Inhibitory Effects of Amlodipine and Fluvastatin on the Deposition of Advanced Glycation End Products in Aortic Wall of Cholesterol and Fructose-Fed Rabbits

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Recent studies suggest that advanced glycation end products (AGEs) can promote the development of atherosclerotic lesions in a similar manner to oxidatively modified low density lipoproteins. As oxidative stress accelerates the formation of AGEs, antioxidant drugs may exert atheroprotective effects via suppression of AGE formation. Although amlodipine, a calcium channel blocker, and fluvastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, show antioxidant and atheroprotective effects, the relation of AGEs to their effects is unknown. We immunohistochemically investigated the inhibitory effects of chronic treatment with amlodipine (5 mg/kg per day) or fluvastatin at a dose insufficient to reduce plasma cholesterol levels (2 mg/kg per day) on the accumulation of AGEs in atherosclerotic aortas of rabbits fed 1% cholesterol diet and 10% fructose containing water. After eight weeks of treatment, AGEs, namely argpyrimidine, carboxymethyllysine and pyrraline, markedly accumulated with intimal thickening in cholesterol and fructose-fed control rabbits, while the drugs inhibited those changes other than the pyrraline deposition without plasma lipid-lowering effects. Enhanced lipid peroxidation was observed in plasma from cholesterol and fructose-fed rabbits only, and lipid peroxidation was not suppressed by the drugs. These results suggest that the atheroprotective effects of the drugs are at least partly due to the suppression of AGE accumulation although the exact mechanism of AGE suppression is ambiguous.

Key word amlodipine; atherosclerosis; advanced glycation end product; fluvastatin; hypercholesterolemia; rabbit

It has been well established that oxidative modification of low density lipoproteins (LDL) plays an important role in atherosclerosis. 1, 2 The uptake of oxidatively modified LDL by macrophages and smooth muscle cells results in the formation of foam cells which accumulate lipid droplets with consequent cell-mediated responses leading to vascular dysfunction, matrix expansion and atherosclerosis. Although the structure of oxidatively modified LDL has not been well characterized, malondialdehyde (MDA)-lysine, 3, 4 4-hydroxy-2-nonenal (HNE)-protein 4 and oxidized phosphatidylcholine 5 have been recognized as the oxidatively modified moieties. Recently, non-enzymatic glycation (Maillard reaction) and the resultant advanced glycation end products (AGEs) such as carboxymethyllysine (CML), 6 pyrraline, 7 and 3-deoxyglucosone (3-DG)-hydromidazolone, 8 have been reported to contribute to the progression of atherosclerosis in humans according to the similar mechanism as in oxidatively modified LDL. 7, 8 It has also been reported that oxidative stress accelerates glycation processes to form AGEs, 8 and conversely glycation reactions generate reactive oxygen species. 10 Furthermore, it has been found that AGEs accelerate oxidative stress via recognition and internalization by their receptors on vascular cells. 11 Thus glycation and oxidative stress are closely linked, and AGEs probably promote the development of atherosclerosis in combination with oxidatively modified LDL. 3

Use of antioxidants is considered to have beneficial effects in the prevention of atherosclerosis because oxidative stress is said to play an integral role in the development of atherosclerotic lesions as described above. 12 Recently, there has been a growing amount of evidence that calcium channel blockers, widely used in the treatment of hypertension, myocardial ischemia and arrhythmia, have atheroprotective effects due to their antioxidant activities. 13 Amlodipine, a long acting and charged dihydropyridine-type calcium channel blocker, exerts antioxidant actions based on not only its electron-donating activity but also the intimate physicochemical interaction with cell membrane. 14 It has shown significant antioxidant activities on isolated membrane vesicles while no effect was observed with other representative calcium channel blockers such as felodipine, verapamil and diltiazem. 14 The inhibitory effects of amlodipine on copper-induced oxidation of LDL in vitro have been also reported. 13, 15 Amlodipine has lowered the level of MDA, an index of lipid peroxidation, in plasma and aorta with reduction in the accumulation of aortic cholesterol in cholesterol-fed rabbits. 6, 7

On the other hand, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors are the most potent antihypercholesterolemic agents, and effective in the prevention of atherosclerosis and its accompanying cardiovascular events. 18, 19 Fluvastatin, the first entirely synthetic HMG-CoA reductase inhibitor, has been reported to suppress atherosclerosis due to its antioxidant activities related to its unique chemical structure 20 and the cholesterol-lowering effects. This drug showed strong free radical scavenging activity, whereas other HMG-CoA reductase inhibitors such as pravastatin, simvastatin, and lovastatin yielded only very weak effects. 21 Fluvastatin but neither pravastatin nor simvastatin showed inhibitory effects on copper-induced oxidation of LDL in vitro. 21, 22 In cholesterol-fed rabbits, fluvastatin at a dose insufficient to reduce plasma cholesterol levels significantly decreased susceptibility of LDL to ex vivo copper-induced oxidation, reduced lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS) in serum, plaque area, formation of superoxide anion and accumulation of macrophages in aortic wall, and reversed the suppression
of acetylcholine-induced relaxation of aortic wall.\textsuperscript{21–23} These inhibitory effects of fluvastatin were not observed in cholesterol-fed rabbits treated with pravastatin.\textsuperscript{22,23}

These studies seem to suggest that amlodipine and fluvastatin help reduce the risk of atherosclerosis via their antioxidant activities. However, no work has been done on their inhibitory effects on the formation and deposition of AGEs related to the development of atherosclerotic lesions. Thus, in the present study, we have investigated the inhibitory effects of these drugs on the deposition of AGEs and progression of atherosclerosis assessed by intimal thickening in aortas of hypercholesterolemic rabbits.

MATERIALS AND METHODS

Chemicals Amlodipine besilate and fluvastatin sodium were supplied by Sumitomo Pharmaceuticals (Osaka, Japan) and Novartis Pharma (Tokyo, Japan), respectively. Phosphate buffered saline powder and Tris–HCl buffer powder were supplied by Sumitomo Pharmaceuticals (Osaka, Japan) and Novartis Pharma (Tokyo, Japan), and dissolved with distilled water to prepare phosphate buffered saline solution (PBS, 10 mM, pH 7.2) and Tris buffer solution (50 mM, pH 7.6), respectively. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Antibodies Vectastain Elite ABC Mouse IgG kit and monoclonal anti-apolipoprotein-B (apo-B) antibody T3 (0.5 mg/ml, clone No. 4C11) were purchased from Funakoshi (Tokyo, Japan). Monoclonal anti-CML antibody (0.25 mg/ml, clone No. CMS-10), anti-pantryal antibody (0.25 mg/ml, clone No. H12) and anti-rabbit macrophage antibody (0.25 mg/ml, clone No. RbM2) were obtained from Trans Genic (Kumamoto, Japan). Monoclonal anti-Arggypirimidine antibody (0.1 mg/ml, clone No. 5B3CMHAH13) was obtained from NOF CORPORATION (Tokyo, Japan).

Animal Studies and Sample Collection Male New Zealand White rabbits weighing 2.5 kg (starting age 10 weeks) were divided into four groups: Group NN=standard chow (n=4); Group CF=standard containing 1% cholesterol and 10% fructose in drinking water (n=6); Group AM=the high cholesterol and fructose diet plus amlodipine, 5 mg/kg body weight per day (n=6); Group FL=the high cholesterol and fructose diet plus fluvastatin, 2 mg/kg body weight per day (n=6). These diets were prepared by mixing amlodipine and fluvastatin directly into each respective high cholesterol diet. Each treatment was continued for eight weeks. About 100 g of food were given to the rabbits every morning. All rabbits were allowed free access to drinking water.

Blood (5 ml) was drawn from an ear vein after 48 h fasting at eight weeks of dietary. A vacuum collection tube containing 7.5 mg of ethylenediaminetetraacetic acid disodium (Venoejct II VP-NA050, Terumo, Tokyo, Japan) for anticoagulation and prevention of autooxidation of lipoproteins was used. Plasma was separated from the blood by centrifugation, and stored in plastic tube until analysis at –80 °C. Total cholesterol and HDL cholesterol in plasma were measured by enzymatic methods using Hitachi (Tokyo, Japan) 7170 automatic analyzer. Lipid peroxides in plasma were assessed by TBARS which was measured by a kit (Wako) and represented in nmol MDA. After blood collection, the rabbits were anesthetized by administration of pentobarbital sodium (25 mg/kg) into the marginal ear vein, disected, and killed by exsanguination from vena cava. The vascular segments (ca. 5 mm length) between the heart and the bifurcation of the carotid arteries were excised, cleared of adhering fat and connective tissue, rinsed with saline, and embedded in Tissue-Tek O.C.T. compound (SAKURA, Tokyo, Japan). The embedded samples were immediately frozen on dry-ice, and stored at –80 °C until histological examination.

Histochemical Methods Thin sections of 5 µm thickness were made of the embedded samples by a cryostat, mounted onto APS-coated slides (Matsunami, Osaka, Japan), fixed with acetone, and rinsed with phosphate buffered saline. The sections were stained with hematoxylin and eosin (HE) following a standard procedure. Intimal thickening of each aorta was estimated as an index of the extent of atherosclerosis by measuring the cross-sectional area of intima and media of five serial sections using an AX80 microscope coupled with a DP50 digital camera system (Olympus Optical, Tokyo, Japan).

Immunostaining of AGEs was performed using the Vectastain Elite ABC Mouse IgG kit based on an avidin/biotin/peroxidase system. The sections were incubated with 10% normal horse serum in PBS at room temperature for 30 min to block nonspecific binding of the second antibody and then reacted with the primary antibodies against different AGE structures, apo-B or macrophages at 4 °C over night. After they were washed in PBS, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol at room temperature for 40 min. They were washed in PBS and exposed to the biotinylated secondary antibody at room temperature for 30 min. After washing in PBS, the specimens were incubated with an avidin–biotin–peroxidase complex at room temperature for 30 min. Deposition was visualized by treating the sections with a solution of 3,3’-diaminobenzenedine tetrahydrochloride (DAB, 30 mg), sodium azide (65 mg) and 30% hydrogen peroxide (18 µl) in Tris buffer (100 ml). After washing with PBS, the sections were stained with methyl green. Control tests for the specificity of immunostaining included the substitution of PBS for the primary antibodies. No significant staining was observed in the control tests.

Statistical Analysis Data were expressed as means± standard deviation (S.D.). Statistical analysis was done using t-test.

RESULTS

Plasma Lipids and TBARS Levels In this study, fluvastatin was administered at a dose insufficient to reduce plasma cholesterol levels to exclude the effects to inhibit atherogenesis through the lipid-lowering property.\textsuperscript{21,23} There was no significant difference in body weight among the different groups of rabbits at the end of experiment. Plasma lipids and TBARS levels were summarized in Table 1. Plasma total cholesterol levels were significantly greater in rabbits fed cholesterol and fructose, whether or not supplementation with amlodipine or fluvastatin was provided. There were no significant differences in plasma HDL cholesterol levels among the different groups of rabbits. The plasma TBARS level of the Group NN rabbits was 1.0±0.1 nmol/ml, while
that of the Group CF rabbits nearly doubled. The decrease in plasma TBARS was not observed by supplementation with amlodipine or fluvastatin.

Morphological Examination of Atherosclerotic Aorta

The representative appearance of HE stained sections of atherosclerotic aortas from the four experimental groups is shown in Fig. 1. Group NN rabbits fed standard chow for eight weeks exhibited no intimal thickening in aortas. A remarkable intimal thickening of aortas was induced by cholesterol and fructose feeding, and it tended to be reduced by supplementation with amlodipine or fluvastatin, the latter being more effective than the former (Fig. 2).

Immunohistochemical Analysis of Atherosclerotic Aorta

The presence of different AGEs including argpyrimidine, CML and pyrraline was immunohistochemically examined in the tissue specimens of the aortas from the four experimental groups. Figures 3, 4 and 5 show the representative appearance of argpyrimidine, CML, and pyrraline immunoreactivity, respectively. Immunostaining of argpyrimidine was observed in both of the intima and media of Group CF rabbits (Fig. 3B). Immunostaining of CML and pyrraline was mainly observed in the intima and the inner media of Group CF rabbits, respectively (Figs. 4B, 5B). Amlodipine and fluvastatin treatment effectively reduced the appearance of argpyrimidine and CML (Figs. 3C, D, 4C, D), and the effects by both drugs were comparable. The effects by these drugs on the deposition of pyrraline were, on the whole, ambiguous although the speckled stain indicated by arrows was suppressed, as shown in Figs. 5C, D. Immunoreactivity of macrophages and apo-B was found in the intima of all rabbits fed cholesterol and fructose, and that of apo-B was also in the media of all groups of rabbits, and the density of immunostaining was similar irrespective of supplementation with amlodipine or fluvastatin (Figs. 6, 7).

DISCUSSION

Deposition of AGEs in Atherosclerotic Lesions

It has been well recognized that AGEs have close connection with the pathophysiological processes of various chronic diseases. The in vivo accumulation of AGEs has been studied in various tissues such as artery, kidney and skin in relation to atherosclerosis, diabetic nephropathy and aging, respectively. Imanaga et al. have demonstrated that CML accumulates in human atherosclerotic lesions. Sakata et al. have shown that CML and MDA-protein mainly colocalize within macrophage/foam cells in human atherosclerotic lesions while pyrraline and apo-B localize in the extracellular matrix. In these studies the AGE structures have been suggested to form on LDL molecules.

Although hypercholesterolemic rabbits have been generally used for the study of atherosclerosis, there had been no report of AGE accumulation in their atherosclerotic aortas before our previous report except for 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole whose presence in vivo is still controversial. In the present study, we examined old well-known AGEs, i.e., CML and pyrraline, and a relatively new AGE structure, argpyrimidine in cholesterol and fructose-fed rabbits. Fructose was given in addition to cholesterol in order to accelerate the formation of AGEs related to atherosclerotic lesions because fructose promotes in vitro AGE formation about 8—10 times faster than glucose. Consequently, CML and pyrraline were found to accumulate in the atherosclerotic aortas of the control cholesterol and fructose-fed New Zealand White rabbits as in humans and cholesterol and fructose-fed Japanese White rabbits. In addition, argpyrimidine remarkably accumulated with intimal thickening. No data has been reported to date on the accumulation of argpyrimidine in atherosclerotic aortas although the AGE structure has been found in retina and media of small artery wall of kidney from diabetic patients. The simultaneous deposition of argpyrimidine and CML, macrophage, and apo-B in the intima is apparently consistent with the proposed mechanism where modified LDL is incorporated into macrophages, which accumulate in the proliferated intima. However, further examination is required to clarify the exact mechanism of AGE deposition in aortic wall because the localization of pyrraline in the intima was ambiguous, and the immunostaining of the three AGEs in the media was considerably different as shown in Figs. 3B, 4B, 5B. The observed accumulation of argpyrimidine, CML and pyrraline accompanied by intimal thickening suggests that the AGES are important factors in the development of atherosclerosis of cholesterol and fructose-fed rabbits, and various AGEs may contribute differently to the development of atherosclerotic lesions.

Recently, carbonyl stress compounds originating from sugars, lipids and amino acids, whose accumulation is mainly accelerated by oxidative stress, have been suggested to play a major role in the formation of chemically modified proteins such as AGEs and advanced lipoxidation end products rather than the conventional glycation pathways accompanying formation of Amadori compounds, and exhibit direct actions to cardiovascular systems. The carbonyl stress compounds comprise of glyoxal, methylglyoxal, 3-DG, MDA, HNE etc. In particular, the former three compounds, namely 3-ketoaldehydes, have lately attracted special interest as the main carbonyl stress compounds because they have higher chemical reactivity to form various AGEs such as argpyrimidine, CML and pyrraline and direct actions to cardiovascular systems. The latter two compounds are of importance in the sense that they are well-known as lipid peroxidation products and the precursors of MDA-lysine and HNE-protein recognized as the oxidatively modified LDL. Argyrimidine and pyrraline are exclusively formed from methylglyoxal and 3-DG, respectively, while CML is formed by the condensa-

Table 1. Effects of Amlodipine and Fluvastatin on Plasma Lipids and TBARS in High Cholesterol and Fructose-Fed Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cholesterol (mg/dl)</th>
<th>HDL cholesterol (mg/dl)</th>
<th>TBARS (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group NN</td>
<td>34±12</td>
<td>18±3.11</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Group CF</td>
<td>943±252*</td>
<td>19.2±13.9</td>
<td>2.3±0.7*</td>
</tr>
<tr>
<td>Group AM</td>
<td>1312±744*</td>
<td>39.4±40.2</td>
<td>1.9±0.5*</td>
</tr>
<tr>
<td>Group FL</td>
<td>1380±412*</td>
<td>19.3±12.7</td>
<td>2.3±0.6*</td>
</tr>
</tbody>
</table>

a) Data of Group NN and other groups are represented as mean±S.D. for four and six rabbits, respectively, except for the HDL cholesterol value (n=5) of Group AM. Group NN=standard chow only; Group CF=high cholesterol and fructose; Group AM=high cholesterol and fructose plus amlodipine; Group FL=high cholesterol and fructose plus fluvastatin; TBARS=thiobarbituric acid reactive substances. ∗p<0.01 versus Group NN.
Fig. 1. Comparative HE Staining of Atherosclerotic Aorta Wall
A, Group NN=standard chow only; B, Group CF=high cholesterol and fructose; C, Group AM=high cholesterol and fructose plus amlodipine; D, Group FL=high cholesterol and fructose plus fluvastatin. Original magnification is ×100 for all photographs.

Fig. 2. Inhibitory Effects of Amlodipine or Fluvastatin Treatment on Intimal Thickening in Thoracic Aorta of Rabbits Fed High Cholesterol and Fructose for 8 Weeks
Data are represented as mean±S.D. Group CF=high cholesterol and fructose (n=5); Group AM=high cholesterol and fructose plus amlodipine (n=6); Group FL=high cholesterol and fructose plus fluvastatin (n=6); Group NN=standard chow only (n=4). Intima/media ratio is the ratio of intimal area to medial area.

Fig. 3. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for Argpyrimidine
The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was positive in the intima and media (B, arrows). Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

Fig. 4. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for CML
The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was positive in the intima (B, arrows). Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.
Fig. 5. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for Pyrraline

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was positive in the inner media (B, arrows). However, that in the intima was slight. Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

Fig. 6. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for Rabbit Macrophages

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was observed in the intima (B, C, D). Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.

Fig. 7. Comparative Immunohistochemical Staining of Atherosclerotic Aorta Wall with a Monoclonal Antibody for apo-B

The abbreviations are the same as in Fig. 1. The presence of antigen is indicated by a red-brownish color. Immunoreactivity was observed in the intima and media. Original magnification is ×100 for all photographs. L=lumen; I=intima; M=media.
tion of glyoxal with proteins as well as the oxidative degradation of Amadori compounds.\(^8,9\) Thus, these AGEs may be useful as the integrative biomarkers for carbonyl stress. The observed accumulation of these AGEs in the control cholesterol and fructose-fed rabbits probably reflects the increase in the concentrations of the corresponding \(\alpha\)-ketoaldehydes.

The increase of plasma TBARS indicated the acceleration of oxidative stress in the Group CF rabbits compared with the Group NN rabbits. Similar acceleration of oxidative stress has been observed previously in cholesterol-fed rabbits.\(^6,7,17,21,23\) This enhanced oxidative stress has been reported to be explained by the increase of NADPH oxidase which forms superoxide anion in artery.\(^30\) Oxidative stress not only accelerates formation of some AGEs including CML from Amadori products but also promotes formation of glyoxal and suppresses detoxification of \(\alpha\)-ketoaldehydes by glyoxalase.\(^29\) leading to the acceleration of carbonyl stress. In addition, fructose fed to rabbits contributes to the accumulation of the above AGEs because it is converted to Amadori compounds and carbonyl stress compounds including methylglyoxal and 3-DG.\(^6,9,25\) The accelerated oxidative and carbonyl stress are thus considered to be responsible for the marked deposition of argpyrimidine, CML and pyrraline.\(^9\) Needless to say, cholesterol feeding increases the level of plasma LDL so that formation of AGE-modified LDL accelerates.\(^9\)

**Inhibitory Effects of Amlodipine and Fluvastatin on the Deposition of AGEs in Atherosclerotic Lesions**

The deposition of argpyrimidine and CML in the aortic walls of the cholesterol and fructose-fed control rabbits was remarkably suppressed by amlodipine and fluvastatin, independent of their lipid-lowering effects. These results are the first presentation of pharmacological effects to inhibit the deposition of AGEs in atherosclerotic aortas. In the literature,\(^17,21,23\) atherosclerotic lesions are suppressed at the dose used in the present experiments. Treatment by the drugs showed, in fact, a tendency to reduce intimal thickening although the reduction was statistically insignificant. Thus, the observed inhibitory effects on the deposition of AGEs are likely associated with their atheroprotective effects.

The suppressive effects for AGEs were thought to be due to the reduced formation of AGEs by the antioxidant activities of the drugs. However, no significant decrease in plasma TBARS, an index of lipid peroxidation in the circulation, was observed. Turgan et al.\(^17\) have reported that amlodipine (5 mg/kg/d) reduces plasma MDA in New Zealand White rabbits fed 1% cholesterol for eight weeks. Bandoh et al.\(^23\) have reported that fluvastatin (2 mg/kg/d) reduces serum TBARS in Japanese White rabbits fed 0.5% cholesterol for 17 weeks. The contradiction concerning lipid peroxidation between this paper and the literature is probably due to the differences in the various experimental conditions including the additional feeding of fructose in the present experiments and the index of lipid peroxidation. As mentioned above, oxidative stress increases the levels of argpyrimidine and CML. It should be noted that CML has been proposed as a marker of oxidative stress.\(^31—33\) Thus, the suppressive effects for AGE deposition by amlodipine and fluvastatin may suggest that the drugs exerted their local antioxidant activities in the aortic walls.

Amlodipine and fluvastatin have been reported to show indirect antioxidant activities other than the direct quenching of reactive oxygen species. Amlodipine induced antioxidant nitric oxide production accompanied by increased inducible nitric oxide synthase mRNA expression and protein accumulation in rat vascular smooth muscle cells.\(^34\) Fluvastatin upregulated endothelial nitric oxide synthase mRNA expression and reduced production of superoxide anion,\(^35\) and suppressed the increase of lipid peroxides and NADPH oxidase component mRNA expression,\(^30\) in aortas of 0.5% cholesterol-fed rabbits. These biological activities may be partly responsible for the local antioxidant effects of the drugs.

In conclusion, the present results demonstrate the inhibitory effects of amlodipine and fluvastatin on the deposition of AGEs in atherosclerotic aortas. These effects seem to be associated with the atheroprotective effects although the exact mechanism of AGE suppression is ambiguous. The inhibitory effects on the deposition of AGEs in artery wall of hypercholesterolemic animal models might be a significant indication to evaluate the atheroprotective effects of candidate drugs.

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