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ORIGINAL ARTICLE

Expression of vascular endothelial growth factor in dura mater of patients with moyamoya disease

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Abstract Vascular endothelial growth factor (VEGF) has been found to be involved in vasculogenesis in different intracranial lesions. We investigated meningeal cellularity and VEGF expression in dura mater of patients with and without moyamoya disease. Nine dural specimens from nine cerebral hemispheres of seven patients with moyamoya disease and four control dural specimens from four non-moyamoya patients were collected during surgery and investigated. Dural specimens were immunohistochemically stained with VEGF antibody, and then meningeal cellularity and VEGF expression in dural tissue were analyzed. The mean±standard error (SE) of total number of meningeal cells (meningeal cellularity) in dural tissue was 21.5±3.0 in the moyamoya disease patients, whereas it was 2.7±0.7 in control patients. The mean±SE of VEGF expression was 51.1±4.9% in the moyamoya disease patients, whereas it was 13.8±5.9% in control patients. The meningeal cellularity and VEGF expression were statistically significantly higher in the moyamoya group in comparison to control group ($p<$ 0.0001). Meningeal cellularity and VEGF expression are significantly increased in dura mater of the patients with moyamoya disease.

Keywords Dura mater · Moyamoya disease · Vascular endothelial growth factor

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Introduction

Moyamoya disease is characterized by the angiographic findings of arterial stenosis and occlusion of the circle of Willis [14]. After reduced cerebral perfusion due to arterial stenosis and occlusion, ischemic attacks may occur. Direct and/or indirect bypass surgeries are performed to enhance the development of natural collaterals and to prevent the progression of the disease [4, 8, 13, 16]. After surgical bypass, it has been found that the ischemic attacks diminish significantly, and revascularization develops from dural arteries as has often been observed on follow-up cerebral angiography [11]. On the other hand, vascular endothelial growth factor (VEGF) has been found to be involved in vasculogenesis and vascular permeability in different intracranial lesions. In ischemic disease without corresponding moyamoya disease, ischemia leads to cerebral angiogenesis by the release of VEGF [1, 6]. The extent of VEGF expression in the dura mater of patients with moyamoya disease has not yet been reported. In this study, we investigated expression of VEGF in dura mater of patients with and without moyamoya disease.

Materials and methods

The clinical characteristics of patients are summarized in Table 1. Seven patients with moyamoya disease were enrolled in this study. In moyamoya group, the patients included five women and two men ranging in age from 6 to 36 years (mean age, 18.0 years). The patients included four pediatric and three adult cases. All patients were diagnosed as having moyamoya disease on the basis of angiographic findings. Cerebral angiograms of moyamoya patients were classified according to the angiographic staging system

Table 1 Summary of seven patients with moyamoya disease and four control patients

Patient No.	Age/sex	Disease	Symptom	Side	Angiographic stage	Operation
Moyamoya disease patients						
1	15/F	Moyamoya	TIA	Rt	2	STA–MCA–EMS
2	24/F	Moyamoya	TIA	Lt	3	EDAS
3	13/F	Moyamoya	TIA	Rt	3	EDAS
				Lt	3	EDAS
4	36/M	Moyamoya	TIA	Rt	3	STA–MCA–EMS
5	22/F	Moyamoya	TIA	Lt	3	STA–MCA–EMS
6	10/M	Moyamoya	TIA	Lt	2	EDAS
7	6/F	Moyamoya	TIA	Rt	3	EDAS
				Lt	3	EDAS
Control patients						
1	57/M	Aneurysm	Incidental	Lt		Clipping
2	53/M	Aneurysm	Incidental	Rt		Clipping
3	78/F	Aneurysm	Incidental	Lt		Clipping
4	65/F	Aneurysm	Incidental	Lt		Clipping

No. Number, *F* female, *M* male, *TIA* transient ischemic attack, *Rt* right, *Lt* left, *EDAS* encephalo-duro-arterio-synangiosis, *STA–MCA–EMS* encephalo-myo-synangiosis procedure in combination with superficial temporal artery–middle cerebral artery anastomosis

proposed by Suzuki and Takaku [14]. Nine hemispheres of seven patients were studied: two were classified as stage 2 and seven were classified as stage 3 moyamoya. The clinical symptoms were transient ischemic attacks (TIAs), such as transient hemiparesis and sensory disturbance, in all patients. Regarding surgical bypass procedure for the middle cerebral artery (MCA) territory, three of nine hemispheres underwent encephalo-myo-synangiosis (EMS) procedure in combination with superficial temporal artery (STA)–MCA anastomosis (STA–MCA–EMS), and six of nine hemispheres underwent encephalo-duro-arterio-synangiosis (EDAS) [9, 10, 17]. Similarly, all patients underwent encephalo-galeo-synangiosis [3] as a surgical bypass procedure for the anterior cerebral artery territory. After surgical bypass, all the patients were found to be symptom free till the last follow-up.

A small piece of dura mater was resected from the edge of the dural incision at the time of STA-MCA-EMS or EDAS procedure. The dural defect was covered with the STA strip in case of EDAS and with temporal muscle flap in case of STA-MCA-EMS. From seven patients, nine small specimens of dura mater of the MCA territory were obtained.

As control specimens, dura maters of four patients with intracranial aneurysms, ranging in age from 53 to 78 years (mean age, 63.2 years), were studied. A small piece of dura mater was resected from the edge of the dural incision in fronto-temporal region while undergoing craniotomy for the clipping surgery. The dural defect was repaired with the fascia of temporalis muscle. Four dural specimens were obtained from four patients. All the specimens were obtained with prior informed and written consent from patients or their guardians according to the rule of hospital.

Immunohistochemistry

Sections of dura mater were fixed in 4% buffered paraformaldehyde and embedded in paraffin, and 4-μm-thick sections were cut. The slides with tissue section were deparaffinized with xylene, the process of antigen retrieval carried out and endogenous peroxidase blocking performed. The slides were rinsed with phosphate-buffered saline solution, pH 7.5, three times for 5 min each after each step. Streptavidine biotine (SAB) method was employed for antibody incubation using histofine SAB kit (Nichirei Company, Tokyo, Japan). The primary antibody used for VEGF was a goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution [18]. Negative control for immunostaining was included by omitting primary antibody. Paraffin-embedded specimens of glioblastoma were used as positive controls for VEGF.

The slides were evaluated for meningeal cellularity and VEGF labeling index (LI) by counting all the cells first, which represents cellularity, and then only the VEGF-positive cells out of them which represents VEGF expression, and thus VEGF LI. At least ten randomly selected fields of each sample were counted under ×400 magnifications of light microscope and photographs taken (Fig. 1a–d). The VEGF LI was calculated as a percentage of total meningeal cells, i.e., number of VEGF-positive cells per 100 meningeal cells, for that particular specimen.

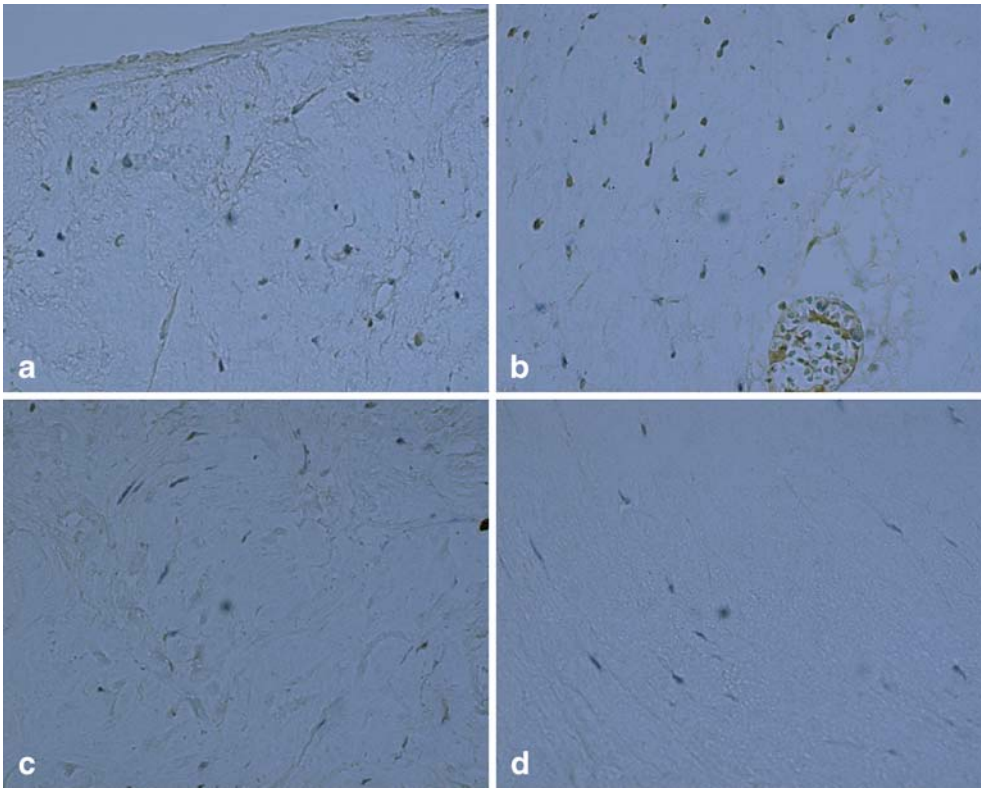

Fig. 1 **a** Picture of VEGF immunostaining of the dura mater of a patient with moyamoya disease (original magnification, ×400). VEGF LI was 23%. **b** Picture of VEGF immunostaining of the dura mater of a patient with moyamoya disease (original magnification, ×400). VEGF LI was 46%. **c** Picture of VEGF immunostaining of the dura mater of a control patient (original magnification, ×400). VEGF LI was 15%. **d** Picture of VEGF immunostaining of the dura mater of a control patient (original magnification, ×400). VEGF labeling index was 0%

Statistical analysis

Statistical analysis was performed using the software package of StatView, version 5.0. The meningeal cellularity and VEGF LI of two groups, dura mater of moyamoya disease patients and dura mater of control patients, were compared by using Mann–Whitney *U* test. The values of $p < 0.05$ were regarded as statistically significant.

Results

The immunostaining results of nine dural tissue specimens of moyamoya patients and four specimens of control patients are summarized in Table 2. The mean±standard error (SE) of total number of meningeal cells (meningeal cellularity) in dural tissue was 21.5±3.0 (range, 3 to 95) in the moyamoya disease patients, whereas it was 2.7±0.7

Table 2 Immunostaining results of dura specimens of seven moyamoya patients and four control patients

Patient No.	Age/sex	Disease	Side	No. of VEGF-positive cells	Meningeal cellularity	VEGF LI (%)
Moyamoya disease patients						
1	15/F	Moyamoya	Rt	27.0	28.2	95.7
2	24/F	Moyamoya	Lt	1.3	6.3	20.0
3	13/F	Moyamoya	Rt	5.3	19.5	26.9
			Lt	13.0	28.3	46.0
4	36/M	Moyamoya	Rt	5.3	19.5	26.9
5	22/F	Moyamoya	Lt	6.8	46.0	14.7
6	10/M	Moyamoya	Lt	14.8	21.2	69.8
7	6/F	Moyamoya	Rt	16.6	18.8	88.3
			Lt	4.4	8.4	52.4
Mean±SE				11.0±1.8	21.5±3.0	51.1±4.9
Control patients						
1	57/M	Aneurysm	Lt	0.6	1.2	50.0
2	53/M	Aneurysm	Rt	0.8	6.0	12.5
3	78/F	Aneurysm	Lt	0.4	1.4	28.6
4	65/F	Aneurysm	Lt	0.8	3.0	25.0
Mean±SE				0.6±0.2	2.7±0.7	13.0±5.9

No. Number, *F* female, *M* male, *Rt* right, *Lt* left, *VEGF* vascular endothelial grow, *LI* labeling index, *SE* standard error

(range, 0 to 10) in control patients. The mean±SE of number of VEGF-positive cells in dural tissue was 11.0± 1.8 (range, 0 to 47) in the moyamoya disease patients, whereas it was 0.6±0.2 (range, 0 to 3) in control patients. Similarly, the mean±SE of VEGF LI was 51.1±4.9% (range, 0 to 95.7%) in the moyamoya disease patients, whereas it was 13.8±5.9 (range, 0 to 50%) in control patients. The meningeal cellularity and the number of VEGF-positive cells (VEGF LI) in the moyamoya group were statistically significantly higher in comparison to control group ($p<0.0001$).

Discussion

Angiogenic factors, such as VEGF and fibroblast growth factor (FGF), lead to pathologic vessel formation [1]. In cases of moyamoya disease, only the expression of FGF in the dura mater has been studied [5]. Hoshimaru et al. [5] reported that the dura mater obtained from patients with moyamoya disease revealed more intense FGF expression in meningeal and vascular cells as revealed by immunohistochemical staining. Regarding VEGF expression in moyamoya disease, Takekawa et al. [15] reported VEGF expression in autopsy specimens of adult moyamoya disease. They observed VEGF expression mainly in the peripheral areas of ischemic change in the left external capsule and claustrum, chiefly in glial cells. However, the expression of VEGF in dura mater of moyamoya disease has not been reported yet. Therefore, this is the first study to observe the VEGF expression in moyamoya dura. VEGF has been found to have its effect on vasculogenesis, endothelial cell proliferation and migration, vascular permeability, and stromal degradation through the activation of proteolytic enzymes that are involved in angiogenesis [18]. In our study, the total meningeal cellularity and VEGF expression in the meningeal cells in the moyamoya dura mater was statistically significantly higher than those in control patients ($p<0.0001$). This observation of increased VEGF expression and increased meningeal cellularity, in combination with the previous report of increased basic FGF expression, may be related to the enhanced angiogenesis of the dura mater in moyamoya disease. Increased VEGF expression might increase migration and proliferation of vascular cells and induce the abundant neovascularization that is usually conspicuous after indirect bypass surgery for moyamoya disease [5]. However, collateral vessels from the middle meningeal artery (MMA) and its branches are not seen as abundantly as expected in moyamoya disease [2].

The dura mater consists of three layers: periosteal, meningeal, and the dural border-cell layers [7]. The periosteal or outer layer has fewer fibroblasts and more extracellular collagen, whereas the meningeal or inner layer has more fibroblasts and less collagen. The dural border-cell layer forms a transitional zone between the dura and arachnoid. The outer layer contains more numerous blood vessels compared to the inner layer. Although the MMA and its branches are in proximity to the ischemic cortex, collateral vessels from this source have not been found to that degree as might be expected in moyamoya disease [2]. It has been recognized that the inner meningeal layer of the dura must serve as an impediment to collateral vessel ingrowth from the extremely vascular outer periosteal dura into the brain. Therefore, Dauser et al. [2] proposed that the inner layer of the dura acted as a natural anatomical barrier between the internal carotid artery territory and the external carotid circulation, thus explaining the relative lack of natural collateral circulation between the MMA circulation and the ischemic brain in this disease.

Some limitations in this study have to be acknowledged. This is small study comprising only 13 samples of dural tissue which may not be enough to make any concrete speculation. Similarly, age of the control group has not well matched with that of moyamoya patients. Thus, VEGF expression and cell density of dura mater might have been influenced by the age of the patients. Besides, it would have been better if expression of other growth factors or cytokines along with VEGF were studied. Recently, Nanba et al. [12] studied the cerebrospinal fluid (CSF) levels of hepatocyte growth factor (HGF), a strong inducer of angiogenesis, in development of moyamoya disease. The study revealed that CSF level of HGF markedly elevated in moyamoya disease. Based on the result, they speculated that HGF might be a key protein in pathogenesis of moyamoya disease. Therefore, correlation between HGF in CSF and VEGF in dural tissue can be another scope of research and study.

In conclusion, our study showed that the total meningeal cellularity and VEGF expression in the moyamoya dura was statistically significantly higher in moyamoya group as compared to control group. From this result, we can assume that the natural anatomical barrier of dura might be strong even in patients with moyamoya disease.

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Comments

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The pathogenesis of moyamoya disease is still unknown. There have been various approaches attempted to open this hard gate. Those included the microsattelite analysis of gene using the familial pedigree cases of moyamoya disease, gene expression analysis of the tissue sample from the patient, and investigation of the substance specific to this disease. However, so far, the survey of the gene closely related to moyamoya disease has not reached to confirmation of specific gene. In addition, there is no particular pattern of gene expression reported using the sample of moyamoya disease.

Among these reports, there are some cytokines, FGF, VEGF, HGF, that are suggested to be related to moyamoya disease. In this paper, the VEGF expression is significantly increased in the dura mater of the patients with moyamoya disease. In addition, the cellularity of the dural tissue was significantly high in moyamoya disease.

These facts are quite remarkable because those suggested the pathological process of moyamoya disease reaches not only to the intracerebral vascularity but also meningeal (extracerebral tissue). However, as it is true for other paper similar to this paper, the increase in VEGF expression and increased cellularity of dural tissue is not always considered the primary mechanism of this disease. On the other hand, it is conceivable that these increases may be a result of the specific ischemia of the moyamoya disease.

This paper has added novel fact to the hints to consider the pathogenesis of moyamoya disease. However, it is true that it is very far from the core mechanism of this unusual disease that the primary lesion is the centripetal narrowing of the anterior part of the Willis ring.

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Sakamoto and colleagues compared dural tissue specimens in patients with and without moyamoya disease and demonstrated increased meningeal cellularity, greater numbers of VEGF-positive cells, and higher VEGF labeling indices in moyamoya patients as compared to aneurysm patients. In addition, STA–MCA bypass with encephalo-myo-synangiosis was more effective than encephalo-duro-synangiosis, with greater revascularization responses observed on follow-up angiography. Involvement of VEGF in the brain's response to moyamoya disease is not surprising—increased VEGF expression has been demonstrated in many conditions of altered cerebral blood flow. VEGF expression in the dura is an intrigui`ng finding, but one that is difficult to interpret without similar measures of VEGF expression in the brain, where there is active tissue ischemia. If brain specimens could have been collected simultaneously, VEGF expression in brain would probably have been higher than in dura. The authors speculate that a natural anatomical barrier in the dura limits the response of the middle meningeal artery to brain ischemia, and accounts for angiographic results with direct bypass that are superior to indirect bypass. I am not sure about the existence of this anatomical barrier, but nonetheless favor direct bypass in my moyamoya patients.