Quantitative Analysis of Gene Expressions Related to Inflammation in Canine Spastic Artery After Subarachnoid Hemorrhage

Yasuo Aihara, MD; Hidetoshi Kasuya, MD; Hideaki Onda, MD; Tomokatsu Hori, MD; Jun Takeda, MD

Background and Purpose—The possible role of inflammatory reaction of the cerebral artery in the pathogenesis of cerebral vasospasm has been noted in recent studies. We quantitatively measured the levels of expression of genes related to inflammation in the spastic artery in a canine double-hemorrhage model.

Methods—Twenty dogs were assigned to 4 groups: group D0, control; group D2, dogs killed 2 days after cisternal injection of blood; group D7, dogs given double cisternal injections of blood and killed 7 days after the first injection; and group D14. Angiography was performed twice: on the first day and before the animals were killed. Total RNA was extracted from the basilar artery. The expressions of interleukin (IL)-1α, IL-6, IL-8, IL-10, tumor necrosis factor-α, E-secretin, fibronectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule-1, transforming growth factor-β, basic fibroblast growth factor, and collagen types I, III, and IV were examined with TaqMan real-time quantitative reverse transcription–polymerase chain reaction.

Results—Prolonged arterial narrowing peaking on 7 day was observed. There was a significant difference in vessel caliber between D0, D2, D7, and D14 groups (P < 0.0001). There were significant differences in mRNA expression in the basilar artery for IL-1α, IL-6, IL-8, ICAM-1, and collagen type I between D0, D2, D7, and D14 groups (P = 0.0079, 0.0196, 0.0040, 0.0017, and < 0.0001, respectively). The average level of mRNA was highest in D7 for IL-1α, IL-6, IL-8, and ICAM-1 (17-, 16-, 131-, and 1.7-fold compared with those of D0, respectively) and in D14 for collagen type I (10.9-fold).

Conclusions—Increased expression of genes related to inflammation in the spastic artery suggests that inflammatory reaction of the cerebral artery is associated with sustained contraction. (Stroke. 2001;32:212-217.)

Key Words: cerebral vasospasm ■ cytokines ■ inflammation ■ subarachnoid hemorrhage

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From the Department of Neurosurgery (Y.A., H.K., H.O., T.H.), Tokyo Women’s Medical University, Tokyo, Japan, and the Department of Cell Biology (Y.A., H.O., J.T.), Laboratory of Molecular Genetics, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan.

Correspondence to Hidetoshi Kasuya, MD, Department of Neurosurgery, Tokyo Women’s Medical University, Kawada-cho 8-1, Shinjuku-ku, Tokyo 162-8666, Japan. E-mail hkasuya@nij.twmu.ac.jp

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212
### Nucleotide Sequences of Dog-Specific Primers and Probes for TaqMan Real-Time Quantitative RT-PCR

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<th>Gene</th>
<th>Sense Primer</th>
<th>Probe</th>
<th>Antisense Primer</th>
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<td>AGCCGACATCTGGAAGCATAGACCTTCCA</td>
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<td>AGATTGCCAAGGATGATGCACCTTCTCAATA</td>
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*Sequenced in this study.

surgical procedures were performed according to the standards of Tokyo Women's Medical University Protocol on Laboratory Animals. Twenty mongrel dogs weighing between 17 and 28 kg were anesthetized with intravenous sodium pentobarbital (25 mg/kg). In 15 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 was injected into the cisterna magna at a rate of 5 mL/min. The dogs were tilted with the tail up for 15 minutes. The catheter then was removed, and the animals were allowed to recover. The 20 dogs were divided into 4 groups with 5 animals in each group. Group D0 comprised control animals, killed on day 0 without cisternal blood injection. Group D2 animals were killed on day 2 after the first injection on day 0. Group D7 and D14 animals were killed on days 7 and 14 after injections on days 0 and 2. Angiography of the cerebral arteries of each animal was performed on day 0 and before the animal was killed. The diameters of the basilar arteries were measured on angiographic film for evaluation of vasospasm.

### RNA Isolation

The animals were killed by injection of 100 mg/kg pentobarbital, exsanguinated, and perfused with 1500 to 2000 mL normal saline. Total RNAs were extracted from individual basilar arteries from each group with the use of TRIzol (GIBCO BRL), according to the manufacturer’s instructions. Possible traces of genomic DNA contaminating RNA preparations were removed by DNase I (Promega) digestion.

### Preparation of Dog-Specific Primers and Probes for Quantitative RT-PCR

The dog sequences of mRNAs for interleukin (IL)-1α, IL-6, IL-8, and IL-10, tumor necrosis factor (TNF)-α, E-secretin, fibronectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and collagen types I and IV were obtained by a database search with the Entrez program at NCBI (http://www.ncbi.nlm.nih.gov/Entrez). The dog sequences of transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF), collagen type III, and β-actin were not known, so partial nucleotide sequences for these genes were determined. The cDNA was synthesized from 1 μg total RNA extracted from canine middle cerebral arteries by the murine myeloma leukemia virus RT (Gibco BRL) and oligo (dT) primer (Gibco BRL). The cDNAs were amplified by PCR with degenerate oligo primers that were designed on the basis of the human and mouse nucleotide sequences of the target genes. The PCR products were directly sequenced with an Applied Biosystem DNA Sequencer model 377 with a Taq Dye Deoxy Termination Cycle Sequence kit (Perkin Elmer). The dog sequences obtained, which represented >90% identity to the human sequences, were subsequently used to design the dog-specific primers and probes for quantitative RT-PCR (Table).

### Quantitative RT-PCR With Real-Time TaqMan Technology

To evaluate the expression level of the target genes, quantitative RT-PCR was performed with real-time TaqMan technology with a Sequence Detection System model 7700 (Perkin Elmer). Five serial dilutions of each total RNA sample (100, 50, 25, 12.5, and 6.25 ng total RNA) were analyzed for each target gene. The dog-specific detection probes were labeled with a reporter fluorescent dye, FAM (6-carboxylfluorescein), on the 5’ nucleotide and a quenching fluorescent dye, TAMRA (6-carboxy-tetramethyl-rhodamine), on the 3’ nucleotide. Amplification reactions (50 μL) contained total RNA samples, 1× TaqMan EZ buffer, 300 μmol/L dATP, dCTP, and dGTP, 600 μmol/L dUTP, 3 mmol/L manganese acetate, 5.0 U rTth DNA polymerase, 0.5 AmpErase uracil-N-glycosylase (UNG), 200 nmol/L each primer, and 100 nmol/L of each detection probe. The thermal cycling conditions were as follows: 2 minutes at 50°C for the initial step, 30 minutes at 60°C for reverse transcription, 5 minutes at 95°C for deactivation of UNG, 40 cycles of 20 seconds at 94°C for denaturation, and 1 minute at 60°C for annealing and extension. Ct values corresponded to the cycle number at which the fluorescent emission monitored in real time reached the threshold, which was set at 10 SD above the mean of baseline emission calculated from cycles 5 to 15. The Ct values decreased linearly with increasing target quantity and could be used as a quantitative measurement of the input target number. Total RNA concentrations from each sample were normalized by the quantity of β-actin mRNA, and the expression levels of target genes were evaluated by the ratio of the number of target mRNA to β-actin mRNA.

### Statistical Analysis

Statistical comparisons of arterial diameters and levels of mRNA in target genes between groups were evaluated by ANOVA and the Kruskal-Wallis test. Probability values of <0.05 were considered statistically significant.
Results

Vasospasm Model

Figure 1 shows the change in vessel caliber of basilar arteries after cisternal injection of blood. A significant reduction in vessel caliber was recognized on angiograms in the D2, D7, and D14 groups, compared with the D0 baseline. There was no difference in vessel caliber at baseline between groups. There was a significant difference in vessel caliber between D0, D2, D7, and D14 groups ($P<0.0001$).

Expression Levels of Cytokine/Chemokine (IL-1$\alpha$, IL-6, IL-8, IL-10, and TNF-$\alpha$)

The ratios of these mRNA to $\beta$-actin in the normal basilar artery were $<0.1$. There were significant differences of IL-1$\alpha$, IL-6, IL-8, and TNF-$\alpha$ mRNA between D0, D2, D7, and D14 groups ($P=0.0075$, 0.1039, 0.0310, and 0.0354, ANOVA; $P=0.0079$, 0.0196, 0.0040, and 0.0949, Kruskal-Wallis). The patterns of difference in each mRNA were similar; the level of these gene expressions in the basilar artery was highest in D7. The average level of IL-8 mRNA in D7 was extremely high (131-fold) compared with that of D0: 17-fold for IL-1$\alpha$, 16-fold for IL-6, and 2.2-fold for TNF-$\alpha$ (Figure 2).

Expression Levels of Adhesion Molecule (E-secretin, fibronectin, ICAM-1, and VCAM-1)

The ratios of fibronectin and ICAM-1 mRNA to $\beta$-actin in the normal basilar artery were $>1$, whereas those of E-secretin and VCAM-1 were $<0.1$. There was a significant difference of ICAM-1 mRNA between D0, D2, D7, and D14 groups ($P=0.0017$, ANOVA; $P=0.0095$, Kruskal-Wallis). The average level of ICAM-1 mRNA was highest in D7 (1.67-fold) compared with that of D0 (Figure 3). The patterns of difference in E-Secretin and VCAM-1 mRNA were similar to that of ICAM-1, but there were no significant differences between groups. There was also no significant difference of fibronectin mRNA between groups.

Cytokine/Chemokine

Figure 2. Levels of mRNA expression for cytokine/chemokine (IL-1$\alpha$, IL-6, IL-8, IL-10, and TNF-$\alpha$) in basilar artery after SAH. Expression levels are expressed as relative ratios in Ct values for these genes to $\beta$-actin. Bars represent mean±SD (n=5, each group). There were significant differences of IL-1$\alpha$, IL-6, IL-8, and TNF-$\alpha$ mRNA between D0, D2, D7, and D14 groups ($P=0.0075$, 0.1039, 0.0310, and 0.0354, ANOVA; $P=0.0079$, 0.0196, 0.0040, and 0.0949, Kruskal-Wallis).
Expression Levels of Growth Factor/Extracellular Matrix (TGF-β, bFGF, and Collagen Types I, III, and IV)

The ratios of TGF-β, bFGF, and collagen types I, III, and IV mRNA to β-actin were 0.145, 0.516, 1.343, 0.030, and 0.095, respectively. There was a significant difference in collagen type I mRNA between D0, D2, D7, and D14 groups ($P<0.0001$, ANOVA; $P=0.0016$, Kruskal-Wallis). The average level of collagen type I mRNA was 4.1- and 4.5-fold for D2 and D7, respectively, and highest in D14 (10.9-fold) compared with that of D0 (Figure 4). There were no significant differences of TGF-β, bFGF, and collagen types III and IV mRNA between groups.

Discussion

Inflammatory cytokines, such as IL-1, IL-6, and IL-8, have been reported to be induced in the CSF beginning in the acute stage after subarachnoid hemorrhage (SAH). Increased inflammatory cytokine mRNA expression has been shown to occur after several types of injury to the brain such as ischemia and trauma. Astrocytes, endothelial cells, and neurons can synthesize cytokines, but microglia and macrophages appear to be the dominating sources of these cytokines. Because elevated levels of these cytokines in CSF are correlated with neurological damage, it is likely that they contribute to brain damage after SAH and may not be related to elevated levels of cytokines mRNA in the spastic artery.

Among genes related to inflammation, we showed that the levels of cytokines such as IL-1α, IL-6, and IL-8 that were not expressed much in the normal cerebral arteries were extremely highly elevated in the spastic artery. The pattern of maximum cytokine levels fits remarkably well with the time course of vasospasm. The relation between cytokine synthesis in the spastic artery and cerebral vasospasm could be explained by a causative role of cytokines in the cascade phosphorylation of intracellular kinases. IL-1α, IL-2, and IL-4 were detected in chronic periaortitis by PCR-assisted mRNA analysis but were not detected in normal aorta. Chronic adventitial treatment with IL-1α, IL-1β, and TNF-α was reported to induce selective hyperconstrictive responses to autacoids and coronary arteriosclerosis-like changes. These responses were significantly suppressed in a dose-dependent manner by cotreatment with a selective tyrosine kinase inhibitor, suggesting that tyrosine kinase activation may play an important role in mediating these effects. As general features, the cellular inflammatory cytokine production in response to various cellular stressors provided second-messenger signaling through a cascade of protein phosphorylations involving the mitogen-activated protein kinase pathway. Accumulating evidence shows that the activation of tyrosin kinase and/or the mitogen-activated protein kinase pathway leads to sustained contraction after SAH through actin-regulatory proteins.

ICAM-1 is a member of the immunoglobulin superfamily that is expressed on the endothelial surface in the early phase after tissue injury. ICAM-1 expression can be stimulated by various cytokines including lipopolysaccharides, TNF-α, interferon-γ, and IL-1. ICAM-1 mediates adherence and transendothelial migration of neutrophils in the area of tissue injury. The increased expression of endothelial ICAM-1 was reported in response to the deposition of blood around arteries in the rat.
femoral artery model of vasospasm. Endothelial ICAM-1 expression increased 3 hours after blood deposition, remained elevated for 24 hours, and returned to baseline levels by 48 hours. Antibodies to ICAM-1 administered intracisternally and systemically inhibited vasospasm in a rabbit single-hemorrhage and rat femoral artery model. It is conceivable that cellular adhesion is an important step in the initiation of vasospasm, but it is not necessarily important throughout the entire duration of the phenomenon. However, Handa et al reported that there was greater expression of ICAM-1 on the endothelial layer of the basilar artery in SAH rats and that the expression was observed also in the medial layer of the artery from 2 to 5 days after SAH. The current results are in accordance with this observation and did not indicate upregulation of ICAM-1 in the endothelium in the early stage of SAH, probably because the mRNA extracted from endothelial cells was negligible.

We previously observed that the expression of procollagen types I and III mRNA was increased in rat femoral arteries exposed to periarterial blood on day 7 and day 14. We now observed in the canine double-hemorrhage model that collagen type I mRNA expression started increasing at day 2 (4.1-fold), and reached its peak (10.9-fold) at day 14. Onoda et al reported that the application of antisense oligonucleotides for collagen type I gene inhibited arterial contraction in the rat femoral artery model and resulted in a marked decrease in α1(I) procollagen mRNA expression. These findings are consistent with the concept of increased collagen deposition in the vessel wall after SAH, on the basis of light and electron microscopic observations. Although the peak of increased expression of collagen type I mRNA was later than that of vasospasm, the expression had already elevated 4-fold at days 2 and 7. Increased synthesis of collagen type I may be related to increased artery wall stiffness, which may contribute to the sustained arterial narrowing of cerebral vasospasm.

The expression of the collagen type I gene was upregulated by cytokines such as IL-1, interferon-γ, TNF-α, and TNF-β and growth factors such as platelet-derived growth factor, TGF-β, in human vascular smooth muscle cells. We previously reported that TGF-β gene expression in rat femoral artery is stimulated after exposure to blood. The expression increased >3-fold 3 days after the application of blood, at which time the procollagen gene expression remained unchanged. The gene expression of TGF-β in the current model may have been elevated more before 2 days after SAH. Because of the stimulatory effects of IL-1α and TNF-α on collagen formation, the increase in IL-1α and TNF-α at 7 days is probably related to the maximum increase in collagen type I gene expression 14 days after SAH.

In conclusion, expression of genes related to inflammation, especially cytokines, was increased in the spastic artery, suggesting that inflammatory reaction of the cerebral artery may cause sustained contraction of the cerebral artery, probably through both the process of signal transduction to contraction and the change of components in the cerebral artery.

Acknowledgments

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Mechanisms that mediate vasospasm after subarachnoid hemorrhage (SAH) have been studied fairly widely. Despite this effort, changes that occur within the blood vessel wall at the molecular level are poorly defined. For example, relatively few studies have examined changes in expression of multiple genes in vessels from experimental models of SAH.

Previously, it has been difficult to accurately and reproducibly quantify mRNA levels using RT-PCR. The recent development of real time PCR methodology has eliminated much of this variability, and should allow for more routine and reliable quantification of PCR products. This method has recently been applied for studies of changes in gene expression in extracranial vessels and in brain tissue after experimental stroke.

In the present study, real time RT-PCR was used to quantify changes in expression of several inflammatory related genes in the basilar artery after SAH. The results suggest that expression of mRNA for several genes, including the cytokines IL-1alpha, IL-6, and IL-8, is increased markedly after SAH. Expression of these genes was relatively low in vessels under control conditions.

This work appears to be the first using real-time RT-PCR in cerebral vessels. The functional importance of the observed changes in gene expression was not evaluated in this study. However, the results support the concept that expression of components of the inflammatory cascade may contribute to vasospasm after SAH.

Frank M. Faraci, PhD, Guest Editor
Department of Internal Medicine
Cardiovascular Division
University of Iowa College of Medicine
Iowa City, Iowa

References
4. Aihara et al Gene Expressions After Subarachnoid Hemorrhage 217

Guest Editor
Frank M. Faraci, PhD,
Department of Internal Medicine
Cardiovascular Division
University of Iowa College of Medicine
Iowa City, Iowa

References
4. Aihara et al Gene Expressions After Subarachnoid Hemorrhage 217

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Frank M. Faraci, PhD,
Department of Internal Medicine
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University of Iowa College of Medicine
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References
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Frank M. Faraci, PhD,
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University of Iowa College of Medicine
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