membrane over the frontal cortex or Sylvian fissure, 1 to 2 milliliter of CSF was collected. CSF samples were also obtained from patients with ICA occlusion during bypass surgery and were obtained from patients with cervical spondylosis by lumbar tap during myelography. All CSF samples were filtered through a 0.22-mm filter (Millipore Co) and stored at −80°C until subsequent assay. HGF in the samples was measured with an enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer’s instructions.

In this study, we analyzed correlation between CSF level of HGF and patients’ clinical features. Continuous data were expressed as mean ± SD and statistically analyzed by using Student \( t \) test between 2 groups or 1-factor ANOVA followed by post hoc Fisher’s protected least significant difference among >3 groups. Categorical variables were compared by using \( \chi^2 \) test. All analysis was performed using StatView version 5.0 (SAS Institute Inc). Values of \( P < 0.05 \) were considered statistically significant.

Immunohistochemical Analysis in the Carotid Fork

The expression of HGF and c-Met in the intracranial arteries from 2 patients with moyamoya disease was evaluated. One moyamoya disease specimen was obtained from a 30-year-old man who died of unknown cause 10 years after bilateral bypass surgery. The second moyamoya disease specimen was obtained from a 62-year-old woman who developed subarachnoid hemorrhage because of the rupture of basilar artery aneurysm. The aneurysm was clipped through trans-Sylvian approach. During surgery, the nearly occluded segments of the ICA and middle cerebral artery (MCA) were removed as a specimen.

Two control specimens were obtained in autopsy from a 50-year-old woman and a 51-year-old man who died of pulmonary embolism and intracranial hemorrhage, respectively. The specimens were fixed in buffered formalin (4%) and were embedded in paraffin. Four-um thick cross-sections were prepared for the subsequent staining. Hematoxylin and eosin (H&E) staining was used to observe the anatomical structure of the specimens. The cross-sections were also stained with primary antibodies against HGF and c-Met. The sections were autoclaved with 10 mM citric acid (pH 6.0) for 15 minutes. The specimens were treated with PBS at room temperature for 30 minutes and then with 1% hydrogen peroxide for 10 minutes. Nonspecific binding was blocked by treatment with 10% normal goat serum for 15 minutes. After rinsing in PBS, the specimens were incubated with primary antibody against HGF (mouse monoclonal anti-human HGF, NeoMarkers, Lab Vision Corp, Fremont, Calif; 1:100) or c-Met (rabbit polyclonal anti-human c-Met p140 antibody, Santa Cruz Biotechnology Inc, Santa Cruz, Calif; 1:400) at 4°C for overnight. They were then rinsed \( 3 \times \) in PBS and incubated with biotinylated secondary antibody for 30 minutes at room temperature. After washing in PBS, the specimens were incubated with biotinylated secondary antibody for 30 minutes at room temperature. After washing with distilled water, they were counterstained with hematoxylin. To quantitatively analyze the expression of HGF and c-Met in the specimens, we counted the number of HGF- or c-Met–positive cells in 3 individual fields at a magnification of \( \times 20 \) and calculated the proportion of HGF- or c-Met–positive cells to total cells.

All experimental procedures were approved by Ethical Committee at Hokkaido University Graduate School of Medicine, and all samples were obtained with patients’ agreement.

Results

CSF Level of HGF in Moyamoya Disease

CSF level of HGF was 408.2 ± 201.6 pg/mL in patients with cervical spondylosis and 443.2 ± 193.5 pg/mL in patients with ICA occlusion. There was no significant difference between 2 control groups. CSF level of HGF was 820.3 ± 319.0 pg/mL in patients with moyamoya disease, being significantly higher than those in 2 control groups (\( P < 0.01 \), Figure 1a).

When moyamoya patients were divided into pediatric and adult groups, CSF levels of HGF were 872.3 ± 324.4 pg/mL and 736.9 ± 302.1 pg/mL, respectively. Their CSF levels of HGF were significantly higher than those in both control groups (\( P < 0.01 \) and \( P < 0.05 \), respectively; Figure 1b). There was also no significant correlation between CSF level of HGF and patients’ age (\( R^2 = 0.022, P > 0.05 \)). CSF levels of HGF in male and female patients were 858.7 ± 355.1 pg/mL and 796.2 ± 299.6 pg/mL, respectively, showing no significant difference between genders (\( P > 0.05 \)).

When correlation between CSF level of HGF and onset pattern was analyzed, the values were 811.2 ± 262.7 pg/mL in TIA, 978.0 ± 397.2 pg/mL in cerebral infarction, and 428.8 ± 249.6 pg/mL in intracranial bleeding (Figure 1c). Therefore, patients with intracranial bleeding had significantly lower CSF level of HGF than those with TIA (\( P < 0.05 \)) and with cerebral infarction (\( P < 0.01 \)). Correlation between CSF level of HGF and Suzuki’s angiographic stage was also evaluated.\textsuperscript{1} Because angiographic stage is different between sides, a more advanced stage was chosen for analysis. CSF levels of HGF in patients with stage 2 (\( n = 1 \)), stage 3 (\( n = 17 \)), stage 4 (\( n = 17 \)), and stage 5 (\( n = 4 \)) were 556.9 pg/mL, 889.5 ± 333.0 pg/mL, 748.5 ± 277.5 pg/mL, and 896.6 ± 446.7 pg/mL, respectively. Statistical analysis disclosed no significant difference among them. To evaluate the effect of cerebral infarction on CSF level of HGF, the values were also compared between moyamoya patients with and without cerebral infarction. The values were 768.9 ± 285.2 pg/mL (\( n = 10 \)) and 969.3 ± 378.6 pg/mL (\( n = 29 \)) in patients with and without cerebral infarction, respectively. Presence of cerebral infarction had no significant effect on the values.

Postoperative angiography, which was performed 3 months after surgery, revealed good revascularization through indirect synangiosis in 36 of the 39 patients (good collateral group). However, little revascularization was...
observed in the other 3 patients (poor collateral group). The CSF level of HGF in the good collateral group was 848.6±312.1 pg/mL, which was slightly higher than 480.1±201.6 pg/mL in the poor collateral group (P=0.0533).

Expression of HGF and c-Met in the Carotid Fork
On H&E staining, the terminal portion of the ICA and the proximal portion of the MCA had severe asymmetrical stenosis of the lumen. The intima was markedly thickened. The internal elastic lamina was also thickened and severely curved. There was no significant thickening in the media. These were the typical histological findings in moyamoya disease (Figure 2). Control specimens exhibited typical findings of atherosclerosis, including thickening of the intima, on H&E staining (data not shown).

On immunohistochemistry, the HGF-positive cells were widely distributed in the media of the intracranial arteries

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obtained from 2 patients with moyamoya disease (Figure 3a). The HGF-positive cells were also distributed sparsely in the intima. However, a much smaller number of the HGF-positive cells could be observed in the control specimens (Figure 3b). Quantitative analysis revealed that \( \approx 75\% \) and \( 20\% \) of cells were positive for HGF in the media of moyamoya and control specimens, respectively.

Likewise, the c-Met-positive cells were widely observed in the media and intima of the intracranial arteries obtained from 2 patients with moyamoya disease, although there were few c-Met-positive cells in the control specimens (Figure 4). Quantitative analysis revealed that \( \approx 60\% \) and \( 5\% \) of cells were positive for c-Met in the media of moyamoya and control specimens, respectively.

**Discussion**

**Role of HGF in Development of Atherosclerosis**

HGF is a mesenchyme-derived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells and stimulates endothelial cell growth and migration of vascular smooth muscle cells (VSMCs), being known as a strong inducer of angiogenesis.\(^{18}\) Both HGF mRNA and c-Met mRNA were detected in endothelial cells and VSMCs of human aorta.\(^{14}\) HGF is secreted from endothelial cells and VSMCs and is speculated to play a key role in maintaining the vascular structure in an autocrine-paracrine manner under physiological conditions.\(^{19}\) Hayashi et al reported that overexpression of HGF in endothelial cells or VSMCs strongly stimulates endothelial cell growth.\(^{20}\) Recently, Taher et al reported that HGF/c-Met signaling pathway might promote the VSMC migration.\(^{21}\) Serum HGF concentration also correlates with blood pressure.\(^{17}\) Therefore, HGF is most likely involved in the pathogenesis of atherosclerosis.

In this study, immunocytochemical analysis revealed that HGF is strongly expressed in the media of the carotid fork in moyamoya disease. HGF was also positive in the thickened intima. The findings were quite different from those in the atherosclerotic intracranial arteries where only faint and scattered HGF immunoreactivity was observed. The findings were also distinct when compared with the distribution of bFGF immunoreactivity, which was positive only in the single layer of endothelial cells in the carotid forks of moyamoya disease.\(^{9}\) These findings strongly suggest that enhanced expression of HGF in the VSMC may promote intimal thickening and migration of VSMCs into the intima in the carotid fork of moyamoya disease and be closely related to pathogenesis of moyamoya disease.

**Role of HGF in Moyamoya Disease**

HGF mRNA and protein have been found in the brain. Its cellular receptor c-Met is also expressed in the brain. Cerebral ischemia upregulates both HGF and c-Met.\(^{22,23}\) Nagayama et al reported that
HGF is expressed in the reactive astrocytes in the peri-infarct region after permanent MCA. Treatment with HGF reduces infarct volume in the rat model of transient focal ischemia. Furthermore, overexpression of HGF gene stimulates angiogenesis and ameliorates tissue damage due to permanent MCA. These findings strongly suggest that HGF may function both as a neurotrophic and angiogenic factor in the brain.

According to previous studies, CSF level of HGF is normally \( \approx 350 \, \text{pg/mL} \) and is approximately half of its serum level. The value was very similar to that determined in this study. It has been reported that all patients with bacterial meningitis have elevated CSF level of HGF. Tsuboi et al also reported the CSF level of HGF in patients with Alzheimer disease is elevated. Therefore, moyamoya disease is the third unique disease in which CSF level of HGF is differentially elevated.

We have previously determined CSF levels of various kinds of angiogenic factors, including bFGF, interleukin-8, platelet-derived growth factor, endothelial growth factor, and vascular endothelial cell growth factor, in moyamoya disease. Of these, only bFGF was significantly higher in moyamoya disease than in the controls. Therefore, HGF may be an important specific protein involved in the development of moyamoya disease as well as bFGF, soluble adhesion molecules, and CRABP-1.

This study revealed that patients with moyamoya disease had significantly higher CSF level of HGF than the controls. Close correlation between CSF level of HGF and onset pattern was observed. The finding is quite different from previous results showing no significant difference in CSF levels of bFGF in patients with ischemic and hemorrhagic onset. Although the finding should be carefully confirmed because the number of patients with intracranial bleeding was small (n=3), the result strongly suggests that elevated HGF may be involved in rapid progression of the disease, causing ischemic symptoms in moyamoya disease. Alternatively, cerebral ischemia itself may secondarily elevate CSF level of HGF.

Although the difference was borderline, CSF level of HGF was somewhat higher in the patients with good development of collateral circulation through indirect synangiosis than in those with poor results. The finding is similar to our previous results, in which bFGF was significantly higher in the patients with good results. In the future, therefore, CSF levels of both bFGF and HGF could be reliable indicators to predict the efficacy of revascularization after indirect bypass surgery.

In this study, we did not measure serum level of HGF in patients with moyamoya disease. However, serum level of HGF may also be elevated in them, because moyamoya disease sometimes involves the vessels of the other organs.

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**Figure 3.** 3a, Photomicrographs of immunohistochemistry stained with primary antibody against HGF in a moyamoya disease specimen. Original magnifications: left, \( \times 4 \); right, \( \times 100 \). 3b, Photomicrographs of immunohistochemistry stained with primary antibody against HGF in a control specimen. Original magnifications: \( \times 40 \).

**Figure 4.** 4a, Photomicrographs of immunohistochemistry stained with primary antibody against c-Met in a moyamoya disease specimen. Original magnifications: \( \times 40 \). 4b, Photomicrographs of immunohistochemistry stained with primary antibody against c-Met in a control specimen. Original magnifications: \( \times 20 \).
**Infection**  
* Genetic factors  
* Unknown factors  

**Carotid fork**  
* Media  
* Endothelium  

**HGF**  
* Intimal thickening migration  
* CSF  
  * Perforating artery  
  * Indirect bypass  

**Figure S. Diagram illustrating the hypothesis of HGF in development of moyamoya disease.**

**Summary**

Based on these observations, we speculate the role of HGF in moyamoya disease as shown in Figure S. Some unknown factors such as genetic abnormality and infection may increase the expression of HGF in the media of the carotid forks, inducing intimal thickening and VSMC migration into the media. This process may promote the occlusive change in the carotid fork. Increased amounts of HGF in the media, in turn, may elevate its CSF level and develop the moyamoya vessels by stimulating the proliferation of the intima of the perforating artery (“angiogenesis”). Elevated CSF level of HGF may also contribute to the development of collateral circulation through indirect pial synangiosis through their function as a strong inducer of “vascugelization.” Therefore, HGF may be a key protein in pathogenesis of moyamoya disease and provide an important clue for prevention and treatment of the disease.

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