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Original Article

Histidine-rich Glycoprotein Could Be an Early Predictor of Vasospasm after Aneurysmal Subarachnoid Hemorrhage

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Cerebral vasospasm (CVS) is a major contributor to the high morbidity and mortality of aneurysmal subarachnoid hemorrhage (aSAH) patients. We measured histidine-rich glycoprotein (HRG), a new biomarker of aSAH, in cerebrospinal fluid (CSF) to investigate whether HRG might be an early predictor of CVS. A total of seven controls and 14 aSAH patients (8 males, 6 females aged 53.4 ± 15.4 years) were enrolled, and serial CSF and serum samples were taken. We allocated these samples to three phases (T1-T3) and measured HRG, interleukin (IL)-6, fibrinopeptide A (FpA), and 8-hydroxy-2'-deoxyguanosine (8OHdG) in the CSF, and the HRG in serum. We also examined the release of HRG in rat blood incubated in artificial CSF. In contrast to the other biomarkers examined, the change in the CSF HRG concentration was significantly different between the non-spasm and spasm groups ($p < 0.01$). The rat blood/CSF model revealed a time course similar to that of the human CSF samples in the non-spasm group. HRG thus appears to have the potential to become an early predictor of CVS. In addition, the interaction of HRG with IL-6, FpA, and 8OHdG may form the pathology of CVS.

Key words: biomarker, histidine-rich glycoprotein, predictor, subarachnoid hemorrhage, vasospasm

Aneurysmal subarachnoid hemorrhage (aSAH) is a devastating type of stroke with high morbidity and mortality. The mortality is approx. 60%, with 30% of survivors having significant morbidity. Cerebral vasospasm (CVS) and microvascular dysfunction associated with aSAH can decrease the cerebral blood flow, leading to delayed cerebral ischemia (DCI) and hypoxia in brain tissues and ultimately to severe neurological dysfunction [1]. These are major contributors to the high morbidity and mortality of aSAH patients. A CVS usually occurs at around 4 days after the onset of aSAH and reaches a peak in incidence and severity at 7-10 days [2].

There is substantial evidence to suggest that oxidative stress, inflammation, alterations in the coagulation system, and subsequent vascular endothelial injury are significant in the development of acute brain injury and CVS following aSAH. The pathogenesis of CVS has not been fully clarified. Various cytokine and protein profiles in serum or CSF have been investigated to elucidate this pathogenesis. Although some biomarkers have

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shown a relationship with CVS, an effective early predictor has not been found. In addition, the interactions between biomarkers are still unknown.

Histidine-rich glycoprotein (HRG) is a plasma glycoprotein produced in and secreted from the liver. Since HRG binds to a diverse range of ligands, it has been suggested to be involved in the regulation of coagulation/fibrinolysis [3-5], immune response [6], and angiogenesis [7-10]. HRG was also recently shown to have important effects on intravascular elements. HRG inhibits the tight attachment of neutrophils, sustains rheological stability, and inhibits vascular endothelial cell activation [11, 12]. This suggests that a loss of HRG might be involved in the endothelial injury that leads to a CVS. However, HRG in cerebrospinal fluid (CSF) has not been investigated.

The primary aims of the present retrospective study were to determine whether there is a difference in HRG concentration between patients who incurred an aSAH and those who did not, and between patients who developed a vasospasm and those who did not. We also investigated the usefulness of HRG as an early predictor of CVS. A second goal was to determine whether HRG interacts with other potential biomarkers. We measured and evaluated interleukin (IL)-6, an inflammatory marker, fibrinopeptide A (FpA), a coagulation marker, and 8-hydroxy-2'-deoxyguanosine (8OHdG), an oxidative stress marker.

Materials and Methods

Ethical approval. For this type of retrospective study, formal consent of the patients was not required. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and all of the procedures in the present animal experiments were in accordance with the ethical standards of our institution.

Background characteristics. We retrospectively analyzed the cases of patients with aSAH and controls. They were of comparable age and gender. We classified the aSAH patients into spasm and non-spasm groups. The aSAH patients were evaluated both at their admission with the Hunt and Kosnik grading (H & K grading) and the World Federation of Neurological Surgeons SAH scale (WFNS scale), as well as at discharge using the modified Rankin Scale (mRS).

Aneurysmal neck clipping or coil embolization to prevent re-rupture was performed in the patients who were of treatment adaptation. In addition, in the spasm group, we evaluated whether the patients developed a symptomatic spasm and/or DCI. All assigned patients received medical treatment in our hospital's intensive care unit for ≥2 weeks. Standard prophylactic treatments were performed to prevent vasospasm or DCI. Each CVS was detected by digital subtraction angiography (DSA) or magnetic resonance angiography (MRA) 7-9 days after its onset. DCI was confirmed by computer tomography (CT) or magnetic resonance imaging (MRI) with proper timing. When it was thought necessary for CSF drainage, a spinal tube was inserted appropriately. Each patient's sample study was gathered at the same time that the CSF was submitted for a clinical examination.

We enrolled the total of 40 aSAH patients who were admitted to Kagawa University Hospital from September 2014 to September 2016. CSF and serum samples were gathered several times per patient between day 0 and day 11. We allocated these samples to three phases (T1-T3). We defined T1 as the period within 48 h following the patient's SAH. The T2 phase was after T1 and before the examinations for spasm. T3 was the phase after the spasm check. Thus, T1 reflects the period immediately after spasm onset, T2 the early or pre-spasm phase, and T3 the severe spasm phase.

We selected the cases of the patients in whom CSF samples could be gathered during T1 and T2 with or without T3, and we investigated several biomarkers including HRG. Serum HRG concentrations were calculated in some of the enrolled patients who had stored samples. We excluded the cases in which CSF samples were not obtained in the appropriate phase, the cases in which the treatment for preventing re-rupture was not performed, and the cases of patients who died during their follow-up or suffered severe meningitis. The sample collection and retrospective analysis were approved by the Ethics Committee of Kagawa University. We also investigated relationship the between the value of T1 and the hematoma volume. The semiquantitative classification of hematoma is as follows. Each of 10 basal cisterns and fissures were graded separately on a semiquantitative scale according to the amount of extravasated blood, from 0 to 3. The total amount of subarachnoid blood (sum score) was calculated by adding the 10 scores, and the possible sum thus ranged from 0 to 30. There was a dose-dependent relationship

between the T1 value and semiquantitative classification of the hematomas.

CVS detection. At our facility, all aSAH patients undergo preoperative DSA. Thus, when follow-up DSA is performed to check for a spasm, the images obtained before and after treatment can be compared. We classified the patients into a non-spasm group and a spasm group based on their DSA findings. We further divided the spasm group into the subgroup of patients who had experienced an angiographic spasm (*i.e.*, a CVS around the circle of Willis or a peripheral artery, without neurological defect, including patients who had slight stenosis compared with preoperative findings) and the subgroup of patients who had experienced a symptomatic spasm (*i.e.*, a CVS with a neurological defect such as consciousness disorder, paralysis, and dysarthria).

Handling of CSF and serum samples. Within 15 min of collection, the CSF samples were centrifuged at 1,000 *g* for 15 min, and the supernatants were collected and stored at −80°C. Before each assay was performed, high-molecular-weight proteins were removed by filtration (COSMOSIL, Kyoto, Japan). Serum samples were first left to stand for 30 min and then centrifuged at 300 *g* for 10 min. We did not use a reservoir for serum samples. The samples were stored as for CSF.

Concentrations of HRG, IL-6, FpA, and 8OHdG. We measured the targeted proteins and cytokines in the patients' CSF and serum with commercial quantitative sandwich enzyme-linked immunosorbent (ELISA) assay kits. HRG was measured by a kit from CUSABIO/CusAb (College Park, MD, USA), IL-6 was measured by a kit from (R&D Systems, Minneapolis, MN, USA), FpA measured by a kit from CUSABIO/CusAb, and 8OHdG measured by a kit from JaICA (Shizuoka, Japan).

Standards were prepared and the appropriate volume of samples or standards were added to 96-well polystyrene microtiter plates pre-coated with a monoclonal antibody to HRG, IL-6, FpA, or 8OHdG. The plates were incubated for the time recommended by the ELISA kit's manufacturer. Each well was then aspirated, and the plates were washed with the buffered surfactant provided. An enzyme-linked polyclonal antibody against each element was then added, and the plates were incubated and washed. Substrate solution was added to each well, and the optical density was read at a wavelength of 450 nm.

Rat preparation and the incubation of blood/CSF. To investigate the time course of the HRG concentration in CSF, we made a rat blood/CSF model. Three 8-week-old Sprague-Dawley rats weighing approx. 250 g had a femoral artery exposed and a catheter inserted in the aorta to obtain 0.5 ml of arterial blood. These aliquots were mixed with 9.5 ml of artificial CSF (Funakoshi, Tokyo) and placed in an incubator at 37°C and aged under sterile conditions in the dark for ≤240 h. At 3, 48, 96, 144, 192 and 240 h, 1.0 ml of blood/CSF was harvested. Each sample was centrifuged at 1,000 *g* for 15 min, and the supernatant collected and stored at −80°C. The HRG concentration was then measured with an ELISA kit from CUSABIO/CusAb.

ROC analysis. We obtained non-parametric receiver operating characteristic (ROC) curves and the area under the curve (AUC) to quantify how well HRG discriminated between the spasm and non-spasm groups. The AUC can be considered a simple measure of the probability that a randomly selected case would have a higher biomarker value than a control.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). The statistical analyses were done with StatMate V software (ATMS, Tokyo, Japan). We used Welch's *t*-test or an analysis of variance (ANOVA) which is supported by LSD (least significant difference) to analyze the data. Statistical significance was defined as $p < 0.05$.

To clarify the precise utility of HRG, we calculated the sensitivity, specificity, and AUC by obtaining the (ROC) curves with the use of JMP 13 software (SAS, Cary, NC, USA).

Results

Table 1 summarizes the background characteristics of the patients. Of the 40 patients who suffered an aSAH during the target period, 26 were excluded. A spinal tube was not inserted in 15 patients. CSF was not collected in 10 patients with the proper timing. One patient suffered from bacterial meningitis because of the spinal tube during observation. No patient was prevented from undergoing treatment for re-rupture prevention, and no patient died during their observation period.

The 14 enrolled patients were 8 males and 6 females aged 53.4 ± 15.4 years (range 32-84 years). Of the aSAH group, 7 patients incurred a CVS. Of these, 3 patients' condition developed to an angiographical CVS and 4 to

Table 1 Patient demographics

	Control group	aSAH group		*p* value
N	7	14		
		spasm	nonspasm	
		7	7	
Age	66.9 ± 20.6	57.3 ± 16.2	56.4 ± 15.0	NS
Gender (male: female)	5 : 2	4 : 3	4 : 3	NS
SAH grading				
H&K grade				NS
I		0	0	
II		4	3	
III		1	1	
IV		1	2	
V		1	1	
WFNS gradc				NS
I		2	1	
II		3	2	
III		0	0	
IV		0	2	
V		2	2	
TI (day)		0.57 ± 0.53	0.50 ± 0.55	NS
T2 (days)		4.71 ± 1.38	5.17 ± 1.47	NS
T3 (days)		8.71 ± 1.98	8.67 ± 1.21	NS
Surgical procedure (coil: clipping)		5 : 2	6 : 1	NS
Symptomatic spasm		4		
DCI		1		
mRS		2.57 ± 1.6	2.00 ± 1.5	NS

a symptomatic CVS. Of the symptomatic CVS group, 1 patient received an intra-arterial injection of fasudil hydrochloride hydrate, and 3 underwent a balloon angioplasty. However, notwithstanding this aggressive treatment, 1 patient developed DCI.

The non-spasm patients and the spasm patient did not differ significantly by age, gender, SAH grade (H&K grading, WFNS grading), surgical procedure (coil/clip rate) or mRS at discharge. The sampling interval for CSF allocated to the T1, T2 and T3 phases also did not differ between these 2 patient groups. Among the patients who suffered an aSAH, 10 (4 spasm patients, 6 non-spasm patients) had their serum HRG concentrations measured in addition to their CSF HRG levels.

Seven patients (5 males, 2 females) were enrolled as the control group. Five had idiopathic normal pressure hydrocephalus (iNPH) and two had a benign brain tumor. The controls were older than the aSAH patients at 66.9 ± 20.6 years.

Fig. 1 compares the CSF HRG concentrations in the aSAH groups and controls in the initial T1 time period.

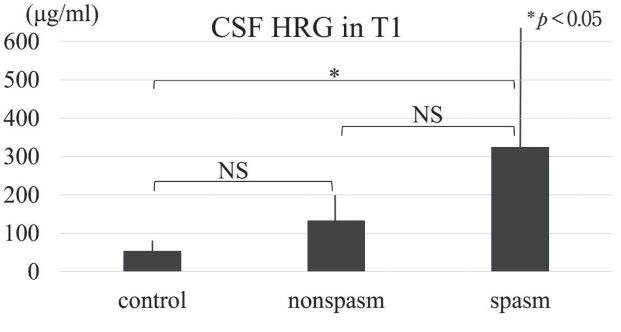

Fig. 1 The CSF HRG concentrations during the T1 period in the control, non-spasm, and spasm aSAH patients. The HRG levels were significantly higher in the spasm aSAH patients compared to the controls and higher in the spasm group than the non-spasm group.

Significantly higher HRG levels were revealed in the aSAH group (324.9 ± 300.5 μg/ml) compared to the control group (54.3 ± 19.3 μg/ml; $p < 0.05$), and the spasm group's HRG levels were higher than those of the non-spasm group (133.4 ± 68.5 μg/ml), but not significantly so.

We also investigated whether there is a relationship

between the T1 value and hematoma volume. Each of 10 basal cisterns and fissures were graded separately on a semiquantitative scale, according to the amount of extravasated blood from 0 to 3. The total amount of subarachnoid blood (sum score) was calculated by adding the 10 scores, providing a possible total score from 0 to 30 [13]. The results indicated a positive correlation between the T1 value and hematoma volume (Fig. 2).

Fig. 3A, B shows the time courses of the patients' CSF and serum HRG concentrations during phases T1-T3 in the aSAH group. The standard value of serum concentration was set based on a previous report [6], and we calculated the standard value of the CSF concentration based on the average value of control group. In both the aSAH and control groups, the serum HRG concentrations were almost the same as the standard, and the amount of change with time was small. In contrast, the CSF HRG concentrations were extremely high in both aSAH groups compared to the average of the control group.

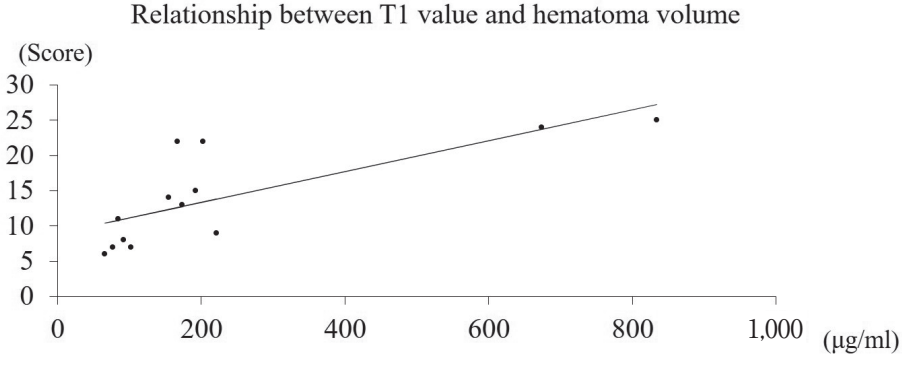

Fig. 2 The relationship between the T1 value and the hematoma volume. The results indicate a positive correlation ($p < 0.01$).

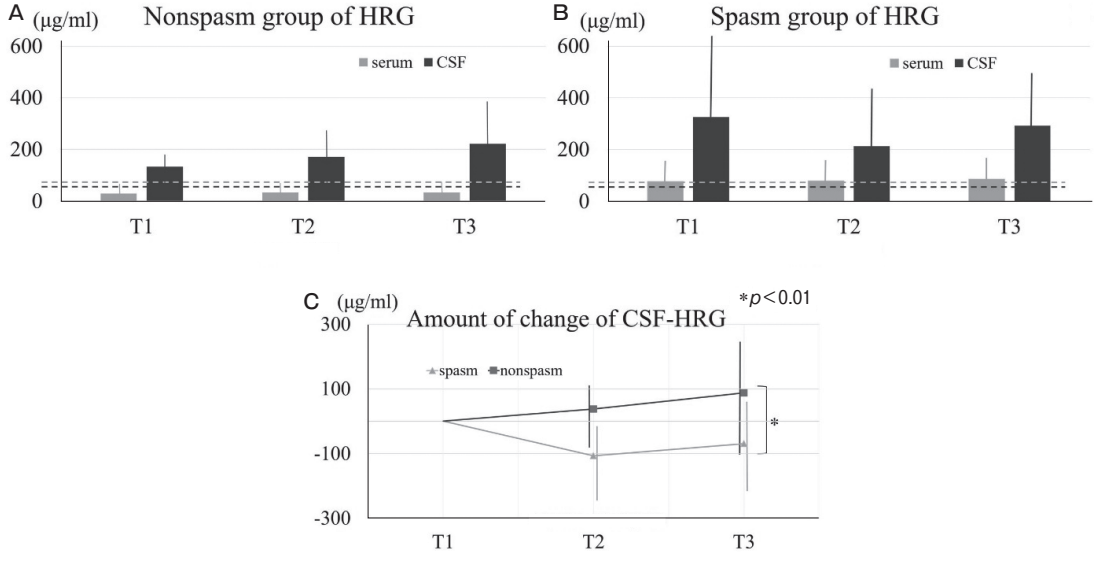

Fig. 3 The CSF HRG concentrations in the T1-T3 phases in the non-spasm (**A**) and spasm (**B**) aSAH groups. *Black dotted line*: The average CSF concentration in the control patients. *Gray dotted line*: The mean serum HRG concentration. In both the non-spasm and spasm groups, the serum HRG concentrations were almost the same as those of the control patients, and the amount of change with time was small. In contrast, the CSF HRG concentrations were much higher in both aSAH groups compared to the controls, and this difference persisted with time. **C**, The changes in the CSF HRG concentration at T2 and T3 compared to T1 (set as 0). In the non-spasm aSAH group, the T2 and T3 values were higher than the T1 values, whereas they were significantly lower in the spasm group ($p < 0.01$).

We use the CSF data to calculate the change in the HRG level with time, with T1 set as zero (Fig. 3C). In the non-spasm group, the T2 and T3 values were higher than the T1 values (changes from T1 at T2: 37.7 ± 86.1 μg/ml, at T3: 87.3 ± 184.2 μg/ml). In contrast, the T2 and T3 values were lower in the spasm group compared to the T1 values (changes from T1 at T2: −107.2 ± 113.2 μg/ml, T3: −69.6 ± 136.2 μg/ml). There were significant differences among the groups ($p < 0.01$). However, there was no difference between the angiographic spasm and symptomatic spasm groups (data not shown). The detailed data for each patient are shown in Fig. 4.

Fig. 5 shows the time courses of the IL-6, FPA, and

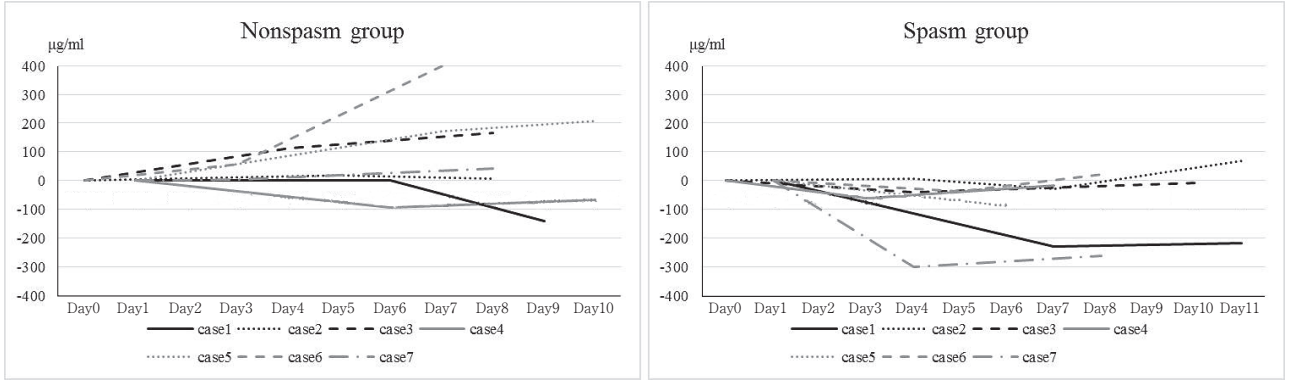

Fig. 4 Each patient's HRG concentration changes. The HRG concentration tended to rise in the non-spasm group and tended to decline in the spasm group.

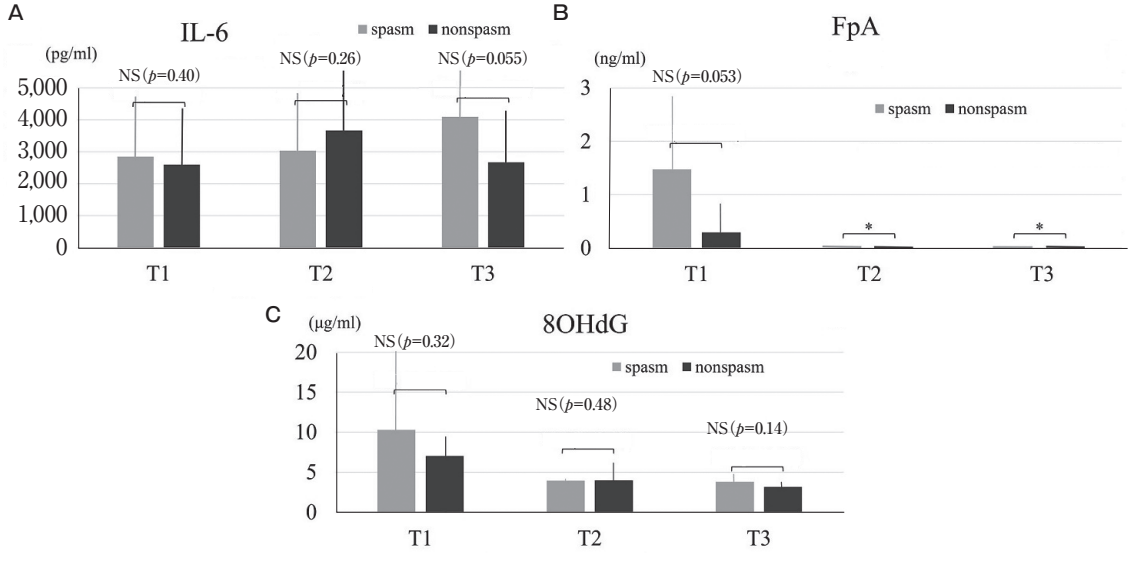

Fig. 5 The time courses of potential biomarkers in the CSF in the spasm and non-spasm aSAH patients. **A**, The IL-6 concentrations during phases T2 and T3 were higher than those in T1 in both groups. In the spasm group, the concentration reached a maximum in T3. In the non-spasm group, after the concentration reached a maximum during T2, the concentration decreased. There were no significant differences between the spasm and no-spasm groups at any time point, although at T3 the spasm group was nearly significantly higher ($p = 0.055$); **B**, The CSF FpA concentration in both groups was highest in T1, and rapidly decreased. All T2 and T3 samples in both groups were below the measurement sensitivity ($p < 0.039$ ng/ml). In T1, the CSF FpA concentration was higher in the spasm group, but this was not quite significant ($p = 0.053$) *Below the measurement sensitivity; **C**, In both the spasm and non-spasm groups, the CSF 8OHdG concentrations were highest in T1 and gradually decreased. There were no significant differences between the two groups at any time point.

8OHdG concentrations in CSF. As with HRG, these concentrations were measured at the T1, T2 and T3 time periods. Regarding IL-6, in both groups, the CSF concentrations at T2 and T3 were higher than at T1 (spasm group, T1: 2,840±1,884 pg/ml, T2: 3,028±1,825 pg/ml, T3: 4,089±1,274 pg/ml; non-spasm group, T1: 2,593±1,766 pg/ml, T2: 3,662±1,768 pg/ml, T3: 2,666±1,626 pg/ml). The Il-6 concentration gradually increased and reached a maximum at T3 in the spasm group.

In contrast, in the non-spasm group, the Il-6 concentration decreased after it reached a maximum at T2. During T3, the IL-6 concentration was higher in the spasm group than the non-spasm group, but this did not quite reach significance ($p=0.055$, Fig. 5A). The concentrations of FpA were highest at T1 (spasm group: 1.47±1.33 ng/ml, non-spasm group: 0.29±0.57 ng/ml) and then rapidly decreased. All of the T2 and T3 samples in both groups were below measurement sensitivity (<0.039 ng/ml). The T1 concentration of FpA was higher in the spasm group, but this did not quite reach significance ($p=0.053$, Fig. 5B). As for 8OHdG, the T1 concentrations were the highest (spasm group: 10.3±10.2 μg/ml, non-spasm group: 7.0±2.1 μg/ml), and the concentrations then gradually decreased (spasm group, T2: 3.9±0.1 ng/ml, T3: 3.8±0.7 ng/ml; non-spasm group, T2: 4.0±2.2 ng/ml, T3: 3.2±0.5 ng/ml). However, there were no significant differences between the spasm and non-spasm groups (Fig. 5C).

Fig. 6 shows the time course of the HRG concentrations in the rat blood/CSF incubation model. The concentration was measured at 3, 48, 96, 144, 192, and 240 h after blood/CSF incubation. The HRG concentration peaked at 48 h and decreased thereafter. However, all of the values after 48 h were higher than the 3 h value. This HRG time course was similar to that found for CSF in the samples from the non-spasm patients.

Fig. 7 shows the ROC curves regarding the prediction of CVS at arbitrarily chosen HRG cutoff values. We used the amount of change in the T2 values with T1 set as zero. The cutoff value of −36.6 μg/ml for HRG showed 85.7% sensitivity and 71.4% specificity for the prediction of a CVS during the T2 phase. The AUC of this ROC was 85.7%.

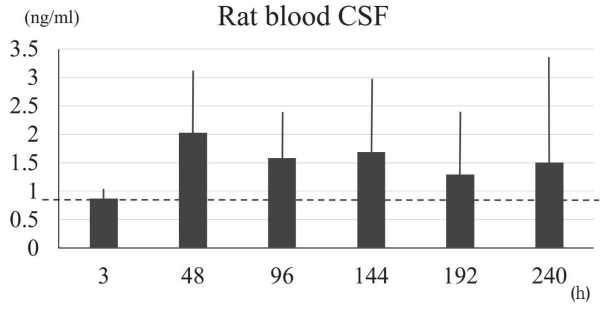

Fig. 6 The HRG concentration time course after blood/CSF incubation. The concentration peaked at 48 h and decreased thereafter. However, all of the samples after the 48-h value were still higher than the 3-h value.

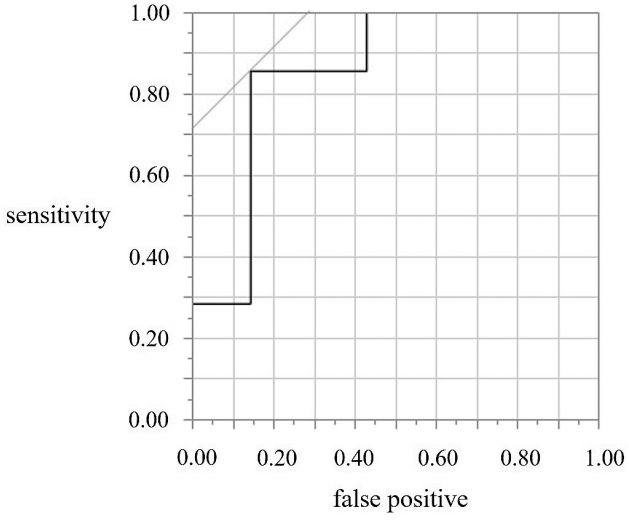

Fig. 7 ROC curves regarding the prediction of CVS.

Discussion

We consider the following hypothesis regarding the etiology of CVS. As the first step, a strong coagulation disorder occurs, which leads to a decrease in HRG that is caused by the consumption. The decrease in HRG also induces inflammation. A series of cascades causes a vascular endothelial injury, resulting in a CVS.

HRG is a 75-kDa plasma glycoprotein produced in and secreted from the liver [14]. It is thought to be involved mainly in the regulation of coagulation and fibrinolysis. HRG inhibits the contact pathway activation of plasma in vitro, presumably because the histidine-rich region of HRG binds to negatively charged surfaces and prevents the auto-activation of FXII (coagulation factor XII) [3, 4]. In support of this concept, HRG-deficient mice exhibit shortened clotting times [5]. On the other hand, it was also reported that HRG par-

ticipates in the immune response [6] and angiogenesis [7-10]. Interestingly, Wake *et al.* have clearly shown a novel and important role of HRG in controlling neutrophil shape, adhesiveness, and basal reactive oxygen species (ROS) producing activity [8]. The neutrophil shape maintained by HRG should be suitable for the passage of neutrophils through capillary vessels, sustaining rheological stability, and preventing the unnecessary activation of endothelial cells that can lead to vascular injury. Thus, decreased HRG may lead to vascular injury and the production of inflammatory cytokines such asIL-6.

Plasma HRG concentrations in healthy humans are reported to be constant at approx. 1 mM [6]. However, the CSF concentrations of HRG have never been reported. In the present study, we considered the average value of the control group as a standard value, approx. 0.7 μM. The HRG concentrations in the CSF of our aSAH patients were extremely high for the entire study period compared to the standard value. In contrast, the drastic changes observed in the CSF were not seen in the patients' serum. It is likely that we were not able to detect changes in the serum because pathological changes in aSAH occur locally in the subarachnoid space. Thus, for further examinations of the importance of HRG, CSF concentrations are more useful than those in serum.

The relationship between biomarkers and CVS. Understanding the pathophysiology of CVS and early risk assessment are still major challenges in SAH. A CVS occurs due to the continuous contraction of one or several areas of smooth muscle in the intracranial vasculature, or vascular injury, which leads to changes in the luminal morphology and intracranial vascular stenosis or spasm [15]. Various biomarkers (*e.g.*, inflammatory, oxidative stress, and coagulation system markers) have been investigated in SAH, and we examined such biomarkers to judge their usefulness compared with HRG. We selected IL-6 as an inflammatory marker, FpA as a coagulation system marker, and 8OHdG as an oxidative stress marker.

IL-6 is a pleiotropic inflammatory cytokine of low molecular weight [16]. After an aSAH, the CSF concentration of IL-6 peaks at 6-7 days and declines by approx. 10 days [17]. These findings suggested that a severe inflammatory response occurs in the brain. Unfortunately, inflammation in CSF can induce a persistent and severe CVS [18]. Emerging evidence indicates that high concentrations of IL-6 in the plasma or in the CSF are associated with a significant high risk of CVS, DCI, and poor outcome after aSAH [19-24]. It was also indicated that IL-6 not only reflects a current vasospasm but may also be an early biomarker for predicting vasospasm after SAH [25,26]. Our present results support this idea. In our spasm group, the maximum IL-6 concentrations were confirmed at the T3 phase, suggesting persistent inflammation in CVS cases.

Fibrinopeptide A is cleaved from fibrinogen by thrombin during coagulation [27-29]. When an aSAH occurs, the balance between the coagulation and fibrinolytic systems in the CSF is altered. It is thus understandable that various coagulation factors were reported to be more strongly activated within 3 days after an aSAH [30]. Kasuya *et al.* observed extremely high FpA levels in the CSF of patients during the immediate post-aSAH phase compared to healthy humans [31]. Higher concentrations of FpA have been reported in DCI compared to non-DCI patients [32-34]. These changes occurred within 48 h of aSAH onset. Subsequently, the FpA concentration rapidly decreased in both groups [34]. In the present study, the FpA concentrations during the T1 period were higher in the spasm group, indicating that the coagulopathy in the CSF was more marked in this group. As in previous reports, the FpA concentration decreased rapidly. Thus, the changes in coagulopathy in the CSF during the immediate phase may be related to CVS indirectly or directly.

8-Hydroxy-2'-deoxyguanosine, derived from a hydroxyl radical attack of deoxyguanosine residues, is the most representative product of DNA oxidative modification [35,36]. Oxidative stress plays a significant role in the processes of acute brain damage after SAH as well as cerebral vasospasm [37]. It was shown that 8OHdG peaks at day 2 after SAH and then gradually decreases and correlates to CVS and following DCI [38,39]. We observed no clear difference in the 8OHdG concentration between our non-spasm and spasm groups. However, the finding that the peak CSF 8OHdG concentration occurred during the immediate (T1) period suggests that oxidative stress occurs in the immediate phase after an aSAH.

The relationship between HRG and CVS. This is, to the best of our knowledge, the first publication studying HRG concentrations in the CSF during the acute phase of aSAH. Our extensive literature search revealed only one article reporting an influence of HRG

on cerebral arteries; those researchers reported that HRG causes a dose-dependent dilation of porcine cerebral arterioles [40]. A decrease in HRG may thus result in cerebral vessel contraction. We observed that the CSF HRG concentration in the T1 period correlated with the hematoma volume. Considering that the HRG concentrations in T1 were higher in the spasm group, we speculate that a hematoma in the subarachnoid space affects the development of a CVS, as other researchers have suggested. There were also more remarkable differences among the groups during the T2 phase; the HRG concentration was decreased in the spasm group whereas it increased in the non-spasm group. During T3, a similar tendency was observed.

In the blood/CSF rat model, the HRG concentration followed a time course that was similar to that of the CSF HRG concentration in the human non-spasm group. The increased HRG concentration may be due to the release of free HRG found in plasma as well as the release of HRG stored in the α-granules of platelets [41, 42]. We thus suggest that the consecutive release of HRG from an aging clot caused the local increases in HRG in the human non-spasm group and in the blood/CSF model. Samantha *et al.* measured the time course of the HRG release from aging clots [40], and they found that the HRG concentration was significantly elevated during 24-72 h of culture, which is in agreement with our results. The decrease in CSF HRG with time in our group of spasm patients may be related to pathological changes triggered in that group.

Our evaluation of biomarkers in CSF related to CVS in aSAH patients demonstrated that increased FpA at T1, decreasing levels of HRG at T2, and increased IL-6 levels at T3 occurred in the spasm group. In addition, although no clear differences between the spasm and non-spasm groups were observed, increased 8OHdG in the immediate phase was confirmed in the spasm group.

A proposed pathological mechanism underlying CVS based on these results is as follows. In the CSF of aSAH patients who develop a CVS, first, a strong coagulation disorder occurs, and then HRG decreases due to metabolism/clearance and induces inflammatory cytokines. These cytokines cause vascular endothelial injury, resulting in a CVS. This proposed pathway suggests that HRG may form a part of a pathologic pathway in CVS. To test these hypotheses, additional experiments are necessary.

The usefulness of HRG as an early predictor of spasm. We examined the usefulness of the CSF level of HRG for predicting a spasm, in a comparison with three other biomarkers. Our results suggested but did not prove a relationship between the FpA and IL-6 concentrations with CVS. On the other hand, we did not detect a difference in the CSF 8OHdG levels between the spasm and non-spasm groups. Various biomarkers — including those in the present study — have been examined, but no useful early predictor for CVS has been established. In our study, the CSF's level of HRG decreased at the T2 phase in the spasm group, with a significant difference between the spasm and non-spasm aSAH groups. We examined whether HRG might become an early predictor of CVS. The usefulness of HRG during the T2 period was statistically excellent. The T2 period is before DSA or MRA is performed to check for a spasm in angiographic spasm patients, and before suffering from neurological deficits in the symptomatic spasm group. HRG may thus become a biomarker that can detect changes in the early or preliminary phase of a CVS, and its diagnostic usefulness may be extremely high.

Regarding possible limitations of the present study, the number of samples and the collection days were not uniform, as the CSF samples of aSAH patients were examined retrospectively. The pathophysiology of aSAH can vary by individual, and the onset day of a neurological deficit with a symptomatic spasm can also differ. Therefore, we do not know whether we can allocate such diversity into three time periods uniformly. In addition, in the patients with a mild SAH, the hematoma was washed out around onset day 10 and their HRG markedly decreased, causing unevenness in the data. A prospective study is needed to resolve these issues. In such a study, we will increase the number of samples per patient and sample at fixed intervals.

In conclusion, we investigated the time course of HRG in the cerebrospinal fluid after aSAH patients for the detection of a cerebral vasospasm. The results suggest that the CSF concentration of HRG has the possibility to become an early predictor of CVS. In addition, the interactions of HRG with IL-6, FpA and 8OHdG may form part of the pathology of CVS. A future prospective study will investigate the relationship between the pathology of CVS and HRG.

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References

1. Sabri M, Ai J, Lakovic K and Macdonald RL: Mechanisms of microthrombosis and microcirculatory constriction after experimental subarachnoid hemorrhage. Acta Neurochir Suppl (2013) 115: 185–192.
2. Loch MR: Management of cerebral vasospasm. Neurosurg Rev (2006) 29: 179–193.
3. Vestergaard AB, Andersen HF, Magnusson S and Halkier T: Histidine-rich glycoprotein inhibits contact activation of blood coagulation. Thromb Res (1990) 60: 385–396.
4. MacQuarrie JL, Stafford AR, Yau JW, Leslie BA, Vu TT, Fredenburgh JC and Weitz JI: Histidine-rich glycoprotein binds factor XIIa with high affinity and inhibits contact initiated coagulation. Blood (2011) 117: 4134–4141.
5. Tsuchida-Straeten N, Ensslen S, Schafer C, Woltje M, Denecke B, Monser M, Gräber S, Wakabayashi S, Koide T and Jahnen-Dechent W: Enhanced blood coagulation and fibrinolysis in mice lacking histidine-rich glycoprotein (HRG). J Thromb Haemost (2005) 3: 865–872.
6. Poon IK, Hulett MD and Parish CR: Histidine-rich glycoprotein is a novel plasma pattern recognition molecule that recruits IgG to facilitate necrotic cell clearance via FcgammaRI on phagocytes. Blood (2010) 115: 2473–2482.
7. Doñate F, Juarez JC, Guan X, Shipulina NV, Plunkett ML, Tel-Tsur Z, Shaw DE, Morgan WT and Mazar AP: Peptides derived fromthe histidine-proline domain of the histidine-proline-rich glycoprotein bind to tropomyosin and have antiangiogenic and antitumor activities. Cancer Res (2004) 64: 5812–5817.
8. Wake H, Mori S, Liu K, Takahashi HK and Nishibori M: Histidine-rich glycoprotein inhibited high mobility group box 1 in complex with heparin-induced angiogenesis inmatrigel plug assay. Eur J Pharmacol (2009) 623: 89–95.
9. Dixelius J, Olsson AK, Thulin A, Lee C, Johansson I and Claesson-Welsh L: Minimal active domain and mechanism of action of the angiogenesis inhibitor histidine-rich glycoprotein. Cancer Res (2006) 66: 2089–2097.
10. Lee C, Dixelius J, Thulin A, Kawamura H, Claesson-Welsh L and Olsson AK: Signal transduction in endothelial cells by the angiogenesis inhibitor histidine-rich glycoprotein targets focal adhesions. Exp Cell Res (2006) 312: 2547–2556.
11. Shannon O, Rydengård V, Schmidtchen A, Mörgelin M, Alm P, Sørensen OE and Björck L: Histidine-rich glycoprotein promotes bacterial entrapment in clots and decreases mortality in a mouse model of sepsis. Blood (2010) 116: 2365–2372.
12. Wake H, Mori S, Liu K, Morioka Y, Teshigawara K, Sakaguchi M, Kuroda K, Gao Y, Takahashi H, Ohtsuka A, Yoshino T, Morimatsu H and Nishibori M: Histidine-Rich Glycoprotein Prevents Septic Lethality through Regulation of Immunothrombosis and Inflammation. EBioMedicine (2016) 9: 180–194.
13. Hijdra A, Brouwers PJ, Vermeulen M and van Gijn J: Grading the amount of blood on computed tomograms after subarachnoid hemorrhage. Stroke (1990) 21: 1156–1161.
14. Koide T, Foster D, Yoshitake S and Davie EW: Amino acid sequence of human histidine-rich glycoprotein derived from the nucleotide sequence of its cDNA. Biochemistry (1986) 25: 2220–2225.
15. Anderson GB, Ashforth R, Steinke DE and Findlay JM: CT angiography for the detection of cerebral vasospasm in patients with acute subarachnoid hemorrhage. Am J Neuroradiol (2000) 21: 1011–1015.
16. Brown JM, Grosso MA and Harken AH: Cytokines, sepsis and the surgeon. Surg Gynecol Obstet (1989) 169: 568–575.
17. Tiit M, Birger A, Annika L and Hans VH: Increased interleukin-6 levels in cerebrospinal fluid following subarachnoid hemorrhage. J Neurosurg (1993) 78: 562–567.
18. Chaichana KL, Pradilla G, Huang J and Tamargo RJ: Role of inflammation (leukocyte-endothelial cell interactions) in vasospasm after subarachnoid hemorrhage. World Neurosurg (2010) 73: 22–41.
19. Tang QF, Lu SQ, Zhao YM and Qian JX: The changes of von willebrand factor/a disintegrin-like and metalloprotease with thrombospondin type I repeats-13 balance in aneurysmal subarachnoid hemorrhage. Int J Clin Exp Med (2015) 8: 1342–1348.
20. Kao HW, Lee KW, Kuo CL, Huang CS, Tseng WM, Liu CS and Lin CP: Interleukin-6 as a Prognostic Biomarker in Ruptured Intracranial Aneurysms. PLoS ONE (2015) 10: e0132115.
21. Fassbender K, Hodapp B, Rossol S, Bertsch T, Schmeck J, Schutt S, Fritzinger M, Horn P, Vajkoczy P, Kreisel S, Brunner J, Schmiedek P and Hennerici M: Inflammatory cytokines in subarachnoid haemorrhage: association with abnormal blood flow velocities in basal cerebral arteries. J Neurol Neurosurg Psychiatry (2001) 70: 534–537.
22. Niwa A, Osuka K, Nakura T, Matsuo N, Watabe T and Takayasu M: Interleukin-6, MCP-1, IP-10, and MIG are sequentially expressed in cerebrospinal fluid after subarachnoid hemorrhage. J Neuroinflammation (2016) 13: 217.
23. Osuka K, Watanabe Y, Yamauchi K, Nakazawa A, Usuda N, Tokuda M and Yoshida J: Activation of the JAK-STAT signaling pathway in the rat basilar artery after subarachnoid hemorrhage. Brain Res (2006) 1072: 1–7.
24. Nakahara T, Tsuruta R, Kaneko T, Yamashita S, Fujita M, Kasaoka S, Hashiguchi T, Suzuki M, Maruyama I and Maekawa T: High-mobility group box 1 protein in CSF of patients with subarachnoid hemorrhage. Neurocrit Care (2009) 11: 362–368.
25. Ni W, Gu YX, Song DL, Leng B, Li PL and Mao Y: The relationship between IL-6 in CSF and occurrence of vasospasm after subarachnoid hemorrhage. Acta Neurochir Suppl (2011) 110: 203–208.
26. Osuka K, Suzuki Y, Tanazawa T, Hattori K, Yamamoto N, Takayasu M, Shibuya M and Yoshida J: Interleukin-6 and development of vasospasm after subarachnoid haemorrhage. Acta Neurochir (Wien) (1998) 140: 943–951.
27. Blomback B: Fibrinogen and fibrin-proteins with complex roles in hemostasis and thrombosis. Thromb Res (1996) 83: 1–75.
28. Weisel JW: Fibrin assembly. Lateral aggregation and the role of the 2 pairs of fibrinopeptides. Biophys J (1986) 50: 1079–1093.
29. Weisel JW, Veklich Y and Gorkun O: The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. J Mol Biol (1993) 232: 285–297.
30. Yong JI, Meng QH and Wang ZG: Changes in the coagulation and fibrinolytic system of patients with subarachnoid hemorrhage. Neurol Med Chir (Tokyo) (2014) 54: 457–464.
31. Kasuya H: Complement components in cerebrospinal fluid and vasospasm after subarachnoid hemorrhage. Journal Tokyo Woman's Medical University (1990) 60: 63–68 (in Japanese).
32. Kasuya H, Shimizu T, Okada T, Takahashi K, Summerville T and Kitamura K: Activation of the coagulation system in the subarachnoid space after subarachnoid hemorrhage: Serial measurement of fibrinopeptide A and bradykinin of cerebrospinal fluid and plasma in patients with subarachnoid hemorrhage. Acta Neurochir (1988)

91: 120–125.
33. Blat Y and Seiffert D: A renaissance for the contact system in blood coagulation? Thromb Haemost (2008) 99: 457–460.
34. Colman RW: Are hemostasis and thrombosis two sides of the same coin? J Exp Med (2006) 203: 493–495.
35. Kasai H: Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. Mutat Res (1997) 387: 147–163.
36. Lunec J, Holloway KA, Cooke MS, Steve F, Helen RG and Mark DE: Urinary 8-oxo2'-deoxyguanosine: redox regulation of DNA repair in vivo? Free Radic Biol Med (2002) 33: 875–885.
37. Ayer RE and Zhang JH: Oxidative stress in subarachnoid haemorrhage: significance in acute brain injury and vasospasm. Acta Neurochir (Suppl) (2008) 104: 33–41.
38. Zhao M, Ikeda Y, Jimbo H, Fukuda A, Sugiyama K, Ishihara K and Matsumoto K: The correlation between DNA damage and cell membrane damage based on the analysis of the new oxidative stress marker in patients with subarachnoid hemorrhage. J Showa Univ Soc (2002) 62: 50–56 (in Japanese).
39. Mori T, Nagata K, Town T, Tan J, Matsui T and Asano T: Intracisternal increase of superoxide anion production in a canine subarachnoid hemorrhage model. Stroke (2001) 32: 636–642.
40. Steelman SM, Hein TW, Gorman A and Bix GJ: Effects of histidine-rich glycoprotein on cerebral blood vessels. J Cereb Blood Flow Metab (2013) 33: 1373–1375.
41. Leung LL, Harpel PC, Nachman RL and Rabellino EM: Histidine-rich glycoprotein is present in human platelets and is released following thrombin stimulation. Blood (1983) 62: 1016–1021.
42. Blank M and Shoenfeld Y: Histidine-rich glycoprotein modulation of immune/autoimmune, vascular, and coagulation systems. Clin Rev Allergy Immunol (2008) 34: 307–312.