Delayed Postischemic Treatment With Fluvastatin Improved Cognitive Impairment After Stroke in Rats

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Background and Purposes—Recent clinical evidences indicate that statins may have beneficial effects on the functional recovery after ischemic stroke. However, the effect of delayed postischemic treatment with statins is still unclear. In the present study, we evaluated the effects of fluvastatin in the chronic stage of cerebral infarction in a rat model.

Methods—Rats exposed to permanent middle cerebral artery occlusion were treated for 3 months with fluvastatin beginning from 7 days after stroke. MRI, behavioral analysis, and immunohistochemistry were performed.

Results—Two months of treatment with fluvastatin showed the significant recovery in spatial learning without the decrease in serum total cholesterol level and worsening of infarction. Microangiography showed a significant increase in capillary density in the peri-infarct region in fluvastatin-treated rats after 3 months of treatment. Consistently, BrdU/CD31-positive cells were significantly increased in fluvastatin-treated rats after 7 days of treatment. MAP1B-positive neurites were also increased in the peri-infarct region in fluvastatin-treated rats. In addition, rats treated with fluvastatin showed the reduction of superoxide anion after 7 days of treatment and the reduction of Aβ deposits in the thalamic nuclei after 3 months of treatment.

Conclusions—Thus, delayed postischemic administration of fluvastatin had beneficial effects on the recovery of cognitive function without affecting the infarction size after ischemic stroke. Pleiotropic effects of fluvastatin, such as angiogenesis, neuritogenesis, and inhibition of superoxide production and Aβ deposition, might be associated with a favorable outcome. (Stroke. 2007;38:3251-3258.)

Key Words: angiogenesis ■ cerebral infarct ■ microcirculation ■ statins

Despite conflicting data correlating cholesterol level with stroke, 2 early trials of HMG-CoA reductase inhibitors (statins) in patients after myocardial infarction patients showed a reduction in stroke risk as a secondary end point.1 A meta-analysis of 9 statin intervention trials, which enrolled patients with coronary artery disease or those at high risk for coronary disease, demonstrated a 21% relative risk reduction for stroke after 5 years of treatment.2 Another clinical evidence suggests that the commencement of statins within 4 weeks of a stroke results in a favorable 90-day outcome.3 To clarify the effects of postischemic statin treatment, previous studies in which atorvastatin was started 1 day after stroke in rodents showed improvement of sensory motor deficit through induction of angiogenesis, neurogenesis, and synaptogenesis.4,5 These pleiotropic effects of statins were shown to be the result of induction of vascular endothelial growth factor or brain-derived neurotrophic factor.4 Additionally, the microvascular dysfunction in the posttreatment of stroke with recombinant human tissue-type plasminogen activator could be reduced by statins in rodent model.6 However, the effect of delayed treatment with statins after ischemic stroke is still unknown. From this viewpoint, we investigated whether chronic statin treatment beginning 7 days after ischemic stroke had influences on neurological deficits and pathophysiology after the permanent middle cerebral artery occlusion (MCAo) model in rats.

Materials and Methods

Surgical Procedure

Male Wistar rats (270 to 300 grams; Charles River; Kanagawa, Japan) were used in this study. The right MCA was occluded by placement of poly-L-lysine–coated 4-0 nylon, as described previously.7

Protocol for Treatment and Behavioral Tests

Ten rats were only anesthetized (sham operation) and 32 rats were subjected to MCAo (day 1). Based on neuromuscular function on day 7, the rats were divided equally into saline-treated (n=16) or fluvastatin-treated (n=16) groups. Fluvastatin (5 mg/kg per day;
provided by Novartis Pharma) or saline was given by gavage from day 7 to 100. We chose the dose (5 mg/kg per day), because a previous report showed that this dose could effectively induce angiogenesis in ischemic limb.6 On day 55, neuromuscular function and locomotor activity were evaluated in the surviving rats. Then, cognitive function was examined by Morris water maze from day 56 to 63, because the effects of neuronal regeneration could be detected not in the early stage but in the chronic stage of ischemic brain such as 49 to 53 days after the insult.9 On day 96, MRI was performed. On day 100, microangiography was performed.

MRI
High-resolution T1-weighted fast spin echo sequence images (repetition time [TR]=1500 ms; echo time [TE]=10.3 ms; field of view [FOV]=4×3 cm; matrix=256×192; slice thickness=1.5 mm; slice gap=0.5 mm; number of slices=16; number of excitation=10; total time=9:39 min) were obtained using a 3-T MRI scanner (Signa LX VA/II; GE).

Sensory Motor Deficit and Locomotor Activity
Although there are various batteries for testing sensory motor deficit, we used a simple protocol.10 For forelimb flexion, rats were held by the tail on a flat surface. Paralysis of the forelimbs was evaluated by the degree of left forelimb flexion. For torso twisting, rats were held by the tail on a flat surface. The degree of body rotation was checked. For lateral push, rats were pushed either left or right. Rats with right MCA occlusion showed weak or no resistance against a left push. For hind limb placement, one hind limb was removed from the surface. Rats with right MCA occlusion showed delayed or no replacement of the hind limb when it was removed from the surface.

Spontaneous activity was measured via the open field (0.69 m²). We set the sensor, which also put beams on the field, at 30 cm above the field. The number of count, which is when the animal crosses the beam, was measured for 30 minutes.

Morris Water Maze Task
A cylindrical tank 1.5 m in diameter was filled with water (25°C), and a transparent platform 15 cm in diameter was placed at a fixed position in the center of 1 of the 4 quadrants (O’Harra & Co, Ltd). In the hidden platform trials, we performed the tests 4 times per day for 4 days. When the rat could not reach the platform, the latency was set at 60 sec. In the visible platform trials, the tests were performed 4 times per day for 4 days. The acquired data were averaged per day.

Evaluation of Capillary Density
Using a recently developed microangiographic technique,11 capillary density and blood–brain barrier leakage were evaluated in the cerebral cortex after MCA occlusion. The area or length of vessels was analyzed with an angiogenesis image analyzer (version 1.0; Kurabo).

Immunohistochemical Study:
Bromodeoxyuridine Labeling
To identify newly formed DNA, saline-treated (n=5) and fluvastatin-treated (n=5) rats received injections of bromodeoxyuridine (BrdU, 50 mg/kg; Sigma-Aldrich, Saint Louis, Mo) intraperitoneally starting on day 7 twice per day until day 13. Rats were euthanized on day 14. After the sections (8-μm thickness) were fixed in 10% formaldehyde/MoOH neutral buffer solution and blocked, they were incubated with mouse monoclonal anti-rat CD31 antibody (1:100; BD Biosciences; San Jose, Calif), goat polyclonal anti-doublecortin (anti-DCX; Santa Cruz) antibody (1:100; Santa Cruz, Calif), mouse monoclonal anti-NeuN antibody (1:1000; Chemicon, Temecula, Calif), or mouse monoclonal anti-MAP1B antibody (1:100; Sigma-Aldrich), followed by anti-mouse goat fluorescent antibody (1:1000 for NeuN and MAP1B, 1:400 for CD31, Alexa Flour 546, Molecular Probes; Eugene, Ore) or anti-goat donkey fluorescent antibody (1:1000 for DCX Alexa Fluor 546). For double immunostaining, these sections were fixed again and incubated in 2 N HCl at 37°C for 30 minutes. After blocking, they were incubated with rat monoclonal anti-BrdU antibody (1:200; Abcam, Cambridge, UK) followed by anti-rat goat fluorescent antibody (1:1000, Alexa Fluor 488). For immunohistochemical staining for Aβ, sections were pretreated for 30 minutes with hot (85°C) citrate buffer as described before.12 Confocal images were acquired using an FV-300 (Olympus).

Quantitative Histological Analysis
To quantify the immunoreactivity for MAP1B and Aβ, the acquired image was analyzed by Image J (version 1.32; NIH).

Detection of Superoxide Anion in Brain Sections
Superoxide anion was detected on day 14 as described previously.13 Because intact cortex showed red fluorescence, we calculated the ratio of fluorescence as follows: ratio of fluorescence intensity in ischemic core or peri-infarct region/[fluorescence intensity in intact region].

Statistical Analysis
All values are expressed as mean±SEM. To analyze the differences in the type of cerebral infarction, χ² test was performed. The latency, path length, and mean speed in Morris water maze and sensory motor deficits were analyzed by a 2-factor repeated-measure ANOVA. Post hoc analyses were performed, and the Scheffe test was applied to control the inflation in type I error. The value of the serum total cholesterol, the blood pressure, and the spontaneous activity was analyzed by Scheffe rules. The differences in the immunohistochromatic intensity and the volume of infarction were assessed by Mann–Whitney U analyses. In all cases, P<0.05 was considered significant.

Results
Effects of Fluvastatin on Cognitive Impairment
To confirm the severity of cerebral infarction, all rats were examined by T1-weighted MRI after 89 days of treatment. Although the total volume of infarction calculated in T1-weighted images was not different between rats treated with

| Table. Infarction Volume Calculated by MRI, Blood Pressure, and Serum Total Cholesterol |
|---------------------------------|-----|-----|-----|-----|
| Infarction volume in total rats (mm³) | Sham | MCAo+S | MCAo+F | P    |
| Day 7 | 116.1±5.4 | 123.7±6.0 | 115.5±7.3 | 0.654 |
| Day 56 | 146.5±4.7 | 148.3±2.7 | 136.1±5.2 | 0.132 |

Systolic blood pressure (mm Hg) in type A rats
Day 7 | 9.39±0.5 mm | 322.8±15.0 | 327.0±18.8 | 0.758 |
Day 56 | 12 11 | 12 11 | 12 11 |

Serum total cholesterol (mg/dl) in type A rats
Day 7 | 85.9±5.6 | 75.3±3.5 | 73.5±2.7 | 0.949 |
Day 56 | 20 5 | 20 5 | 20 5 |

Type A, low-intensity area seen in the dorsolateral and lateral portions of the neocortex and the entire caudate putamen; type B, low-intensity area seen in the dorsolateral and lateral portions of the neocortex and in part of the caudate putamen; type C, low-intensity area seen in part of the lateral neocortex and caudate putamen. MCAo+S, saline-treated rats after MCAo; MCAo+F, fluvastatin-treated rats after MCAo.

P, saline vs fluvastatin.
saline and fluvastatin (Table), the pattern of cerebral infarction was divided into 3 groups: type A, low-intensity area seen in the dorsolateral and lateral portions of neocortex and the entire caudoputamen; type B, low-intensity area seen in the dorsolateral and lateral portions of neocortex and in part of the caudoputamen; and type C, high-intensity area seen in part of the lateral neocortex and caudoputamen. Sensory motor deficit (b). Spontaneous locomotor activity (c). Hidden platform test in Morris water maze. Each figure showed latency (d), path length (e), and mean speed (f). Days 1 to 4 indicate the trial day in the hidden platform test (56 to 59 days after middle cerebral artery occlusion). Visible platform test in Morris water maze. Each figure showed latency (g), path length (h), and mean speed (i). Days 1 to 4 indicate the day in the visible platform test (60 to 63 days after middle cerebral artery occlusion). MCAo+S indicates rats treated with saline after middle cerebral artery occlusion; MCAo+F, rats treated with fluvastatin after middle cerebral artery occlusion.

Sensory motor deficit had spontaneously recovered to some extent by 8 weeks in both groups, and there was no difference (Figure 1b). Locomotor activity in rats subjected to MCAo was increased as compared with that in sham-operated rats, as described before, but there was no significant difference between fluvastatin-treated and saline-treated rats (Figure 1c). In Morris water maze (Figure 1d-i), which examines spatial learning, there were significant differences...
in the latency and path length in hidden platform test among the groups (supplemental Table I, available online at http://stroke.ahajournals.org). A significant difference was observed on day 4 between fluvastatin-treated and saline-treated rats (supplemental Table I). Also, there was a significant difference between sham and saline-treated rats (supplemental Table I). There was no significant difference both in swimming speed and visible platform test, which excluded the possible influence of visual loss, sensory motor deficit, and motivation on the results. These data suggest that impaired spatial learning was improved by fluvastatin.

**Histological Changes by Fluvastatin**

Next, we studied whether fluvastatin had some influences on the histology. Initially, we focused on neurogenesis and angiogenesis. To examine neurogenesis, we measured BrdU-incorporated cells after injecting BrdU from day 7 to day 13. Although BrdU-positive cells were observed in the peri-infarct cortex (a), peri-infarct basal ganglia, and subventricular zone, these cells did not express NeuN (a), but expressed DCX in the subventricular zone (b). Fluvastatin-treated rats showed some BrdU/CD31-positive cells (arrows, c), although most BrdU-positive cells were negative for CD31 in saline-treated rats (d). The number of BrdU-positive cells (e), NeuN-positive cells (f), BrdU/DCX-positive cells (h), DCX-positive cells (i), and BrdU/CD31-positive cells (j); the percentage of BrdU/DCX-positive cells (g) or BrdU/CD31 cells (k) in total BrdU-positive cells. PC indicates peri-infarct cortex; PBG, peri-infarct basal ganglia; IC, infarcted cortex; IBG, ischemic basal ganglia; ISVZ, subventricular zone on infarcted side; CC, contralateral cortex, CSVZ, subventricular zone on contralateral side; CBG, contralateral basal ganglia (n=5 in each group, *P<0.05, bar=100 μm).
peri-infarct cortex and subventricular zone (Figure 2a). Although some BrdU-positive cells expressing DCX, a marker for migrating neuroblasts, could be detected in subventricular zone (Figure 2b), the percentage in total BrdU-positive cells (Figure 2g) and the number (Figure 2h) did not differ between the groups. Also, the number of DCX-positive cells was same in the both groups (Figure 2i). There were no BrdU-positive cells expressing DCX in the cerebral cortex. Unexpectedly, these data suggest that neurogenesis was not enhanced by fluvastatin.

Thus, we further examined whether angiogenesis was affected by fluvastatin. In the peri-infarct cortex and basal ganglia, BrdU-positive cells that were positive for CD31 as a marker of endothelial cells could be detected (Figure 2c,2d). The number of BrdU/CD31-double-positive cells was significantly increased in fluvastatin-treated rats (Figure 2j). The percentage of BrdU/CD31-double-positive cells in total BrdU-positive cells was also increased in fluvastatin-treated rats (Figure 2k). Consistently, microangiography using FITC-conjugated albumin also showed that microvessels were significantly increased in fluvastatin-treated rats only in the peri-infarct cortex and basal ganglia, without destruction of the blood–brain (Figure 3a to 3h). Quantitative analysis showed that the length and area of microvessels were also increased in the peri-infarct region, but not in the contralateral cortex and contralateral basal ganglia, in rats treated with fluvastatin, at 3 months after stroke (Figure 3i,j).

Because recent reports showed that neurite outgrowth was observed in the peri-infarct region from 7 to 14 days after cerebral infarction, we next examined the effect of fluvastatin on neurite outgrowth. Immunohistochemical staining showed that treatment with fluvastatin significantly increased the immunoreactivity of MAP1B, a marker of neurite outgrowth, in neurites (Figure 4), although the number of MAP1B-positive cells was the same in both groups. These data implied that the fluvastatin might promote angiogenesis, resulting in improvement of the microcirculation, and neurite outgrowth.

One possible explanation for the enhanced angiogenesis and neurite outgrowth is a decrease in oxidative stress by fluvastatin. To assess oxidative stress, we evaluated superoxide production using dihydroethidium staining (Figure 5a to 5e). Superoxide anion was increased in the ischemic core as compared with the contralateral region at 2 weeks after MCA occlusion (Figure 5a,5c). However, rats treated with fluvastatin showed a significant reduction in superoxide anion especially in the ischemic core region, but not in the peri-infarct cortex and basal ganglia (Figure 5b,5d,5e).

Finally, we examined Aβ deposition in the thalamic nuclei, because previous reports showed that Aβ deposits in the...
thalamic nuclei persisted as long as 9 months after focal cerebral ischemia. Although immunohistochemical staining showed marked deposition of Aβ in the ventrolateral and ventromedial thalamic nuclei at 3 months after stroke, the area of Aβ deposits was significantly decreased in fluvastatin-treated rats (Figure 5f to 5h). In other regions, such as cortex or basal ganglia, there was no Aβ deposits in both groups as reported before.

Discussion
Although several laboratories have shown that long-term pretreatment with a statin reduces infarct size in rodents, no articles have reported the effects of delayed postischemic treatment with statins. The present study demonstrated that statin treatment beginning 7 days after ischemic stroke resulted in significant improvement of spatial learning at 8 weeks after stroke, without any change in the plasma cholesterol level and infarct size.

Fluvastatin-treated rats showed a significant increase of MAP1B in neurites in the peri-infarct region. Considering that MAP1B is especially prominent in extending neurites and related to functional recovery after ischemic stroke, one of the possible effects of fluvastatin is to enhance neurite outgrowth, neuritogenesis, in the early stage of treatment. This speculation might be supported by the recent study demonstrating that neurite outgrowth is accelerated by pravastatin via inhibiting the activity of geranylgeranylated proteins such as RhoA.

As BrdU/CD31-positive cells were increased 14 days after MCAo and microvessels were also increased in the peri-infarct region 100 days after MCAo, fluvastatin enhanced angiogenesis and resulted in improvement of microcirculation in the peri-infarct region. Although the relationship between the improved microcirculation and behavior is still unclear, a recent report demonstrated that the restoration of perfusion by collateral growth and new capillaries in the
ischemic border zone around a cortical infarct supported long-term functional recovery in rats. Additionally, others reported that some patients who received tissue plasminogen activator therapy with no immediate clinical improvement despite early recanalization showed delayed clinical improvement. From these viewpoints, it is likely that the improvement of microcirculation is an important factor for the functional recovery.

Of importance, fluvastatin reduced deposition of Aβ in the ventrolateral–ventromedial thalamic nuclei in the chronic stage of ischemic stroke, although rats subjected to focal cerebral ischemia develop deposition of Aβ in the ventroposterior lateral and ventroposterior medial nuclei for as long as 9 months. This might be similar with precious reports showing that statins reduced the production of Aβ in Alzheimer disease. The mechanism of the reduction of Aβ by fluvastatin should be further investigated.

Thus, the rats treated with fluvastatin showed enhancement of angiogenesis and neurite outgrowth in the peri-infarct cortex and reduced deposition of Aβ in the ventrolateral–ventromedial thalamic nuclei. Because those regions are important sites for spatial learning, we speculate that the enhancement of functional recovery by fluvastatin might be dependent on those regions.

The other histological difference was the reduction of superoxide anion in the ischemic core in fluvastatin-treated rats. Because cerebral blood flow in the ischemic cortex remained to be reduced for 48 hours and restored to some extent 9 days after permanent MCAo, we speculate that fluvastatin could reach the ischemic core and show the antioxidative effects. On the contrary, in the peri-infarct region, superoxide anion was not detected even in the control group and no effect of fluvastatin might be observed. This effect of statin is similar with the previous report showing that cerivastatin prevented the production of superoxide anion in the cerebral parenchyma in stroke-prone spontaneously hypertensive rats. Also, fluvastatin is reported to possess antioxidative properties in other cells.

The association of neurogenesis is also the center of interest, because previous reports showed an increase in neurogenesis after atorvastatin treatment beginning at 1 day after stroke. However, we speculate that neurogenesis might not have contributed to the favorable outcome in the present study, because the volume of infarction was not decreased by fluvastatin, and the density of mature neurons (NeuN-positive cells) and proliferative immature neurons (BrdU/DCX-positive cells) was the same in both groups. From the viewpoints, the timing of treatment seems important for the enhancement of neurogenesis and the beginning of statin 7 days after MCAo might be too late to enhance neurogenesis.

The limitation of the present study is that there is no data demonstrating that fluvastatin crossed over the blood–brain barrier and acted on neurons directly. Blood–brain barrier permeability differs among statins and correlates in part with their respective lipophilicity. Considering that pretreatment with pravastatin and rosuvastatin, whose lipophilicity is 0.84 and 0.33, respectively, shows significant effects on reducing infarction volume, fluvastatin, whose lipophilicity is 1.27, might penetrate blood–brain barrier and have some direct effects on neurons. Otherwise, fluvastatin could penetrate the brain because of the disruption of blood–brain barrier after MCAo. One of other limitations in the present study is no...
examination of the characteristics of BrdU positive-cells other than CD31, DCX, or NeuN. In addition, how these histological changes in fluvastatin-treated rats were mecha-
nistically linked to improved outcome was not clarified. Further study is necessary to clarify these points.

Summary
Overall, delayed postischemic chronic fluvastatin treatment showed beneficial effects on the recovery of cognitive im-
pairment after stroke by enhancement of neurogenesis and of angiogenesis and a decrease in Aβ deposition and super-
oxide anion production. Further studies might show potential clinical utility to treat cognitive impairment in patients with ischaemic stroke.

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