Role of p38 Mitogen- Activated Protein Kinase on Cerebral Vasospasm After Subarachnoid Hemorrhage

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Background and Purpose—Inflammatory cytokines are involved in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH). This study was conducted to examine the role of p38 mitogen-activated protein kinase (MAPK) in the development of vasospasm and cytokine production.

Methods—We measured the expression levels of genes and proteins related to inflammation in human vascular smooth muscle cells (hVSMCs) treated with hemolysate and FR167653 (FR) (1 μmol/L), a selective p38MAPK inhibitor, for 48 hours by TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR) and ELISA. Twenty-one dogs were assigned to 3 groups of 7 animals: control, placebo, and FR-treated (1 mg/kg/d) groups in a double-hemorrhage model. The effects were assessed through the caliber of the basilar artery, and the changes in gene expressions and the activation of p38MAPK were assessed by Western blot analysis.

Results—Treatment of hVSMCs with hemolysate induced significant upregulation of interleukin (IL)-1α, IL-1β, and IL-8 gene and protein expressions, which was suppressed significantly with FR. The mean vessel caliber on day 7, as a percentage of that of day 0, was 49% in the placebo, and 74% in the FR group (P<0.0001). The gene expression levels of IL-1α, IL-1β, and IL-8 in the arterial wall were extremely elevated in the placebo, and significantly suppressed in the FR group (P=0.0027, 0.0002, and 0.0073). p38MAPK phosphorylation was stimulated in the placebo and hemolysate in vitro, and suppressed in the FR group.

Conclusions—These results suggest that p38MAPK is activated in the arterial wall after SAH, leading to the development of vasospasm, possibly through the upregulation of inflammatory cytokines. (Stroke. 2004;35:1466-1470.)

Key Words: cytokines ■ inflammation ■ mitogen-activated protein kinase kinases ■ subarachnoid hemorrhage ■ vasospasm, intracranial

Cerebral vasospasm after subarachnoid hemorrhage (SAH) is a major clinical problem causing cerebral ischemia and infarction. The pathogenesis of cerebral vasospasm remains unclear even though prolonged smooth muscle contraction, histological changes of the arterial wall, and an inflammatory or immunological reaction may be involved.1 Several signal transduction pathways, including mitogen-activated protein kinase (MAPK), have been proposed to explain prolonged smooth muscle cell contraction after SAH.2,3

Previously, we quantitatively measured genes related to inflammation in the spastic artery in a canine double-hemorrhage model and found that the number of inflammatory cytokines/chemokines was markedly elevated during vasospasm. The pattern of maximum cytokine level fits remarkably well with the time course of the vasospasm.1,4 The activation of p38MAPK is involved in intracellular signaling pathways that regulate the production of several cytokines or chemokines, such as IL-1β, IL-6, IL-8, TNF-α and MCP-1,5,6,7 IL-1 is known to be a potent activator of MAPK pathways.8,15 Therefore, we hypothesized that SAH induces the activation of p38MAPK in the smooth muscle cells of the cerebral artery, eventually leading to the development of cerebral vasospasm by the induction of inflammatory cytokines/chemokines. In this study, FR167653 (FR), a selective inhibitor of p38MAPK, was used to examine the effects in vitro and in vivo on the gene expressions of inflammatory cytokines and cerebral vasospasm after SAH.9,10,11,12,13

Materials and Methods

Hemolysate Preparation
Arterial blood collected from humans was centrifuged at 2500g for 15 minutes, and the supernatant was discarded. The erythrocyte fraction was washed 3 times with saline (volume of saline/erythrocyte fraction ratio, 1:3). After the erythrocytes were disintegrated by ultrasonic waves, the particulate material was centrifuged at 15 000g for 90 minutes, and the supernatant (hemolysate) was collected and stored at −80°C. The oxyhemoglobin concentration was 473±50 μmol/L.2
Cell Culture of Human Vascular Smooth Muscle Cells (hVSMCs)
The human arterial smooth muscle cells from umbilical artery, dedifferentiated type (Cell Systems) were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air in 100-mm dishes. The growth medium was Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The medium was changed twice a week. The cells were treated for 48 hours with hemolyase at a concentration of 10%, and preincubated with FR167653 (1-[4-fluorophenyl]-1,2,3,4-tetrahydro-8-(4-pyridyl) pyrazolo [5,1-c] [1,2,4] triazin-2-yl; MW=543.53) at 1 μmol/L 30 minutes before adding hemolyase. After the treatment for 48 hours, the supernatants were collected, centrifuged at 15 000g for 10 minutes at 4°C, and the supernatants and stored at ~80°C.

Cytokine Production by hVSMCs
IL-1α, IL-1β, and IL-8 in the supernatants were determined with specific ELISA kits (Biosource International, R&D Systems), according to the manufacturer’s instructions.

Immunoprecipitation and p38MAPK Activity Assay
p38MAPK activity was measured with use of the p38MAPK assay kit (New England Biolabs), according to the manufacturer’s instructions. After the treatment for 48 hours, the cells were washed with cold PBS on ice, scraped, and extracted in 0.5 mL of cold lysis buffer. The extracts were sonicated and centrifuged at 15 000g for 10 minutes at 4°C, and the supernatants were stored at ~80°C. The immunoreactivity was detected with enhanced chemiluminescence immunoreactivity was detected with enhanced chemiluminescence with enhanced chemiluminescence imaging system (Amersham). The precipitated proteins were washed with TBS-T, 0.1% Tween 20 (TBST) for 1 hour. The membranes were blocked using 5% nonfat milk in Tris-HCl buffered saline containing 0.1% Tween 20 (TBST) for 1 hour. The membranes were washed with TBS-T for 5 minutes and 0.1% Tween 20 (TBST) for 1 hour. The membranes were washed with TBS-T for 10 minutes, and the supernatants were collected, centrifuged at 15 000g for 10 minutes at 4°C, and the supernatants were stored at ~80°C.

RNA Isolation From hVSMCs
Total RNA was extracted from culture cells using TRizol (GIBCO-BRL), according to the manufacturer’s instructions. Possible traces of genomic DNA contaminating the RNA preparations were removed by DNase I (Promega) digestion.

Quantitative RT-PCR
To evaluate the expression level of the target genes, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using real-time TaqMan technology with a Sequence Detection System, model 7700 (Perkin Elmer) as described previously. 4 Quantitative RT-PCR primers were designed using Primer Express V.2.0 (Applied Biosystems). Primers were synthesized using sequences from a database search at NCBI for human IL-1α, IL-1β, IL-8, and β-actin as follows: IL-1α sense primer: TGGGAT-TACAGGGCTGTAGC; IL-1α antisense primer: AGTGCCCTGGTG-CACAGTGAAGA; IL-1β sense primer: TCAGGAGAAGCAGT-CAGCTCT; IL-1β antisense primer: GAGGCGTGGCTCA-ACAAAG; IL-8 sense primer: AAGGAAACATCTCACTGTT-GTAAAC; IL-8 antisense primer: ATACGAGAAGGCTGCAA-GAG; β-actin sense primer: TCACCGAGCGGCTGCT; β-actin antisense primer: TAATGTCACGCACGATTCCC.

Canine Double-Hemorrhage Model
The experimental protocol used for animals was evaluated and approved by the Institutional Animal Care and Use Committee of Tokyo Women’s Medical University. Care of the animals and surgical procedures were performed according to the standards of Tokyo Women’s Medical University Protocol on Laboratory Animals. Twenty-one dogs were randomly assigned to 3 groups of 7 animals; control, placebo, and FR-treated groups in a double-hemorrhage model. Dogs weighing between 7.5 and 17 kg were anesthetized with intravenous sodium pentobarbital (25 mg/kg). In 14 dogs, the cisterna magna was punctured percutaneously, and 0.3 mL/kg of cerebrospinal fluid (CSF) was removed by spontaneous egress. Subsequently, 0.5 mL/kg of fresh autologous arterial nonheparinized blood, from the femoral artery through the sheath for the angiography catheter, was injected into the cisterna magna at a rate of 5 mL/min. The dogs were positioned with the tail up for 15 minutes. The catheter was then removed, and the animals were allowed to recover. Control group animals were killed on day 0 without cisternal blood injection. Placebo group animals were injected with physiological saline and continuously infused from the jugular vein using an osmotic pump (Alzet). FR group animals were injected with FR167653 (0.25 mg/kg) and continuously infused (1 mg/kg/d). The procedures were performed in a blind fashion. These animals were killed on day 7 after injections of blood on days 0 and 2. An angiography of the cerebral arteries of each animal was performed on day 0 and before the animal was killed.

Vessel Caliber Assessment
With a calibrated optical micrometer, the diameters of the basilar arteries were measured on the angiographic films at 3 separate, equally spaced locations along the artery by an experienced person who was blinded to the treatment groups. The average diameter value at each location was used.

RNA Isolation From the Canine Basilar Artery
The animals were killed by injecting 100 mg/kg pentobarbital, exsanguinated, and perfused with 2500 to 3000 mL physiologic saline. Total RNA was extracted from individual basilar arteries of each group using TRIzol, according to the manufacturer’s instructions. Possible traces of genomic DNA contaminating the RNA preparations were removed by DNase I (Promega) digestion.

Preparation of Dog-Specific Primers and Probes for Quantitative RT-PCR
The canine sequences of mRNAs for interleukin (IL)-1α, IL-1β, IL-8, and β-actin were obtained by a database search of NCBL. 4 The canine sequence of IL-1β was not known, thereby, the partial nucleotide sequence of this gene was determined. The cDNA was synthesized from 1 μg total RNA extracted from canine middle cerebral arteries by ThermoScript RT-PCR System (Invitrogen). The cDNA was amplified by PCR with degenerate oligonucleotides based on the human and mouse nucleotide sequences of the target genes. The PCR products were directly sequenced with an Applied Biosystem DNA Sequencer, model 377. The canine sequences obtained, which had >90% identity to the human sequences, were subsequently used to design the dog-specific primers and probe for quantitative RT-PCR (IL-1β sense primer, AACAAGAITGCTGAGGCTTGC; antisense primer, AGCCGAAGCCTCATGAGTAC).

Western Blot Analysis
The frozen arteries were minced and homogenized ultrasonically (10 seconds, 3 times) in an extraction buffer (50 mmol/L Tris buffer pH 7.5, containing 100 mmol/L NaCl, 1% Nonidet P-40, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, and 50 μg/mL each of leupeptin, aprotinin, and PMSF). Lysates were then centrifuged at 13 000g at 4°C for 10 minutes, and the supernatants were transferred to fresh tubes and stored at ~80°C until required. The protein concentrations of the lysates were determined using Coomassie protein assay reagent (Bio-Rad). The samples (30 μg protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 4% to 12% Bis-Tris Gel (Invitrogen). After electrophoretic transfer of the separated polypeptides to nitrocellulose membranes, the membranes were blocked using 5% nonfat milk in Tris-HCl buffered saline containing 0.1% Tween 20 (TBST) for 1 hour. The membranes were then washed with TBST and incubated overnight at 4°C in a 1:1000 dilution for a mouse anti-p38MAPK antibody and antiphosphorylated p38MAPK antibody (Cell Signaling). These antibodies cross-react with canine p38MAPK and phosphorylated p38MAPK. Nitrocellulose membranes were later washed with TBST and incubated with a 1:10 000 dilution of a sheep anti-mouse G (Ig G) antibody linked with horseradish peroxidase for 2 hours at room temperature. An enhanced chemiluminescence system (Amersham) was used to visualize the protein bands.
Figure 1. Effect of FR 167653 on gene expression levels of interleukin (IL)-1α, IL-1β, and IL-8 in hemolysate-treated human vascular smooth muscle cells (hVSMCs). C indicates saline-treated control for 48 hours; H, hemolysate-treated for 48 hours; H+FR, preincubated with FR 167653 for 30 minutes (1 μmol/L), and then treated with hemolysate for 48 hours. Gene expression levels are expressed as relative ratios in cycle threshold (CT) values for these genes to β-actin. Bars represent the mean ± SD (n=4, each group). There were significant differences among groups (P<0.0001, P=0.0144, and 0.574, ANOVA; P=0.0073, 0.0073, and 0.0125, Kruskal–Wallis).

Statistical Analysis
Statistical comparisons of arterial diameters and levels of mRNA in target genes among groups were evaluated by ANOVA, the Fisher PLSD posthoc test, and the Kruskal–Wallis test. Results were considered significant if probability values were less than 0.05.

Results
Effect of Hemolysate and FR on Gene Expressions of IL-1α, IL-1β, and IL-8 in Cultured hVSMC
There were significant differences in the gene expression levels of IL-1α, IL-1β, and IL-8 among the control, hemolysate, and hemolysate/FR groups (P<0.0001, P=0.0144, and 0.574, ANOVA, respectively; P=0.0073, 0.0073, and 0.0125, Kruskal–Wallis, respectively). The average expression levels of IL-1α, IL-1β, and IL-8 in the hemolysate groups were 18, 25, and 270 times higher than those in the control group. The gene expression levels of IL-1α, IL-1β, and IL-8 were significantly stimulated with hemolysate. FR significantly suppressed the hemolysate-stimulated upregulation of IL-1α, IL-1β, and IL-8 (Figure 1).

Production of IL-1α, IL-1β, and IL-8 in the Supernatant of Cultured hVSMC
The production of IL-1α, IL-1β, and IL-8 was significantly stimulated with hemolysate. FR significantly suppressed the hemolysate-stimulated production of IL-1α, IL-1β, and IL-8 (Table). The level of IL-8 was much higher than levels of IL-1α and IL-1β.

Effect of Hemolysate and FR on p38MAPK Activity in Cultured hVSMC
The amount of p38MAPK activation by hemolysate in cultured hVSMC was 4.7±2.2-fold that measured in unstimulated controls. Hemolysate-stimulated p38MAPK activity was significantly inhibited by FR to 1.9±1.5-fold of unstimulated controls (Table).

Canine SAH Model
Change in Vessels Caliber
There was no difference in vessel caliber at the baseline among the control, placebo, and FR groups. All the placebo SAH animals developed severe vasospasm of the basilar artery. FR-treated SAH animals had mild or no vasospasm (Figure 2). There was a significant difference in vessel caliber among the groups (baseline of control, day 7 of placebo and FR groups) (P=0.0001, ANOVA; P=0.0023; Kruskal–Wallis) (Figure 3). The mean diameter of the basilar arteries on day 7, as a percentage of that on day 0, was 49.2±20.0% in the placebo group and 74.8±23.0% in the FR group.

Gene Expression Levels of IL-1α, IL-1β, and IL-8
There were significant differences in the gene expression levels of IL-1α, IL-1β, and IL-8 among the control, placebo, and FR groups (P=0.0023, 0.0002, and 0.0073, respectively; Kruskal–Wallis). The average expression levels of IL-1α, IL-1β, and IL-8 in the placebo group were 28, 437, and 9 times higher than those in the control group. The gene expression levels of IL-1α, IL-1β, and IL-8 were significantly stimulated after cisternal injections of blood, which was suppressed when treated with FR (Figure 4).

Table: Effect of FR167653 on Productions of Interleukin (IL)-1α, IL-1β, and IL-8 and Activation of p38MAPK in Hemolysate-Treated Human Vascular Smooth Muscle Cells

<table>
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<tr>
<th></th>
<th>N (pg/ml)</th>
<th>C (pg/ml)</th>
<th>H (pg/ml)</th>
<th>FR (pg/ml)</th>
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<tr>
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<td>0.24±0.10</td>
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<tr>
<td>IL-8</td>
<td>4</td>
<td>13±6</td>
<td>203±151</td>
<td>41±24</td>
</tr>
<tr>
<td>p38MAPK</td>
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<td>1</td>
<td>4.7±2.2</td>
<td>1.9±1.5</td>
</tr>
</tbody>
</table>

IL-1α, IL-1β, and IL-8 in the supernatants were determined with specific ELISA kits (the mean±SD). C indicates saline-treated control for 48 hours; H, hemolysate-treated for 48 hours; H+FR, preincubated with FR167653 for 30 minutes (1 μmol/L), and then treated with hemolysate for 48 hours. There were significant differences among groups (P=0.0110, 0.0330, and 0.0289, ANOVA; P=0.0450, 0.0284, and 0.0125, Kruskal–Wallis). Although there was no significant difference of p38MAPK activity among the control, hemolysate, and hemolysate/FR groups (n=3, P=0.0642, ANOVA; P=0.0992, Kruskal–Wallis), there was a significant difference of p38MAPK activity between the control and hemolysate (P=0.0027, Fisher PLSD).

Figure 2. Representative angiograms obtained from control animals killed after angiography (C), hemorrhage animals treated with saline for 7 days (H) (placebo), and hemorrhage animals treated with FR 167653 (1 mg/kg/d) for 7 days (H+FR).
Figure 3. Graph showing changes in the vessel caliber of basilar arteries in a canine double-hemorrhage model. C indicates control animals killed after angiography; H, hemorrhage animals treated with saline for 7 days (placebo); and H+FR, hemorrhage animals treated with FR 167653 (1 mg/kg/d) for 7 days. Bars represent the mean±SD (n=7, each group). There was a significant difference in vessel caliber between C, H, and H+FR (P<0.0001, ANOVA; P=0.0023, Kruskal-Wallis).

Western Blotting of p38MAPK and Phosphorylated p38MAPK
Lysate (30 μg protein) from each group was randomly selected and separated by SDS-PAGE on the same membrane. The bands of anti-p38MAPK could be identified at 38-kDa and the density of the bands appeared similar among the 3 groups. The extract from canine basilar arteries contained a 38-kDa band and a band of lower molecular weight, a possible degradation product of p38MAPK, when an antiphosphorylated p38MAPK antibody was used. This tendency was more obvious in animals of the double-hemorrhage model. The bands of the antiphosphorylated p38MAPK antibody were dense in the order of placebo, FR, and control groups (Figure 5). Three experiments were performed and similar results were obtained.

Discussion
MAPK has been reported to play an important role in intracellular signal transduction in the proliferation, differentiation, and cellular response to stress. Recent studies have indicated that there are at least 3 types of MAPK, namely: extracellular signal-regulated protein kinase (ERK1/2; p42/p44); c-Jun N-terminal protein kinases (JNK and p54); and p38MAPK. p38MAPK regulates cellular responses to a variety of cellular stresses, such as heat shock, hyperosmolarity, ultraviolet radiation, the endotoxin lipopolysaccharide (LPS), and hypoxia. The activation of p38MAPK leads to the production of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α, and MCP-1 by various types of cells. We examined the role of p38MAPK on vasospasm because the gene expressions of a number of inflammatory cytokines/chemokines were markedly elevated in the spastic artery of the canine double-hemorrhage model.

In this study, we demonstrated that 48 hours of treatment with hemolysate, a causative agent proposed for vasospasm, activated p38MAPK and induced a prominent upregulation of the gene and protein expressions for IL-1α, IL-1β, and IL-8 in the hVSMCs. We focused on these particular inflammatory cytokines/chemokines because they were upregulated most significantly in the arterial wall during vasospasm in previous experiments. FR167653 significantly inhibited p38MAPK and suppressed the upregulation of these genes and proteins in vitro, indicating the possibility that FR could suppress the upregulation of cytokines in the artery and prevent vasospasm in an animal model.

We next demonstrated that the intravenous administration of FR inhibits the activation of p38MAPK and the upregulation of inflammatory cytokines in the spastic artery, and prevents cerebral vasospasm in the canine double-hemorrhage model. FR167653 inhibits p38α kinase activity in a dose-dependent manner, and significant inhibition is observed at 0.1 μmol/L, or 0.01 μmol/L in vitro. We maintained 100 ng/mL (approximately 0.2 μmol/L) of FR concentration in serum based on the data of the dynamics of serum concentration changes in dogs. Our procedure is acceptable because the average concentration of FR in serum on days 2 and 7 was 86 ng/mL measured by high-performance liquid chromatography (data not shown). Because FR is a potent inhibitor of cytokine production, these results suggest that FR prevents cerebral vasospasm by inhibition of...
the upregulation of inflammatory cytokines through the p38MAPK.

FR is a low-molecular pyrazolotriazine derivative and has been characterized as a potent suppressant of TNF-α and IL-1β production at the transcriptional and translational levels. The pharmacological characteristics and chemical structure of FR resemble those of pyridinylimidazole inhibitors of p38MAPK, such as SB203580 and VK199111. FR has been reported to effectively suppress TNF-α and IL-1β production and attenuate symptoms in a lipopolysaccharide-induced–disseminated intravascular coagulation model in rats, a pulmonary ischemia-reperfusion model in dogs, an ischemic reperfusion injury model of canine hearts, neointimal hyperplasia after vascular injury in rats, and a model for Helicobacter pylori-induced gastritis in Mongolian gerbils.

We report for the first time that the inhibition of p38MAPK prevented cerebral vasospasm by inhibiting the upregulation of inflammatory cytokines IL-1α, IL-1β, and IL-8. FR did not reduce the hemolysate-induced contraction of the artery in an isometric tension study (data not shown), suggesting that p38MAPK may not be involved directly in signal transduction to sustained contraction of smooth muscle cells. A positive feedback system was reported between cytokine synthesis and cascade phosphorylation of intracellular kinases in the arterial smooth muscle cells. Activation of p38MAPK is reported to phosphorylate downstream signaling pathways such as heat shock protein 27, which promotes actin remodeling by enhancing actin polymerization. These mechanisms may be involved in a complex pathological process of cerebral vasospasm characterized by persistent contraction of arterial smooth muscle and morphological changes in the arterial wall.

In conclusion, hemolysate induced the prominent upregulation of IL-1α, IL-1β, and IL-8 in hVSMCs in vitro, which was suppressed by FR, a selective inhibitor of p38MAPK. We also found that the intravenous administration of FR prevents cerebral vasospasm by inhibiting the upregulation of inflammatory cytokines. FR attenuated the levels of phosphorylated p38MAPK in hemolysate-stimulated cultured hVSMC and during vasospasm. These results suggest that p38MAPK is activated in cerebral arterial walls after SAH, leading to the development of vasospasm, possibly through the upregulation of inflammatory cytokines.

Acknowledgments
This work was supported in part by a grant-in-aid for scientific research (C) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (to H.K.); the Japanese Ministry of Health, Labor, and Welfare (to H.K. and I.I.); and a Research for the Future Program Grant from the Japan Society for the Promotion of Science (I.I.). We thank Fujisawa Pharmaceutical Co, Ltd, for generously providing the FR167653 needed to perform the experiments.

References
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Stroke. 2004;35:1466-1470; originally published online April 29, 2004;
doi: 10.1161/01.STR.0000127425.47266.20
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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