Modified Citrus Pectin Prevents Blood-Brain Barrier Disruption in Mouse Subarachnoid Hemorrhage by Inhibiting Galectin-3

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Background and Purpose—Plasma levels of galectin-3—a matricellular protein—are increased after aneurysmal subarachnoid hemorrhage (SAH), but the functional significance remains undetermined. This study was conducted to evaluate whether modified citrus pectin (MCP; galectin-3 inhibitor) prevents post-SAH early brain injury, focusing on blood-brain barrier disruption.

Methods—C57BL/6 male adult mice (n=251) underwent sham or filament perforation SAH modeling, followed by a random intracerebroventricular injection of vehicle or drug at 30 minutes post-modeling. First, vehicle-treated and 0.8, 4, 16, or 32 µg MCP-treated mice were assessed by neuroscore and brain water content at 24 and 48 hours post-modeling. Second, Evans blue extravasation, Western blotting, coimmunoprecipitation and immunostaining were performed in vehicle-treated or 4 µg MCP-treated mice at 24 hours post-modeling. Third, vehicle or R-galectin-3 (recombinant galectin-3) was administered to SAH mice simultaneously with vehicle or MCP, and neuroscore and Evans blue extravasation were evaluated at 24 hours post-modeling. Fourth, vehicle or R-galectin-3 was administered to MCP-treated SAH mice at 24 hours, and neuroscore and IgG immunostaining were evaluated at 48 hours post-SAH.

Results—Among tested dosages, 4 µg MCP showed the best neuroprotective effects as to preventing neurological impairments and brain edema at 24 to 48 hours post-SAH. Four micrograms MCP attenuated post-SAH blood-brain barrier disruption and galectin-3 upregulation in brain capillary endothelial cells, associated with inactivation of ERK (extracellular signal-related kinase) 1/2, STAT (signal transducer and activator of transcription)-3, and MMP (matrix metalloproteinase)-9, and the consequent preservation of a tight junction protein ZO-1 (zonula occludens-1). Coimmunoprecipitation assay demonstrated physical interactions between galectin-3 and TLR (Toll-like receptor) 4. R-galectin-3 blocked the neuroprotective effects of MCP.

Conclusions—MCP prevents post-SAH blood-brain barrier disruption possibly by inhibiting galectin-3, of which the mechanisms may include binding to TLR4 and activating ERK1/2, STAT-3, and MMP-9. This study suggests galectin-3 to be a novel therapeutic target against post-SAH early brain injury.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2018;49:2743-2751. DOI: 10.1161/STROKEAHA.118.021757.)**

Key Words: blood-brain barrier ■ galectin-3 ■ pectins ■ subarachnoid hemorrhage ■ Toll-like receptor 4

Subarachnoid hemorrhage (SAH) because of aneurysmal rupture remains to be one of the most devastating diseases, despite advances in the diagnosis and treatment.[1] Accumulating evidences suggest that early brain injury (EBI) plays a pivotal role in poor outcomes after SAH.[2] EBI is defined as acute pathophysiological events that occur in brain before onset of cerebral vasospasm within 72 hours of SAH.[3] Although EBI consists of any type of brain insults or pathophysiologies other than iatrogenic brain injuries, the molecular mechanisms are complex and not well understood.[2] Previous studies reported that various substances produced by SAH, such as heme, fibrinogen, intracellular components, and inflammation-related proteins, stimulate cell surface receptors, activate several inflammatory pathways, and cause EBI.[3,4] Our previous studies revealed that MAPK (mitogen-activated protein kinase) pathway is one of the major pathways causing EBI through the cell surface receptors, including TLR (Toll-like receptor) 4.[3,5,6] In addition, matricellular proteins, such as tenascin-C, periostin, and osteopontin, have been implicated in the development of EBI in terms of blood-brain barrier (BBB) disruption and neuronal apoptosis.[5–7]

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Galectin belongs to the β-galactoside–binding lectin superfamily composed of 1 or 2 carbohydrate recognition domains (CRDs) and is classified as a matricellular protein.[8] Galectin-3—the only chimera-type galectin—comprises 1 CRD and N-terminal non-CRD for carbohydrate binding and exists both inside and outside cells.[9] Galectin-3 is reported to act as an endogenous paracrine TLR4 ligand through its CRD.[10] In a clinical setting, higher admission plasma galectin-3 levels were correlated with more severe SAH and worse 6-month outcomes in patients with aneurysmal SAH.[11] Our recent study also showed that higher plasma galectin-3 levels at days 1 to 3 post-SAH were independent predictors of delayed cerebral ischemia without cerebral vasospasm in nonsevere aneurysmal SAH patients.[12] However, no experimental studies have been conducted to reveal the functional significance of galectin-3 in SAH. Modified citrus pectin (MCP) binds to galectin-3 CRDs, blocks its activity, and is known as a galectin-3's inhibitor.[13–15] The aim of this study was thus to evaluate whether MCP prevents post-SAH EBI by inhibiting galectin-3, focusing on BBB disruption, and if so, the possible mechanisms.

Materials and Methods

The authors declare that all supporting data are available within the article and the online-only Data Supplement.

Animals

The Animal Ethics Review Committee of Mie University approved all procedures. The study complies with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines for reporting in vivo experiments (Table I in the online-only Data Supplement). C57BL/6 male adult mice (age, 10–12 weeks; weight, 25–30 g; SLC, Hamamatsu, Japan) were used for this study. Because this study was intended to clarify the pathophysiology rather than therapeutic intent, we used only male animals. After SAH induction, animals were randomly assigned to one of the treatment or control groups by drawing lots. Data collection and analyses were performed by a researcher blinded with respect to the treatment group.

Study Protocol

First, to assess the dose-response relationships of MCP—a galectin-3 inhibitor—on EBI, 100 mice were randomly divided into the sham+vehicle (n=12), SAH+vehicle (n=26), SAH+0.8 μg MCP (n=19), SAH+4 μg MCP (n=22), SAH+16 μg MCP (n=9), and SAH+32 μg MCP (n=12) groups. The drug was intracerebroventricularly administrated at 30 minutes post-modeling. Neuroscore, SAH severity, and brain water content were evaluated at 24 and 48 hours post-modeling. These time points were selected based on our previous study, in which post-SAH BBB disruption and neurological impairments peaked at 24 and 48 hours, respectively.[16]

Second, to elucidate the mechanisms of MCP against EBI, 71 mice were randomly divided into the sham+vehicle (n=20), SAH+vehicle (n=27), and SAH+4 μg MCP (n=24) groups. The drug was intracerebroventricularly administrated at 30 minutes post-modeling. Neuroscore, SAH severity, Evans blue dye extravasation, Western blotting, coimmunoprecipitation assay, and immunohistochemical staining were evaluated at 24 hours post-modeling.

Third, to confirm the implication of galectin-3 in post-SAH BBB disruption, 19 SAH mice randomly underwent an intracerebroventricular injection of 4 μg MCP (n=9) or 4 μg MCP+0.2 μg R-galectin-3 (recombinant galectin-3; n=10) at 30 minutes post-SAH. As a separate study, 16 SAH mice also randomly received vehicle (n=8) or 0.2 μg R-galectin-3 (n=8) intracerebroventricularly at 30 minutes post-SAH. Neuroscore, SAH severity, and Evans blue dye extravasation were evaluated at 24 hours post-SAH.

Fourth, to reconfirm the implication of galectin-3 in MCP's anti-EBI effects, 24 surviving SAH mice that received 4 μg MCP intracerebroventricularly at 30 minutes post-SAH randomly underwent the second intracerebroventricular injection of vehicle (n=12) or 0.2 μg R-galectin-3 (n=12) at 24 hours post-SAH. Neuroscore, SAH severity, and IgG immunostaining were evaluated at 48 hours post-SAH.

SAH Modeling and Intracerebroventricular Injection

The endovascular filament perforation SAH model was produced as described previously.[6] Sham mice underwent the same procedure except that the suture was only advanced 3 mm into the left internal carotid artery without perforating the artery (Methods in the online-only Data Supplement).

At 30 minutes or 24 hours post-modeling, surviving mice underwent an intracerebroventricular injection as described previously.[6] Sterile 2 μL PBS (vehicle), MCP (0.8, 4, 16, or 32 μg; molecular weight, ≤10 kDa; PectaSol-C MCP; Econugenics, CA), 0.2 μg R-galectin-3 (molecular weight, 27.3 kDa; 9039-GA; R&D Systems, MN), or 4 μg MCP with 0.2 μg R-galectin-3 in 2 μL PBS was injected at a rate of 1 μL/min. R-galectin-3 of 0.2 μg was selected based on the datasheet and our preliminary study, in which the administration of 0.05, 0.1, and 0.2 μg R-galectin-3 had no effects on physiological parameters and neuroscores in sham-operated mice (Table II in the online-only Data Supplement).

Neurobehavioral Test

Neurobehavior functions were blindly assessed using the modified Garcia neurological score system as described previously.[6] Animals were given a total score of 2 to 18 in 1-number steps, and higher scores indicated better function (Methods in the online-only Data Supplement).

SAH Grade

The severity of SAH was blindly evaluated using high-resolution pictures of the base of the brain taken at each sacrifice.[6] The basal cistern was divided into 6 segments and was allotted a score of 0 to 3 depending on the amount of subarachnoid blood clot in each segment. The animals received a total score ranging from 0 to 18 after adding the scores from all 6 segments.

Brain Water Content

Brain edema was determined using the wet/dry method as reported previously.[4] The percentage of water content was calculated according to the following formula: ([wet weight−dry weight]/wet weight)×100% (Methods in the online-only Data Supplement).

Evans Blue Dye Extravasation

BBB disruption was assessed by measuring the extravasation of Evans blue dye as described previously[5] (Methods in the online-only Data Supplement).

Western Blotting

Western blotting was performed using the left cerebral cortex as described previously.[6] The following primary antibodies were used: goat polyclonal anti–galectin-3 (R&D Systems, Minneapolis, MN), rabbit polyclonal anti-TLR4 (Thermo Fisher Scientific, Waltham, MA), rabbit polyclonal antiphosphorylated JAK (Janus kinase)-1, rabbit monoclonal antiphosphorylated JAK2, rabbit monoclonal antiphosphorylated STAT (signal transducer and activator of transcription)-1, rabbit monoclonal antiphosphorylated STAT-3 (Tyr705), rabbit monoclonal antiphosphorylated STAT-3 (Ser727), rabbit monoclonal antiphosphorylated NF-κB (nuclear factor-κB), rabbit monoclonal antiphosphorylated ERK (extracellular signal-related kinase) 1/2, rabbit monoclonal antiphosphorylated p38 (Cell Signaling

Technology, Danvers, MA), mouse monoclonal antiphosphorylated JNK (c-Jun N-terminal kinase; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti–MMP (matrix metalloproteinase)-9 (EMD Millipore, Billerica, MA), and rabbit polyclonal anti–ZO-1 (zonula occludens-1; Thermo Fisher Scientific) antibodies. β-tubulin (Cell Signaling Technology) was blotted on the same membrane as a loading control (Methods in the online-only Data Supplement).

Immunoprecipitation

Immunoprecipitation was performed as described previously[17] with a slight modification. The left cerebral cortex was used with the same method as Western blotting. The immunoprecipitate was obtained using rabbit polyclonal anti-TLR4 antibody (Thermo Fisher Scientific) followed by incubation with goat polyclonal anti–galectin-3 antibody (R&D Systems; Methods in the online-only Data Supplement).

Immunohistochemical Staining

Immunohistochemical staining was performed as reported previously.[5] Four-micrometer-thick coronal sections at 1.0 mm posterior to the bregma were cut and incubated with biotinylated horse anti-mouse polyclonal IgG (Vector Laboratories, Burlingame, CA), goat polyclonal anti–galectin-3 (R&D Systems), or rabbit monoclonal anti–Iba-1 (ionized calcium-binding adapter molecule 1; Abcam, Cambridge, MA) antibodies. To evaluate the amount of IgG extravasation (BBB disruption) or Iba-1 expression, 4 continuous pictures of the left temporal cortex were photographed under light microscope (×200), and the relative quantity was calculated by Image Pro Plus 6.0 software (Media Cybernetics, Inc, Rockville, MD)[6] (Methods in the online-only Data Supplement).

For double immunolabeling for galectin-3 and MMP-9 or galectin-3 and VWF (von Willebrand factor—an endothelial cell-specific marker),[18] sections were first labeled with anti–galectin-3 antibody (R&D Systems) using peroxidase/diaminobenzidine system. Next, sections were incubated with anti–MMP-9 antibody (EMD Millipore) or anti-VWF antibody (Abcam). Color reaction was developed through alkaline phosphatase system. Vector blue (Vector Laboratories) was used as chromogen.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining was performed using commercially available kit (In Situ Cell Death Detection Kit, version 15; Roche, Mannheim, Germany) as described previously.[19] Five continuous pictures of the left temporal base cortex at ×200 magnification were photographed, and the percentage of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive capillary endothelial cells (the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive endothelial cells/the total number of endothelial cells×100) was calculated.[20]

Statistical Analysis

In the statistical analysis, we calculated the power of the tests. The number of animals per group necessary to reach the desired power of 0.800 was in the range of 4 to 6. Mortality was compared by Fisher exact tests. Other data were expressed as the median±25th to 75th percentiles. SAH grade and neurological scores were analyzed by Mann-Whitney *U* tests or Kruskal-Wallis tests, followed by Steel-Dwass multiple comparisons. For other values, unpaired *t* tests or 1-way ANOVA with Tukey-Kramer post hoc tests were used when each population being compared followed a normal distribution using Shapiro-Wilk W tests. A P value <0.05 was considered significant.

Results

Dose-Response Relationships of MCP on EBI

Physiological parameters among the groups showed no significant differences. None of sham-operated mice died for the observation period. Among SAH groups, the mortality of the SAH+32 μg MCP mice (58.3%) was significantly higher than that of the SAH+vehicle (7.7%), SAH+0.8 μg MCP (10.5%), and SAH+4 μg MCP (9.1%) groups at 24 hours (Table III in the online-only Data Supplement). Seven mice (SAH+vehicle, 1 mouse; SAH+0.8 μg MCP, 2 mice; SAH+4 μg MCP, 1 mouse; SAH+16 μg MCP, 1 mouse; and SAH+32 μg MCP, 2 mice) were excluded because of mild SAH grades ≤7. SAH grades of the remaining animals were similar among the groups at 24 hours (Figure 1A). Neurological scores of SAH mice were significantly worse than those of sham-operated mice, and only 4 μg MCP significantly prevented post-SAH neurological impairments (Figure 1B). As to brain edema at 24 hours, both 0.8 and 4 μg MCP significantly inhibited post-SAH brain edema formation (Figure 1C). Thus, effects of 0.8 and 4 μg MCP were also evaluated at 48 hours post-SAH.

At 48 hours, the mortality was not significantly different among the SAH groups (Table III in the online-only Data

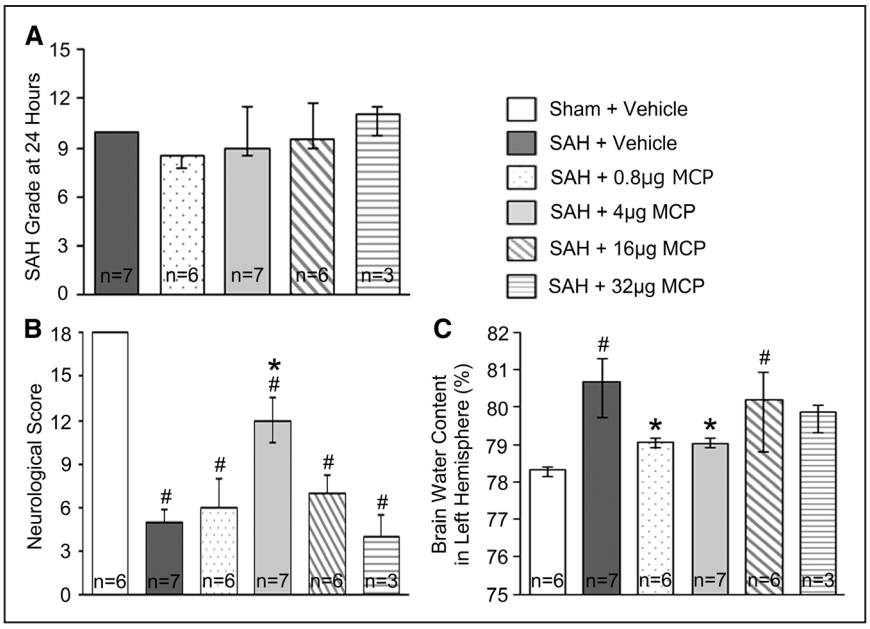

Figure 1. Effects of modified citrus pectin (MCP) treatment on the severity of subarachnoid hemorrhage (SAH; **A**), neuroscores (**B**), and brain water content in the left cerebral hemisphere (**C**) at 24 h after SAH. Data are expressed as median±25th to 75th percentiles. #P<0.05 vs sham group, *P<0.05 vs SAH+vehicle group; Kruskal-Wallis tests (**A** and **B**) and ANOVA (**C**).

Supplement). Four mice (SAH+vehicle, 2 mice; SAH+0.8 μg MCP, 1 mouse; and SAH+4 μg MCP, 1 mouse) were excluded because of mild SAH grades ≤7. SAH grades of the remaining animals were similar among the groups (Figure 2A). Four micrograms MCP also significantly suppressed post-SAH neurological impairments (Figure 2B) and tended to decrease post-SAH brain edema (Figure 2C). Thus, 4 μg MCP treatment was selected for the following experiments.

MCP Prevents Galectin-3 Induction and BBB Disruption After SAH

In the second experiment, 6 mice in the SAH+vehicle group and 5 mice in the SAH+4 μg MCP group died, and 1 mouse in the SAH+vehicle group and 1 mouse in the SAH+4 μg MCP group was excluded because of mild SAH grades ≤7 (Table IV in the online-only Data Supplement). SAH grades of the remaining animals were similar between the groups, and 4 μg MCP significantly prevented post-SAH neurological impairments as above (Figure I in the online-only Data Supplement). Four micrograms MCP also significantly inhibited a post-SAH increase in Evans blue dye extravasation in the left cerebral hemisphere (Figure 3A) and IgG extravasation in the left temporal cortex (Figure II in the online-only Data Supplement). Western blot analyses showed that the expression of galectin-3 was significantly upregulated in SAH+vehicle mice, whereas the administration of MCP significantly inhibited the post-SAH induction of galectin-3 compared with SAH+vehicle mice (Figure 3B). MCP also significantly prevented post-SAH MMP-9 activation and consequently preserved a tight junction protein ZO-1 (Figure 3C and 3D), although pro-MMP-9 levels were unchanged among the groups (Figure III in the online-only Data Supplement). Immunohistochemistry showed that SAH induced galectin-3 and MMP-9 in brain capillary endothelial cells in the left temporal cortex, which was suppressed by MCP (Figures IV through VI in the online-only Data Supplement). Iba-1–positive cells and terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive capillary endothelial cells were also increased after SAH but not decreased by MCP (Figures VII and VIII in the online-only Data Supplement).

Post-SAH Induction of Galectin-3 Activates ERK1/2 and STAT-3 Possibly via TLR4

Western blotting also showed that expression of TLR4 was neither affected by SAH nor MCP, but ERK1/2 was activated by SAH and inhibited by MCP (Figure 4A and 4B). However, coimmunoprecipitation assays revealed the physical interaction between galectin-3 and TLR4, suggesting that galectin-3 activated ERK1/2 via binding to TLR4 (Figure 4C). Other 2 major isoforms of MAPKs, JNK, and p38, as well as another major downstream signaling pathway of TLR4, NF-κB, were not activated after SAH (Figure IX in the online-only Data Supplement). Meanwhile, although expression levels of phosphorylated JAK1 and JAK2 were not different among the groups, STAT-1 and STAT-3 were significantly activated in the SAH+vehicle group (Figure X in the online-only Data Supplement). In addition, the administration of MCP significantly prevented a post-SAH increase in phosphorylated STAT-3 (Figure XD and XE in the online-only Data Supplement), suggesting the cross talk between ERK1/2 and STAT-3.

R-Galectin-3 Blocks MCP's Neuroprotective Effects

In the third experiment, the mortality was not different among the SAH+vehicle, SAH+4 μg MCP, SAH+4 μg MCP+0.2 μg R-galectin-3, and SAH+0.2 μg R-galectin-3 groups, and 2 mice were excluded because of mild SAH grades ≤7 (Table V in the online-only Data Supplement). SAH grades and physiological parameters in the remaining animals were similar among the groups (Figure 5A). The addition of 0.2 μg

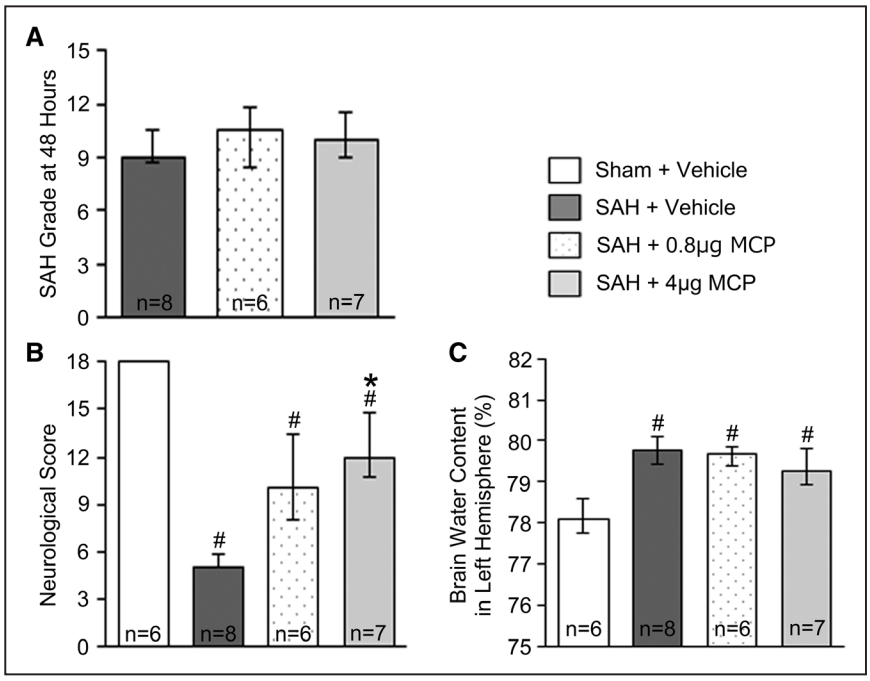

Figure 2. Effects of modified citrus pectin (MCP) treatment on the severity of subarachnoid hemorrhage (SAH; **A**), neuroscores (**B**), and brain water content in the left cerebral hemisphere (**C**) at 48 h after SAH. Data are expressed as median±25th to 75th percentiles. #*P*<0.05 vs sham group, **P*<0.05 vs SAH+vehicle group; Kruskal-Wallis tests (**A** and **B**) and ANOVA (**C**).

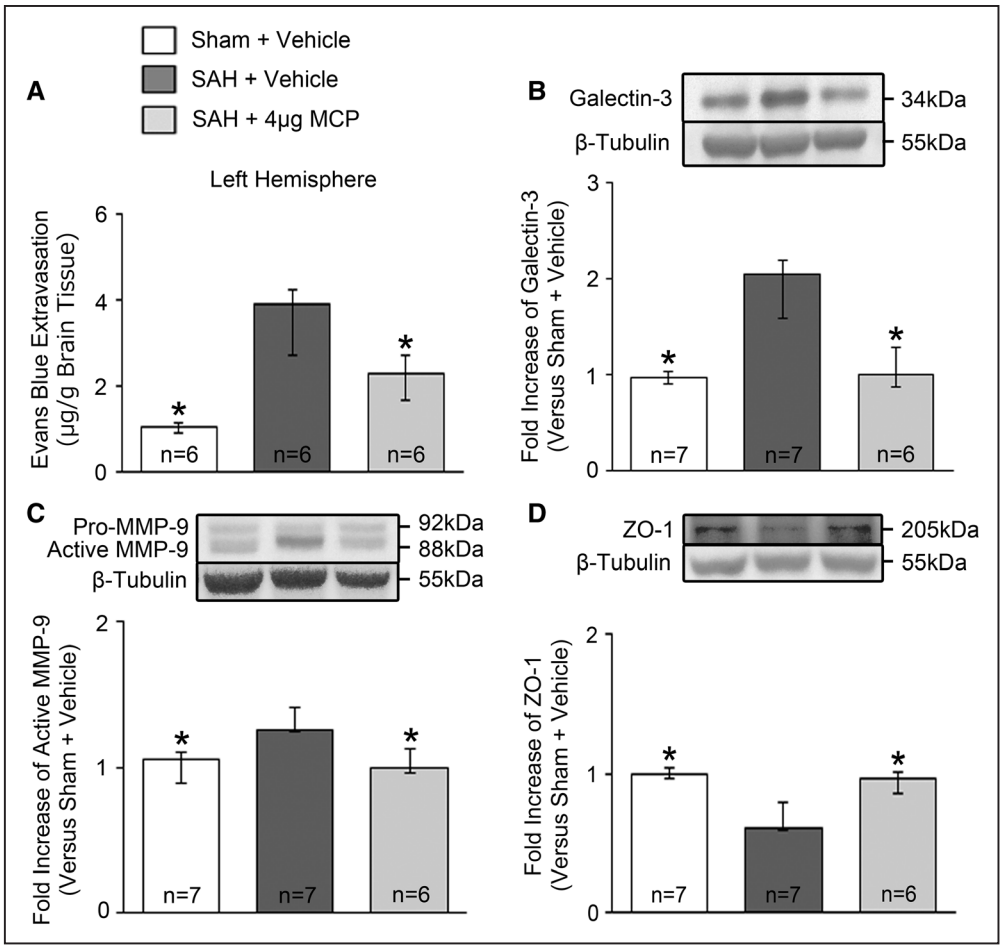

Figure 3. Effects of modified citrus pectin (MCP) treatment on Evans blue dye extravasation in the left cerebral hemisphere (**A**) and expression of galectin-3 (**B**), active MMP (matrix metalloproteinase)-9 (**C**), and ZO-1 (zonula occludens-1; **D**) in the left cerebral cortex at 24 h after subarachnoid hemorrhage (SAH). **B–D**, **Top**, Representative Western blots, and each protein level is expressed as a ratio of β-tubulin levels for normalization. Data are median±25th to 75th percentiles. *P<0.05 vs SAH+vehicle group, ANOVA.

R-galectin-3 to 4 μg MCP-treated SAH mice significantly reaggravated post-SAH neurological impairments and BBB disruption in the left cerebral hemisphere, whereas the addition of R-galectin-3 to vehicle-treated SAH mice did not affect the findings (Figure 5B and 5C).

In the fourth experiment, SAH mice that intracerebroventricularly received 0.2 μg R-galectin-3 at 23.5 hours after 4 μg MCP administration at 30 minutes also had significantly worse neuroscores and BBB disruption compared with the SAH+4 μg MCP+vehicle mice at 48 hours post-SAH irrespective of similar SAH grades (Figure 6; Table VI in the online-only Data Supplement; Figure XI in the online-only Data Supplement).

The numeric data for all groups/experiments are provided in Tables VII through XIX in the online-only Data Supplement.

Discussion

Recently, matricellular proteins, such as tenascin-C, osteopontin, and periostin, have been a focus of interest in the central nervous system because they have various biological effects.[8,19,21–24] Although higher plasma levels of another matricellular protein galectin-3 in an acute phase were associated with more severe SAH, more frequent subsequent development of delayed cerebral infarction without cerebral vasospasm, and worse outcomes in patients with aneurysmal SAH,[11,12] the molecular mechanisms of galectin-3 in SAH have never been investigated. The present study first demonstrated that galectin-3 was upregulated in brain capillary endothelial cells after experimental SAH in mice and that MCP attenuated neurological impairments, brain edema, and BBB disruption through the downregulation of galectin-3, associated with inactivation of ERK1/2, STAT-3, and MMP-9 and the consequent preservation of a tight junction protein ZO-1. In addition, it was suggested that galectin-3 caused post-SAH BBB disruption via TLR4.

However, the findings in the third and fourth experiments in this study were somewhat conflicting. That is, when R-galectin-3 was added simultaneously with or at 23.5 hours after MCP administration, R-galectin-3 reaggravated post-SAH neurological impairments, and BBB disruption at 24 and 48 hours post-SAH, respectively: the latter experiment excluded the possibility that the effect of MCP was neutralized in the premade injection solution before the administration rather than the biological effect of galectin-3 at the injury sites, reinforcing the possible involvement of galectin-3 in post-SAH BBB disruption. In contrast, SAH mice that

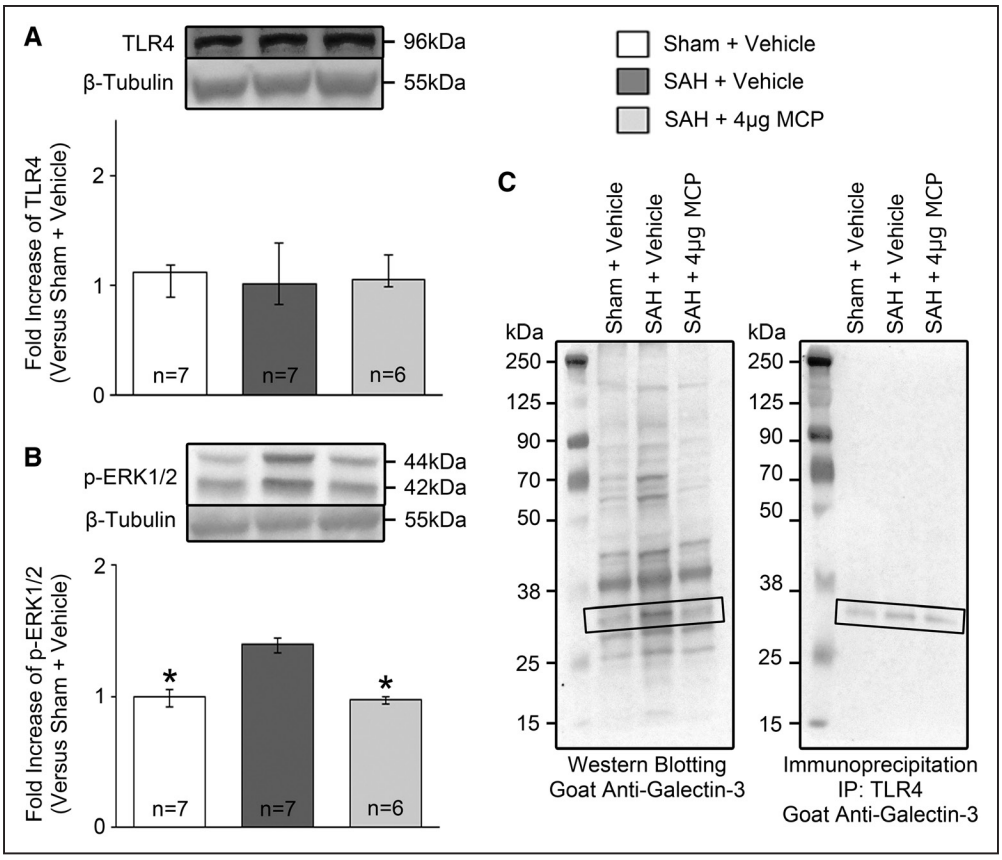

Figure 4. Representative Western blots, effects of modified citrus pectin (MCP) treatment on expression of TLR4 (Toll-like receptor 4; **A**) and p-ERK1/2 (phosphorylated extracellular signal-related kinase 1/2; **B**), and immunoprecipitation (IP) assays using anti-TLR4 antibody followed by incubation with anti–galectin-3 antibody (**C**) in the left cerebral cortex at 24 h after subarachnoid hemorrhage (SAH). Data are median±25th to 75th percentiles. *P<0.05 vs SAH+vehicle group, ANOVA (**A** and **B**).

received R-galectin-3 at 30 minutes post-SAH did not show aggravation of neuroscores or BBB disruption compared with vehicle-treated SAH mice. The reason is unclear, but excess quantities of galectin-3 might not work effectively because the receptor TLR4 might not be induced enough to react with exogenous galectin-3 in addition to endogenous galectin-3 in such an acute phase of SAH.[25]

MCP is a water-soluble indigestible polysaccharide binding to galectin-3 and predominantly antagonizing functions linked to its CRD.[13,14] MCP can reduce galectin-3 expression and severity in some experimental non-neurological diseases.[14,15,26] However, these studies used daily oral intake of MCP,[14,15,26] and there are few reports using intrathecal injections of MCP. Ma et al[13] reported that daily intrathecal administration of MCP for 2 weeks via lumbar puncture decreased galectin-3 expression and proinflammatory mediators, attenuating neuropathic pain in spinal nerve ligation models in rats. The present study revealed a bell-shaped dose-response relationship of an intracerebroventricular injection of MCP against post-SAH neurological impairments and brain edema and found 4 μg of MCP to be the most effective, but the effects of MCP at 48 hours were limited, needing the improvement of the treatment regimen, for example, such as multiple injections. In this study, MCP prevented neurological impairments but not brain edema formation at 48 hours post-SAH, suggesting the existence of MCP's pleiotropic neuroprotective action, such as antineuroinflammatory and antiapoptotic effects, other than protecting BBB through inhibiting galectin-3[2, 13]; however, this study failed to detect MCP's beneficial effects against microglial activation and endothelial cell apoptosis at 24 hours, and thus, further studies are needed to clarify MCP's other neuroprotective effects. In addition, possible mechanisms of toxicity in higher dosages of MCP remain unknown: one possibility is nonspecific effects associated with an intracerebroventricular injection of excessive MCP, and other possibilities include that higher dosages of MCP may have apoptosis-promoting effects in SAH.[27] In this regard, further studies are also needed.

Recent studies have suggested that BBB disruption is an important pathological manifestation of EBI after SAH, which is mediated by neuroinflammation.[3,6,16] Increasing evidence has shown that TLR4 signaling plays an important role in SAH-induced EBI and may be an important therapeutic target for post-SAH neuroinflammation.[3] TLR4 is expressed on various cell types in the central nervous system, including brain capillary endothelial cells.[28] Yan et al[29] demonstrated that a selective inhibition of myeloid differentiation primary-response protein, which is 1 of 2 distinct adaptor proteins of TLR4, alleviated SAH-induced inflammatory responses and EBI through the downregulation of MAPKs and NF-κB signaling pathways. Both MAPKs and NF-κB, which induce MMP-9 and cause BBB disruption, are activated in TLR4

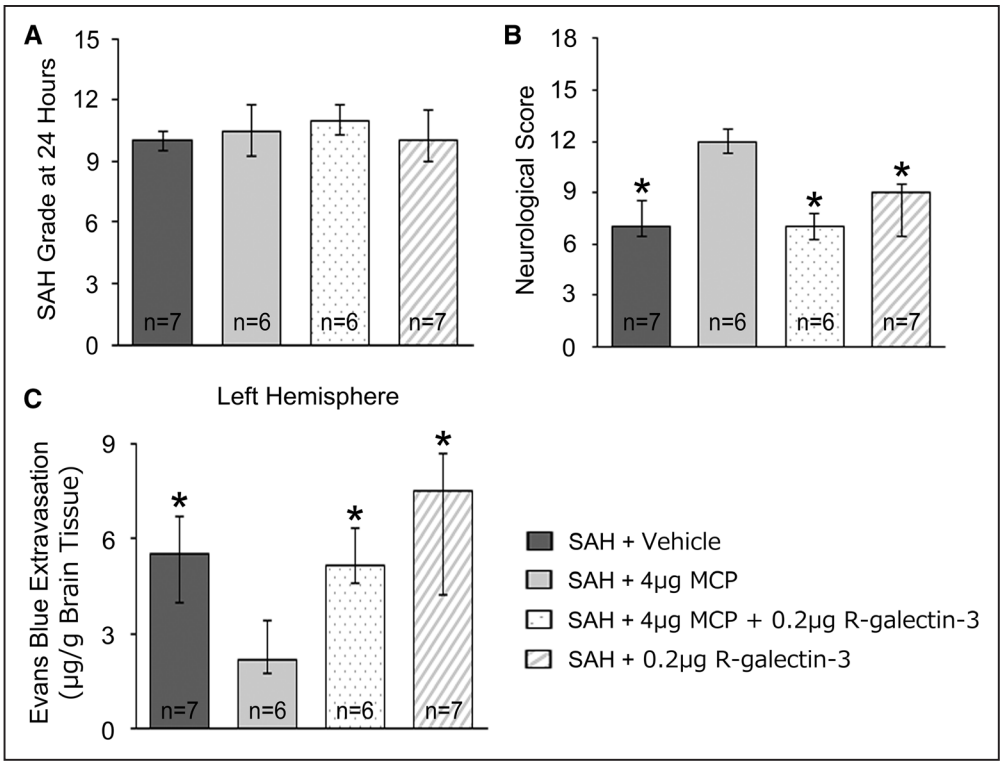

Figure 5. Effects of simultaneous administration of R-galectin-3 (recombinant galectin-3) with vehicle or modified citrus pectin (MCP) on the severity of subarachnoid hemorrhage (SAH; **A**), neuroscores (**B**), and Evans blue dye extravasation in the left cerebral hemisphere (**C**) at 24 h post-SAH. Data, median±25th to 75th percentiles. *P<0.05 vs SAH+4 μg MCP group, Kruskal-Wallis tests.

signaling[3] and have been identified as possible major inflammatory pathways in EBI.[3,5,6] Galectin-3 is reported to bind to TLR4 and act as a TLR4 ligand through its CRD, contributing to sustained neuroinflammation.[10] In this study, galectin-3 was upregulated in brain capillary endothelial cells after SAH. It remains unknown how SAH upregulates galectin-3, but free radicals are important candidates because there are several sources for the excessive generation of free radicals after SAH

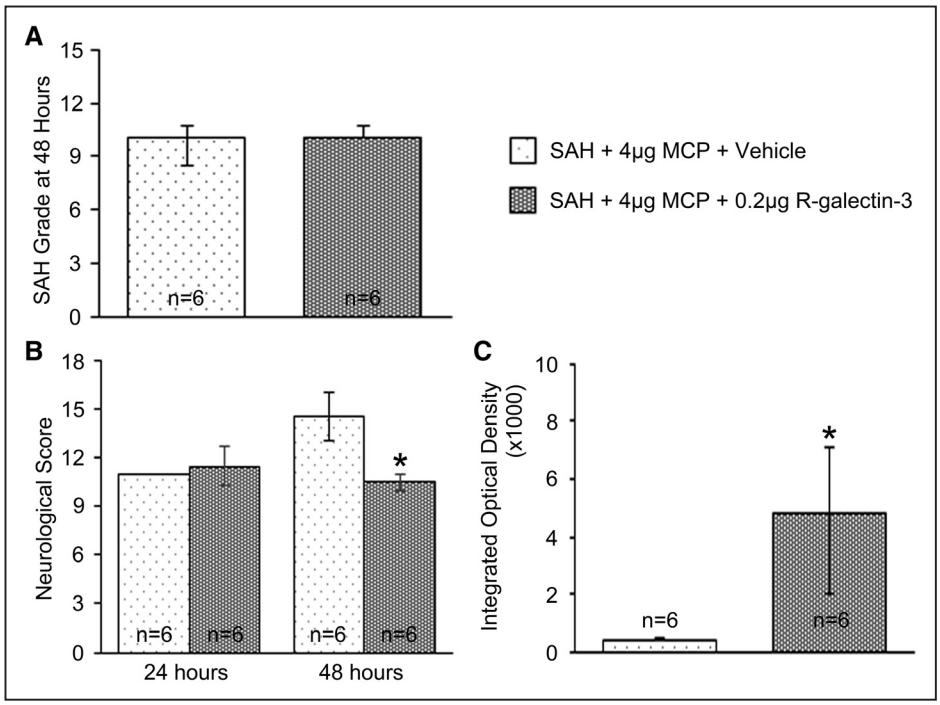

Figure 6. Effects of R-galectin-3 (recombinant galectin-3) administered at 23.5 h after modified citrus pectin (MCP) treatment on the severity of subarachnoid hemorrhage (SAH; **A**), neuroscores (**B**), and IgG immunostaining (integrated optical density) in the left temporal cortex (**C**) at 48 h post-SAH. Data are median±25th to 75th percentiles. *P<0.05 vs SAH+4 μg MCP+vehicle group, Mann-Whitney U tests (**A** and **B**) and unpaired t tests (**C**).

and endothelial cells are susceptible to oxidative stress.[30] MCP might block interaction of galectin-3's CRD with TLR4 and, therefore, post-SAH activation of a major MAPK ERK1/2 and the downward proinflammatory mediator MMP-9, preventing BBB disruption (Figure XII in the online-only Data Supplement). TLR4–NF-κB pathways were not implicated in MCP's preventive effects against MMP-9–mediated BBB disruption in this study.

On the contrary, the non-CRD of galectin-3 can bind to interferon-γ receptor 1 and induce direct phosphorylation of JAK2 and several STATs, thereby activating neuroinflammation.[31] Phosphorylation of STATs depends on the phosphorylation of JAKs, and in particular, activation of JAK2 has been linked with phosphorylation of STAT-1, STAT-3, and STAT-5.[32] Activation of STAT-3 or JAK-STAT pathway upregulates MMP-9 in glioblastoma cell lines[33] and aortic vascular smooth muscle cells[34] and causes BBB disruption because of inflammation-induced endothelial cell injuries.[35] However, MCP used in this study cannot block the interaction of galectin-3's non-CRD with the binding partners.[13,14] In addition, JAKs were not activated in this study. Thus, activation of STATs shown in this study is considered to occur not through interferon-γ receptor 1. As the mechanisms of activation of STATs in this study, the cross talk between ERK1/2 and STAT-3 can be considered: according to Suman et al,[36] ERK1/2 acts as a kinase to phosphorylate STAT-3 at serine residue 727. Taken together, our study suggested that galectin-3 activates ERK1/2 MAPK pathway through TLR4, which in turn activates both MMP-9 and STAT-3 in cross talk manner; and the STAT-3 further activates MMP-9, eventually resulting in the degradation of ZO-1 and BBB disruption.

This study has some limitations. First, long-term outcomes of a galectin-3 blockage were not studied. Some studies suggest that galectin-3 contributes to postischemic tissue remodeling, including angiogenesis and neurite outgrowth in a delayed fashion, and that long-term deletion of galectin-3 exacerbates postischemic damage.[17,37,38] Second, although the main established mechanism of action for MCP comes from antagonizing galectin-3,[27] MCP's galectin-3–unrelated action cannot be excluded. In addition, effects of MCP on MMP-9 and microglial activation were examined, but MCP's or galectin-3's action on other inflammatory reactions remains unknown. Lastly, in this study, signaling mechanisms were studied in crude brain tissue lysates, and the proposed mechanisms in endothelial cells were conceived by the extrapolation of existing evidence from the literature, as well as findings in this study. Therefore, further studies are needed to elucidate the role and mechanisms of galectin-3 in SAH.

Conclusions

The present study suggested that a matricellular protein galectin-3 causes post-SAH BBB disruption by the activation of ERK1/2, STAT-3, and MMP-9 through TLR4. MCP exerted neuroprotective effects by inhibiting post-SAH upregulation of galectin-3 and its downstream pathways. These findings show that galectin-3 may be a novel target to develop new therapies against post-SAH EBI.

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Disclosures

None.

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