Preventive Effect of MK-733 (Simvastatin), an Inhibitor of HMG-CoA Reductase, on Hypercholesterolemia and Atherosclerosis Induced by Cholesterol Feeding in Rabbits

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Abstract—MK-733 was found to prevent an increase of serum cholesterol levels in cholesterol-fed rabbits, and lovastatin also markedly inhibited their increase. MK-733 and lovastatin inhibited the increase of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol, and it slightly affected the high density lipoprotein (HDL) cholesterol levels. MK-733 and lovastatin suppressed the increase of serum phospholipid levels and slightly affected the triglyceride levels. MK-733 suppressed the development of atherosclerosis in coronary arteries and aorta, and lovastatin also diminished their development.

Atherosclerosis is thought to be caused by many factors. These include obesity, smoking, hypertension, diabetes, lack of exercise, stress, hypertriglyceridemia and hypercholesterolemia. It is now generally accepted that elevation of serum cholesterol is a major risk factor in coronary heart disease and presumably also in other atherosclerotic diseases (1). Results (2) of the Lipid Research Clinics Coronary Primary Prevention Trial demonstrated that a reduction in plasma LDL cholesterol was associated with a reduced incidence of coronary heart disease in men at risk due to elevated LDL cholesterol. MK-733 (simvastatin) is a chemical derivative of lovastatin (MK-803), which is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme in the cholesterol biosynthetic pathway. The chemical structure of MK-733 is [1S-[1α,3α,7β,8β(25*,4S*)]. 8αβ]-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)ethyl]-1-naphthalenyl 2,2-dimethylbutanoate. L-654,969, the active open acid form of this compound, inhibited HMG-CoA reductase with an IC50 of 0.94×10^{-3} M, and it is approximately twice as active as L-154,819, the active open acid form of lovastatin (3). MK-733 inhibited cholesterol synthesis from [14C]acetate in different animal cell lines and reduced the serum cholesterol levels in normal and cholestyramine-primed dogs (4). MK-733 is now under clinical investigation for the treatment of hyperlipidemic diseases including heterozygous familial hypercholesterolemia. Cholesterol in humans is derived from the diet (200–400 mg/day) and from endogenous synthesis (750–1000 mg/day) (5). It was reported that the feeding of cholesterol produced hypercholesterolemia and atherosclerosis in rabbits (6). This animal model is very useful for evaluating the effect of hypolipidemic agents and/or anti-atherogenic agents on dietary hypercholesterolemia. Therefore, we have examined the preventive effect of MK-733 on hypercholesterolemia and atherosclerosis in cholesterol-fed rabbits. Lovastatin was used as a control drug because this drug has been used clinically in the U.S.A.

Materials and Methods

1. Animals: Male Japanese white rabbits
aged 18 to 19 weeks and weighing 2.2 to 2.9 kg were used in this study. These animals were purchased at the age of 14 to 15 weeks from Clean Experimental Animals Co., Ltd., Saitama, Japan, and they were maintained under the following environmental conditions: room temperature, 23±2°C; relative humidity, 55±15%; illumination, 12 hr from 6 A.M. to 6 P.M. The rabbits were housed in metal cages individually and were given a normal chow pellet (RC-4, Oriental Yeast, Tokyo, Japan) and water ad libitum for 4 weeks in order to acclimatize them.

2. Drugs: MK-733 (Lot No.: L-644,128-000U053; purity, 99.0%; Merck Sharp and Dohme Research Laboratories (MSDRL), Rahway NJ, U.S.A.) and lovastatin (Lot No.: L-154,803-00G110; purity, 99.0%; MSDRL) were used. The structures of MK-733, lovastatin and L-654,969 are shown in Fig. 1.

3. Dosage levels and methods for administration: Dosage levels and number of animals used in this experiment are shown in Table 1.

Either MK-733 or lovastatin was incorporated into a diet (RC-4, Oriental Yeast) containing 1% cholesterol at a concentration of 0.03% (w/w). Both diets were stored at -20°C until use. The drug concentration in the diet was adjusted with the diet including 1% cholesterol, according to the dose level.

4. Experimental procedures: Fifty-six rabbits were selected by pretreatment values of serum total cholesterol and body weight, and they were divided into 6 groups. All rabbits were fed 100 g of diet per 3 kg of body weight daily in the morning for 12 weeks. Food intake and body weight were measured daily and weekly, respectively.

Fasting blood samples were collected from an ear artery. An aliquot of serum was provided for analysis of lipid parameters. Total, VLDL, LDL and HDL cholesterol, phospholipids and triglyceride were determined after 0, 1, 2, 4, 6, 8, 10 and 12 weeks of the treatment. After 12 weeks of the treatment, all

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage level</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control (1% Cholesterol diet)</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>MK-733</td>
<td>10 mg/kg</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>MK-733</td>
<td>5 mg/kg</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>MK-733</td>
<td>2.5 mg/kg</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>Lovastatin</td>
<td>5 mg/kg</td>
<td>10</td>
</tr>
<tr>
<td>F</td>
<td>Non-fed control (Normal diet)</td>
<td>—</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 1. Structures of MK-733, lovastatin and L-654,969.

Table 1. Experimental design
animals were fasted overnight and exsanguinated the following morning under general anesthesia (sodium pentobarbital, 20–30 mg/kg, i.v.). The heart was removed, weighed, immersed in saline and fixed in 10% (v/v) phosphate-buffered formalin. A part of the liver (lobus sinister lateralis) was removed and frozen for analysis of cholesterol content. The aorta of each rabbit was removed from the aortic valve to the iliac artery and rinsed with saline. The aorta was dissected free from perivascular fat. Two to three mm of ascending aorta from the aortic valve was trimmed for the lipid staining. The remaining aorta was opened on the dorsal side, and the aortic surface was copied on a commercial Xerox machine. The aorta was divided in half longitudinally. One half was fixed in 10% (v/v) phosphate-buffered formalin, and the other half was frozen for analysis of cholesterol content.

5. Measurement of serum lipids and lipoprotein cholesterol: Phospholipids and triglyceride were measured by an autoanalyzer (Centrifichem, Encore, Baker Instrument Co., Allenton PA, U.S.A.), using P-L Nagase (Nagase and Co., Tokyo, Japan) and the Triglyceride G-FA Test Wako (Wako Pure Chemical Co., Osaka, Japan), respectively. Serum lipoprotein fractions were separated by ultracentrifugation into a combined HDL+LDL fraction with d=1.006 g/ml and a HDL fraction with d=1.063 g/ml. Density was adjusted with KBr solution (7). Centrifugation was carried out in a Hitachi SCP70H ultracentrifuge using an RPL42T rotor at 194,100×g for 3 hr at 10°C. Cholesterol concentration in the serum and each fraction was measured by an enzymatic method (Determiner TC555, Kyowa Medex Co., Tokyo, Japan). The cholesterol level in VLDL was calculated by subtracting cholesterol in the LDL+HDL fraction from the total cholesterol level. The LDL cholesterol level was calculated by subtracting the HDL cholesterol level from the LDL+HDL cholesterol level.

6. Measurement of cholesterol content in the aorta and liver: Lipids in the aorta and liver were extracted according to Folch et al. (8). The lipid extract was evaporated in vacuo, and redissolved with isopropyl alcohol. Cholesterol concentrations were determined as described earlier.

7. Determination of fatty streaks in area percent: The outline of the aortic surface and intimal lesion on the Xerox copy were traced onto thin transparent graph paper ruled into 1 mm squares. The number of 1 mm squares in the traced boundaries were counted according to Hata et al. (9).

8. Histological examination: The frozen specimen of ascending aorta was sliced into sections by a cryotome, stained with Oil Red-O. The lipid deposition was observed microscopically. Three specimens (1 cm length) were obtained from each aorta at 2–3 mm (ascending) from the aortic valve, adjacent first branch of the intercostal artery (descending) and near the renal artery (abdominal). These strips of aorta fixed in phosphate-buffered formalin were embedded in paraffin, cut into sections and stained with hematoxylin-eosin, elastica Van Gieson (for elastic fiber), Azan Mallory (for collagen fiber and smooth muscle cell), and Kossa (for calcium). All the preparations were scored as follows: negative to slight, − − +; moderate, ++; and severe, ++++. The transverse specimen of the heart was taken just below the coronary sinus, embedded in paraffin, cut into sections, and stained with hematoxylin-eosin and Masson's trichrome. The frequency of atherosclerotic lesions on coronary arteries was analyzed in the transversed section of heart. The numbers of coronary arteries with or without lesions, which were larger than 80 μm in outer diameter, were counted. The results were expressed in percentage. The examiner who scored these aortic and coronary sections did so in a “blind fashion”.

9. Statistical methods: Statistical analysis was performed using Student's t-test (measured data) (10) and the chi-square test (frequency data) (11).

Results

1. General observation: There was no remarkable difference in the body weight among the groups. The food intakes in the drug treated groups were comparable to those in the control group. Two rabbits that had received MK-733 at the dose level of 10 mg/kg died at 6 and 9 weeks after the treatment due to possible respiratory infections. The
data obtained from these rabbits, therefore, were excluded from the final results.

2. Serum lipid levels: As shown in Fig. 2, total cholesterol levels were increased markedly by the feeding of a 1% cholesterol diet and reached 2263 mg/dl at 12 weeks in the control group. MK-733, on the other hand, markedly prevented this increase; MK-733 at 10 mg/kg almost completely inhibited the increase. MK-733 at the dose levels of 5 mg/kg and 2.5 mg/kg inhibited the increase of total cholesterol levels to 445 mg/dl and 982 mg/dl, respectively. Lovastatin also prevented the increases of total cholesterol levels to 1130 mg/dl. The phospholipid levels in the serum were also increased by the treatment of cholesterol feeding and reached 787 mg/dl at 12 weeks. MK-733 prevented the increase of phospholipid levels. Lovastatin also suppressed the increase of phospholipid levels. The suppressive effect of 5 mg/kg of lovastatin on serum cholesterol and phospholipid levels was found to be almost equal to that of 2.5 mg/kg of MK-733. The triglyceride levels in serum were slightly increased by the feeding of cholesterol. MK-733 at the dose level of 5 and 10 mg/kg prevented the increase of triglyceride levels.

![Fig. 2. Preventive effects of MK-733 and lovastatin on levels of serum total cholesterol, phospholipids, and triglyceride in 1% cholesterol-fed rabbits.](image)

![Fig. 3. Effects of MK-733 and lovastatin on levels of serum VLDL-, LDL- and HDL-cholesterol in 1% cholesterol-fed rabbits.](image)
As shown in Fig. 3, VLDL cholesterol and LDL cholesterol levels were increased remarkably by cholesterol feeding and reached 1464 mg/dl and 771 mg/dl, respectively, at 12 weeks. HDL cholesterol levels were slightly increased by cholesterol feeding. MK-733 at the dose level of 5 and 2.5 mg/kg inhibited the increase of VLDL cholesterol more significantly than that of LDL cholesterol, whereas MK-733 at 10 mg/kg inhibited the increase of VLDL and LDL cholesterol equally. The effect of 5 mg/kg of lovastatin on lipoprotein cholesterol levels was almost the same as that of 2.5 mg/kg of MK-733. MK-733 at 10 mg/kg reduced HDL cholesterol levels significantly, but these levels were significantly higher than those in the non-fed control group.

3. Atherosclerosis: The atherosclerosis in the main trunks of coronary arteries was scarcely developed even in the control group. The numbers of coronary arteries with or without lesions, which were larger than 80 μm in outer diameter, were counted. As shown in Fig. 4, the coronary atherosclerosis with severe stenosis in small and medium sized vessels appeared with high frequency (34.5%) in the control group. MK-733 reduced the frequency of the coronary atherosclerosis to 0.9% at the dose level of 10 mg/kg, 3.5% at 5 mg/kg and 15.8% at 2.5 mg/kg. Lovastatin also inhibited the development of coronary atherosclerosis.

Aortic fatty streaks were recognized in all rabbits in the control group. As shown in Fig. 5, fatty streak involvement in the aorta was 43.5% of the total area in the control group. MK-733 suppressed the development of aortic fatty streaks: its development was inhibited almost completely with 10 mg/kg of MK-733, but reduced to 8.4% with 5 mg/kg of MK-733, to 18.7% with 2.5 mg/kg of MK-733, and to 11.3% with 5 mg/kg of lovastatin. Histological findings in aortae are summarized in Table 2. The incidence of atherosclerotic lesions found in 3 sections of each aorta was 29/30 in the control group. MK-733 prevented the formation of the lesions. MK-733 at the dose level of 10 mg/kg completely inhibited the development of lesions. The frequencies of atherosclerotic lesions with MK-733 at the dose levels of 5 mg/kg and 2.5 mg/kg...
Table 2. Histological findings, incidence of atherosclerosis lesions in aortic specimens and changes of components in lesions

<table>
<thead>
<tr>
<th>Group: Treatment</th>
<th>(N)\textsuperscript{a}</th>
<th>Incidence of atherosclerotic lesion</th>
<th>Foam cell</th>
<th>Collagen fiber</th>
<th>Elastic fiber</th>
<th>Smooth muscle cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Control</td>
<td>(10)</td>
<td>29/30\textsuperscript{c}</td>
<td>0\textsuperscript{d}</td>
<td>15</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>B: MK-733 10 mg/kg</td>
<td>(8)</td>
<td>0/24\textsuperscript{**}</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C: MK-733 5 mg/kg</td>
<td>(10)</td>
<td>15/30**</td>
<td>2</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>D: MK-733 2.5 mg/kg</td>
<td>(10)</td>
<td>20/30**</td>
<td>2</td>
<td>9</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>E: Lovastatin 5 mg/kg</td>
<td>(10)</td>
<td>18/30**</td>
<td>1</td>
<td>14</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>F: Non-fed control</td>
<td>(6)</td>
<td>1/18**</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} number of animals in parenthesis. \textsuperscript{b} grade, −−+: negative to slight, ++: moderate, +++: severe. \textsuperscript{c} 3 parts of the aorta were observed in each aorta. \textsuperscript{d} distribution of grade in atherosclerotic lesions. Significantly different from the control (chi-square test). **P<0.01.
kg were 15/30 and 20/30, respectively. Lovastatin also decreased the frequency of atherosclerotic lesions to 19/30. Only one lesion was detected in the non-fed control group. However, this lesion was not thought to be an atheromatous lesion, because it did not include foam cells. The accumulation of foam cells and proliferation of collagen fibers, elastic fibers and smooth muscle cells in the atheroma were observed in almost all the aortic sections in the control group. Both MK-733 and lovastatin prevented these alterations. The calcium deposition, destruction of internal elastic lamella, and necrosis in the atheroma and media of the aorta were relatively weak even in the control group (data, not shown). The foam cell formation, and proliferations of fibers and smooth muscle cells in the atheroma in the 2.5 mg/kg and 5 mg/kg of MK-733 groups were weaker than those in the control group. The atheroma formed in the drug treated groups was not qualitatively different from those in the control group.

As shown in Table 3, the cholesterol content in the aorta was 1.1 mg/g tissue (wet weight) in the non-fed control group, and 11.4 mg/g tissue (wet weight) in the control group. MK-733 prevented the increase of the aortic cholesterol content. MK-733 at the dose level of 10 mg/kg reduced the cholesterol content in the aorta to less than that in the non-fed control group. The cholesterol content in the liver was 2.0 mg/g tissue (wet weight) in the non-fed control and increased to 25.7 mg/g tissue (wet weight) by the feeding of 1% cholesterol. MK-733 diminished the accumulation of cholesterol in the liver. Lovastatin also inhibited the accumulation of cholesterol in the aorta and liver with a potency almost equal to that of MK-733 at 2.5 mg/kg. In the control group, the aortic lipid deposition stained with Oil Red-O was observed in both atheromatous and non-atherosclerotic regions. Most of the aortic lipids accumulated in the foam cells of the atheroma, and a less amount of lipids infiltrated the extracellular space of smooth muscle cells in the media. Both MK-733 and lovastatin prevented microscopic lipid deposition in the aorta (data, not shown). No lipid deposition was observed in the aorta of rabbits receiving MK-733 at the dose level of 10 mg/kg.

### Table 3. Effects of MK-733 and lovastatin on cholesterol contents of aorta and liver in rabbits fed a 1% cholesterol diet

<table>
<thead>
<tr>
<th>Group: Treatment</th>
<th>Cholesterol content (mg/g wet weight) (N)*</th>
<th>Liver (mg/g wet weight) (N)</th>
<th>Serum total cholesterol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Control</td>
<td>11.3±0.23 (8)</td>
<td>25.7±0.18 (10)</td>
<td>[2263 ± 499.4]</td>
</tr>
<tr>
<td>B: MK-733 10 mg/kg</td>
<td>0.7±0.09** (8)</td>
<td>3.6±0.13*** (8)</td>
<td>[94.5 ± 89.0]</td>
</tr>
<tr>
<td>C: MK-733 5 mg/kg</td>
<td>1.6±0.89** (10)</td>
<td>9.7±0.34*** (10)</td>
<td>[445.2 ± 199.9]</td>
</tr>
<tr>
<td>D: MK-733 2.5 mg/kg</td>
<td>2.7±1.43** (9)</td>
<td>18.2±0.68* (10)</td>
<td>[981.6 ± 521.7]</td>
</tr>
<tr>
<td>E: Lovastatin 5 mg/kg</td>
<td>3.4±0.94** (9)</td>
<td>16.5±11.27 (10)</td>
<td>[1130 ±1020]</td>
</tr>
<tr>
<td>F: Non-fed control</td>
<td>1.1±0.10** (6)</td>
<td>1.9±0.13*** (6)</td>
<td>[20.3 ± 5.6]</td>
</tr>
</tbody>
</table>

* Number of animals in parenthesis. Each value represents the mean±S.D. Significantly different from the control (Student's t-test). **P<0.05, ***P<0.01, ****P<0.001.

### Discussion

MK-733 (simvastatin) is a chemical derivative of lovastatin, which was isolated from culture filtrates of Aspergillus terreus (12) as a competitive inhibitor of HMG-CoA reductase. Lovastatin has been used as an anti-hyperlipidemic drug since September of 1987 in the U.S.A. Lovastatin is prescribed for the reduction of elevated total and LDL cholesterol levels in patients with primary hypercholesterolemia. Lovastatin reduces total cholesterol and LDL cholesterol, and it elevates HDL cholesterol.

In the present experiment, cholesterol feeding markedly increased total, VLDL and LDL cholesterol and phospholipid levels, and it slightly increased HDL cholesterol and triglyceride levels. In comparison with normal VLDL, in which triglyceride is the predominant
lipid, the VLDL of cholesterol-fed rabbits contains cholesterol ester as the major lipid (13). Because this lipoprotein shows an abnormal \( \beta \)-mobility on electrophoresis, it is called \( \beta \)-VLDL (14). A mechanism for the increase in \( \beta \)-VLDL is thought to be the impaired removal of \( \beta \)-VLDL from plasma in cholesterol-fed rabbits. This impaired removal of \( \beta \)-VLDL is caused by the reduced number of high affinity, saturable cell surface receptors (15). The elevation of VLDL levels may accompany triglyceride elevation. MK-733 was found to prevent an increase of serum cholesterol levels, and lovastatin also markedly inhibited their increase. MK-733 and lovastatin inhibited the increase of VLDL and LDL cholesterol. MK-733 at 10 mg/kg reduced HDL cholesterol levels compared to the control, but these values were significantly higher than those in the non-fed control group. MK-733 at 10 mg/kg inhibited the increase of total cholesterol levels by 96%. This may cause the inhibition of increase of HDL cholesterol. MK-733 and lovastatin suppressed the increase of serum phospholipid levels, and it slightly affected the triglyceride levels.

Kritchevsky et al. (16) reported that lovastatin was effective on hypercholesterolemia and atherosclerosis induced by cholesterol feeding in rabbits. The results from our present study about lovastatin have confirmed their observation.

There are, theoretically, three mechanisms to control the expansion of the cholesterol pool: 1) decreased absorption of dietary cholesterol; 2) decreased synthesis of cholesterol; and 3) increased excretion of either or both cholesterol and its metabolites, the bile acids (17).

Serum cholesterol levels in cholesterol-fed rabbits are very markedly higher than those in normal ones. A major part of the serum cholesterol is thought to be derived from the exogenous source in cholesterol-fed rabbits. So, we examined the effect of MK-733 on \([^{3}H]\)-cholesterol absorption in cholesterol-fed rabbits (18). The multiple treatment with MK-733 (10 mg/kg/day) clearly reduced the serum \([^{3}H]\)-radioactivity in the cholesterol-fed rabbits. The area under the serum radioactive concentration-time curve (AUC) of \([^{3}H]\)-cholesterol calculated from the serum radioactivity in the MK-733 group was about 9.6% of that in the control group. The cumulative excretion of the fecal radioactivity of \([^{3}H]\)-cholesterol in the MK-733 group was higher than that in the control group. From these results, it is thought that MK-733 inhibits the absorption of cholesterol from the gastrointestinal wall in the cholesterol-fed rabbits.

These results may show that the decreased levels of VLDL and LDL cholesterol were caused by the reduction of cholesterol absorption.

It is not clear whether MK-733 affects the excretion of cholesterol and/or bile acids. So, we plan to examine the effect of MK-733 on the cholesterol and bile acid excretion.

Despite the fact that the mechanism of action of MK-733 on cholesterol transport and metabolism is not yet known at this time, it seems that MK-733 has a profound effect on cholesterol absorption and can thereby prevent exogenously induced hypercholesterolemia.

Total serum cholesterol levels (94.5 mg/dl) in the MK-733 (10 mg/kg) group were 5 times higher than those (20.3 mg/dl) obtained for the non-fed control group at the end of treatment and yet the concentration of total cholesterol in the wall of the aorta was less in the MK-733 (10 mg/kg) group than in the non-treated group. It is possible, therefore, that MK-733 may also affect the distribution of cholesterol between the serum compartment and the peripheral tissue.

MK-733 prevented the development of atherosclerosis in the aorta and coronary arteries. Moreover, the anti-atherosclerotic potency of MK-733 was found to be similar to its hypolipidemic potency. So, it was thought that hypolipidemic effect of MK-733 is the main mechanism for preventing the development of atherosclerosis. These results seem to support the view that hypercholesterolemia is a major risk factor in atherosclerosis.

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