SYSTEMIC ADMINISTRATION OF SIMVASTATIN AFTER THE ONSET OF EXPERIMENTAL SUBARACHNOID HEMORRHAGE ATTENUATES CEREBRAL VASOSPASM

Matthew J. McGirt, M.D.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Gustavo Pradilla, M.D.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Federico G. Legnani, M.D.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Quoc-Anh Thai, M.D.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Pablo F. Recinos, B.A.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Rafael J. Tamargo, M.D.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Richard E. Clatterbuck, M.D., Ph.D.
Department of Neurosurgery,
The Johns Hopkins University
School of Medicine,
Baltimore, Maryland

Reprint requests:
Richard E. Clatterbuck, M.D., Ph.D.,
Assistant Professor of
Neurological Surgery,
Director of Cerebrovascular Research,
The Johns Hopkins Hospital,
600 North Wolfe Street,
MEYER 8-181,
Baltimore, MD 21287.
Email: rclatte1@jhmi.edu

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OBJECTIVE: Experimental evidence suggests that intercellular adhesion molecule-1 mediated leukocyte extravasation contributes to the pathogenesis of cerebral vasospasm. Simvastatin, an HMG-CoA reductase inhibitor, decreases intercellular adhesion molecule-1 expression and competitively inhibits leukocyte intercellular adhesion molecule-1 binding. We hypothesized that administration of simvastatin after the onset of subarachnoid hemorrhage (SAH) would attenuate perivascular granulocyte migration and ameliorate cerebral vasospasm in a rabbit model of SAH.

METHODS: New Zealand white rabbits (n = 15) underwent injection of autologous blood into the cisterna magna or sham surgery followed by subcutaneous injection of simvastatin (40 mg/kg) or vehicle 30 minutes, 24 hours, and 48 hours after SAH or sham surgery. Seventy-two hours later, basilar artery lumen diameter was measured by in situ perfusion/fixation and image analysis. CD-18 monoclonal antibody stained perivascular granulocytes and macrophages were counted under light microscopy.

RESULTS: In vehicle treated rabbits, mean ± standard deviation basilar artery diameter was reduced 3 days after SAH (n = 5) versus sham (n = 5) rabbits (0.49 ± 0.08 mm versus 0.75 ± 0.03 mm, $P < 0.01$). After SAH, mean ± standard deviation basilar artery diameter was greater in simvastatin (n = 5) treated rabbits versus vehicle (n = 5) (0.63 ± 0.04 mm versus 0.49 ± 0.08 mm, $P < 0.01$). In vehicle treated rabbits, SAH resulted in an increase in the mean ± standard deviation perivascular CD18 cell count (sham-vehicle, 2.8 ± 2; SAH-vehicle 90 ± 27; $P < 0.01$). Subcutaneous administration of simvastatin attenuated this increase in perivascular CD18-positive cells after SAH (SAH statin, 41.6 ± 13; SAH vehicle, 90 ± 27; $P < 0.001$).

CONCLUSION: Subcutaneous administration of simvastatin after the onset of SAH attenuates perivascular granulocyte migration and ameliorates basilar artery vasospasm after experimental SAH in rabbits. 5-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, such as simvastatin, may potentially serve as agents in the prevention of cerebral vasospasm after SAH.

KEY WORDS: Cerebral vasospasm, Intercellular adhesion molecule-1, Simvastatin, Subarachnoid hemorrhage

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Delayed cerebral vasospasm is a major cause of morbidity and mortality in patients with aneurysmal subarachnoid hemorrhage (SAH) (20). Current medical treatments fail to prevent vasospasm, and the pathogenesis of this disease remains incompletely understood (21). The role of inflammation in the pathogenesis of cerebral vasospasm has been previously recognized. However, the importance of leukocytes has only recently been appreciated (3, 4, 6, 7, 9, 12, 13, 28, 31, 34–37). Leukocyte migration into the central nervous system is initiated by interleukin (IL) and chemokine-stimulated leukocyte activation, proliferation, and chemotaxis (17). Leukocyte function antigen (LFA)-1 binding to endothelial intercellular adhesion molecule (ICAM)-1 is necessary to initiate leukocyte migration through the vascular media and subsequent migration into the central nervous system (17).

Experimental and clinical evidence suggests that ICAM-1 mediated leukocyte migration may play a crucial role in the pathogenesis of cerebral vasospasm (6, 7, 13, 31, 36, 37). SAH results in increased endothelial ICAM-1 expression (1, 18, 30, 36) and resultant perivascular leukocyte migration (37). Furthermore, serum ICAM-1 level correlates with the onset of cerebral vasospasm (25). Perivascular chemokine-activated inflammatory cells synthesize and release endothelin-1, a potent vasoconstrictor, as well as superoxide free radicals, leading to inactivation of nitric oxide (NO) and vasoconstriction (11, 27). Anti-ICAM-1 antibodies decrease leukocyte migration and attenuate cerebral vasospasm after SAH (2, 7, 26).

Vasoconstriction also seems to be perpetuated by an imbalance in the availability of periadventitial NO. NO synthase, the enzyme responsible for NO production, metabolizes L-arginine to NO and citrulline, and constitutive expression of its endothelial isoform (eNOS) is normally observed in cerebrovascular endothelium. After SAH, eNOS messenger ribonucleic acid (mRNA) and protein decrease significantly (29). Although the mechanism of eNOS reduction is not known, it causes a reduction in NO production that results in disruption of endothelial vasodilation after SAH.

Simvastatin, an 5-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, decreases ICAM-1 expression and competitively inhibits LFA-1/ICAM-1 interaction (38, 39). Statins, such as simvastatin, interfere with multiple steps of leukocyte recruitment and migration by inhibiting monocyte and endothelial production of ILs, chemokines, and matrix metalloproteinase-9 (3, 8, 15, 22, 32, 33). The fact that inhibition of LFA-1/ICAM-1 interaction alone attenuates posthemorrhagic vasospasm (2, 7, 26) suggests that an agent affecting multiple steps in leukocyte migration, such as simvastatin, may have more profound effects on cerebral vasospasm. Furthermore, simvastatin directly up-regulates eNOS expression in human endothelial cells exposed to hypoxia by increasing the stability of eNOS mRNA and prolonging its half-life (23). An increase in eNOS mRNA and protein, eNOS enzymatic activity, and cerebral blood flow have been observed after statin administration (10). Similarly, administration of simvastatin to mice before and after SAH increased protein levels of eNOS 72 hours after hemorrhage (24).

The administration of simvastatin for 14 days before SAH attenuated cerebral vasospasm in a murine model of SAH (24). The effect of simvastatin administered after the onset of SAH remains unknown. We hypothesized that systemic administration of simvastatin after the onset of SAH would attenuate perivascular leukocyte migration and ameliorate cerebral vasospasm in a rabbit model of SAH.

MATERIALS AND METHODS

All experiments were approved by the Johns Hopkins University Animal Care and Use Committee. Simvastatin (Zocor; Merck and Co., Inc., West Point, PA) was chemically activated by alkaline hydrolysis, as previously described (16, 23). New Zealand white rabbits (*Oryctolagus cuniculus*; Robinson Co., Winston Salem, NC) weighing 1.5 to 2.5 kg were subcutaneously injected once daily 30 minutes, 24 hours, and 48 hours after sham or SAH surgery with simvastatin (40 mg/kg, 5 ml) or a corresponding volume of vehicle. Fifteen rabbits were randomized to three groups: vehicle-sham (n = 5), vehicle-SAH (n = 5), and simvastatin-SAH, (n = 5).

SAH Model

We have previously described the surgical technique for the rabbit SAH model (14). Animals were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg; 100 mg/ml; ketamine HCL; Abbot Laboratories, Chicago, IL) and xylazine (10 mg/kg; 100 mg/ml; Xyla-ject; Phoenix Pharmaceutical, St. Joseph, MO). After induction of anesthesia, ceftriaxone intramuscularly (20 mg/kg) was administered, and through a midline incision, the atlanto-occipital membrane was exposed. Animals in the sham group were irrigated with saline solution and closed. In the other two experimental groups (SAH-vehicle and SAH-simvastatin), 1 ml of cerebrospinal fluid was aspirated by cisternal puncture. Nonheparinized blood from the central ear artery, 1.5 ml, was then injected into the cisterna magna. After 30 minutes in the head-down position, the animals were allowed to recover.

Basilar Artery Lumen Measurement

Peak vasospasm in the rabbit model of SAH is present at 72 hours after blood injection into the cisterna magna. All animals were killed at 72 hours after induction of experimental SAH by intraperitoneal injection of sodium pentobarbital (200 mg/kg), and in situ perfusion/fixation was performed. Transcardiac perfusion was performed with 300 ml of normal saline solution followed by 500 ml of ice-cold freshly depolymerized 4% paraformaldehyde in 0.1 mol/L phosphate buffer. Solutions were delivered by a Watson Marlow peristaltic pump at 100 rpm (25 ml/min).

The basilar arteries and the brainstem were harvested en bloc and cryoprotected in 20% sucrose in 0.1 mol/L phosphate buffer for 3 days at 4°C. After snap-freezing in dry ice equilibrated isopentane cooled to −60°C, the specimens were stored at −80°C. The specimens were mounted in tissue freezing compound (Triangle Biomedical Sciences, Durham, NC) and sectioned transversely in 10 μm slices with a microtome cryostat (Microm GmbH, Walldorf, Germany) at 200 μm intervals beginning at the basilar termination. The tissue slices were mounted on Superfrost Plus slides (Fisher Scientific, Hanover Park, IL) and stained with hematoxylin-eosin followed by coverslip mounting with Permount medium (Fischer Chemicals, Fairlawn, NJ).

Vessel patency was quantified by measuring the basilar artery circumference with the use of a computerized image analysis system (MCID; Imaging Research, St. Catharines, Ontario, Canada). To correct for vessel deformation and off-transverse sections, the internal circumferences of six different sections of each vessel separated by 200 μm were measured and averaged. The luminal cross-sectional area of each vessel

was estimated with the use of the calculated radius (r) value obtained from the measured circumference (r = measured circumference/2π; area of circle = πr^2).

Perivascular Granulocyte Count

Frozen sections from the basilar artery were cut in 20 μm sections and mounted as described above. Slides were left to dry at room temperature for 20 minutes and were then fixed with methanol for 10 minutes. After fixation, slides were washed with phosphate-buffered saline and blocked for 1 hour with normal goat serum and triton x-100. Primary antibody incubation was performed at room temperature overnight using anti-CD-18 antibody (Veterinary Medical Research and Development, Inc., Pullman, WA) at a dilution of 1:200. Slides were then washed and incubated with secondary antibody. The immune reaction was developed with diaminobenzidine tetrahydrochloride used as a chromogen (Vector Laboratories, Inc., Burlingame, CA).

Positively stained macrophages and granulocytes were counted by light microscopy (Olympus BH-2, Optical Elements Corp, Melville, NY) with the ×40 objective, and results expressed as number of cells per high-powered field. A representative area in the adventitia from vessel sections was chosen, and perivascular granulocytes and macrophages were counted and averaged from three sections (≥100 μm apart) of each vessel.

Statistical Analysis

The mean diameter of the basilar arteries was calculated after digital measurement of the cross sectional circumference. The mean lumen patency of basilar arteries was also expressed as percent lumen patency obtained by dividing the mean luminal cross sectional area of each SAH group by the mean luminal cross sectional area of the sham group. The mean number of perivascular macrophages and granulocytes and vessel diameters were compared using an analysis of variance followed by a Newman-Keuls post hoc analysis. Statistical analysis was performed using Prism 4 for windows version 4.01 (GraphPad Software, Inc., San Diego, CA).

RESULTS

After SAH, five rabbits were treated with simvastatin and five rabbits with vehicle. Body weight did not differ between simvastatin (3.45 ± 0.25 kg) and vehicle-SAH groups (3.33 ± 0.46 kg). Subarachnoid blood was confirmed within the cisterna magna in all cases. No animals died before killing for basilar artery harvesting. Five rabbits underwent sham surgery and vehicle administration.

In vehicle treated rabbits, mean ± standard deviation (SD) basilar artery diameter was reduced 3 days after SAH (n = 5) versus sham (n = 5) rabbits (0.49 ± 0.08 mm versus 0.75 ± 0.03 mm, $P < 0.01$), resulting in a 35 ± 5% reduction in basilar artery lumen patency. After SAH, mean ± SD basilar artery diameter was greater in simvastatin (n = 5) treated rabbits versus vehicle (n = 5; 0.63 ± 0.04 mm versus 0.49 ± 0.08 mm, $P < 0.01$), attenuating the vasospasm reduction in basilar artery lumen patency from 35 ± 5% to 16 ± 2% (*Fig. 1*).

In vehicle treated rabbits, SAH resulted in an increase in the mean ± SD perivascular CD18-positive cell count 72 hours after SAH (sham-vehicle, 2.8 ± 2; SAH-vehicle 90 ± 27; $P < 0.01$). Subcutaneous administration of simvastatin reduced the mean ± SD perivascular CD18-positive cell count 72 hours after SAH (SAH-statin, 41.6 ± 13 versus SAH-vehicle, 90 ± 27; $P < 0.001$) (*Figs. 2* and *3*).

DISCUSSION

In this study, subcutaneous administration of simvastatin after the onset of SAH resulted in a twofold reduction in both morphological cerebral vasospasm and perivascular granulocyte migration resulting from SAH. After SAH, a 35% reduction in lumen patency was observed in rabbits receiving vehicle compared with only a 16% reduction in those receiving simvastatin. Mean perivascular CD18-positive count increased markedly in SAH versus control rabbits. However, SAH induced perivascular migration of granulocytes was reduced significantly with the administration of simvastatin. Although this does not prove a causal relationship between attenuation in inflammation and reduction in vasospasm, the known role of pro-inflammatory cascades in the pathogenesis of cerebral vasospasm suggests that the potent anti-inflammatory effects of simvastatin may underlie its efficacy after SAH.

Several studies have demonstrated elevation of mediators known to induce leukocyte recruitment and migration into the central nervous sytem after SAH. IL-1β, IL-6, and platelet-activating factor have been reported to be elevated in jugular venous blood of patients with SAH (19) and to correlate with the timing and degree of cerebral vasospasm (12) as well as to activate mononuclear cells to synthesize and release endothelin-1, a potent vasoconstrictor, into the cerebrospinal fluid of patients with SAH (11). E-selectin (30), soluble ICAM-1 (25, 30), and soluble vascular cell adhesion molecule-1 (30) are elevated in the cerebrospinal fluid of patients with

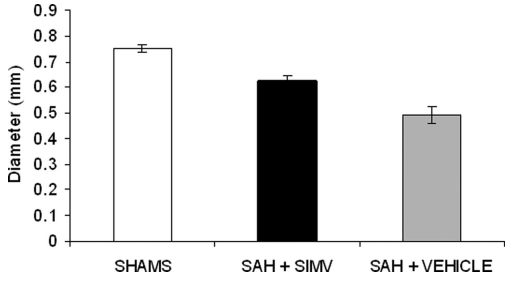

FIGURE 1. *Histogram of vessel diameter. The mean ± standard error of the mean basilar artery lumen diameter after experimental SAH in rabbits treated with simvastatin (40 mg/kg) versus vehicle 30 minutes, 24 hours, and 48 hours after SAH. The mean basilar artery diameter was reduced in SAH versus sham rabbits (0.49 ± 0.08 mm versus 0.75 ± 0.03 mm, P < 0.01). After SAH, the basilar artery diameter was greater in simvastatin versus vehicle treated rabbits (0.63 ± 0.04 mm versus 0.49 ± 0.08 mm, P < 0.01).*

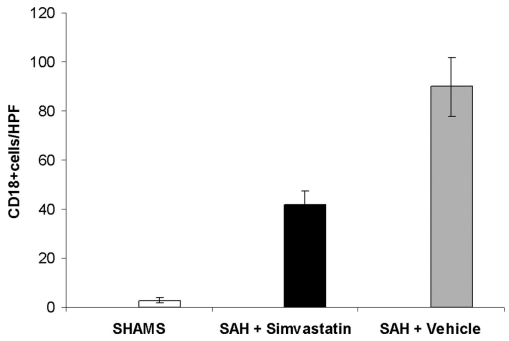

FIGURE 2. *Histogram of perivascular CD18 cell count. The mean ± standard error of the mean perivascular CD18 cell count per high-powered field under light microscopy 72 hours after experimental SAH in rabbits treated with simvastatin (40 mg/kg) versus vehicle 30 minutes, 24 hours, and 48 hours after SAH. Simvastatin versus vehicle significantly reduced the perivascular CD18 cell count after SAH (SAH statin, 41.6 ± 6 versus SAH vehicle, 90 ± 12; P < 0.01).*

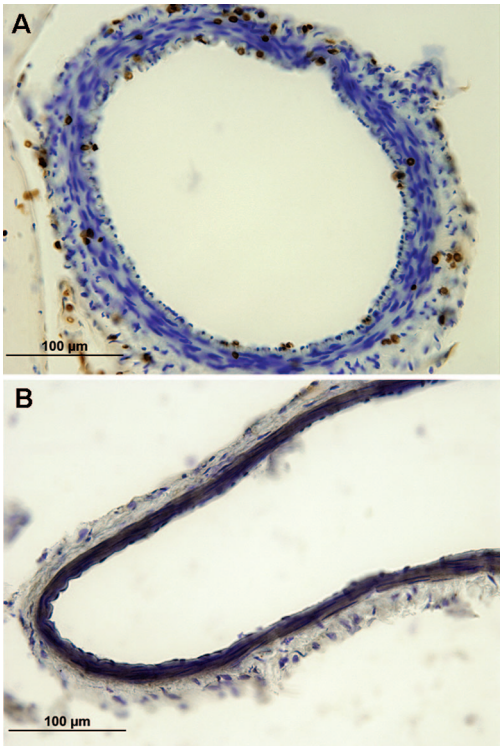

FIGURE 3. *Photomicrographs showing perivascular inflammatory cells. Anti-CD-18 stained basilar artery sections from vehicle (A) and simvastatin (B) treated rabbits as seen under light microscopy (original magnification, ×40) 72 hours after subarachnoid hemorrhage. CD18-positive macrophages and granulocytes are markedly reduced in simvastatin treated animals.*

SAH. These mediators increased and decreased over time, correlating with the onset and resolution of cerebral vasospasm. Posthemorrhagic vasospasm in rat femoral and basilar arteries is associated with ICAM-1 up-regulation (18, 36). Aihara et al. (1) also observed a significant increase in basilar artery expression of IL-1, IL-6, IL-8, and ICAM-1 mRNA after SAH in canines.

In other disease models, statins markedly suppress these key mediators of inflammation, which may underlie the pathogenesis of cerebral vasospasm after SAH. Rezaie-Majd et al. (32) demonstrated that simvastatin decreased serum monocyte expression of IL-6, IL-8, and monocyte chemoattractant protein-1. Statin therapy also reduces interferon induced expression of class II major histocompatibility complexes on antigen-presenting cells (22), decreasing T-cell activation in vivo and inhibiting the release of proinflammatory cytokines (8). Weitz-Schmidt et al. (38) reported that lovastatin inhibits LFA-1/ICAM-1 mediated cell adhesion by directly binding to LFA-1. Statins inhibit monocytes and endothelial cells from producing ILs and chemokines (8, 22, 32, 33), directly block LFA-1/ICAM-1 mediated cell adhesion (38), and inhibit secretion of matrix metalloproteinase-9 (3, 15), thereby interfering with multiple steps of leukocyte recruitment and migration into the central nervous system. The fact that inhibition of the LFA-1/ICAM-1 interaction alone with anti-ICAM-1 or anti-LFA-1 antibodies attenuates posthemorrhagic vasospasm (2, 7, 26) suggests that an agent with broader anti-inflammatory effects may markedly reduce cerebral vasospasm after SAH, as demonstrated in this study.

Subcutaneous administration of simvastatin after the onset of SAH attenuated perivascular leukocyte migration and ameliorated basilar artery vasospasm after experimental SAH in rabbits. 5-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, such as simvastatin, may potentially serve as agents in the prevention of cerebral vasospasm in patients after SAH.

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COMMENTS

McGirt et al. have examined the efficacy of simvastatin in the prevention of cerebral vasospasm after subarachnoid hemorrhage (SAH) in a well-established rabbit model. They demonstrated that simvastatin (40 mg/kg) administered at 30 minutes, and at 24 and 48 hours after SAH attenuated vasospasm as assessed at 72 hours with basilar artery diameter. Furthermore, administration of simvastatin diminished the increase in perivascular leukocytes seen after SAH.

This study provides additional data to a growing body of evidence examining the use of statins in the setting of SAH. The pleiotropic effects of statins have been well recognized for some time, with beneficial cholesterol-independent effects ascribed to reduction of endothelial dysfunction, augmentation of endothelial nitric oxide synthase, inhibition of inflammatory responses, and modulation of procoagulant activity and platelet function (1). Initial data demonstrated that simvastatin augmented endothelial nitric oxide synthase expression and ameliorated vasospasm in a murine model (2). Subsequently, a retrospective review suggested that statin use improved functional

outcome at 14 days and diminished several indices of vasospasm in patients with SAH (3). Recently, Tseng et al. (5) conducted a Phase II randomized controlled trial examining the effects of pravastatin on cerebrovascular regulation and vasospasm-related complications after SAH. Acute treatment with pravastatin seemed to be safe and diminished vasospasm, improved cerebral autoregulation, reduced delayed ischemic neurological deficits, and improved outcome at discharge. Collectively, these results seem promising, although a recent retrospective review suggested that patients with a history of statin use have a higher risk for vasospasm (perhaps from abrupt statin withdrawal after admission) (4). In any event, the acute use of statins in the management of patients harboring ruptured aneurysms requires additional investigation.

It would be interesting to investigate whether the results observed could be totally attributed to cholesterol-independent mechanisms of statins such as reduction in inflammation, as the authors suggest. The short period of administration likely favors cholesterol-independent mechanisms. In summary, the authors have contributed important background scientific data providing impetus for the further study of statins in the setting of SAH.

Aaron S. Dumont
Sagi Harnof
Neal F. Kassell
Charlottesville, Virginia

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This study extends previous experimental work suggesting that 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors or statins prevent or reduce vasospasm after experimental and clinical SAH. These drugs have effects other than the lowering of serum cholesterol, including inhibition of inflammation and upregulation of endothelial nitric oxide synthase. Because inflammation and reduction in endothelial nitric oxide synthase have been postulated to be involved in the pathogenesis of vasospasm, it is of great interest that the statins may reduce vasospasm. Preliminary clinical evidence also has been presented recently (2, 3). The effects of statins on endothelial nitric oxide synthase are interesting in view of data suggesting that polymorphisms in this gene are related to aneurysmal SAH and vasospasm (1). Therefore, this study deserves confirmation in a blinded, randomized study in a larger animal model of chronic vasospasm.

There are some experimental details to discuss as well. The immunohistochemical studies may not have included appropriate positive and negative controls, so the specificity of this staining can be questioned. Proper stereological counting methods (4) were not used to count the cells, and this can introduce significant bias. I am concerned about diminishing group sizes in experimental studies. The authors had five animals per group. The statistical power of the differences is probably extremely low. We would be very skeptical about a clinical trial reporting differences between five treated and five untreated patients. More animals should be included, and the study should be blinded. The authors did use randomization, which also is a critical aspect of experimental design.

R. Loch Macdonald
Chicago, Illinois

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With use of a rabbit model of cerebral vasospasm and a total of 15 animals in a controlled experiment, simvastatin treatment seemed to cause a modest reduction of basilar artery narrowing and an attenuation of perivascular granulocyte accumulation. The investigators can be credited for a succinct and well-prepared report that couples the arterial effect of the investigational drug with some measure of its proposed mechanism of action. In balancing the cumulative evidence behind an inflammatory pathogenesis of cerebral vasospasm and these preliminary but encouraging results, workers in the field will be able to decide whether to pursue this avenue of treatment with an 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor to either duplicate the results or demonstrate them in other animal models of vasospasm.

J. Max Findlay
Edmonton, Canada

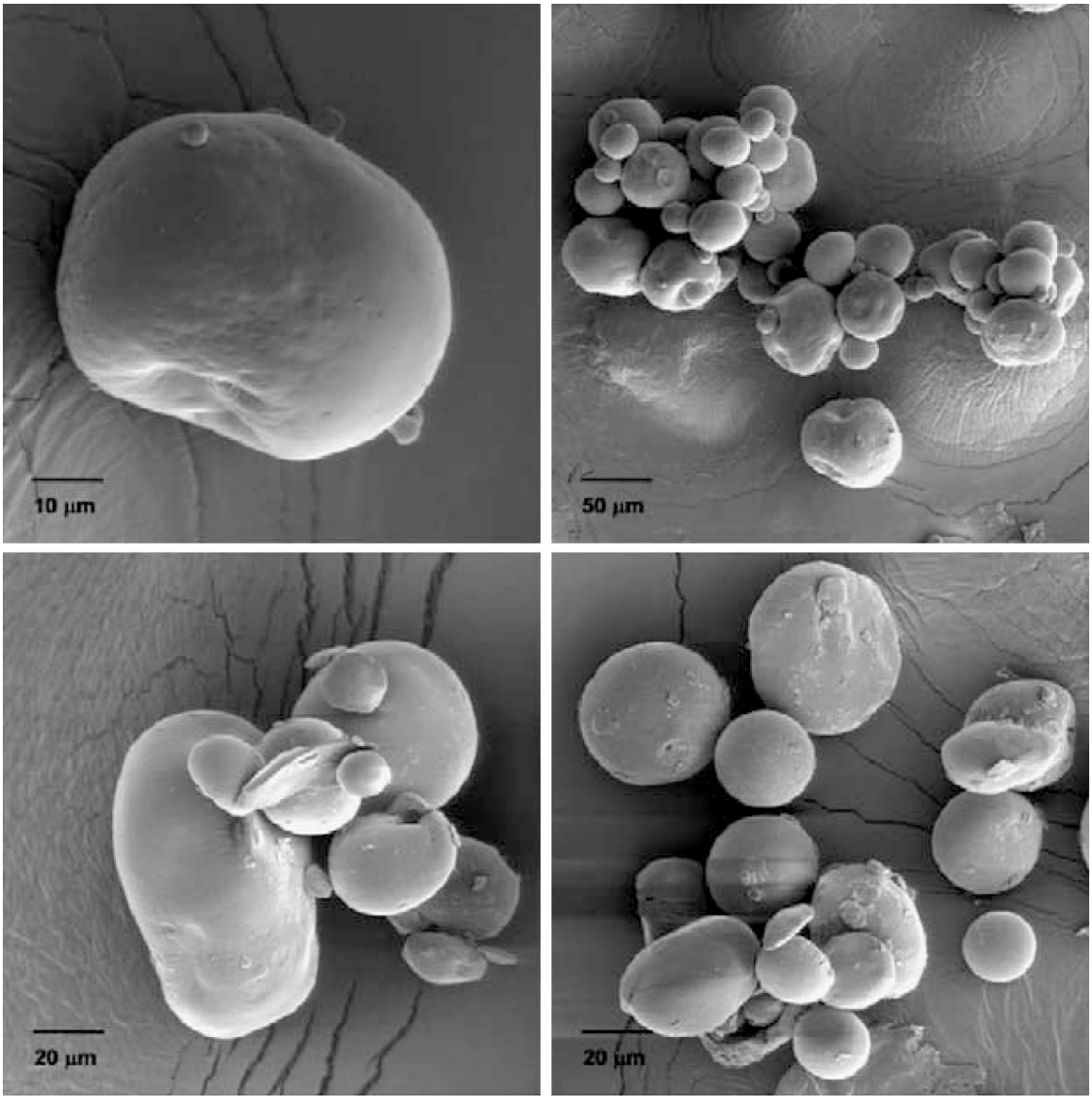
The authors use a well-characterized model of vasospasm to investigate whether a statin (simvastatin) would attenuate vasospasm. Simvastatin is known to decrease intercellular adhesion molecule-1 expression and inhibits leukocyte binding. In other publications, the authors from this laboratory have developed a line of reasoning demonstrating that vasospasm after SAH may in large part be caused by an inflammatory response. This set of experiments represents yet another attempt to block that inflammatory response.

The authors are able to demonstrate a reduction in the degree of vasospasm as measured by percent luminal patency of the basilar artery when simvastatin is used compared with vehicle alone. In addition, the number of inflammatory cells present are diminished in the simvastatin treated animals. The study offers evidence that vasospasm can be attenuated, but not ameliorated, by simvastatin. It is of interest because a growing number of patients and the general population are taking statins on a chronic basis. One question that is currently being looked at by a number of investigators is whether patients who present with SAH and are on statins have a low inci-

dence of vasospasm compared with those who are not. The current authors' experimental evidence, which suggested that patients on statin will fare better after SAH, is a result of vasospasm.

As the statin story unfolds, the implications for vasospasm and prevention of stroke after SAH may be quite important. The authors' current work would certainly indicate this to be the case, and they are to be commended for their contribution.

Christopher S. Ogilvy
Boston, Massachusetts



SEM micrographs of drug/polymer microparticles containing drug nanoparticles. (Thote AJ, Gupta RB. Formation of nanoparticles of a hydrophilic drug using supercritical carbon dioxide and microencapsulation for sustained release. ***Dis Mon.*** *2005 Jun;51(6):362–73.)*