Haptoglobin 2-2 Genotype Determines Chronic Vasospasm After Experimental Subarachnoid Hemorrhage

Kaisorn L. Chaichana, BS; Andrew P. Levy, MD, PhD; Rachel Miller-Lotan, PhD; Sophia Shakur, BS; Rafael J. Tamargo, MD, FACS

Background and Purpose—Chronic cerebral arterial vasospasm is the leading cause of morbidity and mortality after aneurysmal subarachnoid hemorrhage (SAH). Not all cases of SAH, however, develop chronic vasospasm. Inflammation, specifically leukocyte–endothelial cell interactions, appears to be critical in vasospasm development. Haptoglobin (Hp) is a serum protein that limits the extent of inflammation after a hemorrhagic event. An individual’s Hp genotype may predict the severity of the inflammatory response during a hemorrhagic event, and consequently modulate the risk for vasospasm.

Methods—Sixty mice (Hp 1-1, n=30; Hp 2-2, n=30) underwent injection of either autologous blood or normal saline solution into the cisterna magna. An additional 30 mice (15 per genotype) served as controls. The extent and manifestations of vasospasm were assessed by measuring lumen patency, quantifying activity levels, and counting the number of vessel-infiltrated macrophages/neutrophils at 24 hours after injection, which corresponds to the time of peak vasospasm in mice.

Results—Genetically modified Hp 2-2 mice with SAH had significantly lower basilar artery lumen patencies (mean±SEM; 52.9±1.9% vs 82.3±1.3%; P<0.01), reduced activity levels (0.8±0.3 vs 2.4±0.2; P<0.01), and increased macrophage/neutrophil counts in the subarachnoid space (31.2±6.3 vs 8.8±1.7, P<0.01) as compared with wild-type Hp 1-1 mice.

Conclusions—These findings suggest that the Hp 2-2 genotype is critical for the development of severe vasospasm, which typically occurs 24 hours after SAH in mice. (Stroke. 2007;38:3266-3271.)

Key Words: Haptoglobin ■ subarachnoid hemorrhage ■ vasospasm

Cerebral arterial vasospasm is the leading cause of morbidity and mortality after aneurysmal subarachnoid hemorrhage (SAH).1 In humans, cerebral vasospasm is a biphasic phenomenon, where acute vasospasm occurs within hours of the hemorrhage and is followed by a delayed, sustained narrowing of the cerebral arteries 4 to 21 days later.2 This delayed narrowing leads to delayed ischemic neurological deficits, which result in permanent deficits and even death in 20% to 40% of patients.3

There is growing evidence that inflammation and, more specifically, leukocyte–endothelial cell interactions are the root cause of vasospasm after SAH.4–13 Clinical evidence supporting the role of inflammation includes increased body temperature,14 elevated white blood cell counts,15 and increased levels of pro-inflammatory adhesion molecules, namely intercellular adhesion molecule-1 (CD54), in patients in whom vasospasm develops.16,17 Furthermore, inhibition of leukocyte–endothelial cell interactions decreases the incidence of vasospasm in experimental models.4–8,13 Despite these findings, the ability to predict which patients will have vasospasm after SAH is limited. Prospective identification of patients at greater risk for vasospasm would allow for selective early administration of potentially harmful therapies. It may also lead to novel therapeutic approaches aimed at reducing the incidence and severity of vasospasm after SAH.

It has been postulated that there is a genetic predisposition for chronic cerebral vasospasm development, because not all patients who have SAH will have vasospasm.1 After SAH, patients are in 1 of 3 categories: (1) ∼30% of patients have angiographic vasospasm and also clinical symptoms of ischemia; (2) 50% of patients have angiographic vasospasm, but no clinical symptoms; and (3) 20% have neither angiographic nor clinical evidence of vasospasm.3 Recent experimental work suggests that the haptoglobin (Hp) genotype may determine the severity of the inflammatory response to blood products released during a hemorrhagic event.18,19 After a hemorrhage, extracorpuscular hemoglobin (Hgb) is released from red blood cells and acts as a potent inflammatory stimulus.20 This inflammatory response is typically atten-
ated by Hp, which is an abundant serum protein that binds to and clears extracellular Hgb.21 In humans, unlike other mammals, there are 2 alleles for the Hp gene (Hp 1 and Hp 2), which means that an individual can be only 1 of 3 genotypes (Hp 1-1, Hp 2-1, or Hp 2-2).21 Interestingly, recent evidence shows that the Hp 1-1 protein dimer is far superior to the Hp 2-2 cyclical polymer in its immunomodulatory and antioxidative effects.22-25

We hypothesized that the Hp 2-2 genotype predisposes an individual to chronic cerebral vasospasm after SAH. We assessed the role of the Hp 2-2 genotype by comparing the extent of vasospasm, activity level, and microscopic leukocyte counts between wild-type Hp 1-1 mice and genetically modified Hp 2-2 mice, 24 hours after experimentally induced SAH, corresponding to the time of peak vasospasm in mice.26

**Materials and Methods**

**Experimental Design**

The purpose of this study was to assess the role of the Hp genotype in the development of vasospasm after SAH in mice. This was accomplished by: (1) inducing SAH; (2) determining the extent and manifestations of vasospasm; and (3) comparing the extent of vasospasm in Hp 1-1 mice to that of Hp 2-2 mice. The extent and manifestations of vasospasm were assessed by measuring the circumference of the basilar artery to determine lumen patency, quantifying the activity level, and counting the number of vessel-infiltrated macrophages/neutrophils.

Hp 1-1 and Hp 2-2 mice were separately randomized to three experimental groups each to assess the severity of their vasospasm after SAH. For the Hp 1-1 mice, one group underwent injection of autologous blood into the cisterna magna (n = 15), a second group underwent injection of normal saline solution into the cisterna magna (n = 15), and a third group underwent no procedures (n = 15). The setup was the same for the Hp 2-2 mice, in which one group underwent injection of autologous blood into the cisterna magna (n = 15), a second group underwent injection of normal saline solution into the cisterna magna (n = 15), and a third group underwent no procedures (n = 15). In each group, 5 mice were used exclusively for macrophage/neutrophil immunohistochemical studies. The remaining 10 animals underwent activity testing and were subsequently prepared for lumen patency analysis.

**Animals**

C57Bl/6J Hp 1-1 mice (Jackson Laboratories; Bar Harbor, Maine) and C57Bl/6J Hp 2-2 mice (Technion Institute; Haifa, Israel) weighing between 22-27 grams were used. The mice were housed in standard animal facilities with free access to Baltimore, Maryland, water and rodent chow. The Johns Hopkins Animal Care and Use Committee approved all experimental protocols.

**Construction of the Murine Hp 2 Allele**

Wild-type C57Bl/6 mice, along with other nonhuman mammals, are universally Hp 1-1 because the Hp 2 allele is present only in humans.21 We have previously reported the construction of the Hp 2 allele in its normal genomic location. The murine Hp 2 allele was created by producing an intragenic duplication of exons 3 and 4 of the murine Hp 1 allele, analogous to the genetic event that created the Hp 2 allele in humans.21 The Hp serum concentration from Hp 2-2 and Hp 1-1 mice was similar, and the concentration of these proteins was similar to that in humans.18,21 Furthermore, the shape, size, and amino acid homology of the murine Hp 1 and Hp 2 proteins were similar to the human Hp 1 and Hp 2 proteins, respectively.18

**Table. Activity Level**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>0</th>
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<tbody>
<tr>
<td><strong>Posture</strong></td>
<td>Mouse is primarily in nonhuddled position</td>
<td>Mouse is primarily in arched back or huddled position</td>
</tr>
<tr>
<td><strong>Grooming</strong></td>
<td>Fur appears shiny and smooth</td>
<td>Fur appears oily and ruffled</td>
</tr>
<tr>
<td><strong>Ambulation</strong></td>
<td>Mouse is alert, readily ambulates around the cage, and is able to stand on its hind legs</td>
<td>Mouse is nonalert and nonambulatory</td>
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Three-point scale used to assess the mouse’s activity level at 24 hours after induction of SAH, which corresponds to the time of peak vasospasm in mice.

**Anesthesia**

Mice were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg [100 mg/mL; Phoenix Pharmaceutical]) and ketamine (50 mg/kg [100 mg/mL; Phoenix Pharmaceuticals]).

**Surgical Technique**

The surgical protocol was modified from the procedure described by Lin et al.26 Briefly, the animals were placed prone and the atlanto-occipital membrane was exposed with the aid of the operating microscope (Zeiss Co). The animals were then placed supine and 60 µL of autologous blood was withdrawn from the right femoral artery. An equivalent volume of normal saline solution (60 µL) was replaced intraperitoneally after blood removal. The atlanto-occipital membrane was subsequently re-exposed, punctured, and, depending on the experimental group, either 60 µL of autologous blood or 60 µL of normal saline solution was slowly injected into the cisterna magna. The animals were then positioned head-down to confine the blood to the intracranial cisterns. The neck and leg tissues were re-approximated, and the incisions closed with staples.

**Activity Level Assessment**

The mice were evaluated postoperatively for changes in neurological status. Buprenorphine (0.05 mg/kg [0.3 mg/mL, Abbott Laboratories]) was administered prophylactically every 12 hours for analgesia. Because peak vasospasm in mice occurs 24 hours after injection of blood into the cisterna magna,26 the activity level was recorded at 24 hours after surgical procedure. The activity level was assessed using a three point scale that evaluates their posture, grooming, and ambulation (Table). The animals were assessed for 5 minutes by 2 observers who were blinded to their genotype and procedure. The average of the 2 activity level scores was recorded.

**Histology Preparation for Lumen Patency and Macrophage/Neutrophil Infiltration Analysis**

Mice were anesthetized 24 hours after SAH, and intracardiac perfusion-fixation with normal saline solution followed by 4% paraformaldehyde in phosphate-buffered saline was performed. The brain was harvested, and the brain stem with the basilar artery was removed. The section was then fixed, cryoprotected, and frozen. Transverse sections (20 µm) were obtained with a microtome cryostat (Zeiss Co) at 60-µm intervals, from the origin of the basilar artery to its termination. Tissue slices were mounted on glass slides (Fisher Scientific) for either hematoxylin-eosin staining for lumen measurements or immunohistochemical staining for macrophage/neutrophil infiltration analysis.

**Lumen Patency Analysis**

Each animal’s entire basilar artery (comprising ~10 sections) was used for analysis. Hematoxylin-eosin–stained histological sections of the basilar artery were digitized, the lumina were outlined at the transition zone between the internal elastic lamina and the tunica media, and the circumferences were measured using computerized analysis (MCID; Imaging Research, Inc). The circumferences were
measured, instead of the area, to correct for vessel deformation and off-transverse sectioning.

Macrophage/Neutrophil Infiltration Analysis
Each animal’s entire basilar artery (comprising ~10 sections) was used for analysis. Mounted sections were incubated in 3% H₂O₂ blocked in 3% normal goat serum (Vector Laboratories), and incubated with rat anti-mouse Ly-6G/6C primary antibody (1:25; BD Biosciences) to identify macrophages and neutrophils, but not erythrocyte cells or cells of nonhematopoietic origin. The sections were then washed, incubated with biotin-conjugated anti-rat IgG2b secondary antibody (1:100; BD Biosciences), followed by streptavidin-HRP (BD Biosciences). The sections were stained with 3,3′-diaminobenzidine (DAB; BD Biosciences) and counterstained with cresyl violet. Negative controls were prepared by omitting the primary antibody. Each section was evaluated for the number of macrophages/neutrophils per high-powered field.

Statistical Analysis
For lumen patency analysis, the average artery circumference of all obtained sections was determined for each animal. These values were then converted into average area values. Mean cross-sectional areas were then expressed as percentage of lumen patency (% lumen patency) by dividing the mean vessel area of each animal by the mean area of the control group that did not undergo cisterna magna injection. The activity level was assessed using the three point scale detailed in the Table. For macrophage/neutrophil infiltration analysis, all positive staining cells per representative high-powered field in a region adjacent to the adventitia of the basilar artery were counted. For each animal, 3 high-powered fields were counted. The average macrophage/neutrophil count of all obtained sections from each animal was determined.

To determine whether there was a significant difference among the groups, mean vessel areas, activity level, and macrophage/neutrophil infiltration counts were compared using a 1-way, nonparametric ANOVA (Kruskal-Wallis test), with the difference between each group determined by Student-Newman-Keuls multiple comparison test among the Table. For macrophage/neutrophil infiltration analysis, all positive staining cells per representative high-powered field in a region adjacent to the adventitia of the basilar artery were counted. For each animal, 3 high-powered fields were counted. The average macrophage/neutrophil count of all obtained sections from each animal was determined.

For both genotypes, the activity level of the saline-injected group was not significantly different than the controls.

Macrophage/Neutrophil Infiltration After SAH in Hp 1-1 and Hp 2-2 Mice
After SAH, the number of macrophages/neutrophils (mean±SEM) in the subarachnoid space per high-powered field was significantly higher in Hp 2-2 mice as compared with Hp 1-1 mice (31.2±6.3 vs 8.8±1.7; P<0.01; Figures 3 and 4). Whereas the number of macrophages/neutrophils in the subarachnoid space for Hp 1-1 control, saline, and blood-injected groups were 0.2±0.2, 1.2±0.6, and 8.8±1.7, respectively, those of Hp 2-2 control, saline, and blood-injected groups were 0.2±0.3, 2.2±0.7, and 31.2±6.3, respectively (Figure 4). For both genotypes, the macrophage/neutrophil infiltration counts of the saline-injected group were not significantly different than the controls.

Discussion
In this study we assess the role of the Hp genotype in the development of vasospasm after experimentally-induced SAH in mice. We found that after SAH, Hp 2-2 mice had statistically significant lower arterial lumen patencies, reduced activity levels, and increased macrophage/neutrophil counts as compared with Hp 1-1 mice. These findings support the hypothesis that the Hp 2-2 genotype predisposes an individual to severe symptomatic chronic vasospasm after SAH.

SAH after aneurysmal rupture occurs in ~30,000 cases annually in the United States. The most serious complication after aneurysmal SAH is chronic cerebral vasospasm. The pathophysiology of clinical vasospasm has yet to be fully elucidated, and the reason for its development in some patients but not others remains poorly understood. This has
made it difficult to identify susceptible individuals and develop rational preventive therapies.

Increasing evidence shows that inflammation and, more specifically, leukocyte–endothelial cell interactions are the root cause of vasospasm.\textsuperscript{4–13} Cell adhesion molecules necessary for leukocyte–endothelial cell binding, including intercellular adhesion molecule-1, are upregulated after SAH.\textsuperscript{16,17} This upregulation is associated with the extravasation of macrophages/neutrophils into the adventitia of blood-exposed vessels.\textsuperscript{17} Furthermore, the use of monoclonal antibodies against these cell adhesion molecules decreases macrophage/neutrophil infiltration and prevents vasospasm in experimental models.\textsuperscript{5,7,8} In addition, the administration of ibuprofen, which decreases intercellular adhesion molecule-1 expression, results in decreased macrophage/neutrophil transendothelial migration and subsequently less vasospasm.\textsuperscript{4,6}

Hp is a serum protein that limits the extent of inflammation following a hemorrhagic event.\textsuperscript{18,19} After a hemorrhage, blood is extravasated into the interstitial space, leading to the breakdown of erythrocytes and subsequent release of Hgb. Hp binds to free, extracorpuscular Hgb and promotes its clearance via the CD163 scavenger receptor present on macrophages.\textsuperscript{21} The binding and clearance of Hgb neutralizes its oxidative and inflammatory potential.\textsuperscript{20} Extracorpuscular Hgb is a pro-inflammatory stimulus that upregulates the expression of endothelial and leukocyte adhesion molecules, thereby recruiting macrophages and neutrophils to the site of hemorrhage.\textsuperscript{20} Free Hgb also contributes indirectly to inflam-

Figure 2. The activity level was assessed 24 hours after SAH. The activity level was not significantly decreased in Hp 1-1 blood-injected mice as compared with Hp 1-1 saline-injected mice, \( P=0.08 \) (Student-Newman-Keuls). The activity level, however, was significantly decreased in Hp 2-2 blood-injected mice as compared with Hp 2-2 saline-injected mice, **\( P<0.01 \) (Student-Newman-Keuls). More importantly, the activity level was significantly decreased in Hp 2-2 blood-injected mice as compared with Hp 1-1 blood-injected mice, \( P<0.01 \) (Student-Newman-Keuls). Values are the mean±SEM of 10 mice per group.

Figure 3. Immunohistochemical analysis of mouse basilar artery sections demonstrate that (d) blood-injected Hp 2-2 mice have more extensive macrophage/neutrophil infiltration into the subarachnoid space than (b) blood-injected Hp 1-1 mouse. (a) Saline-injected Hp 1-1 mouse. (b) Blood-injected Hp 1-1 mouse. (c) Saline-injected Hp 2-2 mouse. (d) Blood-injected Hp 2-2 mouse. Scale bar, 50 \( \mu \)m.
mation by catalyzing the oxidation of arachidonic acid and promoting prostaglandin synthesis. Free Hgb also binds to nitric oxide and prevents nitric oxide-induced vasodilation. In addition, the heme iron component of Hgb promotes the accumulation of cell-damaging oxygen radicals and lipid peroxides by means of the Fenton reaction. These findings suggest that Hgb plays a pivotal role in neutralizing Hgb-induced inflammation and subsequent vasospasm associated with SAH.

Humans are the only mammalian species that possess 2 alleles for the Hgb gene, which have been designated Hp 1 and Hp 2. The genotype of an individual may therefore be Hp 1-1, Hp 1-2, or Hp 2-2. The Hp 2 allele appears to have been created by an intragenic duplication of exons 3 and 4 of the Hp 1 allele 100,000 years ago. This duplication causes the Hp 2 protein product to be bivalent, unlike the Hp 1 protein, which is univalent. As a result of these differences, the Hp 2 protein is a dimer in Hp 1-1 individuals, a linear polymer in Hp 2-1 individuals, and a cyclical polymer in Hp 2-2 individuals. These structural distinctions confer functional differences as well. The Hp 1-1 protein product, primarily because of its smaller size, can bind to and subsequently clear more Hgb molecules than the cyclical, larger Hp 2-2 protein product. This contributes to Hp 1-1 dimeric protein’s superior anti-inflammatory, immunomodulatory, antioxidant, and vasodilatory effects as compared with the Hp 2-2 cyclical protein.

Figure 4. The number of macrophages/neutrophils per basilar artery section was determined 24 hours after SAH. The number of macrophages/neutrophils was significantly increased in Hp 1-1 blood-injected mice as compared with Hp 1-1 saline-injected mice, **P<0.01 (Student-Newman-Keuls). Likewise, the number of macrophages/neutrophils was significantly increased in Hp 2-2 blood-injected mice as compared with Hp 2-2 saline-injected mice, **P<0.01 (Student-Newman-Keuls). More importantly, the number of macrophages/neutrophils was significantly increased in Hp 2-2 blood-injected mice as compared with Hp 1-1 blood-injected mice, P<0.01 (Student-Newman-Keuls). Values are the mean±SEM of 5 mice per group.

Because only a minority of SAH patients have severe vasospasm, it has been postulated that there is a genetic predisposition to this condition. The Hp genotype may determine the susceptibility to vasospasm, because the Hp 2-2 protein is inferior to Hp 1-1 in its Hgb-clearing capacity, as well as its anti-inflammatory, immunomodulatory, and vasodilatory effects. These differences suggest that after a hemorrhagic event, the inflammation induced by extracorporeal Hgb is more intense in Hp 2-2 individuals. This increased inflammation most likely results in more severe vasospasm and ischemia, causing clinically relevant constitutional and neurologic symptoms.

Based on this premise, we hypothesized that Hp 2-2 individuals may have severe angiographic and clinical vasospasm, Hp 2-1 may develop angiographic vasospasm without symptoms, and Hp 1-1 may have no angiographic or clinical vasospasm after SAH. Interestingly, the distribution of patients who have symptomatic vasospasm (30%), angiographic vasospasm without symptoms (50%), and no angiographic or symptomatic vasospasm (20%) approximates the distribution of these Hp genotypes in the Western world. In Western populations, 36% of individuals are Hp 2-2, 48% are Hp 2-1, and 16% are Hp 1-1. Furthermore, a recent study demonstrated that the presence of the Hp 2 allele is associated with a higher rate of ultrasound-detected vasospasm. The small sample size (n=32) and correspondingly small number of Hp 2-2 individuals (n=11) in this study as well as the use of transcranial Doppler ultrasonography to diagnose vasospasm, however, limited the strength of the conclusions derived from this study.

This Hp 2-2 genotypic association may also explain why animal models of SAH and vasospasm typically show vessel narrowing but often lack related neurological deficits. The Hp 2-2 genotype exists only in humans, yet animals are universally Hp 1-1. The Hp 1-1 protein product suppresses Hgb-induced inflammation more effectively than the Hp 2-2 protein product, thereby reducing the inflammation-associated injury after blood injection. As a result, Hp 1-1 animals have only moderately reduced cerebral artery lumen patencies and usually minimal if any neurological deficits. This absence of symptomatic vasospasm has been observed in several animal models, including monkeys, rabbits, rats, and mice. Another potential limitation is that the time course of vasospasm differs between animals and humans. Vasospasm typically occurs 24 hours after experimentally induced SAH in mice, as compared with 4 to 21 days after aneurysmal SAH in humans. This discrepancy has been attributed in part to the mouse’s ability to clear blood products more rapidly from the subarachnoid space.

Summary

After experimental SAH, genetically modified Hp 2-2 mice have more severe vasospasm than Hp 1-1 wild-type mice at 24 hours, as evidenced by their significantly reduced lumen patencies, decreased activity levels, and increased macrophage/neutrophil counts. These findings suggest that the Hp 2-2 genotype predisposes individuals to development of severe vasospasm after SAH, and that Hp 2-2 may serve as a molecular marker to prospectively identify individuals who are at increased risk. This would allow for the selective early administration of potentially harmful treatments to only those patients with the Hp 2-2 genotype, who would clearly benefit from early aggressive treatment. This finding may also have implications for stroke, in which inflammation is a critical component of pathogenesis, and other conditions associated
with inflammation-induced vasospasm, such as traumatic brain injury, craniotomy for tumors, and meningitis. In time, the use of Hp 2-2 animal models to study vasospasm after SAH may lead to a better understanding of the complex mechanisms underlying this problem, of which vessel narrowing is only one component, and may lead to the development of new therapeutic approaches to reduce the morbidity and mortality associated with this condition.

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**Disclosures**

None.

**References**

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