Aims: Previous studies suggest that both statins and calcium-channel blockers inhibit cardiovascular and cerebrovascular diseases by their pleiotropic effects, such as antioxidation and anti-inflammatory actions. By using stroke-prone spontaneously hypertensive rats (SHRSPs), the isolated effect of each pharmaceutical has been reported, but the effect of a combination of both pharmaceuticals has not been reported. In this study, we evaluated combination therapy of atorvastatin and amlodipine for its efficacy in preventing stroke in the SHRSP model. Main Methods: We initiated treatment of SHRSPs at 8 weeks of age with atorvastatin (2 mg/kg), amlodipine (1 mg/kg), a combination of atorvastatin (2 mg/kg) and amlodipine (1 mg/kg), or vehicle. Measurement of physiological parameters and immunohistochemical assessments for oxidative stress and inflammation were done at each group. Key findings: At 13 weeks of age, the combination therapy group showed greater inhibition of an antioxidation and anti-inflammatory marker than the vehicle group, although there were no differences in blood pressure. Significance: Our study suggests that the combination therapy of atorvastatin and amlodipine may protect against hypertension-induced stroke by the additive effect of the antioxidation and the anti-inflammatory action of the both agents.

Key words: SHRSP, stroke, atorvastatin, amlodipine

Introduction

Stroke is one of the major neurologic disorders and a leading cause of death worldwide. While there are various causes for stroke, hypertension is one of the major risk factors. Chronic hypertension leads to dysfunction and injury of cerebral arteries and induces both cerebral infarction and cerebral hemorrhage.

Persistent hypertensive states cause vascular endothelial dysfunction, resulting in increased oxidative stress, a decline in nitric oxide (NO) production, and accelerated expression of pro-inflammatory cytokines, all of which contribute to the development of arteriosclerosis. Therefore, as oxidative stress and inflammation lay at the root of stroke pathology, suppressing such actions could lead to the prevention of strokes.

The stroke-prone spontaneously hypertensive rat (SHRSP) is a hypertension animal model developed in Japan. SHRSPs develop stroke similar to that of most Japanese hypertensive patients, thereby contributing significantly to understanding pathology and development therapy.

Statins, 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, reduce the cholesterol level by blocking mevalonate synthesis. Statins are...
widely used by patients with hypercholesterolemia to reduce cholesterol. There are many reports that statins have pleiotropic effects except for lowering lipids. Our previous study showed that long-term administration of high-dose atorvastatin suppressed the incidence of stroke and delayed stroke death in SHRSPs without having an influence on serum lipids and blood pressure.

Amlodipine is a dihydropyridine calcium channel blocker (CCB) widely used for the treatment of hypertension. A long-acting dihydropyridine CCB has been shown to prevent the progression of atherosclerosis and decrease the incidence of cardiovascular and cerebrovascular events in humans. Growing clinical and experimental evidence suggests that calcium channel blockers provide beneficial actions beyond the blood pressure-lowering action, such as anti-inflammatory and antioxidative effects.

Based on the foregoing reasons, we hypothesize that a combination of a statin and a calcium channel blocker might provide a favorable effect for the prevention of strokes with a pathogenesis linked to hypertension. Several studies have reported an effect of the combination therapy using an experimental rat model, but there are no reports using SHRSP. This report examines our hypothesis using SHRSP.

**Materials and Methods**

1) **Animals**

Male SHRSPs of the Izumo strain (body weight 250–300 g), aged 8 weeks, were purchased from Japan SLC (Shizuoka, Japan). The animals were provided with water ad libitum and a special chow that consists of 0.39 g sodium chloride per 100 g (Funabashi Farm Co., Ltd., Chiba, Japan) which leads SHRSPs to stroke. When SHRSPs are given this chow, the stroke incidence in the rats is maintained to nearly 100%. All experimental procedures were approved by the Animals Experimental Ethical Review Committee of Nippon Medical School.

2) **Experimental protocol**

The SHRSPs were divided into four groups. These groups were investigated for 5 weeks, each group consisting of 8 rats, respectively. In these four groups, the first group was administered a suspension of atorvastatin (Pfizer Co., Ltd., USA) by gavage at dosages of 2 mg/kg body weight. The second group was administered a suspension of amlodipine (Pfizer Co., Ltd., USA) at dosages of 1 mg/kg body weight. The third group was administered a suspension of atorvastatin at dosages of 2 mg/kg body weight and amlodipine at dosages of 1 mg/kg body weight. The fourth group was administered vehicle at the same volume as given for drug dosing (0.5% sodium methylcellulose in saline). The SHRSPs was administered each medicines orally through a gastric tube every day. Based on the previous report, a 2 mg/kg dose of atorvastatin does not lower serum cholesterol. Similarly, a 1 mg/kg dose of amlodipine does not lower blood pressure.

3) **Observation of histologic examination and immunohistochemistry**

Each animal underwent deep anesthesia using halothane and perfusion fixation was performed with heparinized saline followed by 4% paraformaldehyde. The whole brain was removed and fixed with 4% paraformaldehyde overnight at 4°C. On the next day, the whole brain tissue was subjected to sucrose substitution and cryopreserved at −80°C. The brains were sliced into horizontal sections, cut into 6 µm thick sections starting from the parietal lobe. The sections were stained with hematoxylin-eosin to assess the location of stroke lesions. Immunoreactivities for lectin-like oxidized LDL receptor-1 (LOX-1) and monocyte chemotactic protein-1 (MCP-1) were detected using antibodies against LOX-1 (1:100, Santa Cruz, USA) and MCP-1 (1:100, Santa Cruz, USA). Using anti-von Willebrand factor (vWF) (1:500, DAKO, Glostrup, Denmark) as a marker for vascular endothelium, we performed double stain fluorescence with vWF and the inflammatory markers. After the brain sections were aired dry, these sections were incubated overnight with the appropriate first antibody and vWF with mixing at 4°C. The next day, the brain sections were washed three times with phosphate buffered saline (PBS) and reacted with the mixed secondary antibodies for 30 minutes at room temperature in a darkroom. The sections were washed three times and enclosed with DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Labs, USA). After completing the process, we measured the ratio of the LOX-1 or MCP-1 positive blood vessels to vWF positive blood vessels. The numbers of positive vessels for each marker in 3 randomized 1 mm² areas of a cortex region in each brain section were counted.
The Impact of Combined Treatment with Atorvastatin and Amlodipine in Stroke-prone Spontaneously Hypertensive Rats

Table 1. Physiological parameters

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Atorv</th>
<th>Amlod</th>
<th>Atorv+Amlod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 weeks old age</td>
<td>201±9.7</td>
<td>211±10.5</td>
<td>210±8.9</td>
<td>211.4±7.6</td>
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<tr>
<td>12 weeks old age</td>
<td>278±14.1</td>
<td>256±31.6</td>
<td>257±29.9</td>
<td>258.8±33.2</td>
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<tr>
<td>SBP (mmHg)</td>
<td>162±23.5</td>
<td>168±23.0</td>
<td>169±16.1</td>
<td>163±18.5</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>110±14.9</td>
<td>119±13.1</td>
<td>117±11.1</td>
<td>110±13.1</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>127±16.3</td>
<td>135±15.4</td>
<td>134±10.1</td>
<td>127±11.4</td>
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<tr>
<td>HR (beats/min)</td>
<td>355±28.5</td>
<td>352±35.7</td>
<td>354±38.4</td>
<td>343±32.1</td>
</tr>
</tbody>
</table>

Values are the mean ± S.D. Vehicle group significantly had a small weight in comparison with the medicine administrated group at 8 weeks old age. However, otherwise, there were no significant differences among the groups.

Vehicle, vehicle-treated group; Atorv, atorvastatin-treated group; Amlod, amlodipine-treated group; Atorv+Amlod, atorvastatin+amlodipine-treated group; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; MBP, Mean blood pressure; HR, Heart rate

Fig. 1. Blood pressure of SHRSP after treatment with atorvastatin, amlodipine and combination therapy at different ages (8 and 12 weeks).

Among the four groups, no significant difference was seen in blood pressure at 8 and 12 weeks of age.

Vehicle, vehicle-treated group; Atorv, atorvastatin-treated group; Amlod, amlodipine-treated group; Atorv+Amlod, atorvastatin+amlodipine-treated group.

4) In situ detection of superoxide anion production

We used dihydroethidium (DHE) (10 μmol/L, Life Technologies, Tokyo, Japan) for the evaluation of the production of the reactive oxygen. Frozen brain sections were incubated with DHE for 30 minutes at 37°C in a dark, humidified chamber. Subsequently, the sections were washed three times with PBS and enclosed with glycerine. We counted DHE positive cells in 3 randomized 1 mm² areas of a cortex region in each brain section.

5) Statistical analysis

Data are the mean ± standard deviation (SD). Multiple comparisons were made using one-way analysis of variance followed by the Scheffe post-hoc test. A probability value <0.05 was regarded as statistically significant.

Results

1) Physiological parameters

Physiological parameters at 8 weeks of age are given in Table 1. Body weight was significantly less (p<0.05) in the vehicle group compared with the atorvastatin only group, the amlodipine only group, and the combination therapy group. Despite this, subsequent observations showed satisfactory increases in weight resulting in no significant differences between the vehicle group and the other groups at 12 weeks of age. There were no significant differences in the systolic, diastolic, and mean blood pressures and heart rate among the four groups.

Changes in blood pressure during the observation
period is shown in Fig. 1. Among the four groups, no significant differences were seen in blood pressure at 8 and 12 weeks of age. The calcium antagonist, an orally administered hypotensive drug, did not lower blood pressure at a dose of 1 mg/kg.

2) Evaluation of the stroke lesion

There are no stroke lesions in each brain section stained with hematoxylin-eosin in all groups at 13 weeks of age. Therefore, we observed the randomized cortex areas in each brain section in order to assess the expression of inflammatory markers and the reactive oxygen production.

3) Expression of LOX-1 and MCP-1

LOX-1 positive cells were markedly expressed in vascular endothelium in the vehicle-treated group (Fig. 2A). With respect to the vascular endothelium, the ratio of LOX-1 positive blood vessels in vWF positive vessels was significantly lower in the atorvastatin only, amlodipine only, and the combination therapy group than in the vehicle group (atorvastatin-treated group, \( p<0.0001 \); amlodipine-treated group, \( p=0.0002 \); atorvastatin+amlodipine, \( p<0.0001 \) vs. vehicle-treated group, respectively; Fig. 2B). The combination therapy group showed further lowering of positive blood vessel ratios in LOX-1 compared with the atorvastatin and amlodipine only group (\( p=0.0255 \) vs. atorvastatin-treated group; \( p=0.0001 \) vs. amlodipine-treated group, respectively; Fig. 2B).

MCP-1 positive cells were markedly expressed in vascular endothelium in the vehicle-treated group (Fig. 3A). Immunohistochemical analyses of MCP-1 showed similar results to LOX-1 analyses. With respect to the vascular endothelium, the ratio of MCP-1 positive blood vessels in vWF positive vessels was significantly lower in the atorvastatin only, amlodipine only, and the combination therapy group than in the vehicle group (atorvastatin-treated group, \( p=0.0037 \); amlodipine-treated group, \( p=0.0102 \); atorvastatin+amlodipine-treated group, \( p<0.0001 \) vs. vehicle-treated group, respectively; Fig. 3B). The combination therapy group showed further lowering of positive blood vessel ratios in MCP-1 compared with the atorvastatin and amlodipine only group (\( p=0.0359 \) vs. atorvastatin-treated; \( p=0.0440 \) vs. amlodipine-treated, respectively; Fig. 3B).

Brain sections without the first antibody did not show
**Fig. 3.** Representative photomicrographs showing immunoreactivity with monocyte chemotactic protein-1 (MCP-1) (A). Quantitative analysis of the MCP-1 positive cells (B). Insets denote MCP-1 (green) immunoreactivity colocalized with vWF (red) suggesting that MCP-1 is present on vascular endothelium.

* * *p<0.01 vs. vehicle-treated group. 'p<0.05 vs. atorvastatin or amlodipine-treated group. Scale bar: 100 µm. Vehicle, vehicle-treated group; Atorv, atorvastatin-treated group; Amlod, amlodipine-treated group; Atorv+Amlod, atorvastatin+amlodipine-treated group.

any positive staining for either inflammation marker.

4) **Production of superoxide anion in the brain**

The evaluation of active oxygen production in each group, derived using the DHE method, is shown in Fig. 4. DHE positive cells were remarkably expressed in the vehicle-treated group (Fig. 4A). A significant decrease in active oxygen expression was observed in the atorvastatin only, amlodipine only, and combination therapy group compared with the vehicle group (atorvastatin-treated group, p<0.0001; amlodipine-treated group, p<0.0001; atorvastatin+amlodipine-treated group, p<0.0001 vs. vehicle-treated group, respectively; Fig. 4B). A further significant decrease in active oxygen expression was observed in the combination therapy group compared with the atorvastatin and amlodipine only group (p=0.0053 vs. atorvastatin-treated group; p=0.0015; vs. amlodipine-treated group, respectively; Fig. 4B).

**Discussion**

The present study demonstrated that atorvastatin or amlodipine treatment independently showed anti-inflammatory and antioxidant effect in the hypertension-induced stroke model, and that a combination of the HMG-CoA reductase inhibitor and CCB significantly reduced inflammation and oxidative stress compared with each single treatment.

The primary function of a statin is to reduce serum cholesterol by inhibiting HMG-CoA reductase, a rate-determining step in the cholesterol synthesis pathway, thereby suppressing the development of arteriosclerotic disease, a fact proven in large-scale clinical trials.13, 14 Moreover, a large number of studies have shown that statins have pleiotropic effects independent of lipid lowering actions as follows; a lengthening of endothelial nitric oxide synthase (eNOS) mRNA half-life,15 a suppression of reactive oxygen species (ROS) production originating with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,16, 17 an increase in NO synthase because of stabilization of mRNA of eNOS via inhibition of the Rho-kinase pathway,18 and a reduction of inflammation.19, 20

It has been reported that atorvastatin and simvastatin reduced infarct volume by inhibiting oxidative stress in SHRSPs.21 Kawashima et al. has reported that cerivas-
tatin decreased the incidence and size of stroke because of antioxidative and anti-inflammatory effects in SHRSPs. In addition, our previous study indicated that the long-term pre-treatment of atorvastatin suppressed spontaneous stroke incidence and increased the survival rate in SHRSPs by reducing asymmetric dimethylarginine levels. The present study also showed that the treatment of atorvastatin decreased both immunoreactive markers of oxidative stress and inflammation in SHRSPs.

The long-acting CCB, amlodipine, also has a vasoprotective effect beyond its blood pressure-lowering effect. Amlodipine increases eNOS and decreases NADPH oxidase. Because amlodipine has a high affinity for lipids, amlodipine gets into the cap formation and shows a powerful antioxidation function. Because the dihydropyridine ring that is present in CCBs can easily capture a free electron, CCBs have an antioxidative effect. Previous study has suggested that the infarct volume was reduced by CCBs with antioxidant action after transient middle cerebral artery ischemia in rats. In a study using SHRSP, CCB also prevented oxidative stress through the regulation of Cu/Zn superoxide dismutase activity. Amlodipine also suppresses MCP-1 and vascular cell adhesion molecule-1. As outlined above, the effects of CCB administration on the SHRSP are consistent with the results of anti-inflammatory and anti-oxidation actions observed in this study.

Furthermore, we showed that the combination therapy of atorvastatin and amlodipine significantly decreased inflammatory markers and suppressed superoxide production compared to the administration of the drugs individually, suggesting that the combined treatment may lead to the additive effect of anti-inflammatory and antioxidant as above in a rat based on hypertension.

Recently, the neurovascular unit which is constituted of endothelial cells, astrocytes, microglia and neurons instead of neurons only has been focused as a new therapeutic strategies target of brain protection for ischemic stroke. ROS, especially superoxide anions can cause direct oxidative damage and contribute to blood-brain barrier (BBB) disruption and development of brain edema. Inflammatory process also plays a key role in

Fig. 4. Representative photomicrographs show fluorescent staining of oxidized hydroethidine signals (A). Quantitive analysis of the positive cells (B).

* * p<0.01 vs. vehicle-treated group, * * p<0.01 vs. atorvastatin or amlodipine-treated group. Scale bar: 100 µm. Vehicle, vehicle-treated group; Atorv, atorvastatin-treated group; Amlod, amlodipine-treated group; Atorv+Amlod, atorvastatin+amlodipine-treated group.
BBB extravasation after ischemic stroke. Although the present study showed that the combined therapy of atorvastatin and amlodipine protected against the oxidative stress and the inflammation, the therapeutic benefit of both agents may be involved in some markers of BBB such as matrix metalloproteinases, claudin, occludin and zonula occludens protein 1 after ischemia. Further studies are necessary in order to elucidate the detailed mechanisms of the stroke prevention in this combination treatment.

Conclusions

In conclusion, the combination of atorvastatin and amlodipine showed cumulative protection through the antioxidant and anti-inflammatory effect in SHRS and the present treatment may be a potent therapeutic strategy for hypertension-induced stroke in the clinical field.

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References


