Role of basic fibroblast growth factor in the pathogenesis of moyamoya disease

Kiyohiro Houkin, M.D., Tetsuyuki Yoshimoto, M.D., Hiroshi Abe, M.D., Kazuo Nagashima, M.D., Masahumi Nagashima, M.D., Makoto Takeda, M.D., and Toyohiko Isu, M.D.

Departments of Neurosurgery, Second Pathology, Second Anatomy, Hokkaido University School of Medicine, Sapporo, Japan; and Department of Neurosurgery, Kushiro Rousai Hospital, Kushiro, Japan

The pathogenesis of moyamoya disease is still under investigation. In this study, the authors focus on the role of cytokines in the pathogenesis of moyamoya disease by using immunohistochemical analyses.

The authors examined two specimens in the circle of Willis obtained at autopsy from two patients with moyamoya disease and two additional specimens obtained from control cadavers with atherosclerotic stenosis of the intracranial carotid arteries. Immunohistochemical examinations of the sections of the major intracranial arteries were performed using antismooth muscle cells (SMCs), monocytes, growth factor, cell nuclear antigen, and fragmented DNA antibodies. Basic fibroblast growth factor (bFGF) staining was present only in the endothelial cells of the moyamoya disease specimens and was not seen in control samples. In addition, the endothelial cells and SMCs in the media were positive for terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick-end labeling of fragmented DNA method but not in the SMCs in the intima in moyamoya disease specimens, which indicates that an apoptotic process is active in only SMCs in the media but not in the intima. In conclusion, it is suggested that the presence of bFGF in the media specifically seen in moyamoya disease suppresses the apoptotic process of SMCs in the intima.

Key Words * apoptosis * basic fibroblast growth factor * endothelial cell * moyamoya disease * smooth muscle cell

The pathogenesis of moyamoya disease has been under investigation since the clinical entity was first established in 1970s. There are many specific features in moyamoya disease that are keys to elucidating its pathogenesis. Findings from recent studies have demonstrated several important factors closely related to its pathogenesis. For example, clinically, moyamoya disease tends to be an inheritable, familial disease. Indeed, some researchers have identified a specific chromosome related to this inheritance. However, its histopathological features in the circle of Willis, which resemble but are apparently different from arteriosclerosis, have not been well studied because it is quite difficult to obtain an autopsy specimen of moyamoya disease.
In past studies, we have demonstrated that some cytokines in the cerebrospinal fluid (CSF) are elevated in moyamoya disease.[18,19] Among them, basic fibroblast growth factor (bFGF) was considered to be closely related to the pathogenesis of this disease. However, it is conceivable that elevated bFGF in CSF is the result of cerebral ischemia rather than being the cause of this disease. Therefore, if bFGF is truly involved in the pathogenesis of moyamoya disease, there must be evidence of its role in the specific pathological process of moyamoya disease. To confirm this hypothesis, we submitted specimens obtained at autopsy from two patients with moyamoya disease and two control cases to immunohistochemical examination focusing on bFGF.

MATERIALS AND METHODS

Autopsy Specimens

Specimens of the circle of Willis were obtained at autopsy. There were two specimens obtained in patients with moyamoya disease and two from control cadavers with atherosclerotic stenosis of the intracranial internal carotid arteries (ICAs).

One moyamoya disease specimen was obtained in a 19-year-old man who had experienced repeated transient ischemic attacks and right- and left-sided hemiparesis. After moyamoya disease was diagnosed in this patient (third stage, according to Suzuki's classification in bilateral sides), both direct and indirect vascular reconstructive surgeries were performed bilaterally in 1985, and he made uneventful recovery. He died suddenly from an unknown cause in 1996.

The second moyamoya disease specimen was obtained in a 29-year-old man who had suffered from chronic renal failure since 1990. In 1996 he experienced an abrupt onset of right-sided hemiparesis due to intracerebral hemorrhage. Cerebrovascular angiography demonstrated moyamoya disease bilaterally (third stage, Suzuki's classification), and he experienced a fatal episode of rebleeding 2 weeks later.

Two control specimens were obtained in cadavers with atherosclerotic stenosis of the intracranial ICAs not related to moyamoya disease. The cadaveric specimens were obtained from two male patients (aged 56 and 72 years at death) who died of myocardial infarction and pneumonia, respectively. The circle of Willis was resected for the immunohistochemical study.

Staining Methods

For conventional histological study, the hematoxylin & eosin (H & E) and elastica-Masson's (E-M) staining methods were used to resolve alterations in the anatomical structures of the arteries.

For immunohistochemical study, three factors were selected. For the confirmation of smooth-muscle cells (SMCs), monoclonal mouse antiactin antibody (HHF35) staining, monoclonal mouse antidesmin antibody staining, and monoclonal rabbit antivimentin antibody staining were performed. For the confirmation of monocytes, monoclonal mouse anti-human CD68 antibody testing was performed. For the confirmation of apoptosis, proliferating cell nuclear antigen (PCNA) staining and the terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) of fragmented DNA procedure were performed.

All tissue sections had been immersed in formalin for fixation and then embedded in paraffin. For the immunohistochemical examination, the sections were deparaffinized and washed with distilled water. After overnight incubation with each antibody at 4°C, staining methods were performed using an avidin
biotin complex immunostaining kit according to the manufacturer's instructions.

The TUNEL of fragmented DNA procedure was performed to detect DNA strand breaks in the tissues. Briefly, after rinsing the deparaffinized sections in Tris-HCl buffer, pH 7.4, the sections were incubated in a 20-mg/ml proteinase K solution to remove nuclear proteins, rinsed, and treated for 7 minutes with 2% H₂O₂ to diminish endogenous peroxidase activity. The sections then were immersed in TdT buffer (30 mM Tris-HCl, pH 7.2; 140 mM sodium cacodylate, 1 mM cobalt chloride), and covered with TdT (8 U/ml) and biotinylated 16-dUTP (50 nmol, 50 ml) in TdT buffer, and incubated for 90 minutes at 37°C. The reaction was terminated by placing the slides in citrate buffer (300 mM sodium chloride, 30 mM sodium citrate) for 30 minutes at room temperature. The sections were then immersed in phosphate-buffered saline (PBS), pH 7.4, and treated with 2% bovine serum albumin for 10 minutes at room temperature. Following immersion in PBS, the sections were incubated for 30 minutes at room temperature with peroxidase-conjugated streptavidin, and then washed in PBS. Last, the sections were immersed in a 50 mM Tris-HCl solution, pH 7.6, containing 1 mg/ml 3,3’-diaminobenzidine tetrahydrochloride and 1% H₂O.

Sources of Supplies and Equipment

We obtained the ABC kit from Vector Lab (Burlingame, CA); the monoclonal mouse antiactin antibody (HHF35), monoclonal mouse antidesmin antibody, monoclonal mouse anti-human CD68 antibody, and the proliferating cell nuclear antigen (PCNA) from DAKO (Glostrup, Denmark); the monoclonal rabbit antivimentin antibody from Bioscience Products (Emmenbrücke, Switzerland); and the monoclonal goat anti-bFGF antibody from R&D Systems (Minneapolis, MN). We acquired the TdT buffer from Takara (Osaka, Japan) and the biotinylated 16-dUTP from Boehringer Mannheim (Tokyo, Japan).

RESULTS

Histological Analysis

On the H & E-stained sections of moyamoya disease specimens, the terminal portion of the ICA and the proximal portions of the middle and anterior cerebral arteries demonstrated severe asymmetrical extrinsic stenosis of the lumen (Fig. 1).

![Photomicrograph of the right ICA showing very narrow lumen in a moyamoya disease specimen. H & E, original magnification X 4.](image)
The internal elastic lamina was irregularly thickened (multilaminar) and severely bent (wavelike). The thickened internal membrane and complex internal elastic lamina were considered to be the main cause of the narrow lumen. However, the media was not thickened. Examination of the E-M-stained sections showed that the thickened internal membrane consisted mainly of proliferation of the SMCs as well as collagenous tissue and the complex internal elastic lamina, which coincided well with the H & E findings (Fig. 2 left). On the other hand, the intracranial vertebral and basilar arteries in the moyamoya disease specimens did not show any of these pathological changes. In contrast, the ICA obtained from control specimens with atherosclerotic changes showed partial thickening of the intima and partial thinning of the internal elastic lamina similar to changes observed in moyamoya disease samples (Fig. 2 right).

**Immunohistochemical Examination**

**The HHF35-Positive SMCs.** The HHF35-positive cells were seen in the intima and media in the moyamoya disease specimens (Fig. 3 left). These HHF35-positive cells were believed to be SMCs because they stained positive for vimentin and negative for desmin.
antibody staining, original magnification X 200). Other nuclei of the thickened intima are apparent using H & E staining but not bFGF-antibody staining.

**The bFGF-Positive cells.** The bFGF was positively stained mainly in the endothelium of the major arteries in the moyamoya disease sections (Fig. 3 right) and faintly in the SMC of the intima. However, bFGF was negative in the vertebral artery in the moyamoya disease sections and in the ICAs in the control specimens.

**The CD68-Positive Cells (monocytes).** Few CD68-stained cells were present in the intima or the media, excluding the adventitia in moyamoya disease. In contrast, in the atheromatous intima in the control sections, large clusters of the CD68-staining positive cell, or monocytes (macrophages), were demonstrated.

**The PCNA-Positive and TUNEL-Positive Cells (apoptotic cells).** The PCNA-positive cells were only detected in the endothelial cells and SMCs in the intima and the media in the moyamoya disease specimens (Fig. 4 left) and were not seen in the control sections. The TUNEL staining demonstrated that the endothelium and the SMCs of the media were positive in moyamoya disease specimens (Fig. 4 right). These findings were not demonstrated in the vertebral artery in the moyamoya disease sections or in the control specimens.

![Fig. 4. Photomicrographs. Original magnification X 200. Upper: Left ICA obtained from a moyamoya disease specimen. The PCNA-positive cells were observed in the smooth muscle in the intima and media. PCNA staining. Lower: Right ICA obtained from same moyamoya sample. Apoptotic changes were observed at the endothelium and the smooth-muscle layer in the media. TUNEL staining.](image)

**DISCUSSION**

The irregular thickening of the intima and the internal elastic lamina of the anterior circulation in the circle of Willis demonstrated in this study were different from the pathological changes seen in atherosclerosis in its spatial distribution, although the intimal thickening is partly similar. These pathological features seen in this autopsy study were quite consistent with the classic pathological features of moyamoya disease reported previously.[8,12] The most specific finding in the atherosclerosis specimens was the presence of clusters of macrophages (monocyte) that invaded the intima.[6,13] In this study, few monocytes were seen in the intima or around the internal elastic lamina in moyamoya disease specimens, although it has been reported that T cells and macrophages are sometimes seen even in the media of moyamoya disease.[13] In addition, the alteration of the internal elastic lamina in the
moyamoya disease sections was quite different from that seen in the controls. These findings suggest that
the mechanism of the vascular wall thickness in moyamoya disease is different from that of
atherosclerotic vascular stenosis.

We have previously demonstrated that the bFGF level in the CSF was significantly elevated in patients
with moyamoya disease, which is not seen in other cerebral ischemic diseases.[18,19] This high
concentration of bFGF can explain some clinical features in moyamoya disease. For example, good
neovascularization after indirect revascularization seen in only moyamoya disease can be induced by the
powerful angiogenetic effect of bFGF. In addition, bFGF induces the proliferation of the vascular
endothelial cells, SMCs, and fibroblastic cells, as well as angiogenesis, which may induce stenotic
changes of the major arteries. This hypothesis, which can be called the "bFGF moyamoya disease
theory," is likely to explain most of the specific features found in moyamoya disease. However, the
source of this high bFGF remains unclear.

In general, bFGF exists in neurons, glia, endothelial cells, and vascular SMCs.[1,4,5,9,14,17] However,
in moyamoya disease, bFGF has been reported to be localized in the vascular wall in an autopsy
study[8,15] and in the media of the smooth muscle of the superficial temporal artery[7,16] In our study
we have demonstrated that bFGF was mainly present on the endothelium in the intracranial major
arteries, such as the ICA and the middle and anterior cerebral arteries. Interestingly, it was not present in
the endothelium of vertebrobasilar system in the moyamoya disease specimens nor the ICA in the
atherosclerosis control samples. To our knowledge, in moyamoya disease the presence of bFGF in the
endothelium in the anterior half of the circle of Willis and not in the posterior half has not been
demonstrated. These findings suggest an important role of bFGF in the pathogenesis of the stenotic
change in moyamoya disease.

The presence of PCNA-positive cerebral arteries in moyamoya disease has already been reported by
Masuda, et al.[12] They concluded that PCNA-positive SMCs are characteristic of stenotic arterial
lesions in moyamoya disease but not related to atherosclerotic changes. Our analyses also demonstrated a
proliferation of PCNA-positive SMCs in the intima and media of moyamoya disease specimens, which
was not seen in controls. The TUNEL-positive cells seen in the media were coincident with the
distribution of PCNA-positive staining. However, these TUNEL-positive cells were not seen in the
intima of moyamoya disease specimens. Apoptosis is strongly suggested when both TUNEL and PCNA
staining are positive. Therefore, it is conceivable that apoptotic change progresses in the SMCs in the
media of the intracranial major arteries in moyamoya disease and suppresses the proliferation of SMCs in
the media. On the other hand, the proliferation of SMCs in the intima, which is not controlled by
apoptosis, can induce the pathological feature of "intimal thickening" in moyamoya disease. However,
why is the control of SMC proliferation by apoptosis not seen in the intima and what is the role of bFGF
seen in the intima?

Apoptosis is a genetically programmed process that regulates cell growth, differentiation, and the
maintenance of normal cells. Several genetic factors, cytokines, and growth factors acting through
several proteases are supposed to induce apoptosis. Among them, bFGF is generally considered to inhibit
apoptosis, although the mechanism of this inhibition remains unknown.[2,3,10,11,16] However, specific
presence of bFGF in the intima in moyamoya disease can explain the specific pathological thickening of
the intima in this disease. It is plausible that the apoptotic process of SMCs in the intima is inhibited by
bFGF released from the endothelial cells in moyamoya disease. Consequently the thickening of SMCs is
induced only in the intima, which leads to the intimal thickening that is one of the characteristic features
In moyamoya disease. On the other hand, the absence of bFGF in the media in moyamoya disease and atherosclerotic specimens may induce apoptotic process of SMCs.

In this study, there was no characteristic change seen in the posterior cerebral artery in moyamoya disease. This may be explained by the fact that all specimens were obtained in adult patients. In addition, the number of the patients in this study is not large enough to exclude the possibility that bFGF may play a role in the posterior circulation in pediatric moyamoya disease.

Together, these speculations on the specific pathological features form what we term the bFGF moyamoya disease theory (Fig. 5). However, the details of the molecular biological mechanisms of apoptosis controlled by bFGF remain unknown. In addition, there are many unresolved questions concerning the pathogenesis of moyamoya disease. As we discussed in the introduction, the familial inheritance of moyamoya disease can be explained by some genetic abnormality, which may be related to bFGF. However, factors such as its specific geographical distribution (its incidence in many Japanese and Korean but rarely in Caucasian patients) and age and gender distribution (many in females) have never been explained by any hypothesis, including the bFGF hypothesis. To answer these unresolved questions, further investigation is needed to clarify the role of bFGF in moyamoya disease.

Fig. 5. Diagram depicting the bFGF moyamoya disease hypothesis.

References


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*Address reprint requests to:* Kiyohiro Houkin, M.D., Department of Neurosurgery, Hokkaido University School of Medicine N-15, W-7, Sapporo, Japan. e-mail: khokin@med.hokudai.ac.jp.