Stroke

BASIC AND TRANSLATIONAL SCIENCES

Mast Cell Promotes the Development of Intracranial Aneurysm Rupture

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BACKGROUND AND PURPOSE: Inflammation has emerged as a key component of the pathophysiology of intracranial aneurysms. Mast cells have been detected in human intracranial aneurysm tissues, and their presence was associated with intramural microhemorrhage and wall degeneration. We hypothesized that mast cells play a critical role in the development of aneurysmal rupture, and that mast cells can be used as a therapeutic target for the prevention of aneurysm rupture.

METHODS: Intracranial aneurysms were induced in adult mice using a combination of induced systemic hypertension and a single injection of elastase into the cerebrospinal fluid. Aneurysm formation and rupture were assessed over 3 weeks. Roles of mast cells were assessed using a mast cell stabilizer (cromolyn), a mast cell activator (C48/80), and mice that are genetically lacking mature mast cells ($Kit^{W\text{-}sh/W\text{-}sh}$ mice).

RESULTS: Pharmacological stabilization of mast cells with cromolyn markedly decreased the rupture rate of aneurysms (80% versus 19%, n=10 versus n =16) without affecting the aneurysm formation. The activation of mast cells with C48/80 significantly increased the rupture rate of aneurysms (25% versus 100%, n=4 versus n=5) without affecting the overall rate of aneurysm formation. Furthermore, the genetic deficiency of mast cells significantly prevented aneurysm rupture (80% versus 25%, n=10 versus n=8, wild-type versus $Kit^{W\text{-}sh/W\text{-}sh}$ mice).

CONCLUSIONS: These results suggest that mast cells play a key role in promoting aneurysm rupture but not formation. Stabilizers of mast cells may have a potential therapeutic value in preventing intracranial aneurysm rupture in patients.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: intracranial aneurysm ■ mast cells ■ mice ■ subarachnoid hemorrhage ■ tryptase

Rupture of intracranial aneurysms causes aneurysmal subarachnoid hemorrhage. The 30-day mortality rate after aneurysmal subarachnoid hemorrhage can be as high as 45%.[1] Therefore, surgical clipping or endovascular coiling are offered to patients with unruptured aneurysms for the prevention of aneurysmal rupture. Significant technical advancements and refinements have been made in these invasive treatments. However, the adverse outcome rates resulting from the clipping and coiling of unruptured aneurysms are still not negligible.[2] Therefore, pharmacological prevention of aneurysmal rupture may be an attractive alternative approach in patients with unruptured aneurysms.[3]

Inflammation is increasingly recognized as a critical component in the pathophysiology of intracranial aneurysms.[4–9] Observational studies have shown the presence of inflammatory cells and inflammatory markers in human intracranial aneurysm tissues and serum samples.[5,10] Mast cells have been detected in human intracranial aneurysm tissues,[7,11] and the presence of mast cells was associated with intramural microhemorrhage and wall degeneration of human intracranial aneurysms.[11]

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Mast cells, classically known as key regulators of allergic reactions, have emerged as integral players in cardiovascular diseases.[12–17] By releasing cytokines, including tryptase, chymases, cathepsins, and interleukins, mast cells can affect vascular inflammation and remodeling.[12,18–20] Blocking the cytokine release from mast cells reduces the development and progression of atherosclerosis and abdominal aortic aneurysm in animals.[14,16] Although previous studies suggested an association between mast cell activation and pathological remodeling of aneurysm walls,[21,22] the direct link between mast cell activation and the development of aneurysmal rupture has not been established. Therefore, we tested whether mast cells contribute to the development of aneurysmal rupture using the genetic and pharmacological tools in a mouse model of intracranial aneurysm.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Mouse Model of Intracranial Aneurysm

Experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee. We used C57BL/6J and Kit$^{W\text{-}sh/W\text{-}sh}$ transgenic male mice (Jackson Laboratory, Bar Harbor, Maine). Intracranial aneurysms were induced by combining induced systemic hypertension and a single injection of elastase (35.0 milli-units, unless indicated otherwise) into the cerebrospinal fluid at the right basal cistern as we previously described.[3,23]

A flow chart of all mice used in the current study is included in materials in the Data Supplement (Figure I in the Data Supplement).

Evaluation of Aneurysm Formation and Rupture

To detect aneurysmal rupture, 2 blinded observers performed a daily neurological examination as previously described.[3] Aneurysms are defined as a localized outward bulging of the vascular wall (>150% of the control artery).[24] When mice develop neurological symptoms associated with aneurysmal rupture (neurological score: 1–5), we euthanize them immediately (within 4 hours). In both euthanized and dead mice, we inspect the brain samples and verify the presence of aneurysm and hematoma from subarachnoid hemorrhage by examining the Circle of Willis and its major branches under a dissecting microscope (10×).[3] Our study confirmed the specificity and sensitivity of this approach in detecting aneurysmal rupture.[3] All remaining mice were euthanized 4 weeks after aneurysm induction.[3,25,26] Mice were transcardially perfused with PBS, followed by a gelatin-containing blue dye to visualize cerebral arteries.

Dosing of Drugs

The doses of cromolyn (12.5 or 25 mg/kg per day, intraperitoneal) and C48/80 (2 or 4 mg/kg per day, intraperitoneal) were chosen according to previous published studies in mice.[27,28] Cromolyn is a clinical used drug against diseases such as asthma and systemic mastocytosis. The dose used in the current study is below the dose used in humans according to surface area conversion.[29]

Tissue Collection and Immunohistochemistry

Mast cells were stained in representative aneurysms as previously described.[7,16] We collected aneurysms from mice at 7 days after aneurysm induction, a time point before aneurysmal rupture begins to occur.[3] The brain tissues were fixed with 4% paraformaldehyde for 24 hours and then immersed in 15% sucrose for 24 hours and 30% sucrose for another 24 hours. Then, the tissues were embedded in optimal cutting temperature compound (Tissue-Tek) at −80°C. Sections were immunohistochemically stained with monoclonal antibodies to mast cells using antimast cell tryptase clone AA1 (DakoCytomation, Carpentaria, CA). Sections were incubated in primary antibody overnight at 4°C, followed by incubation with corresponding biotinylated secondary antibodies (Vector Laboratories) and with a complex of avidin-biotin-horseradish peroxidase (Vector Laboratories). Immunoreactivity was visualized by incubating the sections with 0.05% 3,3′-diaminobenzidine (Vector Laboratories). Nuclei were visualized by counterstaining with aqueous hematoxylin.

Real-Time Polymerase Chain Reaction Detection of Cytokines and Mast Cell-Originated Enzymes

We collected total RNA samples from cerebral arteries (Circle of Willis, including aneurysms) 14 days after aneurysm induction as previously described.[30,31] We measured mRNA expression levels of inflammation-related cytokines (angiotensin II type I receptor, interleukin-6, matrix metallopeptidase 9, and tumor necrosis factor-α), cathepsin G, chymase, and tryptase. RNA was extracted using the RNeasy Mini Kit (Qiagen, CA) and transcribed to cDNA using the QuantiTect reverse transcription kit (Qiagen). The mRNA expression levels were determined using SYBR Green technology (Applied Biosystems, CA). Quantitative values were obtained from the threshold cycle value, and the data were analyzed by the $2^{-\Delta\Delta}$CT method. Glyceraldehyde-3-phosphate dehydrogenase expression was quantified and used as an internal RNA control.

Statistical Analysis

Fisher exact test was used to analyze the incidences of aneurysm formation and rupture rates. $P<0.05$ were considered statistically significant. Data are expressed as means±SD.

RESULTS

The Presence of Mast Cells in Intracranial Aneurysms in the Mouse Model

To confirm the presence of mast cells in intracranial aneurysms in this model, we stained representative aneurysms for mast cells as previously described.[16] We collected aneurysms from mice at 7 days after aneurysm induction,

a time point before aneurysmal rupture begins to occur.[3] Consistent with clinical studies that used human intracranial aneurysm tissues,[7,11] intracranial aneurysms in the mouse model exhibited infiltration of mast cells into the adventitial layers and the surrounding tissues (Figure 1).

Pharmacological Stabilization of Mast Cells Prevented the Aneurysmal Rupture

As a first step, we tested whether the stabilization of mast cells can prevent aneurysmal rupture. We used cromolyn (disodium cromoglycate, 25 mg/kg per day) as a mast cell stabilizer as previously described.[16,27,28] We initiated a daily treatment with either cromolyn or vehicle 6 days after aneurysm induction for a total treatment course of 3 weeks (Figure 2A). Previous studies by ours and others found that aneurysm formation occurs during the first 6 to 7 days after aneurysm induction in this model and that aneurysmal rupture begins ≈7 days after aneurysm induction.[3,25,32–35] By treating mice with an experimental agent starting from 6 days after aneurysm induction, it can be tested whether the experimental agent prevents aneurysmal rupture after aneurysms are formed, mimicking the clinical setting.[3,25,26]

As shown in Figure 2, although there was no difference in the overall incidence of aneurysm formation (including both ruptured and unruptured aneurysms) between the vehicle and cromolyn group (59% versus 70%; n=17 versus n=23; *P*=0.52, Figure 2B), the stabilization of mast cells by cromolyn after aneurysmal formation significantly reduced the rupture rate (80% versus 19%; n=10 versus n=16; *P*<0.05, Figure 2C). To test the dose-dependency of cromolyn's protective effect against aneurysm rupture, in a separate experiment, we reduced the dose of cromolyn by 50% (12.5 mg/kg per day). As expected, half-dose cromolyn treatment also reduced the rupture rate as compared with the vehicle-treated group, although this did not reach statistical significance (80% versus 50%; n=10 versus n =12; *P*=0.16; Figure II in the Data Supplement).

As a next step, we tested whether mast cell stabilization affects the formation of aneurysms by administering cromolyn, a mast cell stabilizer, during aneurysm formation. Cromolyn treatment was started one day before aneurysm induction and continued for a week (Figure 3A).

As shown in Figure 3, the stabilization of mast cells during aneurysm formation did not affect the formation of aneurysms (63% versus 89%; n=19 versus n=19; *P*=0.13, Figure 3B). In addition, the mast cell stabilization during aneurysm formation stage did not alter the rupture rate (67% versus 65%; n=12 versus n=17; *P*=0.39, Figure 3C), indicating the unique role of mast cells in promoting aneurysmal rupture after aneurysms are formed. Mast cell stabilization during or after aneurysm formation did not significantly affect blood pressure or body weight (data not shown).

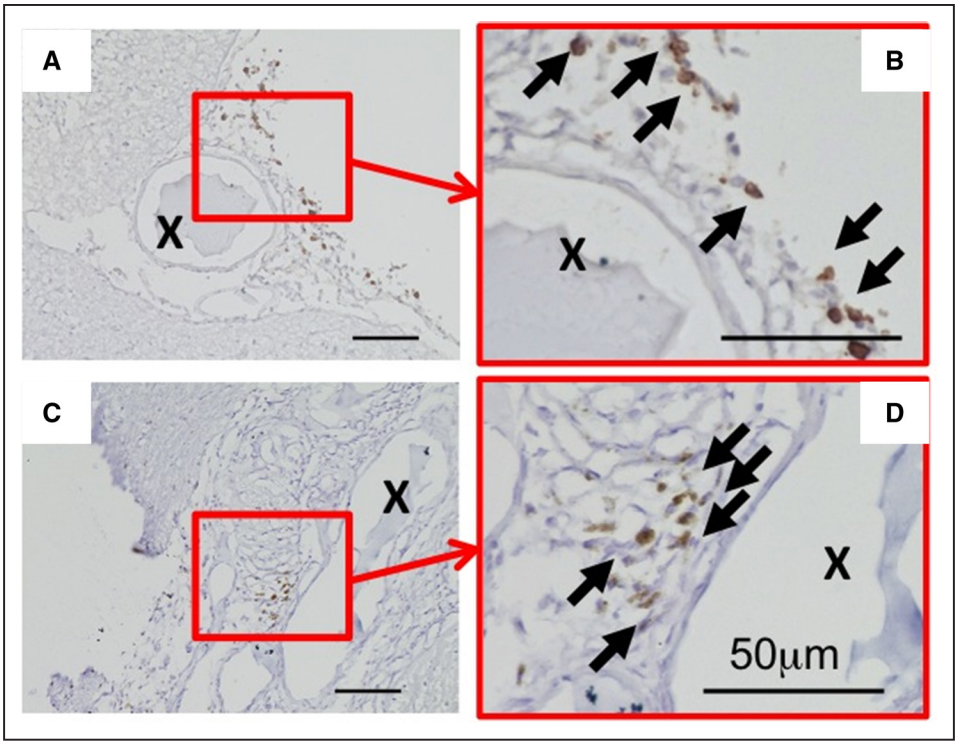

Figure 1. Presence of mast cells in mouse intracranial aneurysms.
Representative images of immunohistochemical staining of mast cells in mouse intracranial aneurysms. Brown color indicates positive staining for mast cells. Arrows indicate mast cells. X indicates the lumen of aneurysms. Scale bar: 50 μm.

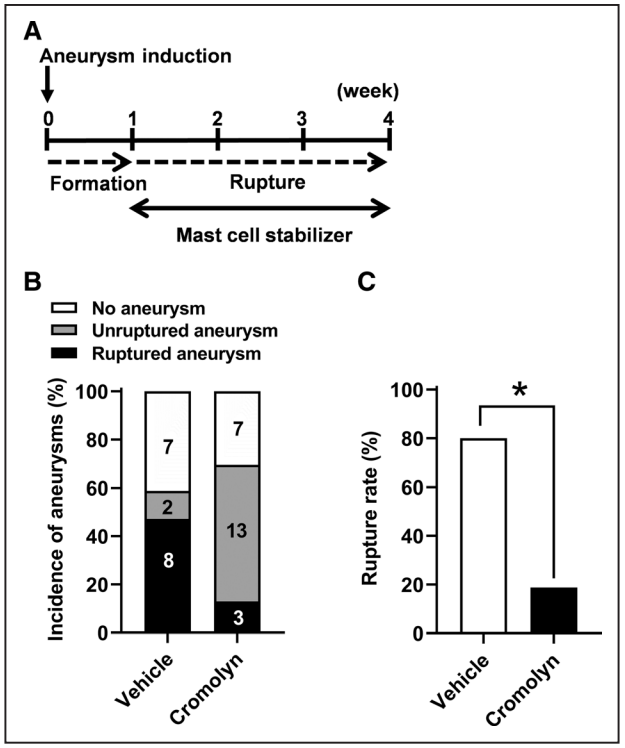

Figure 2. Pharmacological stabilization of mast cells by cromolyn after aneurysm formation prevented aneurysmal rupture.
A, Time window of the mast cell stabilization treatment by cromolyn. **B**, No difference in the incidence of aneurysm between cromolyn-treated and vehicle-treated mice. **C**, Aneurysm rupture rate was significantly decreased in cromolyn-treated as compared with vehicle-treated mice. *P<0.05.

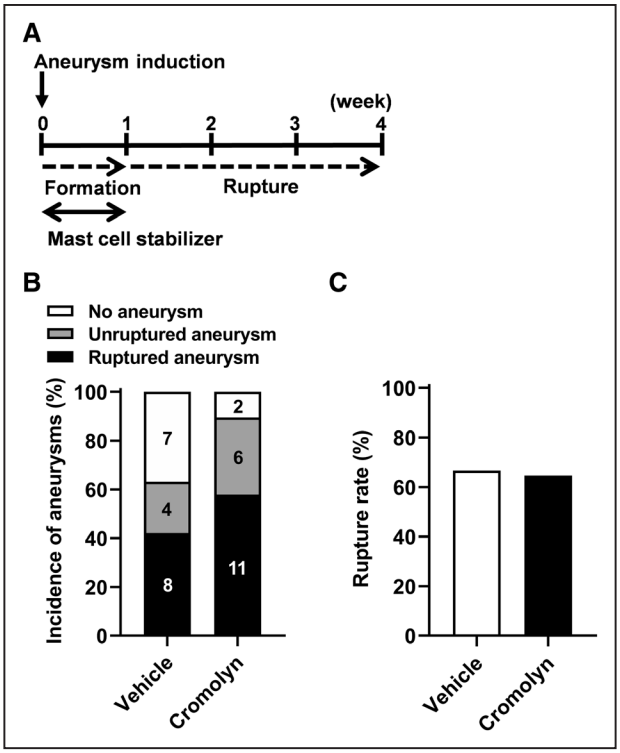

Figure 3. The mast cell stabilization during aneurysmal formation did not affect aneurysm rupture.
A, Time window of the mast cell stabilization treatment by cromolyn. **B** and **C**, No difference in the incidence of aneurysms or rupture rate was found between cromolyn-treated and vehicle-treated mice.

To explore the potential mechanisms underlying the protective effect of cromolyn on aneurysm rupture, we conducted a separate set of experiments to investigate the molecules that may be responsible for the mast cells-mediated effect on aneurysm rupture. We used real-time polymerase chain reaction to quantify the mRNA of inflammatory cytokines, and enzymes with mast cell origin (angiotensin II type I receptor, interleukin-6, matrix metallopeptidase 9, tumor necrosis factor-α, cathepsin G, chymase, and tryptase) in vessels of the Circle of Willis. We found that cromolyn treatment significantly reduced the expression of tryptase as compared with the vehicle-treated group (vehicle versus cromolyn, 1.0±1.09 versus 0.096±0.099, n=4 for both groups, P<0.05; Figure III in the Data Supplement).

Pharmacological Activation of Mast Cells After Aneurysm Formation Promoted Aneurysmal Rupture Without Affecting the Aneurysmal Formation

To further confirm the role of mast cells in the promotion of aneurysmal rupture, we tested whether activation of mast cells promotes aneurysm rupture. Compound 48/80 (C48/80; 4 mg/kg per day, daily intraperitoneal) was used as a mast cell activator as previously described.[28,36] C48/80 promotes mast cell activation and degranulation by activating phospholipase D via heterotrimeric guanosine 5′-triphosphate-binding proteins.[28,37] We used 17.5 milli-units of elastase so that the rupture rate in the vehicle group would be relatively low and that the promotion of rupture by the mast cell activator can be tested.[38]

As shown in Figure 4, while the mast cell activation did not affect the overall incidence of intracranial aneurysms (44% versus 45%; n=9 versus n=11; P=1.00, Figure 4B), it significantly increased the rupture rate (25% versus 100%; n=4 versus n=5; P<0.05, Figure 4C), further confirming the role of mast cells in promoting aneurysmal rupture.

To test the dose-dependency of C48/80's promoting effect on aneurysm rupture, in a separate experiment, we reduced the dose of C48/80 by 50% (2 mg/kg per day). As expected, the mice treated with 2 mg/kg/day C48/80 had a rupture rate that fell between the groups receiving 4 mg/kd/day and vehicle (44% versus 80%; n=9 versus n =15; P=0.07; Figure II in the Data Supplement).

In the real-time polymerase chain reaction quantification of the mRNA of inflammatory cytokines, and enzymes with mast cell origin (angiotensin II type I

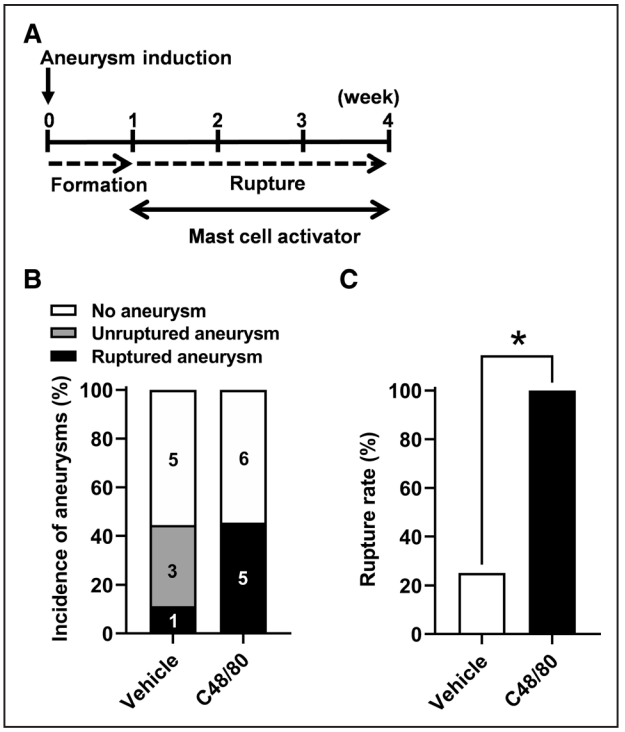

Figure 4. Pharmacological activation of mast cells after aneurysm formation promoted aneurysmal rupture without affecting the aneurysmal formation.

A, Time window of the mast cell activation treatment by C48/80. **B**, No difference in the incidence of aneurysm between C48/80-treated and vehicle-treated mice. **C**, Aneurysm rupture rate was significantly increased in C48/80-treated as compared with vehicle-treated mice. *P<0.05.

receptor, interleukin-6, matrix metallopeptidase 9, tumor necrosis factor-α, cathepsin G, chymase, and tryptase), we did not detect any statistically significant difference between the vehicle-treated and C48/80-treated groups on these molecules (Figure IV in the Data Supplement).

Mast cell activation did not significantly affect blood pressure or body weight (data not shown).

Genetic Deficiency of Mast Cells Reduces Aneurysmal Rupture

To complement the pharmacological experiments, we conducted an experiment using Kit$^{W\text{-}sh/W\text{-}sh}$ mice, mast cell-deficient mice. Kit$^{W\text{-}sh/W\text{-}sh}$ mice genetically lack mature mast cells.[16,39] Kit$^{W\text{-}sh/W\text{-}sh}$ mice in the C57BL/6 background are not anemic or sterile, and they have normal bone marrow and blood neutrophil counts.[19]

Consistent with our findings on pharmacological stabilization of mast cells, mast cell-deficient mice (Kit$^{W\text{-}sh/W\text{-}sh}$ mice) had a significantly lower rupture rate than wild-type littermate mice, indicating that a lack of mast cells prevents aneurysmal rupture (80% for control mice and 25% for Kit$^{W\text{-}sh/W\text{-}sh}$ mice; n=10 versus n=8; P<0.05; Figure 5B). There was no difference in the overall incidence of aneurysms between mast cell-deficient mice and wild-type

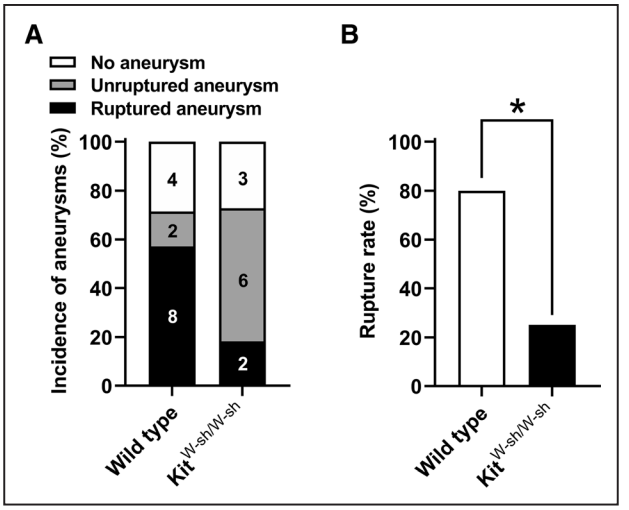

Figure 5. Genetic deficiency of mast cells decreased the aneurysmal rupture rate without affecting the aneurysmal formation.

A, No difference in the incidence of aneurysm between the Kit$^{W\text{-}sh/W\text{-}sh}$ mice and the wild-type mice. **B**, Aneurysm rupture rate was significantly decreased in the Kit$^{W\text{-}sh/W\text{-}sh}$ mice as compared with the wild-type mice. *P<0.05.

mice (71% versus 73%; n=14 versus n=11; P=1.00, Figure 5A). There was no difference in the systolic blood pressure at any time point between the Kit$^{W\text{-}sh/W\text{-}sh}$ mice and the wild-type mice, either (data not shown).

As an additional experiment, we confirmed the specificity of cromolyn's stabilization by treating Kit$^{W\text{-}sh/W\text{-}sh}$ mice with cromolyn. As shown in Figure 6, the treatment with cromolyn did not affect the incidence of aneurysms in Kit$^{W\text{-}sh/W\text{-}sh}$ mice (69% versus 50%; n=16 versus n=8; P=0.41, Figure 6B). However, more importantly, the protective effect of cromolyn against the development of aneurysmal rupture was abolished, as evidenced by the lack of difference in the rupture rate between Kit$^{W\text{-}sh/W\text{-}sh}$ mice treated with the vehicle and Kit$^{W\text{-}sh/W\text{-}sh}$ mice treated with cromolyn (27% versus 25%; n=11 versus n=4; P=0.28, Figure 6C).

DISCUSSION

Mast cells have been detected in human intracranial aneurysm tissues.[7,11,40] Mast cells were more abundant in ruptured aneurysms than in unruptured aneurysms in humans,[7] and the presence of mast cells was associated with the degeneration and microhemorrhage with the aneurysmal walls.[11] However, the causal relationship between mast cell infiltration and development of aneurysmal rupture has not been established.

In this study, using both pharmacological and genetic approaches, we demonstrated that mast cells can promote the development of aneurysmal rupture. For the pharmacological studies, we used both activator and stabilizer of mast cells and showed the link between mast cell activation and the development of aneurysmal

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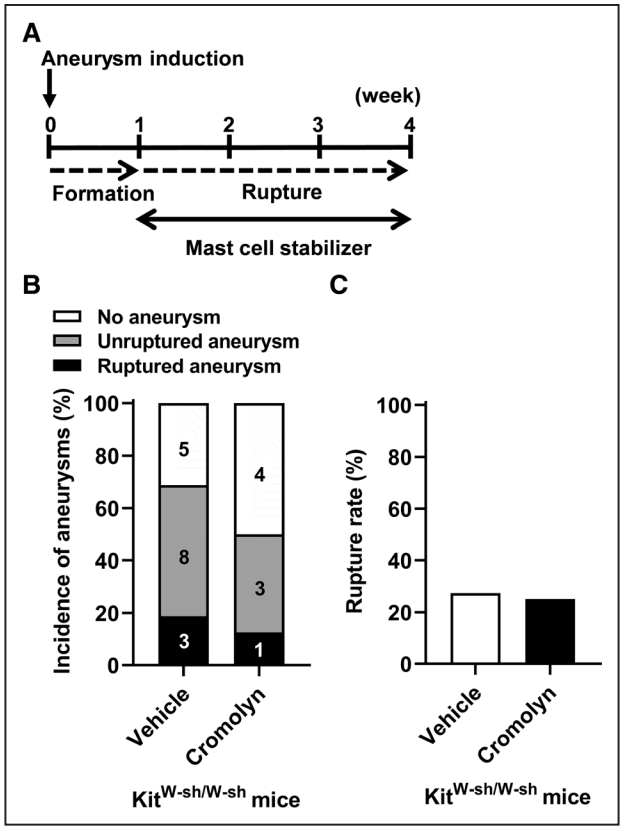

Figure 6. Genetic deficiency of mast cells eliminated cromolyn treatment effects.
A, Time window of the mast cell stabilization treatment by cromolyn in the Kit$^{W\text{-}sh/W\text{-}sh}$ mice. **B** and **C**, No difference in the incidence of aneurysm or rupture rate was found between cromolyn-treated and vehicle-treated groups in the Kit$^{W\text{-}sh/W\text{-}sh}$ mice.

rupture. In addition, our results revealed the unique role of mast cells in the development of aneurysmal rupture. While mast cells promote aneurysmal rupture, they do not seem to play any significant role in the formation of aneurysms. The unique role of mast cells in the development of aneurysmal rupture was confirmed by our experiment that used the mouse that genetically lacks mature mast cells.

Previously, in a rat model of aneurysm initiation that employed the continuous administration of lysyl oxidase inhibitor (β-aminopropyl nitrile), renal hypertension, and unilateral carotid artery ligation, the pharmacological stabilization of mast cells that started the day of aneurysm induction surgery reduced mast cell infiltration and inflammation within the cerebral arteries without affecting the formation of aneurysm.[41] These findings were consistent with our findings that showed no effect of mast cell activation or stabilization on the formation of aneurysms. However, in the study that used the rat model, the potential role of mast cells in the development of aneurysmal rupture could not be assessed.[41] In the mouse model that was used in the current study, spontaneous aneurysmal rupture occurs with a predictable time course, and the aneurysmal rupture can be easily detected by assessing neurological symptoms.[3,23,24,33] This model provided us with a unique opportunity to study the mechanisms of aneurysmal rupture as well as the potential roles of mast cells in the development of aneurysmal rupture. Our findings on the promotion of aneurysmal rupture by mast cells are consistent with our previous studies that showed that mesenchymal stem cells and their microvesicles prevented aneurysmal rupture through the stabilization of mast cells.[21,22] In the current study, we firmly established the link between mast cells and aneurysmal rupture by directly manipulating mast cells using both pharmacological and genetic approaches.

Mast cells can release numerous cytokines and chemokines upon their activation and degranulation. At the same time, a number of cytokines and chemokines can activate mast cells. Many of these cytokines are reported to be involved in the pathophysiology of intracranial aneurysm. For example, tumor necrosis factor-α and hepatocyte growth factor, cytokines that can be released by mast cell, have been shown to play a key role in the promotion of aneurysmal rupture.[42,43] Stromal cell-derived factor-1, a known chemoattractant for mast cells, seem to induce pathological remodeling of aneurysmal walls in animals.[44] Moreover, the conversion of angiotensin I to angiotensin II by mast cell-derived chymases in the aneurysm wall may cause the activation of the vascular renin-angiotensin system that was reported to promote aneurysmal rupture.[25,32] In addition, matrix metalloproteinases can be activated by mast cell-derived chymases and cathepsins.[18]

Tryptase is the major proteinase in mast cells and has been used as a marker for mast cells activation.[45,46] Our data showed that cromolyn treatment reduced the expression of tryptase. This may provide a direct mechanistic explanation for the protective effect of the mast cell stabilizer. It is also likely that concerted effects of multiple cytokines and chemokines mediate mast cell's promotion of aneurysmal rupture. Future studies that use mast cell-specific deletion of cytokines or chemokines may be needed to further elucidate the underlying molecular mechanism. Nevertheless, considering the clinical availability of mast cell stabilizers such as cromolyn and tranilast,[41] our current study may provide the basis for future clinical pilot studies to test mast cell stabilization for the prevention of aneurysmal rupture in humans.

There are other limitations to this study. First, the animal model may not completely replicate biological events that lead to aneurysm formation and growth, as aneurysms were induced, but not spontaneously formed. While many studies indicated the critical roles of vascular inflammation in the pathophysiology of intracranial aneurysms, there may be significant differences in the triggering factors of vascular inflammation between human aneurysms and this model. In addition, the time course of aneurysmal formation and rupture in this model is shorter than that of the human aneurysm. However, the phenotypes of intracranial aneurysms in the model closely mimic that of intracranial

aneurysms in humans.[3,23] More importantly, human intracranial aneurysms and aneurysms in this model share the end phenotypes, aneurysmal rupture and associated neurological symptoms, possibly indicating the common biological processes between human intracranial aneurysms and this mouse model of intracranial aneurysm.[3,25]

Second, we cannot completely exclude potentially confounding effects of unknown development abnormalities of $Kit^{W\text{-}sh/W\text{-}sh}$ mice. We used the $Kit^{W\text{-}sh/W\text{-}sh}$ mice in the C57BL/6 background, because their genetic defects are reported to be limited to mast cells.[19] These mice are not anemic or sterile, and they have normal numbers of bone marrow and blood neutrophils.[19] The $Kit^{W\text{-}sh/W\text{-}sh}$ mice in the C57BL/6 background have been successfully used to study the roles of mast cells in various disease conditions.[14,16,28]

Third, there may be off-target effects of pharmacological agents we used for this study. For example, the mast cell activator, C48/80, may cause mast cell apoptosis.[47] These limitations of using transgenic mice and pharmacological agents underscore the importance of employing both genetic and pharmacological approaches to firmly establish the role of mast cells in the development of aneurysmal rupture.

Lastly, as an initial step to study the contribution of mast cells to aneurysmal rupture, we used only male mice. Sex-dependent differences in the rupture rate of intracranial aneurysms have been observed in both humans and animals.[26,48,49] Further studies will be needed to explore sex-dependent differences in the contribution of mast cells to the development of aneurysmal rupture.

This study showed the role of mast cells in the promotion of the rupture of intracranial aneurysm. Mast cells may serve as a potential therapeutic target for the prevention of intracranial aneurysmal rupture. Further mechanistic and clinical studies are needed to further establish the contribution of mast cells to the development of aneurysmal rupture and subarachnoid hemorrhage.

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Disclosures

None.

Supplemental Materials

Expanded Materials & Methods
Figures I–IV
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