Attenuation of experimental subarachnoid hemorrhage–induced increases in circulating intercellular adhesion molecule–1 and cerebral vasospasm by the endothelin-converting enzyme inhibitor CGS 26303

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Object. Adhesion molecules, including intercellular adhesion molecule–1 (ICAM-1), vascular cell adhesion molecule–1 (VCAM-1), and E-selectin, are important mediators of inflammation, and their levels are elevated in the serum of patients following aneurysmal subarachnoid hemorrhage (SAH). The investigators previously found that CGS 26303 is effective in preventing and reversing arterial narrowing in a rabbit model of SAH. The purpose of the present study was to examine whether levels of adhesion molecules are altered after treatment with CGS 26303 in this animal model.

Methods. New Zealand White rabbits were each injected with 3 ml of autologous blood in the cisterna magna, and intravenous treatment with CGS 26303 (30 mg/kg) was initiated 1 hour later. The compound was subsequently administered at 12, 24, and 36 hours post-SAH. Blood samples were collected at 48 hours post-SAH to measure ICAM-1, VCAM-1, and E-selectin levels. After the rabbits had been killed by perfusion–fixation, the basilar arteries (BAs) were removed and sliced, and their cross-sectional areas were measured.

Treatment with CGS 26303 attenuated arterial narrowing after SAH. Morphologically, corrugation of the internal elastic lamina of BAs was prominently observed in the SAH only and vehicle–treated SAH groups, but not in the CGS 26303–treated SAH group or in healthy controls. There were no significant differences in the levels of VCAM-1 among the four groups. The levels of E-selectin were increased in all animals subjected to SAH (those in the SAH only, SAH plus vehicle, and SAH plus CGS 26303 groups) compared with healthy controls (no SAH); however, the levels of ICAM-1 in the SAH only and SAH plus vehicle groups were significantly elevated (p < 0.001), and treatment with CGS 26303 reduced ICAM-1 to control levels following SAH.

Conclusions. These results show that ICAM-1 may play a role in mediating SAH-induced vasospasm and that a reduction of ICAM-1 levels after SAH may partly contribute to the antispastic effect of CGS 26303.

Key Words • cerebral vasospasm • inflammation • endothelin-converting enzyme inhibitor • E-selectin, intercellular adhesion molecule–1 • vascular cell adhesion molecule–1

Although cerebral vasospasm following SAH has been recognized for longer than a half century, it remains a major complication in patients suffering from SAH.8,11,12,44 As there continues to be no consistently effective treatment for cerebral vasospasm, the pathophysiological mechanisms contributing to arterial dysfunction require further investigation. Basic molecular and cellular research implicates two major hypotheses as key to cerebral vasospasm. One hypothesis centers on the roles of nitric oxide and nitric oxide synthase,21,25,35,40,41 and the other focuses on the role of inflammation.11 In the instance of SAH, a complex series of cellular and molecular events is elicited by the presence of a blood clot in the subarachnoid space and these culminate in a robust inflammatory response. Although the possible role of inflammation in the genesis of cerebral vasospasm has been recognized for some time, its cellular and molecular basis and putative importance have

Abbreviations used in this paper: BA = basilar artery; CSF = cerebrospinal fluid; ECE = endothelin-converting enzyme; ET = endothelin; HRP = horseradish peroxidase; ICAM-1 = intercellular adhesion molecule–1; IEL = internal elastic lamina; PBS = phosphate-buffered saline; PECAM = platelet-endothelial adhesion molecule; SAH = subarachnoid hemorrhage; sE-selectin = soluble E-selectin; sICAM = soluble ICAM; sVCAM = soluble vascular cell adhesion molecule.
Endothelin inhibition, inflammation, and vasospasm

not been clearly defined until recently. A growing body of evidence suggests that various constituents of the inflammatory response, including adhesion molecules, cytokines, leukocytes, immunoglobulins, and complement, may be critical in the pathogenesis of cerebral vasospasm. Adhesion molecules have been implicated in the pathogenesis of various diseases, including cerebral ischemia. The relationship between adhesion molecules and delayed cerebral vasospasm following SAH has also been investigated. Among the adhesion molecules, the immunoglobulin-like superfamily (ICAM-1, -2, and -3, VCAM-1, and PECAMs) and the selectin family have all been studied in the context of SAH-induced vasospasm in both animal and human studies.

Endothelin-1 is a potent vasoconstrictor that has been implicated in the pathogenesis of angiographic vasospasm. It is initially produced as a large prepropeptide composed of approximately 212 amino acids. Multiple steps of post-translational processing are required to produce an active 21–amino acid polypeptide with nanomolar affinity for specific membrane receptors. The final conversion step in this process involves the proteolytic cleavage of a relatively inactive precursor, big ET-1 (~ 40 amino acids in length), to form ET-1. This cleavage is mediated by ECE, a phosphoramidon-sensitive metalloproteinase.

Extracellular ET-1 can alter cerebrovascular tone via interactions with specific ET receptors on the membranes of smooth muscle and endothelial cells. As a result of the key role played by ECE in the production of active ET-1, it is plausible that ECE inhibitors could be of benefit in the treatment of diseases in which ET-1 overproduction or overactivity plays a pathogenic role. We previously investigated the effects of the ECE inhibitor, (S)-2 biphenyl-4yl-1-(1H-tetrazol-5-yl)-ethyl-amino-methyl phosphonic acid (CGS 26303), on SAH-induced cerebral vasospasm. Intravenous bolus injections of CGS 26303 (30 mg/kg) administered twice daily either before or after the establishment of arterial narrowing were effective in the management of cerebral vasospasm pursuant to SAH.

Findings of several studies point to a role for ET-1 in the enhancement of adhesion molecule expression and leukocyte adhesion. Both ICAM-1 and VCAM-1 are constitutively expressed on cerebral microvascular endothelial cell lines derived from the human brain, and the expression of these molecules can be upregulated by ET-1, ET-2, and ET-3 in a dose- and time-dependent manner. Data also indicate that ET-1 treatment induces the expression of E-selectin on these cells. Culturing human coronary artery endothelial cells alone with ET-1 for 4 to 6 hours resulted in increased expression of the endothelial adhesion molecules E-selectin and ICAM-1, and significantly increased the number of adhering neutrophils.

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Tissue Embedding

Basilar arteries were removed from the brainstems and the middle third of each artery was dissected for analysis. The arterial segments were washed several times with 0.1 mol/L PBS (pH 7.4), fixed in 1% osmium tetroxide in PBS for 1 hour at room temperature and then washed again with PBS. The vessel segments were dehydrated and placed in a 1:1 mixture of propylene oxide and epoxy resin overnight. The vessel segments were flat embedded the next day in 100% epoxy resin and allowed to polymerize at 60°C for 48 hours. Cross-sections (0.5 μm in thickness) of the BAs were cut on a Ultratome C ultramicrotome (Reichert), mounted on glass slides, and stained with 0.5% toluidine blue for morphometric analysis.
were analyzed by an investigator blinded to the treatment groups. Automated measurements of the luminal cross-sectional area were made using computer-assisted morphometry (Image 1, Universal Imaging Corp.). Areas of five cross-sections from a given animal were averaged to provide a single value for each animal. Group data are expressed as the means ± standard error of the means. For group comparisons, we performed an analysis of variance with the Bonferroni post-hoc test. Differences were considered significant at a probability value less than 0.05.

**Assays for ICAM-1, VCAM-1, and E-selectin**

We assayed the serum concentrations of ICAM-1, VCAM-1, and E-selectin by using commercially available enzyme-linked immunosorbent assay kits (R&D Systems) in accordance with the supplier’s instructions. The assays involved the simultaneous reaction of sICAM, sVCAM, and sE-selectin (in sample or standard) to a monoclonal antibody precoated on the walls of microtiter plate wells and to an unbound second antibody directed against a different molecular epitope. The second antibody was conjugated to HRP. After removal of unreacted reagents, bound sICAM–HRP, sVCAM–HRP, and sE-selectin–HRP antibodies were detected by a reaction with an HRP-specific substrate (tetramethylbenzidine), which yielded a colored product proportional to the concentrations of sICAM, sVCAM, and sE-selectin (determined relative to an appropriate standard curve). Absorption measurements were obtained at 450 nm using a microtiter plate reader.

**Results**

**General Observations**

Prior to perfusion–fixation, there were no significant differences among the treatment groups in the physiological parameters recorded, including body weight, pH, PaCO₂, PaO₂, and mean arterial blood pressure. A thick subarachnoid clot was observed over the basal surface of the brainstem in each animal subjected to SAH. Morphologically, the BAs in the SAH only and SAH plus vehicle groups exhibited substantial corrugation of the IEL. Corrugation of the IEL was less prominent in animals treated with CGS 26303. Vessels from healthy control animals and CGS 26303-treated animals had similar IELs.

**Cross-Sectional Areas of BAs**

The cross-sectional areas of BAs were significantly reduced in animals subjected to SAH (Fig. 1). The mean cross-sectional areas of BAs in the SAH only and SAH plus vehicle groups were reduced by 72 and 65%, respectively, when compared with the control (no SAH) group. Significant cerebral vasospasm did not develop in rabbits treated with CGS 26303 and the mean cross-sectional area in those animals was similar to that in controls (p = 0.237; Fig. 1). The protective effect of CGS 26303 achieved statistical significance when compared with the SAH only or SAH plus vehicle group (p < 0.001). The mean cross-sectional arterial area in the group receiving CGS 26303 is reduced significantly when compared with the SAH only or SAH plus vehicle group. The mean cross-sectional arterial area in the CGS 26303–treated group does not differ significantly from that in the control group (p = 0.237).

**Levels of VCAM-1, ICAM-1, and E-selectin**

Although there were trends for upregulation of VCAM-1 levels following induction of SAH, there were no statistically significant differences among the four groups (Fig. 2). Levels of E-selectin were increased significantly in all animals subjected to SAH (SAH only, SAH plus vehicle treatment, and SAH plus CGS 26303 treatment group) when compared to levels of E-selectin in healthy controls (p < 0.01, p < 0.005, and p < 0.005, respectively; Fig. 3). Treatment with CGS 26303 had no effect on the increased levels of E-selectin after SAH. Levels of ICAM-1 in the SAH only and SAH plus vehicle groups were reduced when compared with the levels of ICAM-1 in the control group and significantly elevated when compared with the CGS 26303–treated group (p < 0.001; Fig. 4). Treatment with CGS 26303 reduced ICAM-1 to a mean level not significantly different from that of the control group (p = 0.299).

**Discussion**

Adhesion molecules have been implicated in the pathogenesis of various diseases, including cerebral ischemia; however, the relationship between adhesion molecules and delayed cerebral vasospasm after SAH has been incompletely investigated. Among the adhesion molecules, members of the immunoglobulin-like superfamily (ICAM-1, -2, -3, VCAM-1, and PECAM), which are expressed by activated endothelium and act via binding to a leukocyte transmembrane protein, have been investigated for their roles in SAH-induced vasospasm in both animal and human studies. Handa et al. demonstrated that ICAM-1 was expressed not only in the endothelial layer but also in the medial layer of the BA, with a correlation between the severity and timing of vasospasm in experimental SAH in rats. In a rat femoral artery model of vasospasm, upregulation of ICAM-1 was an early response, and intraperitoneal administration of anti-ICAM monoclonal antibody significantly decreased the degree of vasospasm and the number of infiltrating leukocytes. Bavbek et al. showed that an intracisternal injection of anti-ICAM-1 and/or anti-CD18 attenuated vasospasm in a rabbit SAH model. Furthermore, administration of a monoclonal antibody against CD11 and CD18, the integrins that interact with ICAM-1, prevented vasospasm in a primate model of vasospasm. After aneurysmal SAH,
In a canine SAH model, increased expression of Endothelins, in particular Endothelin-1, was found to be maximal on Day 7 post-SAH, simultaneously with maximal narrowing of the BA lumen. In a canine SAH model, increased expression of ICAM-1 was found to be maximal on Day 7 post-SAH, simultaneously with maximal narrowing of the BA lumen. In this study, VCAM-1 levels did not differ significantly among groups. However, ICAM-1 levels in the CGS 26303 treatment group were significantly lower than those in the SAH only and SAH plus vehicle groups, and did not differ significantly from ICAM-1 levels in the control group. This finding implies that ECE inhibition may attenuate SAH-induced vasospasm by decreasing ICAM-1 production.

The selectin family of glycoprotein adhesion molecules (P-selectin, L-selectin, and E-selectin) has been implicated in the pathogenesis of cerebral vasospasm. The role of selectin family molecules in ischemic cerebrovascular disease has been widely investigated in both animal and human studies. These findings underscore the importance of the selectins in the pathophysiological characteristics of tissue injury after cerebral ischemia and reperfusion, and imply that blockade of the selectin family may provide therapeutic benefits. Nevertheless, evidence for a role of the selectins in the pathogenesis of delayed ischemic neurological deficit after aneurysmal SAH is scant. Polin et al. demonstrated elevation of levels of E-selectin, ICAM-1, and VCAM-1 in the CSF of patients with severe vasospasm. These findings also further support the hypothesis that pharmacological blockade of E-selectin could partially prevent SAH-induced vasospasm at medium and high doses. These findings also further support the hypothesis that E-selectin may contribute to the pathophysiology of cerebral vasospasm after SAH. Additional experiments are required to fully evaluate the potential use of anti–E-selectin antibody in the treatment of SAH-induced vasospasm. Given the functionally redundant nature of selectin activity, combined blockade of P-selectin and E-selectin might provide more therapeutic benefit than blockade of either molecule separately. Knockout mice with an absence of Adhesion molecules may provide further insight into the role of adhesion molecules in the pathogenesis of cerebral vasospasm after SAH. We have found E-selectin levels to be increased in animals subjected to SAH compared with control animals (unpublished data). Endothelin-converting enzyme inhibition did not decrease the production of E-selectin after SAH in the present experiment.

Mounting evidence has implicated ETs in the pathophysiology of cerebral vasospasm. Endothelins, in particular
ET-1, are among the most potent endogenous vasoconstrictors thus far identified. Clinical studies have shown that elevated levels of ETs are present in the CSF of patients with SAH, suggesting that ET-mediated vasoconstriction contributes to vascular constriction after SAH. The potential involvement of ETs in vasospasm has triggered considerable interest in therapeutic strategies that inhibit the biological effects of ET. Such strategies include the following: 1) blocking the biosynthesis of ETs; 2) reducing extracellular ET levels by specific anti-ET antibodies; and 3) antagonizing ET receptors. One promising approach for blocking the biosynthesis of ETs is to suppress the proteolytic conversion of the precursor peptide (big ET-1) to its vasoactive form (ET-1). Consequently, ECE inhibitors represent logical candidate compounds to block the activation of ETs and to limit spastic constriction. Several lines of evidence indicate that targeting the ET function at the level of proteolytic processing can be of therapeutic value in the treatment of cerebral vasospasm.

The effects of ECE inhibitors on the production of ICAM-1, VCAM-1, and E-selectin in the circulation have never been studied. We report the first evidence that an ECE inhibitor did not have effect on the upregulation of E-selectin after SAH. Levels of VCAM-1 displayed no significant changes after SAH, even when treated by an ECE inhibitor. Nevertheless, CGS 26303 treatment significantly lowered the production of ICAM-1 after SAH. Inhibition of ECE may attenuate SAH-induced vasospasm partially by reversal of the upregulation of ICAM-1.

**Conclusions**

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**Disclaimer**

There are no conflicts of interest related to this paper.

**References**

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