Electron Microscopic Studies on the Rabbit Aorta in Acute Hypercholesterolemia*

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The present study is undertaken with electron microscopic technique in order to investigate the effect of dietary magnesium and acute hypercholesterolemia on the early lesions of aorta of rabbits.

Following results were obtained: the initial alteration of atherosclerosis was characterized as the swelling of subintimal space, the fragmentation of internal elastic lamina and the migration of some smooth muscle cells. Though the clear effect of dietary magnesium on the development of atherosclerosis could not be obtained, the endoplasmic reticulum of endothelial cells of aorta tended to expand more frequently in rabbits fed a magnesium deficient diet.

RABBITS fed a diet containing 0.25% cholesterol and 3 mg.% magnesium produced a remarkable increase of aortic lipid deposition in comparison with rabbits fed a diet containing same amount of cholesterol and excess dietary magnesium. However, serum cholesterol level was approximately same between these two groups at the end of experiment.1) The mechanism of the dietary effect of magnesium on the development of atherosclerosis in rabbits remains unknown.

There were a few studies dealing with the electron microscopic changes in the aorta of rabbits produced by an experimental hypercholesterolemia.2-4) The present study is undertaken in order to investigate the effect of acute hypercholesterolemia on the early lesions of aorta of rabbits fed cholesterol-free purified diets with different levels of dietary magnesium for 21 days and given 2.5 Gm. of cholesterol once a day at the 19th, 20th and 21st day. The electron microscopic technique was used with its superior resolving power.

* Supported in part by Grants-in Aid (HE-05249) from the National Institute of Health, U.S. Public Health Service, Bethesda, Maryland, U.S.A.
MATERIALS AND METHODS

Twenty six young weanling albino male rabbits, weighing approximately 500 Gm., obtained from the commercial store of Fukuoka, Japan, were fed a basic diet which consisted of the following in per cent: Purified casein 25; hydrogenated vegetable oil (Crisco) 10; sucrose 20.8; starch 23; wood pulp* 10; salt mixture5) 5.7; vitamin A,D,E mixture** 2.7; potassium acetate 2.5; choline 0.2; inositol 0.1. The following vitamins were added in mg. per Kg. of diet; thiamine HCl 5; riboflavin 5; pyridoxine HCl 5; calcium pantothenate 30; niacin 40; vitamin B12 0.05; biotin 0.1; 2-methyl-1, 4, naphtoquinone (K3) 2. Magnesium in the form of magnesium oxide was added to the basic diet at the level of 0 or 900 mg. per 100 Gm. of diet. An emulsion of cholesterol in 1% albumin solution was given through a stomach tube. An emulsion of cholesterol was prepared as follows: 12.5 Gm. of cholesterol was suspended in 100 ml. of 1% egg-albumin solution and was incubated for 30 min. at 37°C and then stirred by magnetic stirrer for 5 min.

Twenty six rabbits were divided into 16 rabbits fed a magnesium free diet and 10 rabbits fed a magnesium excess diet for 21 days. They were fed and watered (distilled water) ad libitum and housed in individual cages at 22±3°C.

Rabbits of two groups fed diets mentioned above were given 20 ml. of cholesterol emulsion containing 2.5 Gm. of cholesterol through stomach tube once a day at the 19th, 20th, and 21st day. Therefore the exact quantity of cholesterol given to each animal for 3 days was 7.5 Gm. as a total. At the end of 21 days all rabbits were exsanguinated under ether anesthesia.

Cholesterol6) and magnesium7) of blood serum were determined according to the methods described in the reference cited.

The aorta was removed quickly and the part of the aortic arch was dissected into small blocks, and prepared for electron microscopy as described earlier8) except that treatment by uranyl acetate was performed in this experiment. Original magnification ranged from 2,000 to 7,000 diameters.

The rest of the aorta of each animal was stained with Sudan IV as described elsewhere to count an aorta score.13,93

RESULTS

One out of 10 rabbits fed a magnesium excess diet and 5 out of 16 rabbits fed a magnesium deficient diet died during the experimental period. These diseased animals were excluded from the results.

The average gain in weight in rabbits of magnesium excess group and magnesium deficient group was 22 Gm. and 14 Gm. per day, respectively, during the experimental period.

* Obtained from Fukuoka Seishi Co. Ltd.
** A mixture of vitamins A, D and E was prepared by adding 200 mg. of soy bean oil, 1 Gm. of α-tocopherol acetate and 69 Gm. of Halibut liver oil.
1) Cholesterol and magnesium concentration in blood serum were determined at the end of the experiment. The mean value of serum cholesterol of rabbits in magnesium excess group and in magnesium deficient group was 534±291 (mean±s.e.) mg.% and 679±157 (mean±s.e) mg.% respectively. Although the cholesterol level of blood serum of rabbits fed a magnesium deficient diet and then given 2.5 Gm. of cholesterol once a day for 3 days was slightly higher than that of rabbits fed a magnesium excess diet and the same amount of cholesterol, this increase of serum cholesterol level in magnesium deficient rabbits was not statistically significant.

The magnesium concentration of serum of rabbits fed a magnesium deficient diet and a magnesium excess diet was 0.84±0.12 (mean±s.e.) mg.% and 3.13±0.47 (mean±s.e.) mg.% respectively.

2) Histological examination with dissection- and light-microscope

As a histological examination, the left ventricle and remained aorta after using for electron microscopic studies were opened, fixed in formalin and stained with Sudan IV solution. The vascular sudanophilia was judged as described elsewhere.1,9) The early sudanophilic lesions of aorta were present rarely in some animals of both two groups. In the gross, the aortic sudanophilia was involved mainly around the great vessel orifices and the highest total aorta score was below 1.0 among all rabbits of two groups. The microscopic examination of aorta showing the sudanophilia in gross, showed a sudanophilic material in subintimal layer as an initial microscopic lesion. However, no form cell was found in any rabbit of two groups. Sudan IV stain of the microscopic sections of the thoracic aorta revealed no sudanophilia in all rabbits examined. It appeared that the extent and frequency of the lesions were slightly decreased in rabbits fed a magnesium excess diet.

3) Lesions associated with acute hypercholesterolemia

1. Endothelial cells

With the electron microscope, characteristic dense droplets were found deeply in cytoplasm (Fig. 3, 9) and dense materials were found in the upper surface of endothelial cells (Fig. 3). These dense materials were considered to be lipid as described by Buck.3) A few vacuoles were found in cytoplasm (Fig. 2). However, no dense endothelial cap was found in any of rabbits examined. The way of connection of two endothelial cell at the terminal bar was not altered (Fig. 2, 4, 10, 11, 12). The mitochondria and nucleus of endothelial cell of rabbits showing acute hypercholesterolemia maintained their normal structure regardless of the amount of dietary magnesium (Fig. 1, 8). In rabbits showing hypercholesterolemia, the endoplasmic reticulum appeared to be enlarged and swollen (Fig. 8, 9, 11, 12). This enlargement of endoplasmic
reticulum was more frequently found in rabbits fed a magnesium deficient diet comparing with that in rabbits fed a magnesium excess diet. Other pathological changes in endothelial cells were not found between two groups.

2. Elastic tissue

The characteristic alteration was observed with the electron microscope in the internal elastic lamina of all the rabbits given cholesterol regardless of the amount of dietary magnesium. The internal elastic lamina was fragmented, and swollen (Fig. 2, 3, 4, 9, 10, 11, 12). And they were characterized by a decrease in density with loss of its characteristic fibrillar nature (Fig. 9, 10). Those alterations of the internal elastic lamina were the continuous changes, but the characteristic bulges of elastic lamina described by Parker were not found in this experiment. The elastic lamina was fragmented into small pieces, partly swollen and decreased its electron density. The displacement of endothelial cells from the internal elastic lamina was infrequently observed in this experiment (Fig. 3, 4, 10, 11, 12).

3. Subintimal space

The most characteristic lesions induced by acute hypercholesterolemia were found in this area. The characteristic alteration was the enlargement of the subintimal space which was filled with the homogeneous substance and contained a few migrated smooth muscle cells (Fig. 2, 3, 4, 9, 10, 11, 12).

4. Tunica media

The smooth muscle cells were detached from the elastic lamellae which were frequently fragmented, swollen and decreased its electron density. There were no alterations in the smooth muscle cells. Many collagen fibers were found in area surrounding the smooth muscle cells (Fig. 5, 13, 14, 15). No fibroblasts could be identified in tunica media.

5. Tunica adventitia

In tunica adventitia, characteristic alteration could not be found. There were lots of collagen fibers, fibroblasts, vasa vasorum, unmyelinated and myelinated nerve fibers (Fig. 6). The striation of collagen fibers was observed clearly and was considered to be intact (Fig. 7).

DISCUSSION

The addition of cholesterol and cholic acid to a diet containing 24 mg. per cent of magnesium, and amount normally required for normal weanling rats, resulted in magnesium deficiency in rats. The early fat deposition in the rat aorta and heart valves induced by cholesterol and cholic acid feeding were diminished or retarded by increasing the
Fig. 1.* A cross section through aorta of rabbit (No. 7) shows the endothelial cell (EC). Note that mitochondria (M), endoplasmic reticulum (ER) and nucleus (N) maintained normal structure. × 28,500.

Rats fed a diet containing 6 mg. per cent of magnesium and no cholesterol or cholic acid for 24 days and later given a diet containing magnesium intake.\(^9\),\(^10\)

*All figures are electron microscopic photographs of rabbits aorta fixed with 1% osmium tetroxide and stained with 2% uranyl acetate solution.

Figs. 1–7 are the photographs of aorta of rabbits fed a diet containing 900 mg. % of magnesium and intubated cholesterol into the stomach. Figs. 8–15 are the photographs of aorta of rabbits fed a diet containing 0 mg. % of magnesium and intubated same amount of cholesterol into the stomach.

Fig. 16 is the photograph of aorta of rabbits fed a diet containing 900 mg. % of magnesium and intubated 11.5 Gm. of cholesterol suspended in soy bean oil into the stomach in another experiment.

Abbreviations for all figures

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<th>C</th>
<th>collagen fibers</th>
<th>MN</th>
<th>myelinated nerve fiber</th>
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<tr>
<td>CI</td>
<td>caveolae intracellularis</td>
<td>N</td>
<td>nucleus</td>
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<td>EC</td>
<td>endothelial cell</td>
<td>RBC</td>
<td>red blood corpuscle</td>
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<td>EL</td>
<td>elastic lamellae</td>
<td>SM</td>
<td>smooth muscle cell</td>
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<td>endoplasmic reticulum</td>
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<td>I</td>
<td>invagination</td>
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<td>terminal bar</td>
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<td>IEL</td>
<td>internal elastic lamina</td>
<td>UC</td>
<td>undefined cell</td>
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Fig. 2. A cross section through aorta of rabbit (No. 8) shows the endothelial cell showing similar finding like Fig. 1. Note the remarkable change of internal elastic lamina (IEL) and subendothelial space. ×19,500.

Fig. 3. A cross section through aorta of rabbit (No. 8) shows the swelling of the subendothelial and subintimal space, fragmentation of internal elastic lamina and migration of smooth muscle cells (SM). There were some droplets, which were thought to be lipid (L), in the cytoplasm of endothelial cells. ×14,100.
Fig. 4. A cross section from aorta of rabbit (No. 10) shows the remarkable swelling of subintimal space. There were some fragmented internal elastic lamina, and the part of fragmented smooth muscle cell. \( \times 11,400 \).

Fig. 5. A cross section of the tunica media from the rabbit aorta (No. 7) shows the fragmentation of elastic lamellae (EL), especially loosened attachment between the elastic lamellae and smooth muscle cells. \( \times 16,500 \).
Fig. 6. Myelinated nerve fibers in the tunica adventitia from the rabbit aorta (No. 2). × 6,000.

Fig. 7. Collagen fibers in the tunica adventitia from the rabbit aorta (No. 8). No alteration of collagen fibers was found. × 60,000.
Fig. 8. In the endothelial cell of rabbit aorta (No. 8). There was an enlargement of endoplasmic reticulum. × 19,500.

Fig. 9. A cross section shows the swelling of the endoplasmic reticulum of endothelial cells and the expansion of subendothelial space of rabbit aorta (No. 16). × 22,500.
Fig. 10. A cross section through aorta of rabbit (No. 14) shows the expansion of subendothelial space, which was filled with homogeneous substance, the fragmentation and swelling of internal elastic lamina. $\times 14,100$.

Fig. 11. A cross section through aorta of rabbit (No. 7) shows the swelling of the endoplasmic reticulum of the endothelial cells. $\times 6,000$. 
Fig. 12. A cross section through aorta of rabbit (No. 9) shows the swelling of endoplasmic reticulum of endothelial cell, the migration of smooth muscle cells into the subintimal space and the fragmentation and/or swelling of the elastic lamellae. \( \times 6,000 \).

Fig. 13. A cross section shows the tunica media of aorta of rabbit (No. 7). Note the fragmentation of elastic lamellae. \( \times 6,000 \).
Fig. 14. A cross section shows the tunica media of aorta of rabbit (No. 14). Note the fragmentation of the elastic lamellae and loosened attachment between the smooth muscle cells and elastic lamellae. ×6,000.

Fig. 15. A cross section shows the tunica media of aorta of rabbit (No. 15). Note the swelling of elastic lamellae. ×7,800.
cholesterol, cholic acid and 24 mg.% of magnesium produced a marked sudanophilia compared to rats fed a diet containing 96 mg.% magnesium and then given a same amount of cholesterol, cholic acid and magnesium. The preventive effect of the large amount of dietary magnesium on the lipid deposition in the rat aorta occurred despite the fact that serum cholesterol level was not diminished by magnesium feeding. When the diet contained 10% casein, 1% cholesterol and 0.3% cholic acid, there was actually an increase in serum cholesterol levels with higher levels of magnesium feeding. In rabbits experiments feeding 1% cholesterol for 3 weeks, the preventive effect of the large amount of dietary magnesium on lipid deposition in aorta was only observed when the serum cholesterol level was decreased.

However, rabbits fed a diet containing 3 mg.% of magnesium and 0.3% of cholesterol for 21 days, produced a statistically significant increase of aorta lipid deposition, and serum cholesterol was not increased in a statistically significant amount compared to rabbits fed a diet containing 900 mg.% of magnesium and 0.3% of cholesterol. From these results, it was considered that dietary magnesium may cause changes in lipid composition of serum on the one hand, and affect the metabolism of arterial wall on the other hand.

In this experiment, the mean value of serum cholesterol levels of
rabbits fed a diet containing 0 and 900 mg.% of magnesium and later given 2.5 Gm. of cholesterol once a day for 3 days was 679 and 534 mg.%, respectively, at the end of experiment. The distinct effect of prefeeding of magnesium deficient diet for 18 days on the changes of aorta in rabbits which were produced later by an acute hypercholesterolemia, was not demonstrated with electron microscope in this experiment.

Under dissection microscope, it appeared that the prefeeding of a large amount of magnesium inhibited slightly the occurrence of sudanophilia of aorta in gross. In the electron microscopic study, the area around the great vessel orifice where sudanophilia was found, was not used. Further electron microscopic studies on the area around the great vessel orifice of aorta particularly its distal portion will be of interest.

Although the increase of mean value of serum cholesterol in rabbits fed a magnesium deficient diet was not statistically significant, the absorption rate of cholesterol in rabbits fed different levels of dietary magnesium is in progress in our laboratory.

There are a few studies using electron microscope on the early lesions of rabbit aorta produced by feeding cholesterol in short or long period. Buck, Parker and Kato described the fragmentation of internal elastic lamina and swelling of subendothelial space as an early lesion. Those lesions particularly the characteristic fragmentations of internal elastic lamina were confirmed in this experiment.

In different experiment, 2 rabbits fed a diet containing 900 mg.% of magnesium and no cholesterol for 20 days and then given 2.0 Gm. cholesterol, suspended in soy bean oil instead of 1% of albumin, once a day for 4 to 6 days, produced a remarkable enlargement of subendothelial space with relatively intact internal elastic lamina as shown in Fig. 16. Whether the most characteristic enlargement of subendothelial space containing homogeneous unknown substances and the significantly less fragmentation of internal elastic lamina compared with those obtained in the present experiment are produced by the effect of soy bean oil for suspending cholesterol, requires further study. It will be of interest to study whether this homogeneous substance will be acid mucopolysaccharide.

In the present experiment, the lipid deposition on the surface of the endothelial cell was relatively slight compared with the remarkable alterations of the internal elastic lamina and of the subendothelial space. The endoplasmic reticulum of endothelial cell was enlarged and contained homogeneous unknown substance. Similar alterations were described by Buck. Buck postulated that small vesicle might be converted to endoplasmic cysterna. It appeared in this experiment that the enlargement of endoplasmic reticulum was more frequently observed in rabbit
fed a magnesium deficient diet compared with that in rabbits fed a magnesium excess diet. The alteration of endothelial cells except the enlargement of the endoplasmic reticulum was not found.

The mechanism of the development of initial alterations in atherosclerosis of aorta is unknown. The endothelial cells have the pynocytotic activity. The part of mechanism taken lipid into the endothelial cells was explained by pynocytosis.

No dense materials and vacuoles were observed in the subintimal space and in the media in this experiment. Whether the deposition of dense material (lipid) on the cytomembrane of endothelial cells in an initial change and then produce an inhibitory effect on diffusion of nutrients and produce a fragmentation of internal elastic lamina and an enlargement of subintimal space, remains unknown.

**Summary**

Young weanling rabbits were divided into two groups and were fed the purified diets containing 0 or 900 mg.% magnesium for 21 days, and were given 2.5 Gm. of cholesterol once a day at the 19th, 20th and 21st day through stomach tube.

The serum cholesterol levels of rabbits were approximately 600 mg.% regardless of the amount of dietary magnesium. The early lesions of aorta of each rabbit were studied with electron microscope, and the following results were obtained.

(1) The fragmentation of internal and other elastic lamina, the swelling of subintimal space, the loosened connection between smooth muscle cells and elastic lamellae, and the slight deposition of lipid on the endothelial cells were observed.

(2) The distinct accelerating effect of prefeeding of magnesium deficient diet on the development of the early lesions of rabbit aorta could not be obtained. However, it appeared that the endoplasmic reticulum of endothelial cells tended to expand more frequently in rabbits fed a magnesium deficient diet.

**References**