Simvastatin Suppresses the Progression of Experimentally Induced Cerebral Aneurysms in Rats

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Background and Purpose—The pathophysiology of cerebral aneurysms (CAs) is linked to chronic inflammation and degradation of extracellular matrix in vascular walls. Because statins have protective effects on various vascular diseases independent of their lipid-lowering effects, we investigated the effect of simvastatin on CA progression.

Methods—CAs were induced in Sprague-Dawley rats with or without oral administration of simvastatin. The size and media thickness of CAs was evaluated 3 months after aneurysm induction. Expression of macrophage chemoattractant protein-1, vascular cell adhesion molecule-1, endothelial nitric oxide synthase, interleukin-1β, inducible nitric oxide synthase, matrix metalloproteinase-2, and matrix metalloproteinase-9 in aneurysmal walls was examined by reverse transcriptase–polymerase chain reaction and immunohistochemistry. To examine whether simvastatin has a suppressive effect on preexisting CAs, simvastatin administration started at 1 month after aneurysm induction.

Results—Rats treated with simvastatin exhibited a significant increase in media thickness and a significant reduction in aneurysmal size compared with control rats. Treatment with simvastatin resulted in reduced expression of macrophage chemoattractant protein-1 and vascular cell adhesion molecule-1, increased expression of endothelial nitric oxide synthase, and reduced the number of macrophage infiltration. In quantitative polymerase chain reaction and immunohistochemistry, simvastatin significantly inhibited upregulated expression of interleukin-1β, inducible nitric oxide synthase, matrix metalloproteinase-2, and matrix metalloproteinase-9 associated with CA progression. Gelatin zymography revealed decreased activity of matrix metalloproteinase-2 and matrix metalloproteinase-9 in aneurysmal walls by simvastatin treatment. Simvastatin also effectively inhibited aneurysm enlargement and thinning of the media of preexisting CAs.

Conclusions—Treatment with simvastatin suppresses the development of CAs by inhibiting inflammatory reactions in aneurysmal walls. Simvastatin also has a preventive effect on the progression of preexisting CAs. Simvastatin is a promising candidate of a novel medical treatment for the prevention of CA progression. (Stroke. 2008;39:1276-1285.)

Key Words: animal model ■ cerebral aneurysm ■ inflammation ■ macrophage ■ simvastatin

Subarachnoid hemorrhage due to the rupture of cerebral aneurysms (CAs) is a life-threatening disease. Despite the recent technical advancement of microneurosurgery and endovascular surgery, the mortality of subarachnoid hemorrhage is still high because a substantial number of patients die before arriving at a hospital. Therefore, it is important to develop novel therapeutic approaches to prevent the rupture of CAs for public health. Although detailed mechanisms of the initiation, progression, and rupture of CAs are not fully understood, recent investigations have emphasized disease mechanisms involving chronic inflammation and degradation of extracellular matrix in the vascular wall.

Three hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are widely used cholesterol-lowering drugs. In addition to the cholesterol-lowering effect, statins exert vascular protective effects known as “pleiotropic” effects. Statins increase nitric oxide bioavailability by the upregulation and activation of endothelial nitric oxide synthase (eNOS). Statins also inhibit the expression of several matrix metalloproteinases (MMPs), including MMP-2 and MMP-9, by smooth muscle cells and macrophages. Indeed, a large clinical trial has demonstrated that statins reduced cardiovascular events in patients, irrespective of serum cholesterol level.

The purpose of the present study is to determine whether treatment with an hydroxy-3-methylglutaryl coenzyme A reductase inhibitor would have a favorable effect on the progression of CAs and to explore molecular mechanisms underlying it. To address these questions, we examined the effects of simvastatin, one of the most commonly used
hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in clinical practice, on experimentally induced CAs in rats.

**Materials and Methods**

**Experimentally Induced Cerebral Aneurysms**

CAs were induced as previously described by Nagata et al. After the induction of pentobarbital anesthesia (50 mg/kg intraperitoneally), the left common carotid artery and posterior branches of the bilateral renal arteries were ligated at the same time with 10-0 nylon in 7-week-old male Sprague-Dawley rats (Oriental Bioservice, Osaka, Japan). A total of 96 rats were operated on. Animals were fed a high-salt diet containing 8% sodium chloride. Blood pressure was measured by tail-cuff method with rats kept in a small container without any anesthesia. We compared the tail-cuff measurement with invasive measurement in a pilot study to validate our measuring method. Blood pressure was measured at least twice in each animal. Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals.

One or 3 months after aneurysm induction, the anterior cerebral artery/olfactory artery bifurcation was stripped and observed under a light microscope after Elastica van Gieson staining. To evaluate the pathological changes occurring in aneurysmal walls, we analyzed the degeneration of internal elastic lamina (IEL), the thinning of medial smooth muscle cell layer, and aneurysm size. IEL was classified into the following 3 categories: continuous, fragmented, and completely absent. The ratio of the thickness of the media to the thickness of the media in surrounding normal arterial walls. Aneurysm size was calculated as the mean of the maximal longitudinal diameter and the maximal transverse diameter. The images of CA were provided in supplemental Figure I, available online at http://stroke.ahajournals.org.

**Administration of Simvastatin**

Simvastatin was provided by Banyu Pharmaceutical Co Ltd (Tokyo, Japan) and Merck Co Inc (Rahway, NJ). Simvastatin (25 mg/kg per day) was orally given to rats mixed with a high-salt diet. The dose of simvastatin was calculated based on the average daily food consumption (25 g/d) and comparable to doses used in previous studies in rats, in which this amount of simvastatin was proved to adequately inhibit the hydroxy-3-methylglutaryl coenzyme A reductase activity in vivo. The serum concentration of simvastatin was monitored by high-performance liquid chromatography. The serum concentration of simvastatin was measured by Cholesterol E-test (Wako, Tokyo, Japan) according to the manufacture’s directions. To examine the effect of simvastatin on the initiation and progression of CAs, treatment with simvastatin started on the day of aneurysm induction. Control rats were fed only a high-salt diet from the day of aneurysm induction. Simvastatin started on the day of aneurysm induction. Control rats were fed a normal diet after 1 month of aneurysm induction. After aneurysm induction, animals in both groups were fed a normal diet and comparable to doses used in previous studies in rats.

**Immunohistochemistry**

At the designated time point after aneurysm induction, rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. The anterior cerebral artery/olfactory artery bifurcation was stripped and embedded in OCT compound (Sakura, Tokyo, Japan). The anterior cerebral artery/olfactory artery bifurcation was stripped and observed under a light microscope after Elastica van Gieson staining. To evaluate the pathological changes occurring in aneurysmal walls, we analyzed the degeneration of internal elastic lamina (IEL), the thinning of medial smooth muscle cell layer, and aneurysm size. IEL was classified into the following 3 categories: continuous, fragmented, and completely absent. The ratio of the thickness of the media to the thickness of the media in surrounding normal arterial walls. Aneurysm size was calculated as the mean of the maximal longitudinal diameter and the maximal transverse diameter. The images of CA were provided in supplemental Figure I, available online at http://stroke.ahajournals.org.

**RNA Isolation and Reverse Transcription**

At the designated time point after aneurysm induction, rats were euthanized as described previously. Total RNA from the whole Willis ring was isolated using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany). Extraction was performed according to the manufacturer’s directions. Total RNA was converted into cDNA using Sensiscript reverse transcriptase (QIAGEN). The conditions for the cDNA synthesis were 1 hour at 37°C followed by heating at 93°C for 5 minutes.

**Quantitative Polymerase Chain Reaction**

Constructs for IL-1β, iNOS, MCP-1, VCAM-1, MMP-2, MMP-9, eNOS, and β-actin were produced by TOPO TA Cloning (Invitrogen) from cDNA according to the manufacturer’s directions. Quantitative (real-time) polymerase chain reaction was performed using QuantiTect SYBR Green PCR Kit (QIAGEN) and LightCycler quick system 330 (Roche, Basel, Switzerland). Beta-actin was used as an internal control. The primer sets used were: forward 5'-caccctcaagcagcacag-3', reverse 5'-'gggctcattgggaacac-3' for IL-1β; forward 5'-tcagttggctgtttgc-3', reverse 5'-gtgggaat-3' for iNOS; forward 5'-ctcctcactatcagctct-3', reverse 5'-gcagcttgcgtcagcag-3' for MCP-1; forward 5'-ggagaactttgcaggaac-3', reverse 5'-acattaggcgcctg-3' for VCAM-1; forward 5'-ctgtaactcttgctcaacct-3', reverse 5'-cagcaggttttgcctgttt-3' for MMP-2; forward 5'-taagagggtgtttc-3', reverse 5'-ctctgagcctagacccaactta-3' for MMP-9; forward 5'-ageccggactcataactag-3', reverse 5'-gcacccacacccacactctact-3' for eNOS; and forward 5'-aagctctcctaccccagagagagag-3', reverse 5'-aagctctcctaccccagagagagag-3' for β-actin. The conditions for the cDNA synthesis were: 36 cycles of 95°C 20 seconds for the denaturation, 53°C 20 seconds for the annealing, and 72°C 20 seconds for the extension. For quantification, the second derivative maximum method was used for crossing point determination using LightCycler Software 3.3 (Roche). The amount of mRNA was expressed as the ratio to β-actin mRNA.

**Cell Counting for CD68-Positive Cells**

After immunohistochemistry for CD68-positive cells, the number of CD68-positive cells was counted in a 100-μm-square field surrounding a CA.

**Gelatin Zymography**

Total protein from the whole Willis ring was purified by Bio-Plex Cell Lysis Kit (Bio-Rad, Hercules, Calif) according to the manufacturer’s directions. One hundred micrograms of protein was used in one experiment. Gelatin zymography was performed using Gelatin Zymo-Electrophoresis Kit (Primary Cell, Sapporo, Japan) according to the manufacturer’s directions.

**The Effect of Simvastatin on Preexisting Cerebral Aneurysm**

To examine the effect of simvastatin on preexisting aneurysms, simvastatin administration started 1 month after aneurysm induction. After aneurysm induction, animals in both groups were fed a high-salt diet containing 8% sodium chloride for 1 month. After 1 month of aneurysm induction, a normal diet with 25 mg/kg per day of simvastatin was given to the simvastatin-treated group. In the control group, a normal diet was given after 1 month of aneurysm induction. Animals were euthanized 3 months after aneurysm induc-
tion, and aneurysm size and media thickness were examined as described previously.

Statistical Analysis
The score of IEL was expressed as 95% CI for the median and was analyzed by the use of Siegel-Tukey test. Other data (mean±SD) were analyzed by the use of Student t test for 2-group comparison and by one-way analysis of variance followed by a Fisher test for multiple comparison. Differences were considered significant at $P<0.05$.

Results

**The Effect of Simvastatin on Cerebral Aneurysm Formation**
In the control group, 95% CI for the median of IEL score was 0.40 to 1.20 ($n=12$) month after aneurysm induction and 1.19 to 1.89 ($n=11$) after 3 months. Simvastatin treatment reduced the IEL score both after 1 month (0.03 to 0.77, 95% CI for the median, $n=10$; $P<0.01$ versus...
control) and 3 months (1.04 to 1.77, 95% CI for the median, n=10; P=0.014 versus control) (Figure 1A). The media thickness was significantly thicker in the simvastatin-treated group (0.83±0.17, n=10 after 1 month; 0.61±0.17, n=10 after 3 months) compared with the control group (0.57±0.24, n=12 after 1 month, P<0.01; 0.44±0.20, n=11 after 3 months; P=0.040; Figure 1B). The aneurysm size was also smaller in the simvastatin-treated group (23.6±14.7 μm, n=10 after 1 month, 42.7±15.5 μm, n=10 after 3 months) than in the control group (42.0±17.9 μm, n=12 after 1 month, P=0.023; 71.2±24.5 μm, n=11 after 3 months; P<0.01; Figure 1C). In both groups, systemic blood pressure was significantly elevated after aneurysm induction, but there was no significant difference between the control group (166.2±14.8 mm Hg, n=12 after 1 month; 170.6±14.0 mm Hg, n=11 after 3 months) and the simvastatin-treated group (170.6±21.0 mm Hg, n=10 after 1 month; 168.6±14.2 mm Hg, n=10 after 3 months; Figure 1D). Serum cholesterol level was not elevated with time in both groups and there was no significant difference between the control group (70.7±19.4 mg/dL, n=12 after 1 month; 72.6±18.1 mg/dL, n=11 after 3 months) and the simvasta-
The serum concentration of simvastatin measured by high-performance liquid chromatography reached the value sufficient (456±98.0 nM, n=5) for the inhibition of hydroxy-3-methylglutaryl coenzyme A reductase (inhibitory concentration of 50%: 389 nM).

The Effect of Simvastatin on the Expression of Macrophage Chemoattractant Protein-1, Vascular Cell Adhesion Molecule-1, and Endothelial Nitric Oxide Synthase

In quantitative polymerase chain reaction analysis, both MCP-1 and VCAM-1 mRNA expression was upregulated 1 month after aneurysm induction in the control group (MCP-1 \( P<0.01 \), n=6; VCAM-1 \( P<0.01 \), n=6; Figure 2A–B). In the simvastatin-treated group, mRNA levels of these genes after aneurysm induction were lower than those in the control group (MCP-1 \( P=0.011 \) versus control, n=6; VCAM-1 \( P<0.01 \) versus control, n=6; Figure 2A–B). In contrast, the expression level of eNOS mRNA was higher in the simvastatin-treated group than in the control group 1 month after aneurysm induction (\( P<0.01 \), n=6; Figure 2C). In immunohistochemistry, the expression of both MCP-1 and VCAM-1 was induced in endothelial cells of aneurysmal walls in the control group (Figure 2D, F). Simvastatin-treated rats showed a reduced expression of MCP-1 and VCAM-1 in aneurysmal walls (Figure 2E, G). Although eNOS was constitutively expressed in endothelial cells of cerebral arteries, its expression was slightly reduced in aneurysmal walls (Figure 2H). Treatment with simvastatin augmented eNOS expression around CAs (Figure 2I).

The Effect of Simvastatin on Macrophage Infiltration Into Aneurysmal Walls in Rats

The number of macrophages infiltrated into aneurysmal walls per 100-\( \mu \)m square was 4.40±1.65 cells (n=10) in the control group and 2.33±1.58 cells (n=9) in the simvastatin-treated group. Macrophage infiltration was significantly inhibited by simvastatin treatment (\( P=0.012 \); Figure 2L–P).

Figure 3. Quantitative polymerase chain reaction analysis for IL-1\( \beta \) (A), iNOS (B), MMP-2 (C), and MMP-9 (D). Data were analyzed by Student t test. * \( P<0.05 \), ** \( P<0.01 \), 0 mol/L: before aneurysm induction, 1 M: 1 month after aneurysm induction, 3 M: 3 months after aneurysm induction.
The Effect of Simvastatin on the Expression of Interleukin-1β, Inducible Nitric Oxide Synthase, Matrix Metalloproteinase-2, and Matrix Metalloproteinase-9

In quantitative polymerase chain reaction analysis, the expression level of these 4 genes was increased with time in the process of CA formation in the control group (Figure 3A–D). However, the upregulated expression of these genes was significantly inhibited by simvastatin treatment (IL-1β: P<0.01 versus control after 3 months, n=6; iNOS: P<0.01 versus control after 3 months, n=6; MMP-2: P<0.01 versus control after 3 months, n=6; MMP-9: P<0.01 versus control after 1 month, P=0.011 versus control after 3 months, n=6; Figure 3A–D).

In immunohistochemistry, IL-1β, iNOS, MMP-2, and MMP-9 were expressed in aneurysmal walls 3 months after aneurysm induction (Figure 4C, E, G, I). In the simvastatin-treated group, these molecules were less expressed after aneurysm induction than in the control group (Figure 4D, F, H, J).

Gelatinase Activities in Aneurysmal Walls

Gelatinase activities of MMP-2 and MMP-9 measured by gelatin zymography were increased 3 months after aneurysm induction (MMP-2: P<0.01, MMP-9: P<0.01) in the control group. Gelatinase activities in aneurysmal walls were significantly lower in the simvastatin-treated group than in the control group (MMP-2: P=0.011 versus control, n=6; MMP-9: P=0.014 versus control, n=6; Figure 5).

The Effect of Simvastatin on Preexisting Cerebral Aneurysms

In rats without simvastatin treatment, aneurysm size was enlarged from 42.0±17.9 μm (n=10) to 64.3±20.3 μm (n=10) during 2 months (P=0.013). In rats treated with simvastatin, aneurysm size did not significantly change during 2 months (from 42.0±17.9 μm, n=10, to 43.6±21.7 μm, n=10). Aneurysm size 3 months after aneurysm induction was significantly smaller in the simvastatin-treated group than in the control group (P=0.042; Figure 6B). In rats without simvastatin treatment, the media thickness did not significantly change from 1 month (0.57±0.24, n=10) to 3 months (0.46±0.21, n=10; P=0.24). In rats with simvastatin treatment, the media tended to thicken (0.57±0.24 after 1 month, n=10; 0.72±0.17 after 3 months, n=10) during 2 months, but the difference did not reach any statistical significance (P=0.18). The media thickness 3 months after aneurysm induction was significantly thicker in the simvastatin-treated group than in the control group (P<0.01; Figure 6C).

Systemic blood pressure 3 months after aneurysm induction was not different between the control (126.2±15.8 mm Hg, n=10) and the simvastatin-treated group (124.1±16.8 mm Hg, n=10; Figure 6D). Serum cholesterol level 3 months after aneurysm induction was also not different between the control (69.1±10.5 mg/dL, n=10) and the simvastatin-treated group (72.1±17.0 mg/dL, n=10; Figure 6E).

Discussion

Recent studies revealed the contribution of inflammation to CA formation. Macrophages accumulate in cerebral aneurysmal walls both in humans and rats. MCP-1 and VCAM-1, which are prerequisites for macrophage recruitment, were upregulated expressed in the endothelium of rat CAs in the early stage of CA formation. Simvastatin treat-
MMP dramatically inhibited the upregulated expression of MCP-1 and VCAM-1 in aneurysmal walls, consequently reducing macrophage infiltration into aneurysmal walls. Statins inhibit MCP-1 expression and MCP-1-mediated monocyte recruitment in vitro.19,20 Improvement in endothelial function is a hallmark of the pleiotropic effect of statins. Statins increase bioavailability of nitric oxide through the upregulation and activation of eNOS.8 In experimentally induced cerebral aneurysms of rats, mRNA level of eNOS was elevated by the treatment with simvastatin. Endothelial dysfunction is characterized by decreased nitric oxide bioavailability. In atherosclerosis, endothelial dysfunction causes monocyte recruitment by transcriptionally inducing various proinflammatory genes, including MCP-1 and VCAM-1.21 Although the role of endothelial cells in CA formation has not yet been established, the similar mechanism may be involved in the initiation of CA formation.

Another major pathological feature of CAs is thinning of the medial smooth muscle cell layer attributable to extracellular matrix degradation and apoptosis of smooth muscle cells. We previously demonstrated that MMP-2 and MMP-9 degraded extracellular matrix in cerebral aneurysmal walls, thus promoting the progression of CAs.18 Induced expression of MMP-2 and MMP-9 in aneurysmal walls was inhibited by simvastatin. Simvastatin also diminished the proteinase activity of these enzymes in aneurysmal walls. Suppression of MMPs is a consistent effect of statins, which is supported by experimental results showing that statins can inhibit MMP activity in macrophages12,13 and vascular smooth muscle cells.21 Because macrophages mainly secrete MMP-2 and MMP-9 in aneurysmal walls,18 decreased expression and activity of MMP-2 and MMP-9 may, at least in part, result from reduced number of macrophage infiltration.

Thinning of the media is accompanied by increased apoptosis of smooth muscle cells in aneurysmal walls.22 We previously reported that IL-123 and iNOS24,25 induced apoptotic cell death in medial smooth muscle cells and played a role in CA progression. Most previous studies indicated that statins inhibited the expression of these genes,26–28 although a few showed the opposite.29,30 In the present study, expression of these 2 genes was inhibited by simvastatin, suggesting that statins may exert suppressive effect on CA by modulating the expression of IL-1β and iNOS.

The most striking observation of this study is that treatment with simvastatin prevented thinning of aneurysmal walls of preexisting CAs, suggesting that simvastatin does not only inhibit degenerative changes in vascular walls, but also promotes the repair process. Aneurysm enlargement of preexisting CAs was also inhibited by simvastatin. Although we did not examine the effect of simvastatin on CA rupture, these findings suggest the possibility that simvastatin can prevent CA rupture.

Simvastatin also suppresses the development of experimentally abdominal aortic aneurysms (AAA).31,32 In human infrarenal AAA, simvastatin was reported to be associated with a reduced aneurysm growth.33 Although CA shares

Figure 5. MMP-2 and MMP-9 activity measured by gelatin zymography. A, The representative image from 6 independent studies of gelatin zymography 3 months after aneurysm induction with or without simvastatin. B–C, Densitometric analysis (n=6) of MMP-2 (B) and MMP-9 (C) activity. Data were analyzed by one-way analysis of variance followed by a Fisher test. *P<0.05; **P<0.01.
many pathological characteristics with AAA, there is a
fundamental difference between CA and AAA. Most CAs
arise at the arterial bifurcation, whereas AAAs involve
nonbranching sites. The statins' effect on CA also, in part,
differed from that on AAA. Macrophage accumulation is not
influenced by simvastatin in AAA. These data suggest the
pathophysiological difference between CA and AAA.

The pathophysiology of human CA formation involves
multiple factors such as hemodynamic stress, inflammation,
degradation of extracellular matrix, and genetics. At present,
there is no alternative to surgical clipping or endovascular coiling as treatment options for CA. Therefore, the development of medical therapy to prevent the growth and rupture of CAs would be of tremendous benefit to patients with CA. The most important finding of the present study is that simvastatin reduced CA size and stabilized aneurysmal walls. Unfortunately, we cannot examine if simvastatin reduces the frequency of CA rupture, because the rupture rate was only 3% in control animals in our experimental model. This is an innate limitation of the present study. To date, there is no evidence that serum cholesterol level correlates with the incidence of subarachnoid hemorrhage. Low cholesterol level increases the likelihood of intracerebral hemorrhage but not subarachnoid hemorrhage. It remains to be elucidated whether serum cholesterol level influences CA development. Given that serum cholesterol level was not affected in the present study, however, the suppression mechanism of CAs is likely to be independent of lipid-lowering effect of simvastatin.

In the present study, we have demonstrated, for the first time, that simvastatin suppresses CA progression. Simvastatin inhibits macrophage accumulation through its protective effect on vascular endothelium. Simvastatin also prevents degeneration of the medial smooth muscle cell layer by downregulation of gene expression related to extracellular matrix degradation and apoptosis of smooth muscle cells. The findings of this study provide us a novel insight into the pathogenesis of CAs and highlight the preventive effect of simvastatin on the progression of CAs. Simvastatin may be useful as an adjuvant therapy to prevent the growth and rupture of CA. Further study into the effect of statins on reducing CA enlargement and rupture in patients is needed for clinical application of simvastatin to patients with CAs.

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Disclosures
None.

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