Research report

Increased serum production of soluble CD163 and CXCL5 in patients with moyamoya disease: Involvement of intrinsic immune reaction in its pathogenesis

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Abstract

Moyamoya disease (MMD) is a rare cerebrovascular disease characterized by a progressive stenosis at the terminal portion of the internal carotid artery and an abnormal vascular network at the base of the brain. Although its etiology is still unknown, intrinsic immune reactions such as autoimmune response has been implicated in the pathogenesis of MMD. Recently, the RING finger protein 213 (RNF213) was found to be an important risk gene for MMD, and is predominantly expressed in blood cells and the spleen. Thus, we hypothesized that patients with MMD represent an intrinsic autoimmune status mediated by M2-polarized macrophages, which play an important role in tissue remodeling and angiogenesis. We compared the serum level of soluble (s)CD163, an activating marker for CD163+ M2-polarized macrophages that has been implicated in a variety of autoimmune disorders, between MMD patients and healthy controls. We also analyzed serum levels of CXCL5, an augmented cytokines that has been correlated with the severity of autoimmune diseases. As a result, the serum sCD163 levels of MMD patients (281,465 pg/ml) were significantly higher than those of healthy controls (174,842 pg/ml) (p = .004). The serum CXCL5 levels of MMD patients (679.02 pg/ml) were significantly higher than those of healthy controls (401.79 pg/ml) (p = .046). There were no differences in the serum sCD163 and CXCL5 levels between each genotype of the RNF213 polymorphism (wild-type or variant) among MMD patients. Although this is a pilot study and further validation with larger number of samples is necessary, our results indicate that patients with MMD may have increased autoimmune activity, and our results shed light on the pathogenesis of MMD via CD163+ M2-polarized macrophages.

1. Introduction

Moyamoya disease (MMD) is a chronic occlusive cerebrovascular disease characterized by a progressive stenosis at the terminal portion of the internal carotid artery and an abnormal vascular network at the base of the brain (Suzuki and Takaku, 1969; Research Committee on the Pathology and Treatment of Spontaneous Occlusion of the Circle of Willis, Health Labour Sciences Research Grant for Research on Measures for Intractable Diseases, 2012). Although its etiology remains unknown, an autoimmune reaction has been implicated in the pathogenesis of MMD (Kim et al., 2010; Li et al., 2011; Bower et al., 2013; Fujimura et al., 2014). Regarding the genetic basis of MMD, recent advances in genome-wide association studies and linkage analysis of familial MMD patients revealed that the RING finger protein 213 gene (RNF213) in the 17q25-ter region is an important risk gene for MMD among East Asian populations (Kamada et al., 2011; Liu et al., 2011). The role of RNF213 in MMD is still undetermined, while its predominant expression in blood cells and the spleen, in contrast to the lower expression level in central nervous system, may suggest the potential role of an immune reaction in the development of MMD (Kamada et al., 2011; Fujimura et al., 2014). In fact, our most recent study of RNF213-deficient mice under immunologic...
adjuvant administration suggested that RNF213 abnormality might 
compromise immunological self-tolerance, thereby contributing to 
the development of MMD (Kanoke et al., 2016).

Macrophages are functionally polarized into M1 and M2 cells, 
and M2-polarized macrophages are known to have important 
roles not only in parasite infection, but also in tissue remodelling 
and angiogenesis (Satoh et al., 2010). Among M2-polarized 
macrophages, CD163+ macrophages produce chemokines in 
response to cytokine stimuli and microbial and opsonic stimuli 
That recruit immune cells in a variety of diseases including 
autoimmune diseases (Martinez et al., 2006; Gordon and 
Martinez, 2010; Fujimura et al., 2016b). Soluble (s)CD163 is an 
activation marker for CD163+ M2-polarized macrophages that 
is present in the serum as a result of proteolytic shedding 
(Van Gorp et al., 2010), leading to increased serum CXCL5 
derived from M2 macrophages (Fujimura et al., 2016a). Particularly, 
serum sCD163 levels increase in autoimmune diseases including 
rheumatoid arthritis (Greisen et al., 2015), pemphigus 
vulgaris, and bullous pemphigoid (Fujimura et al., 2016a). More 
recently, serum sCD163 and CXCL5 levels are reported as 
biomarkers for autoimmune-like adverse events caused by 
immune checkpoint inhibitors such as nivolumab (Fujimura 
et al., 2017). These reports suggested that serum sCD163 and 
CXCL5 levels may contribute to the pathogenesis of autoimmune 
diseases; however, the roles of M2-polarized macrophages and 
serum sCD163 and CXCL5 levels in MMD patients have not yet 
been examined. We hypothesized that patients with MMD 
represent an intrinsic autoimmune status mediated by CD163+ 
M2-polarized macrophages.

2. Results

2.1. Representative case

A 45-year old woman had presented with a transient ischemic 
attack 4 years before participating in this study. Initial catheter 
angiography demonstrated definitive MMD with a Suzuki’s angiographic 
staging of three bilaterally (Fig. 1A, B). She then underwent 
left superficial temporal artery (STA)-middle cerebral artery (MCA) 
anastomosis on the affected hemisphere without any complications. 
We carefully followed up with yearly magnetic resonance (MR) 
imaging/angiography, by which an effective bypass was well visualized 
(asterisk in Fig. 1C). Four years after the initial surgery, however, 
she presented with a new transient ischemic attack in the right 
hemisphere, and N-isopropyl-p-[123I] iodoamphetamine single-
photon emission computed tomography (123I-IMP SPECT) showed 
decreased cerebral blood flow in the right hemisphere (arrows in 
Fig. 1D). There was no cerebral infarction, and she agreed to partic-
ipate in the present study. She was found to have a heterozygous 
mutant of the RNF213 polymorphism, and serum sCD163 and CXCL5 
levels were remarkably high as 653,139 pg/ml and 1568.25 pg/ 
ml, respectively. She underwent a right STA-MCA bypass without 
any complications, and postoperative MR angiography demon-
strated an apparently patent bypass (arrow in Fig. 1E).

2.2. Incidence of RNF213 polymorphism in MMD patients

Genotyping of the RNF213 SNP, c.14576G > A, p. R4859K, 
showed that 13 patients had the heterozygous variant, one patient 

![Fig. 1. Representative case with high serum production of soluble (s)CD163 and CXCL5. Initial catheter carotid angiograms demonstrating characteristics of definitive moyamoya disease (A, B). Magnetic resonance (MR) angiography after left revascularization surgery (C) indicating a patent bypass (asterisk in C and E). N-isopropyl-p-[123I] iodoamphetamine single-photon emission computed tomography (123I-IMP SPECT) showed decreased cerebral blood flow in the right hemisphere (arrows in D). MR angiography after right revascularization surgery (E) indicating a patent bypass (arrow).](image)
had the homozygous variant, and seven patients had wild-type expression of the gene. This proportion was in accordance with our previous study (Kamada et al., 2011).

2.3. Serum sCD163 and CXCL5 were significantly increased in MMD patients

As shown in Fig. 2, the serum sCD163 levels of MMD patients (281,465 pg/ml) were significantly higher than those of healthy controls (174,842 pg/ml) (p = .004). The serum CXCL5 levels of MMD patients (679.02 pg/ml) were also significantly higher than those of healthy controls (401.79 pg/ml) (p = .046). There were no differences in the serum sCD163 and CXCL5 levels between each genotype of the RNF213 polymorphism (wild-type or variant) among MMD patients (Table 1). Patients’ age and the onset-type (presence or absence of cerebral infarction) were not correlated with the serum sCD163 and CXCL5 levels (Table 1). Regarding Suzuki’s angiographic staging, patients with stage three tended to show relatively higher sCD163 and CXCL5 levels, but differences were not significant (Table 1). Regarding other cerebrovascular diseases, the serum sCD163 levels of unruptured cerebral aneurysm patients (193,096 pg/ml) were compatible to those of healthy controls and were below the levels of MMD patients. The serum CXCL5 levels of unruptured cerebral aneurysm patients (562.82 pg/ml) were also compatible to those of healthy controls, and were below the levels of MMD patients.

2.4. Increased expression of CD163 and CXCL5 in the arachnoid membrane of MMD patients

Immunohistochemical analysis revealed apparent expression of CD163 and CXCL5 in the arachnoid membrane of all the surgical specimens obtained from 10 MMD patients who underwent revascularization surgery (Fig. 3).

3. Discussion

In the present study, we demonstrated, for the first time, that serum sCD163 and CXCL5 levels were significantly elevated in MMD patients compared to those in healthy controls. These results indicate that patients with MMD may have increased autoimmune activity, and our results shed light on the novel pathogenesis of MMD through CD163+ M2-polarized macrophages.

The involvement of an autoimmune reaction in the pathophysiology of MMD has been suggested in light of the association of MMD with a number of autoimmune diseases, including systemic lupus erythematosus, Sjögren’s syndrome, periarteritis nodosa, and anti-phospholipid antibody syndrome, while patients with these diseases have been diagnosed as ‘quasi-MMD’ or ‘akin-MMD’ (Fukui, 1997; Research Committee on the Pathology and Treatment of Spontaneous Occlusion of the Circle of Willis, Health Labour Sciences Research Grant for Research on Measures for Infractable Diseases, 2012; Bower et al., 2013; Fujimura et al., 2014). Besides typical quasi-MMD, recent serological studies strongly suggested the high prevalence of Graves’ disease; autoimmune hyperthyroidism among East Asia patients with MMD (Kim et al., 2010; Li et al., 2011). Kim and colleagues reported that 48.9% of patients with MMD manifested with elevated thyroid autoantibody levels (2010). Li et al. alternatively found that pediatric MMD patients displayed a higher prevalence of increased thyroid function and elevated thyroid autoantibodies in comparison with control subjects (2011). These findings indicate that the relationship between MMD and autoimmune diseases, either clinical or subclinical, is much more common than was previously considered, and a semantic distinction between definitive MMD and quasi-MMD with autoimmune diseases is becoming less significant (Fujimura and Tominaga, 2015). The elevated serum production of sCD163 and CXCL5 in MMD patients may partly explain the high prevalence of autoimmune diseases with MMD, although the causal relationship between enhanced autoimmunity and the development of MMD is still unknown.

While the etiology of MMD is still unknown, both genetic factors and environmental factors are thought to be essential for its development (Fujimura et al., 2014). Regarding the genetics of MMD, our group and others found that RNF213 in the 17q25-ter region was an important risk gene for MMD (Kamada et al., 2011; Liu et al., 2011). We previously reported that the RNF213 SNP, c.14576G > A, p. R4859K, was detected in 95% of cases of familial MMD and 79% of sporadic cases (Kamada et al., 2011). To investigate the role of RNF213 in the development of MMD, we generated mutant mice lacking the Rnf213 gene, and found that they grew normally and did not spontaneously develop MMD (Sonobe et al., 2014). More recently, we also generated knock-in mice with a R4859K mutation of the Rnf213 gene, which also grew
normally and did not have characteristic findings of MMD (Kanoke et al., 2015). These results indicated that environmental factors, in addition to genetic factor, were needed for the development of MMD. This observation is in accordance with the fact that the characteristic SNP of \textit{RNF213} was found among 2% of the general Japanese population (Liu et al., 2011), but the prevalence of MMD is as low as 6.03 per 100,000 people (Kuriyama et al., 2008), indicating a very low penetrance rate of MMD. Considering the predominant expression of \textit{RNF213} in blood cells and the spleen (Kamada et al., 2011), we conducted immunologic adjuvant administration in \textit{RNF213}-deficient mice (Kanoke et al., 2016). Although the administration of immunological adjuvants such as MDP-Lys (L18) or CFA were not sufficient to induce MMD, the ratio of regulatory T cells after the administration of MDP-Lys (L18) was significantly lower in \textit{Rnf213}-deficient mice, suggesting a potential role of \textit{RNF213} abnormality in the differentiation of regulatory T cells, which could thereby compromise immunological self-tolerance (Kanoke et al., 2016). In the present study, there were no differences in the serum sCD163 and CXCL5 levels between each genotype of the \textit{RNF213} polymorphism (wild-type or variant) among MMD patients. Therefore, the role of \textit{RNF213} polymorphism in enhanced autoimmunity in MMD patients is unclear. Autoimmune reactions could alternatively participate in the development of MMD as a strong environmental factor that could implement the development of MMD originating from various types of genetic abnormalities including \textit{RNF213} polymorphism (Fujimura et al., 2014).

**Table 1**

Mean levels of sCD163/CXCL5 according to patient characteristics among moyamoya disease patients (n = 21).

<table>
<thead>
<tr>
<th></th>
<th>sCD163 (pg/ml)</th>
<th>CXCL5 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>RNF213 SNP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Variant</td>
<td>14</td>
<td>269,356</td>
</tr>
<tr>
<td>Wild-type</td>
<td>7</td>
<td>305,684</td>
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<tr>
<td><strong>Age</strong></td>
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</tr>
<tr>
<td>&lt;37 y</td>
<td>7</td>
<td>204,874</td>
</tr>
<tr>
<td>37–45 y</td>
<td>7</td>
<td>360,567</td>
</tr>
<tr>
<td>≥46 y</td>
<td>7</td>
<td>278,556</td>
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<tr>
<td><strong>Cerebral infarction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>226,652</td>
</tr>
<tr>
<td>No</td>
<td>14</td>
<td>308,872</td>
</tr>
<tr>
<td><strong>Suzuki's angiographic staging</strong></td>
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<td></td>
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<tr>
<td>Stage 2</td>
<td>5</td>
<td>246,287</td>
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<tr>
<td>Stage 3</td>
<td>9</td>
<td>339,317</td>
</tr>
<tr>
<td>Stage ≥ 4</td>
<td>7</td>
<td>232,213</td>
</tr>
</tbody>
</table>

| a Probability values were calculated by the Student t-test for comparisons between two groups and by one-way ANOVA for comparisons between three groups.  

**Fig. 3.** Immunohistochemical analysis of sCD163 (A, B) and CXCL5 (C, D) in the arachnoid membrane of moyamoya disease patients. Apparent expression of sCD163 (A) and CXCL5 (B) were evident, while there was no immunoreactivity in the control specimens treated without antibodies (B, D).
We did not find a significant correlation between sCD163 or CXCL5 levels and Suzuki’s angiographic staging due to the relatively small sample size, while these autoimmune-related serum markers tended to be high in patients with ‘stage 3’ Suzuki’s angiographic staging (Suzuki and Takaku, 1969). In light of the fact that the development of abnormal vascular networks at the base of the brain is most prominent in ‘stage 3’ (Suzuki and Takaku, 1969), enhanced serum production of sCD163 and CXCL5 in ‘stage 3’ patients was apparently unique. It was also of great interest to find that the representative case (Fig. 1) with progression of ischemic symptoms showed remarkably high serum levels of sCD163 and CXCL5, suggesting that sCD163 and/or CXCL5 may be useful biomarkers for clinical presentation of MMD patients. Future study with larger number of patients would address this important issue. Alternatively, there are several known inflammatory biomarkers of MMD, such as matrix metalloproteinase-9 and vascular endothelial growth factor (Fujimura et al., 2009; Kang et al., 2010), which are also implicated in autoimmune reactions (Ram et al., 2006). Therefore, it would be interesting to investigate the correlation between serum CD163 or CXCL5 levels and inflammatory biomarkers expression levels in MMD patients with variety of clinical statuses in future studies.

Finally, the exact origin of the characteristic autoimmune signaling response in MMD remains undetermined, but predominant expression of RNF213 in spleen strongly suggests the major contribution of spleen in the pathophysiology of MMD (Kamada et al., 2011; Liu et al., 2011). This observation is supported by the recent findings that neuro-inflammation modulated by spleen plays a critical role in stroke and traumatic brain injury (Acosta et al., 2015; Crowley et al., 2017). More importantly, spleen is reported to contribute significantly to the stem cell-based therapies which promote neuroprotection and neuro-regeneration against stroke and traumatic brain injury (Acosta et al., 2015; Crowley et al., 2017). Further investigation of the intrinsic immune signaling response originating from spleen will clarify the exact pathophysiology of MMD, and may also contribute to establish novel therapeutic strategies including stem cell therapy for MMD.

4. Conclusion

The present study demonstrated that serum sCD163 and CXCL5 levels were significantly elevated in MMD patients compared to those in healthy controls. Although this is a pilot study and further validation with larger number of samples is necessary, these results indicate that MMD patients may have increased autoimmune activity, and our results shed light on the pathogenesis of MMD via CD163+/M2-polarized macrophages.

5. Experimental procedure

5.1. Participants and setting

The study was approved by the ethical committees of Tohoku University Graduate School of Medicine (2016–1-263), and all patients provided informed consent. The present study included 21 patients with definitive MMD aged from 14 to 68 (mean 40.3 years old). The diagnosis was based on the guideline of the Research Committee on Spontaneous Occlusion of the Circle of Willis (Moyamoya Disease) of the Ministry of Health and Welfare, Japan (Fukui, 1997), and the association of active autoimmune diseases was ruled out in all patients. The onset types were as follows: transient ischemic attacks in 12 patients, cerebral infarction in seven patients, intracerebral hemorrhage in one patient, and asymptomatic in one patient. All patients were at a stable neurological state except for the presentation of the transient ischemic attack, and at least one year had passed since the onset of cerebral infarction or intracranial hemorrhage. There were 18 patients who had undergone revascularization surgery, but blood samples were collected at least one year after the last surgery. Suzuki’s angiographic staging of each patient was as follows; stage-2 in five patients, stage-3 in nine patients, stage-4 in four patients, stage-5 in two patients, and stage-6 in one patient (Suzuki and Takaku, 1969).

5.2. Genotyping of the RNF213 single-nucleotide polymorphism (SNP), c.14576G > a p. R4859K

Saliva preserved with an Oragene self-collection kit (OG-500: DNA Genotek Inc., Ontario, Canada) or whole venous blood in EDTA anticoagulant vials were collected from donors. Saliva samples were incubated at 50 °C and then treated with RNase followed by the next step of DNA purification. DNA was extracted from saliva and blood samples using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. SNP analysis for rs112735431 ([https://www.ncbi.nlm.nih.gov/projects/SNP/snp_Ref.cgi?rs=112735431](https://www.ncbi.nlm.nih.gov/projects/SNP/snp_Ref.cgi?rs=112735431)) was performed with TaqMan SNP genotyping assay (Assay ID: C_153120198_10; Applied Biosystems, Foster City, CA, USA) in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) to detect the RNF213 SNP, c.14576G > A, p. R4859K.

5.3. Cytokine ELISA

We analyzed the serum soluble (s)CD163 and CXCL5 in blood collected from 21 patients with MMD and from 15 age-matched healthy controls. The serum levels of sCD163 and CXCL5 proteins were determined by ELISA according to the manufacturer’s protocol (R&D systems), as previously described [15]. We also analyzed the serum sCD163 and CXCL5 in blood collected from 5 patients with unruptured cerebral aneurysm.

5.4. Tissue samples and immunohistochemical staining

The following antibodies (Abs) were used for immunohistochemical staining: mouse monoclonal Abs for human CD163 (Novocastra, Tokyo, Japan) and mouse monoclonal Abs for human CXCL5. Archival, formalin-fixed, paraffin-embedded specimens of arachnoid membranes were collected from 10 patients with MMD during revascularization surgery performed at Tohoku University Hospital.

5.5. Statistical analysis

First, serum sCD163 and CXCL5 levels were compared between MMD patients and healthy controls by Student’s t-test. Second, serum sCD163 and CXCL5 levels were compared according to patient characteristics (genotype of RNF213, age, cerebral infarction, and Suzuki’s angiographic staging) by Student’s t-test or one-way ANOVA among moyamoya disease patients (n = 21). Two-sided values of p < 0.05 or 95% confidence intervals (CIs) not including 1 were considered significant. All analyses were performed with IBM SPSS Statics Desktop, Version 24 (IBM Software Group, Chicago, IL.).

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behalf of all authors, the corresponding author states that there is no conflict of interest.

References


