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Clinical Article

Elevated Cellular Retinoic Acid Binding Protein-I in Cerebrospinal Fluid of Patients with Hemorrhagic Cerebrovascular Diseases : Preliminary Study

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Objective : Elevated cellular retinoic acid binding protein-I (CRABP-I) is thought to be related to the abnormal proliferation and migration of smooth muscle cells (SMCs). Accordingly, a higher CRABP-I level could cause disorganized vessel walls by causing immature SMC phenotypes and altering extracellular matrix proteins which could result in vulnerable arterial walls with inadequate responses to hemodynamic stress. We hypothesized that elevated CRABP-I level in the cerebrospinal fluid (CSF) could be related to subarachnoid hemorrhage (SAH). Moreover, we also extended this hypothesis in patients with vascular malformation according to the presence of hemorrhage.

Methods : We investigated the CSF of 26 patients : SAH, n=7; unruptured intracranial aneurysm (UIA), n=7; arteriovenous malformation (AVM), n=4; cavernous malformation (CM), n=3; control group, n=5. The optical density of CRABP-I was confirmed by Western blotting and presented as mean± standard error of the measurement.

Results : CRABP-I in SAH (0.33±0.09) was significantly higher than that in the UIA (0.12±0.01, p=0.033) or control group (0.10±0.01, p=0.012). Hemorrhage presenting AVM (mean 0.45, ranged 0.30–0.59) had a higher CRABP-I level than that in AVM without hemorrhage presentation (mean 0.16, ranged 0.14–0.17). The CRABP-I intensity in CM with hemorrhage was 0.21 and 0.31, and for CM without hemorrhage 0.14. Overall, the hemorrhage presenting group (n=11, 0.34±0.06) showed a significantly higher CRABP-I intensity than that of the non-hemorrhage presenting group (n=10, 0.13±0.01, p=0.001).

Conclusion : The results suggest that elevated CRABP-I in the CSF could be related with aneurysm rupture. Additionally, a higher CRABP-I level seems to be associated with hemorrhage development in vascular malformation.

Key Words : Cerebrospinal fluid · Arteriovenous malformation · Cavernous malformation · Retinoic acid.

INTRODUCTION

Vascular smooth muscle cells (SMCs) play a vital role in the formation of mature and contractile intracranial vessel walls through SMC proliferation, migration, and differentiation, and in the change in the extracellular matrix (ECM) components[17]. Accordingly, an abnormal SMC production pathway and altered ECM component can contribute to intracranial vascular diseases such as aneurysm[6,11], arteriovenous malformation (AVM) and cavernous malformation (CM)[19]. Kilic et al.[11] showed differently expressed structural proteins and regulatory growth factors among ruptured aneurysms, unruptured intracranial aneurysms (UIAs) and normal arterial walls. In their study, a drop in collagen III, collagen IV, α-smooth muscle actin (α-SMA) and transforming growth factor-α (TGF-α) was observed in patients with ruptured aneurysm and UIA. However, fibronectin in the ruptured aneurysm, which is abundant in immature vascular wall[5,11], was highly expressed compared to the UIA or normal control group. Uranishi et al.[19] reported that SMC differentiation in AVM was different from that in the CM or normal brain. And they suspected that such a difference may lead to different contractility responses to hemodynamic stress.

Various factors such as retinoic acid, TGF-beta, activin A, and platelet-derived growth factor B regulate SMC and ECM proteins. Retinoic acid has an inhibitory effect on SMC proliferation, differentiation and neointimal formation[7,14]. In addition, retinoic acid

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inhibited fibronectin and matrix metalloproteinase-2 (MMP-2)[2]. Retinoic acid is regulated by retinoid acid receptors (RARs) and cellular retinoic acid binding proteins (CRABP-I and II)[9]. In particular, CRABP-I is expressed in various adult tissues[1,12]. The role of CRABP-I was suggested as lowering intracellular concentrations of active all-*trans*-retinoic acid (ATRA) by increasing ATRA metabolism[4]. The association between CRABP-I and intimal hyperplasia was shown in an experimental animal model[15]. Additionally, CRABP-I in the cerebrospinal fluid (CSF) was suggested as a possible candidate for moyamoya disease (MMD)[12]. Theoretically, CRABP-I also may have the potential to cause disorganized vascular walls by altering the structural and adhesive proteins of the ECM as well as causing the formation of SMCs with an immature phenotype which could lead to vulnerable arterial walls with an inadequate response to the hemodynamic stress.

In this pilot study, we hypothesized that high CRABP-I level in the CSF could be related with presentation of hemorrhage in patients with aneurysm. In addition, we also extended this hypothesis in patients with vascular malformation according to the presence of hemorrhage.

MATERIALS AND METHODS

Patients sample

This study was approved by the Institutional Review Board at the participating hospital (H-1103-097-356). This prospective analysis was conducted on patients who underwent surgery for an aneurysm, AVM and CM from April 2011 to March 2012 at a single center. Written informed consent was obtained from every patient or their family before entering the study.

The optical density of CRABP-I was estimated according to the presence of hemorrhage in each disease entity. Then, the CRABP-I difference between the two groups, with and without hemorrhage presentation, was measured in all enrolled patients with an aneurysm, AVM, and CM. CSF samples obtained from a tumor or normal pressure hydrocephalus (NPH) were regarded as the control group[8]. The patients' medical and radiologic data including sex, age, underlying disease, diagnosis, presenting symptoms, location of lesion and size were reviewed.

CSF sample collection and measurement of CRABP-I

CSF samples ranging from 5 to 15 mL were collected from the cortical subarachnoid space including the cortex or sylvian fissure and stored at -80°C. Regarding patients with subarachnoid hemorrhage (SAH), CSF was taken from the cistern of the lamina terminalis (n=2) during the first operation or, the cortical and sylvian fissure during the second operation for concomitant UIA (n=5) to avoid blood contamination. The preparation of CSF samples and Western blot assay were performed according to previously reported methods[8,12]. We carried out protein preparations as follows : 1 mL of the CSF mixed with 250 μL of 50% trichloroacetic acid (Sigma Chemical Co., St. Louis, MO, USA) was centrifuged at 16000 rpm at 4°C for 15 minutes; the pellet was washed with ethyl ether (Merck Co, Darmstadt, Germany) after the supernatant was discarded; then, the protein pellet was dissolved in a solution containing 8 mol/L urea, 4% CHAPS, 40 mmol/L Tris base, and 100 mmol/L dithiothreitol. Determination of the protein concentrations was carried out with the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Western blot analysis was done and repeated as follows. After concentrating the CSF samples with trichloroacetic acid precipitation, 50 μg of the protein was denatured with buffer at 70°C for 10 minutes. Then, the denatured proteins were separated on a Novex NuPAGE 4−12% Bis-Tris gel (Novex, San Diego, CA, USA) and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, NY, USA). After blocking with Tris-buffered saline containing 5% nonfat dry milk, the membrane was incubated with the primary antibody, goat anti-human CRABP-I (1 : 500) (Santa Cruz Biotechnology, Santa Cruz, TX, USA) in TBS containing 0.1% Tween-20 overnight at 4°C. After it was washed, the blot was incubated with horseradish peroxidase-conjugated mouse anti-goat secondary antibody (1 : 1000) at room temperature for 1 hour. Horseradish peroxidase-conjugated anti-human albumin antibody (1 : 2000) (KOMA Biotech Inc, Seoul, Korea) was used as a loading control, and an enhanced chemiluminescence system (Invitrogen, Carlsbad, CA, USA) was used for the detection. The image J program (NIH, Bethesda, MD, USA) was used to analyze the densitometric intensities.

Statistical analysis

The optical density of CRABP-I is presented as mean±standard error of the measurement. A comparative analysis of the CRABP-I according to the presentation of hemorrhage was carried out by the Kruskal-Wallis test and Mann-Whitney U test for all possible pair-wise comparisons. p-values<0.05 were considered statistically significant. Statistics were performed with Statistical Package for the Social Sciences (SPSS) version 19 (SPSS Inc., Chicago, IL, USA).

RESULTS

Enrolled patients

Twenty-six patients were enrolled in this study. This study included aneurysms (n=14), AVM (n=4), CM (n=3), and a control group (n=5). Regarding aneurysms, 7 cases each with SAH and UIA were analyzed. Two cases each of AVM with and without hemorrhage presentation were included. Three cases were placed in the frontal lobe, and one was placed in the cerebellum. Their mean size was measured at 27.4 mm ranging in size from 12.7 to 44.9 mm. Three cases of CM, which were located in the temporal lobe (n=2) and pons (n=1) were enrolled. Two cases presented with hemorrhage. Their mean size was measured at 19.9 mm ranging in size from 11.0 to 30.6 mm (Table 1). The control group included 4 cases of brain tumors and one case of NPH. A case of

Table 1. Baseline characteristics of enrolled patients in this study (n=26)

Case no.	Sex	Age	Diagnosis	Presentation	Association	Optical density
1	F	45	Aneurysm	SAH	HTN	0.13
2	F	47	Aneurysm	SAH		0.27
3	F	46	Aneurysm	SAH		0.14
4	F	53	Aneurysm	SAH		0.79
5	M	44	Aneurysm	SAH	Smoking	0.52
6	F	55	Aneurysm	SAH		0.32
7	M	53	Aneurysm	SAH	DM, HTN, smoking	0.15
8	F	49	Aneurysm	Non-SAH		0.12
9	M	60	Aneurysm	Non-SAH	DM, HTN, smoking	0.09
10	F	50	Aneurysm	Non-SAH	HTN	0.12
11	M	69	Aneurysm	Non-SAH	HTN, dyslipidemia	0.11
12	F	40	Aneurysm	Non-SAH		0.16
13	M	54	Aneurysm	Non-SAH		0.15
14	F	58	Aneurysm	Non-SAH	DM, HTN	0.10
15	M	49	AVM	Hemorrhage		0.30
16	F	21	AVM	Hemorrhage		0.59
17	M	31	AVM	Non-hemorrhage	Smoking	0.17
18	F	16	AVM	Non-hemorrhage		0.14
19	M	20	CM	Hemorrhage	HTN, smoking	0.31
20	M	61	CM	Non-hemorrhage		0.14
21	M	43	CM	Hemorrhage		0.21
22	M	49	Hemangioblastoma	Headache		0.08
23	F	20	Medulloblastoma	Dizziness		0.09
24	M	34	Craniopharyngioma	Headache		0.12
25	F	43	Meningioma	Headache		0.11
26	M	66	NPH	Gait disturbance	HTN	0.10

AVM : arteriovenous malformation, CM : cavernous malformation, SAH : subarachnoid hemorrhage, DM : diabetes mellitus, HTN : hypertension, NPH : normal pressure hydrocephalus

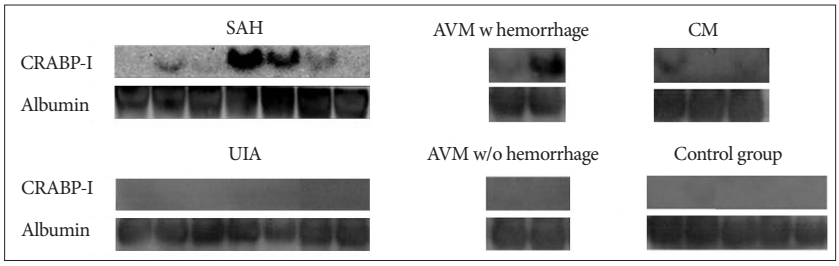

Fig. 1. Western blot analysis of cellular retinoic acid binding protein-I (CRABP-I) expression in cerebrospinal fluid from patients with subarachnoid hemorrhage (SAH), unruptured intracranial aneurysm (UIA), arteriovenous malformation (AVM) presenting with and without hemorrhage, cavernous malformation (CM), and control group. The albumin level was used for normalization.

hemangioblastoma, medulloblastoma, craniopharyngioma and meningioma each was enrolled. Their clinical symptoms were headache, dizziness and gait disturbance. Hemorrhage development was not observed in the control group.

Comparison of CRABP-I according to the presence of hemorrhage

CRABP-I in SAH was highly expressed in the Western blot (Fig. 1). The Kruskal-Wallis test showed that the optical density of CRABP-I was significantly different among the three groups (SAH, 0.33±0.09; UIA, 0.12±0.01; control group, 0.10±0.01; p= 0.004). The Mann-Whitney U test revealed that the optical density in SAH in a ruptured aneurysm was significantly higher than that in UIA (p= 0.033) or the control group (p=0.012). There was no significant CRABP-I difference between the UIA and control group (p=0.447) (Fig. 2). The hemorrhage presenting AVM (mean 0.45, range 0.30−0.59) had a higher CRABP-I level than that in AVM without hemorrhage presentation (mean 0.16, range 0.14−0.17). Two cases of CM with and one case without hemorrhage presentation had CRABP-I optical densities measured at 0.21 and 0.31 and 0.14, respectively (Table 1).

We also measured the CRABP-I intensity between the two groups based on the presence of hemorrhage presentation in all enrolled patients with an aneurysm, AVM and CM. The hemorrhage presenting group (n=11, 0.34±0.06) had a significantly higher CRABP-I intensity than that of the non-hemorrhage presenting group (n=10, 0.13±0.01) (p=0.001).

DISCUSSION

Our preliminary results showed that the elevation of CRABP-I in the CSF could be related to an aneurysmal rupture. Although no statistical analysis was done for vascular malformations due to the small number of enrolled cases, vascular malformation with presentation of hemorrhage appears to have a higher CRABP-I level than that in vascular malformation without hemorrhage presentation. Overall, the hemorrhage presenting cerebrovascular disease group had a higher CRABP-I level than that of the non-hemorrhage group.

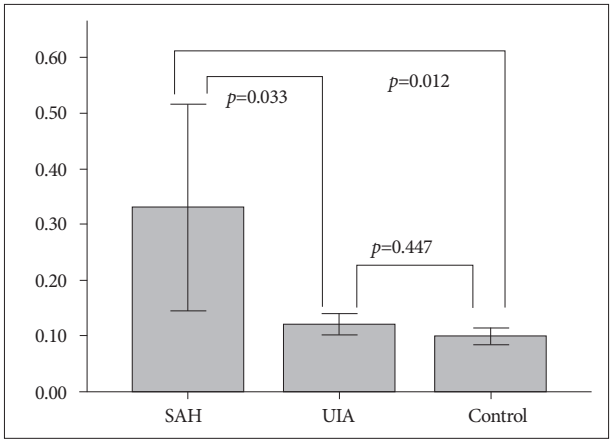

Fig. 2. Graph shows the optical density of cellular retinoic acid binding protein-I (CRABP-I) in the cerebrospinal fluid from patients presenting with subarachnoid hemorrhage (SAH), unruptured intracranial aneurysm (UIA) and the control group. CRABP-I optical density in SAH (0.33±0.09) was significantly higher than that in UIA (0.12±0.01, p=0.033) or the control group (0.10±0.01, p=0.012). There was no significant difference in CRABP-I between UIA and the control group (p=0.447). The bar represents the standard error of the mean.

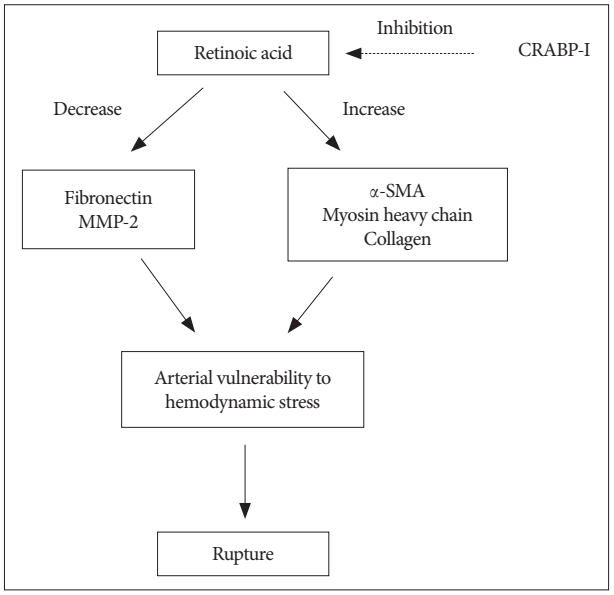

Fig. 3. Potential mechanism between cellular retinoic acid binding protein-I (CRABP-I) and aneurysm rupture. Retinoic acid increases the formation of α-smooth muscle actin (α-SMA) and myosin heavy chain, and the expression of collagen. In contrast, fibronectin and matrix metalloproteinase-2 (MMP-2) are attenuated by retinoic acid. Inhibition of retinoic acid by CRABP-I could attribute to vulnerable aneurysm wall formation in response to hemodynamic stress and may themselves to be more prone to rupture.

Because vascular SMC is not a terminated differentiated phenotype, SMC development and differentiation can be modulated by various factors during the process for aneurysm formation and rupture. A decrease in the number of SMCs by apoptosis has been suggested in aneurysm formation in a rat model[13]. Bygglin et al.[6] reported on two major proteins of α-SMA and calponin and expressed prolyl-4-hydroxylase in typical cultured SMCs from a human intracranial aneurysm. They thought that SMCs with a synthetic phenotype in response to arterial wall injury could attribute to aneurysm formation. MMP-2 secreted from migrating SMCs can result in elastic degradation, and it could cause an aneurysmal rupture[22]. Kilic et al.[11] suspected that aneurysmal rupture could be related to a rise in the ratio of fibronectin to laminin. They thought that fibronectin, which is abundant in immature vessels, could contribute to the improper contractility of the aneurysm wall, which appeared to be more prone to rupture[5,11].

The association between retinoic acid and intracranial aneurysm has not been well established. Neuville et al.[15] reported that ATRA modulates the proliferation and migration of SMCs and α-SMA expression. In that study, ATRA treatment showed an inhibition of DNA synthesis in intimal SMCs and proliferation of intimal and medial SMCs. Additionally, increased cell migration of intimal and medial SMCs was observed after ATRA treatment. Axel et al.[2] showed a decrease in mRNA-expression of the fibronectin and an increase in α-SMA and myosin heavy chain formation after ATRA treatment in human vascular SMCs. In addition, the synthesis of MMP-2 was partially inhibited by a high dose of ATRA treatment. These observations suggested that the retinoic acid signaling pathway could be related to the vulnerability of the aneurysm wall through a remodeling pathway of the SMC and ECM. Retinoic acid is modulated by two proteins, the RARs and CRABP. Boylan and Gudas[4] reported that a higher CRABP-I level facilitates the metabolic rate of retinoic acid by interaction with degradative enzyme cytochrome P450 hydroxylase A1. Accordingly, retinoic acid which interacts with RARs to activate retinoic acid signaling can be deceased in the presence of a higher CRABP-I level, which lessens the inhibitory effect of retinoic acid on growth factors and cytokines for vascular SMCs. Kim et al.[12] suggested highly expressed CRABP-I as a possible candidate for pediatric MMD through inhibition of retinoic acid.

Beyond ATRA's effect on vascular SMCs, it also stimulates ECM production[21]. Accordingly, CRABP-I could contribute to disorganized vascular walls through altered structural and adhesive proteins of ECM which could make the arterial walls vulnerable to hemodynamic stress. Axel et al.[2] thought that ATRA seems to stimulate structural ECM protein such as collagen and elastin but inhibits adhesive proteins of fibronectin and thrombospondin-1. Thrombospondin-1 prevents MMP-2 activation which facilitates ECM degradation and migration of SMCs[18]. However, expression of thrombospondin-1 is observed immediately after

vessel injury[16]. Accordingly, long-lasting expression of fibronectin during arterial wall remodeling[3] could be related with dysmorphic vessel formation secondary to inhibition of retinoic acid by CRABP-I (Fig. 3).

Uranishi et al.[19] reported various stages of SMC differentiation in AVM, CM and normal control brain. α-SMA and myosin heavy chain were universally expressed in AVM, but the contractile property was less in AVM compared with the normal and control brains. Approximately 85% of small CM and 97% of large CM showed α-SMA expression in the subendothelial layer. Additionally, the lesser commonly expressed myosin heavy chain was observed. Smoothelin, which is a marker of SMCs with a contractile phenotype[20], was significantly more expressed in the control brain (40.9%) than in AVM (21.9%) or CM (0%). CM showed more expression of fibronectin and basic fibroblast growth factor in the endothelium than in AVM[10]. These observations could imply that abnormal SMC differentiation and endothelial dysfunction could relate fragile vessel formation vulnerable to hemodynamic stress in vascular malformation. The association between retinoic acid and pathogenesis and its role in hemorrhage presentation in vascular malformation are not well understood. In our pilot study, the mean optical density of CRABP-I in AVM with hemorrhage presentation was higher than that in AVM without hemorrhage presentation. In addition, CM with hemorrhage presentation had a higher CRABP-I optical density than that of CM without hemorrhage presentation. Accordingly, abnormal SMC differentiation through an inhibition of retinoic acid by CRABP-I could be related to hemorrhage development. Therefore, studies on the relationship between retinoic acid and vascular malformation and the CRABP-I difference according to presenting symptoms are needed further.

The possibility of elevated CRABP-I as a result of hemorrhage could be a concern. To the best of our knowledge, an association between CRABP-I and intracranial hemorrhage has not been reported. In addition, we got the CSF sample from the cistern of lamina terminalis during first aneurysm surgery or sylvian cistern during second surgery for concomitant unruptured aneurysm to avoid blood contamination. Accordingly, concern over the correlation between CRABP-I and hemorrhage can be reduced.

There are some limitations in our study. First, we only measured the CRABP-I intensity in 26 patients. Accordingly, further study will be required to verify the relationship between CRABP-I and hemorrhage development in a large number of patients with aneurysm and vascular malformation. Second, we cannot suggest a more precise mechanism of CRABP-I in hemorrhagic cerebrovascular diseases different from that in MMD. Therefore, a study on the role of CRABP-I in the pathogenesis of arterial wall remodeling is required at the molecular level.

CONCLUSION

This pilot study showed that elevated CRABP-I in the CSF could be related to the occurrence of an aneurysm rupture. Moreover, a higher CRABP-I level in the CSF seems to be associated with the presentation of hemorrhage in patients with vascular malformation. A larger study is required to confirm our results.

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