Evidence that the hardness of drinking water inversely correlates with the rate of mortality from apoplectic in various areas of Japan was first provided by Kobayashi. Schroeder and Brattleboro also showed that the annual death rates from atherosclerotic heart disease and cerebrovascular disease were higher in areas of the United States where drinking water was not as hard. Similar findings were also reported in the United Kingdom. Hardness of water is determined by both calcium (Ca) and magnesium (Mg) concentration. As the influence of Ca on water hardness is greater than that of Mg, the beneficial effect of hard drinking water had been attributed to the effect of Ca. Karppanen et al., however, suggested that Mg contributes to the decrease in mortality rate from ischemic heart disease (IHD). They showed that in various countries the ratio of Ca to Mg in the diet significantly correlated with the mortality from IHD. Furthermore, cardiovascular morality has been found to correlate inversely with urinary excretion of Mg. These epidemiologic studies clearly indicate that Mg intake may inhibit the development of atherosclerosis. Actually, a Mg-deficient, high cholesterol (1% to 3%) diet reportedly has caused more extensive lipid deposition in the aortas of monkeys and rats. However, concrete experimental results indicating that dietary Mg exerts an antiatherogenic action are scanty.

The purpose of this study was to investigate the effect of supplementary dietary Mg on the development of atherosclerosis in cholesterol-fed rabbits. Cholesterol feeding in rabbits has been widely used as an experimental model to investigate the effect of various agents on the development of atherosclerosis.

**Methods**

**Animal Experiments**

Thirty-one male New Zealand White rabbits weighing about 2.5 kg each were purchased from the Saitama Animal Laboratory (Saitama, Japan). They were kept individually in stainless steel cages in a room where temperature was maintained at 23°C. The rabbits were divided into five groups and were put on five kinds of diets: 1) a regular diet (n=6), 2) a 1% cholesterol diet (n=6), 3) a 1% cholesterol plus 0.3% Mg diet (n=6), 4) a 1% cholesterol plus 0.6% Mg diet (n=7), and 5) a 1% cholesterol plus 0.9% Mg diet (n=6). Cholesterol, Mg sulfate, or both were added to the regular diet (RC4, Oriental Yeast, Tokyo, Japan), which contained 3% fat. Since the regular diet and 1% cholesterol diet contained 0.4% Mg, diets 3, 4, and 5 actually contained a total of 700, 1000, or 1300 mg, respectively, of Mg per 100 g. These diets contained 1360 mg of Ca per 100 g. The other minerals in 100 g of diet were: 0.54 g phosphorus, 0.16 g sodium, 1.82 g potassium, 24.2 mg iron, 14.5 mg aluminium, 1.01 mg copper, 4.49 mg zinc, 0.07 mg cobalt, and 6.43 mg manganese. The calorie count was 294 kcal per 100 g. The rabbits were allowed free access to tap water, which contained less than 1 mg/dl of Ca and Mg.
less than 2 mg/dl of Mg. Each rabbit received 100 g daily of the assigned diet and was fed the whole diet every day of the experiment. Diets containing additional Mg were well tolerated; no abnormal physical signs such as weight loss, decreased appetite, or diarrhea were noted during the experiment.

Systolic blood pressure was measured once a week by a device (Oiso Ikakikai, Tokyo, Japan) developed by Kawaguchi and Grant and Rothschild. An artery in the ear was compressed with an air-tight pressure capsule, which was connected to a sphygmomanometer. The level at which blood flow resumed during deflation of the air-tight pressure capsule was considered to be systolic blood pressure. We tested the accuracy of the noninvasive method for blood pressure measurement in three male New Zealand White rabbits weighing approximately 3 kg. After the rabbits were anesthetized with pentobarbital, the right femoral artery was cut open, and PE-90 tubing was inserted into the abdominal aorta. The tubing was connected to a strain-gauge transducer (Statham P10EZ, Gould, Oxnard, CA) to measure arterial pressure. The right femoral vein was also cannulated with PE-90 tubing. Blood pressure was increased by intravenous infusion of norepinephrine (Sankyo, Tokyo, Japan) and was decreased by intravenous infusion of nitroglycerine (Nihon Kayaku, Tokyo, Japan) over the range of 60 to 145 mm Hg (by the noninvasive method). Arterial pressure was measured simultaneously. Blood pressure measured by the noninvasive method was well correlated with systolic arterial pressure (r=0.82, n=24, p<0.01); with mean arterial pressure (r=0.88, n=24, p<0.01); and also with diastolic pressure (r=0.93, n=24, p<0.01). Body weight was measured when blood pressure was taken.

At 10 weeks into the study, 10 ml of blood was collected from the ear vein of each animal into a plastic syringe containing 0.13 ml of ethylenediaminetetraacetic acid disodium solution (0.5 M). An additional 10 ml of blood was collected into a plastic syringe, which did not contain anticoagulants. These blood samples were centrifuged at 1500 g for 15 minutes at 4°C. The plasma and serum were stored at -20°C until chemical analysis was performed. After blood sampling, rabbits were exsanguinated under pentobarbital anesthesia.

The total cholesterol concentrations and the high density lipoprotein (HDL) cholesterol concentrations in the plasma were measured with an enzymatic method and a heparin-Mn++ precipitation-enzymatic method, respectively. The plasma triglyceride concentrations were measured with an enzymatic method. The total protein, Mg, and inorganic phosphorus concentrations in serum were measured with an auto-analyzer (Hitachi 736, Tokyo, Japan). Ca concentration in serum was measured with atomic absorption spectrophotometry (Hitachi 180-60).

**Quantifying Atherosclerotic Plaque in Aortic Intima**

Aortas were carefully removed from the aortic root to the bifurcation. The surrounding adventitial tissues were cleaned, and the entire aortas were washed twice with saline. The aortas were longitudinally divided with sharp scissors into anterior and posterior halves of approximately the same size. The posterior half of each aorta was affixed to a plastic board and was fixed by incubation in 3.5% formaldehyde solution (pH 7.0, Muto Pure Chemicals, Tokyo, Japan) for 24 hours. The aortas were then washed with distilled water and stained by incubation in oil red O solution for 20 minutes, as described by Willis et al. Oil red O solution was prepared as follows: Oil red O (Nacalai Tesque, Kyoto, Japan) was dissolved in isopropyl alcohol to a concentration of 5 mg/ml. Six aliquots of the solution were subsequently diluted by adding four aliquots of distilled water. After staining, the intimal surface was photographed. The area of the stained intimal surface was measured with an x-y digitizer connected to a microcomputer (PC-9801 VM2, Nihon Electrics, Tokyo, Japan). The ratio of the stained area to the whole intimal surface of the posterior half of the aorta was considered to be the magnitude of atherosclerosis.

**Measurement of Cholesterol, Calcium, and Magnesium in Aortic Wall**

Cholesterol content was measured in the anterior half of the aorta. The aorta was freeze-dried and weighed. Cholesterol was extracted from the freeze-dried aorta by incubating it in 3 ml of chloroform/methanol (2:1) solution at 4°C for 15 hours by the method described by Hollander et al. Cholesterol was further extracted by incubating the aorta in 3 ml of the same solution at 50°C for 20 minutes. The two aliquots of solution were mixed, and 1 ml of the solution was evaporated with nitrogen gas. The residual substance was dissolved in isopropyl alcohol/Triton X-100 (10%) solution. Cholesterol concentration was measured for the solution by the enzymatic method developed by Alain et al.

The aorta was subsequently homogenized with a polytron homogenizer, and the Ca and Mg concentrations in the homogenate were measured with atomic absorption spectrophotometry (Hitachi 180-60).

**Statistics**

The data were analyzed by using one-factor analysis of variance. If a statistically significant effect was found, the Newman-Keuls test was performed to isolate the differences between groups. Student’s t test for paired data was performed to test the significance of blood chemical data before and after the experiment. A p value of less than 0.05 was considered significant. All data are presented in the text, tables, and figures as the means±SEM.

**Results**

**Blood Pressure, Body Weight, and Blood Chemistry**

Systolic blood pressures and body weights during the experiment are shown in Figure 1. A 1% cholesterol diet with additional Mg did not affect the time course of blood pressure and body weight in the rabbits.

The results of plasma lipid concentration are shown in Table 1. The 1% cholesterol diet significantly increased the total cholesterol concentration in the plasma (regular diet group, 43.7±3.8 mg/dl; 1% cholesterol diet group, 1233.3±89.5 mg/dl, p<0.01). Additional dietary Mg had
no further effect. The 1% cholesterol diet also increased triglyceride (TG) concentration in the plasma, and additional dietary Mg decreased the plasma TG concentration that was increased by the 1% cholesterol diet. On the other hand, the 1% cholesterol diet significantly decreased plasma HDL cholesterol concentration (regular diet group, 25.0±1.0 mg/dl; 1% cholesterol diet group, 12.0±0.6 mg/dl, p<0.01). Again, additional dietary Mg had no further effect.

As shown in Table 2, additional dietary Mg increased the serum Mg concentration, indicating effective absorption of Mg. No significant differences were observed in the total protein concentration in the serum from all groups of rabbits either before or after the experiments. Similar results were obtained for serum Ca concentration, except when the highest dose of Mg was given.

Atherosclerotic Plaque In Intimal Surface of Aorta

As shown in Figure 2, the 1% cholesterol diet significantly increased the oil red O-positive area in the intimal surface of the aortas (regular diet group, 3.2±1.4%; 1% cholesterol diet group, 37.3±1.3%, p<0.01). Although the addition of 0.3% Mg had no effect, the addition of 0.6% Mg and 0.9% Mg significantly reduced the area to 11.7±0.9% (p<0.01 vs. 1% cholesterol diet group) and 9.2±1.5% (p<0.01 vs. 1% cholesterol diet group), respectively.

Cholesterol, Calcium, and Magnesium Contents In Aortic Wall

The cholesterol in the aortic wall was similar to that of the atherosclerotic plaque. There was a significant correlation (r=0.86, n=31, p<0.01). As shown in Figure 3, the 1% cholesterol diet significantly increased the cholesterol content of the aorta: regular diet group, 7.7±0.4 mg/g dry weight (DW); 1% cholesterol diet group, 70.7±6.1 mg/g DW, p<0.01. The addition of 0.3% Mg once again had no effect. The addition of 0.6% and 0.9% Mg, however, significantly reduced the cholesterol content to 42.6±7.3 mg/g DW (p<0.05 vs. 1% cholesterol diet group) and 31.2±2.7 mg/g DW (p<0.05 vs. 1% cholesterol diet group), respectively.

The results of aortic Mg and Ca content are shown in Table 3. The aortic Mg content was similar for the five groups of rabbits. However, aortic Ca content tended to decrease in these groups, although no statistical significance was observed.

Discussion

In the present study, we demonstrated that dietary Mg suppresses the development of atherosclerotic plaque in the intimal surface of the aortas of rabbits on high cholesterol diets. This result strongly suggests that dietary Mg has an antiatherogenic effect; this would support the epidemiological observations that suggest that Mg intake helps decrease mortality from atherosclerotic diseases. Antiatherogenic action was not the result of the effect of Mg on blood pressure or body weight. These parameters were similar in the five groups of rabbits throughout the experiment irrespective of the diet on which they were placed. In the present study, no hypotensive action of Mg was noted, although Mg reportedly has a hypotensive action in hypertensive subjects and in spontaneously hypertensive rats. Dietary Mg might not influence the blood pressure of normotensive rabbits.
Some studies have been conducted on the effect of dietary Mg on vascular lesions. Ito et al.\(^{20}\) reported that the addition of Mg to diet effectively prevented the development of coronary atherosclerosis induced by vitamin D in pigs. Renaud et al.\(^{21}\) reported that the administration of Mg (356 mg per 100 g of diet) to rabbits fed saturated fat slightly decreased both the atherosclerotic lesions of the aortic intima and the cholesterol content of the aorta. Although these results are basically consistent with those we obtained in the present study, dietary Mg seemed to have less of an effect than it did in our present study. This might be because different doses of Mg were administered and different methods were used to evaluate the severity of atherosclerosis. In Renaud’s study, severity was semiquantitatively determined by visual grading. On the other hand, Nakamura et al.\(^{22}\) reported that a deficiency of dietary Mg resulted in enhanced atheroma formation in the aortic intima of rats, indicating the physiological significance of Mg intake.

The exact mechanism of the inhibitory effect of Mg on the development of atherosclerosis is not yet known. In the present study, dietary Mg significantly decreased cholesterol content in the aorta without reducing total cholesterol concentration in plasma. Moreover, Mg sup-

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Table 1. Plasma Lipid Concentrations In Five Groups of Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>T-Chol</th>
<th>HDL-Chol</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>43.2±5.9</td>
<td>24.5±2.1</td>
<td>60.0±17.0</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>43.7±3.8</td>
<td>25.0±1.0</td>
<td>56.7±5.4</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>1233.3±99.5</td>
<td>12.0±0.6</td>
<td>186.0±18.2</td>
</tr>
<tr>
<td>1% Chol</td>
<td></td>
<td>36.8±2.7</td>
<td>26.7±1.0</td>
<td>43.8±4.2</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>36.8±2.7</td>
<td>24.3±2.6</td>
<td>65.3±23.1</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>1143.3±217.0</td>
<td>14.2±0.9</td>
<td>139.4±22.6</td>
</tr>
<tr>
<td>1% Chol+Mg 300 mg</td>
<td></td>
<td>48.2±5.6</td>
<td>27.6±3.3</td>
<td>56.4±5.4</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>49.2±5.6</td>
<td>27.6±3.3</td>
<td>56.4±5.4</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>1207.9±80.8</td>
<td>17.0±2.4</td>
<td>139.4±22.6</td>
</tr>
<tr>
<td>1% Chol+Mg 600 mg</td>
<td></td>
<td>40.5±4.7</td>
<td>25.7±2.2</td>
<td>50.7±6.0</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>40.5±4.7</td>
<td>25.7±2.2</td>
<td>50.7±6.0</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>1038.0±75.2</td>
<td>13.5±5.2</td>
<td>127.5±4.6</td>
</tr>
</tbody>
</table>

Values are given as mg/dl and are the means±SEM. B=before experiment, A=after experiment, T-Chol=total cholesterol, HDL-Chol=high density lipoprotein cholesterol.

\(*p<0.05, **p<0.01 vs. control group fed a regular diet; \(\dagger\)p<0.05, \(\ddagger\)p<0.01 vs. B; \(\ddagger\)p<0.05, \(\ddagger\)p<0.01 vs. 1% cholesterol diet group.

Table 2. Chemical Analysis of Serum In Five Groups of Rabbits before and after Experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>T-protein (g/dl)</th>
<th>Calcium (mEq/l)</th>
<th>Magnesium (mg/dl)</th>
<th>Phosphorus (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>5.6±0.2</td>
<td>6.7±0.2</td>
<td>2.73±0.08</td>
<td>5.73±0.28</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>6.6±0.5†</td>
<td>6.7±0.1</td>
<td>2.52±0.14</td>
<td>5.55±0.34</td>
</tr>
<tr>
<td>1% Chol</td>
<td></td>
<td>6.1±0.1</td>
<td>6.7±0.1</td>
<td>2.25±0.18</td>
<td>6.45±0.36</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>7.4±0.3††</td>
<td>6.9±0.2</td>
<td>2.27±0.08</td>
<td>5.77±0.43</td>
</tr>
<tr>
<td>1% Chol+Mg 300 mg</td>
<td></td>
<td>6.0±0.2</td>
<td>6.5±0.1</td>
<td>2.17±0.06</td>
<td>6.53±0.18</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>7.9±0.3††</td>
<td>6.3±0.1</td>
<td>3.43±0.14††</td>
<td>6.25±0.52*</td>
</tr>
<tr>
<td>1% Chol+Mg 600 mg</td>
<td></td>
<td>6.1±0.1</td>
<td>6.5±0.2</td>
<td>2.85±0.08</td>
<td>7.23±0.25</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>7.0±0.1††</td>
<td>6.5±0.1</td>
<td>5.44±0.18††</td>
<td>7.23±0.38**</td>
</tr>
<tr>
<td>1% Chol+Mg 900 mg</td>
<td></td>
<td>5.9±0.2</td>
<td>6.5±0.1</td>
<td>2.20±0.09</td>
<td>6.80±0.53</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>7.2±0.3††</td>
<td>5.9±0.1**††</td>
<td>6.00±0.44**††</td>
<td>6.66±0.47**</td>
</tr>
</tbody>
</table>

Values indicate means±SEM. B=before experiment, A=after experiment, T-protein=total protein, Chol=cholesterol.

\(*p<0.05, **p<0.01 vs. control group fed a regular diet; \(\dagger\)p<0.05, \(\ddagger\)p<0.01 vs. B; \(\ddagger\)p<0.05, \(\ddagger\)p<0.01 vs. 1% cholesterol diet group.
such as low density lipoprotein (LDL) in the circulating cell layer acts to prevent the entry of macromolecules. Since the vascular endothelial aorta of cholesterol-fed rabbits. Second, Mg may protect Mg possibly prevents the formation of foam cells in the intima in the aorta of cholesterol-fed rabbits. Thus, pathological studies have revealed that many foam cells laden with cholesterol-fed rabbits. Furthermore, the increase in extracellular Mg concentration has been reported to decrease Ca influx in vascular smooth muscles. Altura and Altura found that acute reduction in extracellular Mg concentration increased the Ca content in vascular smooth muscles and that acute elevation had the opposite effect. In the present study, we also observed that dietary Mg tended to decrease Ca content in the aorta of cholesterol-fed rabbits. Furthermore, the increase in extracellular Mg concentration has been reported to decrease the influx of Ca into the aorta and portal veins of rats and also into cultured rat aortic smooth muscle cells. The effect of extracellular Mg is thought to result from the competition with Ca at binding sites in vascular smooth muscle cell membranes. In contrast, White and Hartzell used a patch clamp technique to show that the increase in intracellular free Mg exerted only a small effect on voltage-dependent Ca current in isolated frog cardiac myocytes. Thus, extracellular Mg could have Ca entry-blocking action. This action is somewhat different from that of Ca antagonists, because Mg acts on both voltage-dependent and receptor-operated Ca channels, unlike organic Ca antagonists, which have been thought to act only on voltage-dependent Ca channels. The calcium entry-blocking action of Mg may contribute to the suppression of the development of atherosclerosis in cholesterol-fed rabbits. Interestingly, lanthanum, a trivalent cation, has reportedly suppressed the development of atherosclerosis in cholesterol-fed rabbits. Lanthanum has been shown to block Ca entry by occupying Ca binding sites in various types of cell membranes, and the action is similar to that of Mg.

Table 3. Aortic Calcium and Magnesium Content in Five Groups of Rabbits

```
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Aortic Ca</th>
<th>Aortic Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>551.5±112.9</td>
<td>113.4±23.1</td>
</tr>
<tr>
<td>1% Chol</td>
<td>6</td>
<td>505.9±173.9</td>
<td>138.3±20.3</td>
</tr>
<tr>
<td>1% Chol+Mg 300 mg</td>
<td>6</td>
<td>267.3±9.0</td>
<td>138.4±3.3</td>
</tr>
<tr>
<td>1% Chol+Mg 600 mg</td>
<td>7</td>
<td>332.7±14.3</td>
<td>138.8±6.5</td>
</tr>
<tr>
<td>1% Chol+Mg 900 mg</td>
<td>6</td>
<td>283.7±191.9</td>
<td>139.5±20.0</td>
</tr>
</tbody>
</table>
```

Values are given as μg/mg dry weight and are the means±SEM.

Mg=cholesterol, Mg=magnesium, Ca=calcium.
Solid evidence proving that dietary Mg can influence the development of human atherosclerosis has not been provided. The results of this study might not be applicable to humans because the dose of Mg that proved effective was much higher than the ordinary Mg intake in humans. Moreover, the etiology of atherosclerosis might differ in cholesterol-fed rabbits and in humans. Nevertheless, the role of Mg intake in the prevention of human atherosclerosis should be further investigated, as the beneficial effect of dietary Mg is important from a nutritional standpoint.

In conclusion, we found that an increased amount of dietary Mg suppressed the development of atherosclerotic lesions in the aorta of cholesterol-fed rabbits without affecting plasma total cholesterol and HDL cholesterol concentrations. These findings support the results of epidemiological studies. However, the mechanisms of action and the clinical and nutritional implications should be investigated further.

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Index Terms: atherosclerosis • dietary magnesium • cholesterol diet • oil red O stain • rabbits

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