Original Article

Enhancement Expression of bFGF in Chinese Patients with Moyamoya Disease*

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Abstract

Objective To detect the content of the basic fibroblast growth factor in blood samples of patients with Moyamoya disease, and investigate the relationship between Moyamoya disease and the basic fibroblast growth factor.

Methods This tissue microarray study included 24 cases of superficial temporal artery samples, 15 cases of Moyamoya disease, and 9 cases of normal arteries as control, and bFGF immunofluorescence assay was applied to test the samples. The number of positive cells and total cells of the muscular layer and the endothelium layer were counted separately in every picture, the positive rates were calculated, and the experimental data were analyzed statistically.

Results The bFGF immunofluorescence staining of smooth muscular layer cells, intima cells and endothelial cells from the moyamoya disease group were obviously stronger than that from the control group ($P<0.01$).

Conclusion The enhancement expression of bFGF in the Moyamaya disease group implicates that bFGF plays an important part in the pathogenesis of Moyamoya disease.

Key words: Moyamoya disease; bFGF; Pathogenesis; Superficial temporal artery; Immunofluorescence

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INTRODUCTION

Moyamoya Disease (MMD), known as abnormal vascular network at the skull base, is more common in Asian populations, particularly in Japan and Korea of East Asia. In Japan, the annual incidence rate is about: 0.54 / 100 000[1]. MMD is a kind of cerebrovascular disease with the feature of progressive stenosis or occlusion of bilateral internal carotid arteries and their major branches, with secondary formation of anastomotic collateral capillary network at the base of brain. With the progression of the disease, proximal middle cerebral artery (MCA) and anterior cerebral artery (ACA) may be involved, however, the vertebrobasilar system is rarely involved. Moyamoya disease was first reported in 1957 by Takeuchi and Shimizu[2], and in the year of 1969, Suzuki first described the disease in a comprehensive way and named it "Moyamoya"[3], or "puff of smoke" in Japanese, because of the formation of "puff of smoke" like capillary network at skull base.

With intensive studies going on, MMD has been gradually recognized as a pantosomatous multisystem vascular disease, which involves not only the intracranial internal carotid artery system, but also extracranial superficial temporal artery, pulmonary artery, and renal arteries[4]. One domestic report has stated that lower limb arteries and occipital artery are also involved[5]. There are also undefined systemic processes involved in this vasculopathy[6]. Studies on STA may reflect the status of internal carotid artery terminal, and MCA or ACA. By autopsy of deceased MMD patients it has been found that the pathological changes of STA are similar to the artery circle of the skull base, and

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extracranial arteries and intracranial arteries are involved simultaneously[7].

Since Moyamoya disease was reported in 1957, its etiology has remained unclear in spite of intensive studies.

Our previous studies have shown that basic fibroblast growth factor (bFGF) could be detected in the cerebrospinal fluid of MMD patients[8], and that bFGF is also expressed in the superficial temporal artery (STA) and cerebral dura mater in the immunochemical experiment[9]. Thus, we consider that bFGF may play an important part in the pathogenesis of MMD. This study aims to explore the relationship between bFGF and Moyamoya disease through measurement of bFGF expression of the superficial temporal artery.

MATERIALS AND METHODS

Twenty four post-operative specimens of superficial temporal artery (STA) were obtained for this study, which had been taken from the inpatients of Tiantan Hospital after 2008. Of these specimens, 15 (6 females and 9 males) were assigned to the MMD group, with an average age of 31.6 years ranging from 8 to 67 years, which had been obtained from the STA-MCA anastomosis, while 9 specimens were assigned to the control group (4 females and 5 males), with an average age of 39.1 years ranging from 19 to 62 years, which had been taken from patients of brain injury without history of cerebrovascular diseases. Immunofluorescence (IMF) was used to measure bFGF expression in the cells of muscular layer and tunica intima. This experiment was approved by the ethics committee of the Capital Medical University.

Immunofluorescence

The specimens were placed into 10% formaldehyde solution immediately after they were obtained from operation, and after soaking for about 20 hours they were entrapped with paraffin wax, cut into slice (4 μm thick) with microtome, followed by toasting for 30 min at 63 ^{o}C, deparaffining twice with dimethyl benzene in 10 min period each, dehydration with alcohol, and rinsing with PBS solution and distilled water. Rabbit monoclonal antibody against human FGF-2, (manufactured by Santa Cruz biotechnology, inc) was added at the concentration of 5 mg/mL, overnight at 4 ^{o}C, rinsing with PBS solution again and the goat monoclonal antibody against rabbit IgG marked with FITC was also added. Five specimens were selected randomly from the 24 specimens as the negative control group, processed by the above-mentioned procedures, but without adding in the rabbit monoclonal antibody against human FGF-2. They were eventually made into section, and observed under the XDY-1A-type microscope, at 200 magnifications, (GZJM Precision).

Results of Collection Staining

Under high power lens the tunica intima and muscular layer were observed, and 3 sites were selected randomly to collect the image from each specimen. The number of total cells, positive cells and strong positive cells was counted on each site separately, the positive rate and strong positive rate were calculated subsequently, and the average of 3 sites was regarded as the result of one specimen.

Statistics

Statistical analysis was used to process the positive rate of bFGF expression of the muscular layer cells and endomembrane cells from two groups, rank sum test or *t* test was selected, depending on whether the results were consistent with the distribution of normality, and it was considered statistically significant if *P* value was less than 0.05.

RESULTS

Basic FGF was located in the cytoplasm of the endothelial cells, fibroblast cells and smooth-muscle cells. When intracellular bFGF was at a high level, bright patchy staining structure could be observed under high power field of fluorescence microscope. For lacking of bFGF, the nucleus was observed as dark stained cable-shaped or round-like structures. The number of bright staining structure was equal to the number of positive cells, the number of dark staining structure was equal to the number of nucleus, and the number of nucleus was equivalent to the total number of cells, then the bFGF positive rate could be calculated. When intracellular bFGF was at a higher level than the other cells, the staining would be obviously brighter, and these cells were counted as strong positive cells. For the hyperplastic, stratified and ruptured internal elastic membrane, the structure of theca interma was disordered, cells were difficult to be distinguished, and staining intensity was used to indicate the level of bFGF expression in theca interma. The cell structures of muscular layer and the endothelium cells were relatively clear, and the positive rate was calculated to indicate the level of bFGF expression (Table 1).

Table 1. Results of IMF Staining

Group	Sex	Age	Muscular Layer PR (%)	Muscular Layer sPR (%)	Endothelial Cell PR (%)	Intimal Thickening	Staining Intensity
M1	M	20	67.4	11.8	61.5	NS	+
M2	F	8	53.6	13.8	92.3	S	+++
M3	M	29	52.4	11.2	52.3	S	+++
M4	M	64	52.3	14.8	67.7	S	+++
M5	M	44	71.6	15.7	72.0	S	+++
M6	F	39	41.8	8.6	32.4	NS	++
M7	M	46	53.6	9.0	35.7	S	++
M8	M	37	56.9	8.3	19.4	NS	+
M9	F	23	63.6	7.1	15.7	S	+
M10	M	12	65.2	14.5	42.4	S	+++
M11	F	35	49.3	5.6	44.8	S	+++
M12	F	61	40.2	4.8	15.3	S	++
M13	M	47	44.9	9.6	81.4	S	+++
M14	M	27	58.9	8.3	57.9	S	+++
M15	F	15	57.5	10.8	73.7	S	+++
C1	F	62	9.9	0	32.1	S	+
C2	M	49	23.9	2.1	36.1	S	+
C3	M	34	26.3	3.7	29.1	NS	+
C4	M	51	19.3	0	0	NS	NS
C5	F	36	25.9	0	50.0	S	+
C6	M	19	18.4	0	0	S	NS
C7	F	38	4.1	0	0	NS	NS
C8	F	33	5.0	20.0	0	NS	NS
C9	M	40	16.9	0	0	S	NS

Note. PR = positive rate; sPR = strong positive rate; M = moyamoya disease group; C = control group; +++ = strong staining; ++ = moderate staining; + = weak staining; S = significant; NS = not significant.

Muscular Layer Cells

Specimens were observed under the microscope. In the negative control group without adding in the rabbit monoclonal antibody, only fluorescence background was observed, and the smooth muscle cell structure could not be distinguished (Figure 1①); in the control group, smooth muscle cell structure could be distinguished, without significant positive staining (Figure 1②); in the Moyamoya disease group, patchy bright dying structure could be seen and was significantly positive (Figure 1③). In the MMD group, the positive rate of stained smooth muscle cells ranged between 40.2% and 71.6% (average, 55.3%), with the strong positive rate range of 4.8%-15.7% (10.3% on average), Correspondingly in the control group, the positive rate of stained smooth muscle cells ranged between 4.1% and 26.3% (16.6% on average), with the strong positive rate range of 0-3.7% (0.6% on average). The first normality test was applied to the data of muscular layer cells, and *P* value was greater than K-SZ statistics, so the data of muscular layer positive rate fitted the normal distribution, *t* test could be applied, and *P* value was less than 0.01, so significant differences were found between the two groups, and the expression of bFGF in muscular layer cells of the Moyamoya disease group was higher than that that of the control group.

Endothelial Cells

Cell structures could not be distinguished in either the negative control group or the control group, with negative staining in both groups (Figure 1①). Endothelial cells appeared significantly positive

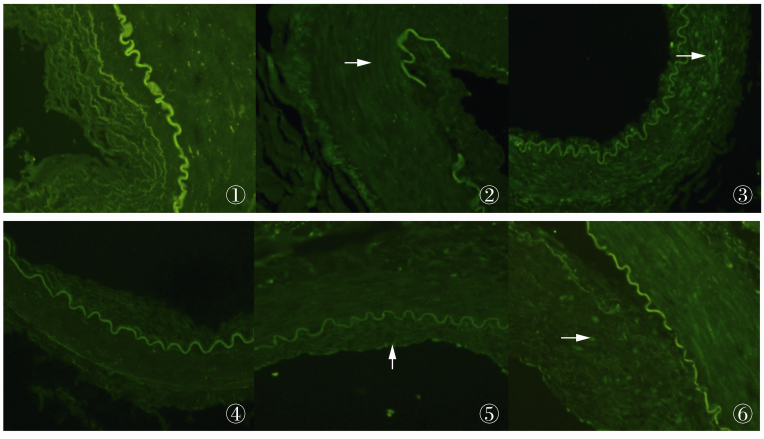

Figure 1. ①Muscular layer structure of specimen of the negative control group without adding in the rabbit monoclonal antibody, the tissue shows the fluorescence ground color, internal elastic lamina autograph under the fluorescence microscope, the structure of muscular layer cells can not be distinguished; ②Muscular layer structure of specimen of the control group, cell structure can be distinguished (as illustrated by the arrow), but staining is not significantly distinguished from fluorescence ground color, may be considered as negative; ③Muscular layer structure of specimen of the MMD group, cell structure was observed in the muscular layer structure, containing heavily stained cells which are obviously distinguished with fluorescence ground color (as illustrated by the arrow); ④Specimen of the control group, the intima is thickening, the intima is weakly stained, and the endothelium is negative; ⑤Specimen of the MMD group, endothelial cells are significantly positive (as illustrated by the arrow); ⑥Specimen of the MMD group, the intima is significantly thickening, the intima is strongly stained, endothelial cells are positive (as illustrated by the arrow). SP ×200.

in the Moyamoya disease group (Figure 1⑤-⑥). In the MMD group, the positive rates of stained endothelial cells ranged between 15.3% and 92.3% (average, 48.1%). Endothelial cells appeared positive only in four cases in the control group, and the positive rates ranged between 0 and 50.0% (average, 10.2%). Because of few endothelial cells in one high power field, very few strong positive endothelial cells were observed, and the error would be obvious if strong positive rate was calculated. As a result, strong positive rate was not analyzed for endothelial cells. The first normality test was applied to the data of endothelial cells, *P* value of the control group was less than K-SZ statistics, so the data of endothelial cell positive rates did not fit the normal distribution, Rank sum test could be applied, and *P* value was less than 0.01, so significant differences were found between the two groups, and the expression of bFGF in endothelial cells of the Moyamoya disease group was stronger than that in the control group.

Tunica Intima Cells

In the negative control group, only internal elastic lamina autograph was observed, without obvious cell structure and significant positive staining (Figure 1①). In the control group, the intima looked thickened and weakly stained (Figure 1④). In the MMD group (Figure 1⑥), the intima was significantly thickening, and strongly stained. Thickened intima was observed in 12 cases among the total 15 cases of MMD, and the rate was 80%. In the twelve specimens with thickened intima, one case was weakly stained, two cases were moderately stained, and other nine were all strongly stained. In the 3 specimens which were not obviously thickened, two cases were weakly stained, and one case was moderately stained. Thickened intima was observed in five cases in the control group which contained 9 specimens, accounting for 55.6%. Four cases were found weakly stained and no obvious staining was observed in the remaining 5 cases in the control group.

No significant statistical difference was found between different genders when comparing data of the two groups.

DISCUSSION

Basic fibroblast growth factor (bFGF)was initially purified out of bovine pituitary by Gospodarowicz in 1974[10]. Because bFGF had significant effect on

mitogenic activity on the fibroblast cells, such as the BALB/C3T3 cells, it was named basic fibroblast growth factor[11]. Basic fibroblast growth factor is a broad-spectrum mitogen with a broad effect on cell proliferation, especially on fibroblasts, vascular endothelial cells, smooth muscle cells and so on. Basic fibroblast growth factor is one of the most effective angiogenesis factors found *in vivo*, and it can effectively contribute to vascular endothelial cells proliferation and division, thus promoting angiogenesis.

For ethical reasons, it is not feasible to obtain skull base vessels samples from patients of Moyamoya disease alive. However, since the pathological changes of superficial temporal artery are similar with skull base vessels in patients of Moyamoya disease, the etiology of the pathological changes of the superficial temporal arteries has some reference value to the etiology of Moyamoya disease. The expression of bFGF in cerebral blood vessels, especially in superficial temporal arteries of Moyamoya disease, has not been reported in domestic literatures. This study is of great significance to reveal the cause of the pathological changes of the superficial temporal arteries.

Normal smooth muscle cells, fibroblasts and vascular endothelial cells may produce bFGF, however the amount of bFGF is small, so no staining or weak staining under fluorescent microscope is observed. In this study the MMD group showed more expression and stronger staining of bFGF than the control group on the smooth muscle cells, endothelial cells and intimal layer cells of STA.

No previous reports dealt with the relationship between the expression of bFGF and age. In our experiment, there is no significant statistical difference between different genders when comparing data of the two groups, so the expression of bFGF in the superficial temporal artery specimens is not considered to be related to gender. Also, there is no statistical difference between cases of elder/younger than 30 years, so the expression of bFGF in the superficial temporal artery specimens appears unrelated to age. However, considering the fact that the quantity of specimen is limited, and most specimens are taken from adult patients, the finding of the present study provides some information only for reference.

In addition, it was also noticed that bFGF staining of the specimens with intimal thickening was stronger than that of specimens without intimal thickening in the Moyamoya disease group. Taking into account the role of bFGF, a certain correlation might possibly exist between the levels of the bFGF and the extent of intimal thickening in Moyamoya disease. In the control group among the four weak staining specimens intimal thickening could be found in three cases, yet, no obvious staining of bFGF was observed in two cases of intimal thickening. The control group did not fully meet the above assumption, probably because of the overall low level of bFGF in the control group, and the intimal thickening being affected by a variety of systemic and local factors.

It was found via autopsy of patients of the Moyamoya disease that thickened intima at the terminal of internal carotid arteries and the basilar arteries circle were mainly composed of smooth muscle cells And immunohistochemical analysis to these arteries found that bFGF staining was also positive, and there was no relevant reports on the expression of bFGF in the skull base abnormal vascular network. bFGF has a role of promoting the smooth muscle cells proliferation and differentiation, and in the process of angiogenesis, bFGF is also essential because of the role of promoting vascular endothelial cell movement, proliferation and differentiation. Therefore, it is speculated in the pathogenesis of Moyamoya disease that bFGF not only promotes the proliferation of vascular endothelium, vascular smooth muscle, fibroblast, eventually leading to internal carotid artery and its major branches stenosis and occlusion, but also promotes the formation of abnormal vascular network at the skull base.

bFGF is a systemic factor, rather than a partial factor in the pathogenesis of Moyamoya disease. Previous studies showed that the concentration of bFGF in cerebrospinal fluid of moyamoya disease significantly increased, and bFGF was seen increased in dura, superficial temporal artery and the skull basilar artery[4-5,12], bFGF was also found in extracellular matrix of smooth muscle cells of stenotic part of terminal internal carotid artery and basal artery circle. Therefore, it was speculated in patients of Moyamoya disease, for certain reasons, that bFGF expression increased in nerve cells in brain, glial cells, endothelial cells and vascular smooth muscle cells, and bFGF not only acted on their own, but also was secreted or released into the cerebrospinal fluid, and was spread extensively via cerebrospinal fluid. The reason why bFGF resulted in two different pathological changes - stenosis of the

internal carotid artery terminal and the formation of abnormal vascular network in the skull base, might be related to the different concentration of bFGF in the two positions.

There are two possible mechanisms for enhancement of bFGF expression in STA of the MMD group - primary enhancement and secondary enhancement. The primary bFGF expression enhancement is mainly because of genetic changes which results in the enhanced bFGF expression, which was not reported previously. The secondary bFGF expression enhancement is mainly because of bFGF responsive increasing, which may be associated with two possibilities. First, more bFGF may be secreted by various types of brain cells in response to stenosis and ischemia of the arteries circle of skull base during the process of MMD pathogenesis, as confirmed in the experiment of animal model of cerebral ischemia that bFGF amount obviously increases in the surrounding area of cerebral ischemia issue[13]. Second, apoptosis plays a role. Yasushi found, compared to the patients of internal carotid artery or middle cerebral artery (MCA) occlusion, the MCA vessel wall of MMD patients showed distinct apoptosis, and mainly focused on the smooth muscle cells of middle layer[14]. The injury of the vessel wall cells may cause increased bFGF expression to improve proliferation and differentiation of intimal cell, smooth muscle cell and fibroblast as compensation and reparation. Globally uncontrolled smooth muscle cell proliferation, in the absence of environmental risk factors, may be a primary etiologic event leading to Moyamoya disease[15].

The pathological changes are more significant on the skull base arteries than STA, which is attributed, as we consider, to the fact that skull base arteries have one situation different with STA, basic fibroblast growth factor increased in the cerebrospinal fluid of MMD patients, as found in recent studies[16]. The fact that bFGF increases in the cerebrospinal fluid and bFGF is expressed on arteries themselves, cause the pathological changes of skull base arteries of Moyamoya disease .

In this study, it was observed that the STA showed more expression of bFGF than the control group on the smooth muscle cells, endothelial cells and intimal layer cells in the Moyamoya group, which indicates that STA is one of the involved vessels in Moyamoya disease. At present, superficial temporal artery - middle cerebral artery anastomosis is a major approach to the surgical treatment of Moyamoya disease, and the anastomosis can improve the symptoms of cerebral ischemia in short term, however, since there is pathological change in superficial temporal artery per se, and there is large amount of bFGF in blood and cerebrospinal fluid of patients of Moyamoya disease, the reconstructed vessels may still in the process of Moyamoya disease pathogenesis, and the effect of anastomosis needs long-term follow-up.

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