Effect of Vitamin E on Vascular Integrity in Cholesterol-Fed Guinea Pigs

Yan Qiao, Munehiro Yokoyama, Kohji Kameyama, Goro Asano

This study was designed to clarify the effects of vitamin E on the alterations in proteoglycan distribution and vascular permeability, which were examined by immunohistochemical and ultrastructural techniques in the aortas of cholesterol-fed guinea pigs. The animals were divided into three groups: a control group, a cholesterol group, and a vitamin E group. Serum levels of cholesterol, triglyceride, low-density lipoprotein, high-density lipoprotein, and thiobarbituric acid–reactive substances were measured. An increase in thiobarbituric acid–reactive substances was observed in the cholesterol group compared with control and vitamin E groups. Intimal atheromatous lesions of the aorta were significantly decreased in the vitamin E group compared with the cholesterol group. Histochemically, an increased distribution of proteoglycans such as chondroitin, dermatan, and heparan sulfates and ruthenium red reaction products in the intima; decreased glycoalyx on the endothelial surface; and increased permeability to horseradish peroxidase were revealed in the cholesterol group compared with the vitamin E group. Hypercholesterolemia, resulting in superoxide production, may have contributed to the endothelial damage and increased permeability to plasma proteins and lipids in the vascular wall of the cholesterol group. However, vitamin E administration inhibited lipid deposition and development of this abnormal permeability associated with an irregular distribution of proteoglycan. These results suggest that vitamin E preserves the morphological and functional integrity of the vascular wall and may contribute to the inhibition of atherogenesis in cholesterol-fed guinea pigs. (Arterioscler Thromb. 1993;13:1885-1892.)

KEY WORDS • hypercholesterolemia • atherosclerosis • vascular permeability • proteoglycans • vitamin E

Endothelial cells act as a barrier to anionic substances such as albumin and low-density lipoprotein (LDL). Vascular proteoglycans containing chondroitin sulfate, dermatan sulfate, and heparan sulfate glycosaminoglycans have been characterized by chemical and physicochemical procedures and may contribute to the viscoelasticity and selective permeability of the aorta. The physical and morphological integrity of the plasma membrane and proteoglycans in the extracellular matrix may be influenced by factors such as hypercholesterolemia and vitamin E. Furthermore, vitamin E has antiatherogenic activities as a regulator of endothelial cells, an inhibitor of membrane lipid peroxidation, and a preventer of lipoprotein oxidative modification. This study was designed to elucidate whether or not vitamin E affects the morphological and functional integrity of the hypercholesterolemic guinea pig aorta and to clarify the relations among vitamin E, proteoglycans, vascular permeability, and hypercholesterolemia.

Methods

The experiments were performed on Hartley guinea pigs weighing 250 to 300 g. Sixty animals were allocated to three groups: a control group fed the control diet (RC4, Oriental Yeast Co, Ltd); a cholesterol group fed a cholesterol diet containing 2.5% cholesterol, 0.25% cholic acid, and 7.5% beef fat; and a vitamin E group that received the cholesterol diet plus vitamin E. In plasma samples from these animals, the levels of cholesterol, triglyceride, LDL, and high-density lipoprotein were measured, and the extent of lipid peroxidation was measured in terms of thiobarbituric acid–reactive substance (TBARS) expressed as malondialdehyde equivalents. For evaluation of vascular permeability, 115 mg horseradish peroxidase (HRP type II, Sigma Chemical Co, Ltd) in normal saline and adjusted to pH 7.3 with sodium hydroxide, was injected intravenously into guinea pigs under pentobarbital sodium (30 mg/kg) anesthesia 5 minutes before perfusion of the animal with a fixative solution containing 2% paraformaldehyde and 0.25% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 15 minutes. After 1, 2, or 3 months, the entire aorta from animals in each group was excised and dissected free of fat and connective tissue. The aorta was then opened longitudinally along its ventral margin and fixed in 10% buffered formalin. After fixation, the aortic lesions were enhanced by Sudan III staining using Daddi’s method. Lesions were assessed throughout the entire length of the aorta from the aortic arch to the abdominal region and compared morphometrically among the three groups; vascular segments for examination were also removed from other thoracic regions of each group.
**Lipid and Thiobarbituric Acid–Reactive Substances Levels of Serum**

<table>
<thead>
<tr>
<th>Group/Month</th>
<th>Total Serum Cholesterol, mg/100 mL</th>
<th>Serum Triglyceride, mg/100 mL</th>
<th>Serum LDL, mg/100 mL</th>
<th>Serum HDL, mg/100 mL</th>
<th>Serum TBARS, mol/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62.5±6.3</td>
<td>85±13.1</td>
<td>50.2±6.2</td>
<td>8±0.9</td>
<td>2.17±0.3</td>
</tr>
<tr>
<td>1</td>
<td>99±7</td>
<td>63±10</td>
<td>75±7</td>
<td>21±2</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>2</td>
<td>53±11.8</td>
<td>72.3±20.5</td>
<td>25.3±10.3</td>
<td>13.3±2.9</td>
<td>2.4±0.25</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>505±32.4</td>
<td>93.2±10</td>
<td>477±34.9</td>
<td>22.5±2.9</td>
<td>3.35±0.19</td>
</tr>
<tr>
<td>1</td>
<td>425±89</td>
<td>75.3±14.4</td>
<td>386±87</td>
<td>34±2.5</td>
<td>4.5±0.8</td>
</tr>
<tr>
<td>2</td>
<td>504.7±80.8</td>
<td>104.3±18.3</td>
<td>463.8±80.3</td>
<td>15.1±2</td>
<td>4.6±1.3</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>450±28.3</td>
<td>66±6.2</td>
<td>401.5±17.9</td>
<td>19.5±0.9</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>1</td>
<td>424±34.3</td>
<td>79.4±11.6</td>
<td>378.4±36.3</td>
<td>35±2.3</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>2</td>
<td>439±37</td>
<td>93±10.9</td>
<td>403±36.3</td>
<td>11.6±1.5</td>
<td>2.1±0.1</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and TBARS, thiobarbituric acid–reactive substances. Values are mean±SEM. Increases in serum cholesterol, lipoproteins, and TBARS were observed in the cholesterol group compared with the other two groups. The triglyceride level did not differ from the control value in the cholesterol group.

**Light and Electron Microscopy Observations**

Vascular segments from the thoracic aorta were bisected, and one half was fixed with 10% formalin and embedded in paraffin for light microscopy observation. The other half was fixed with 2.5% glutaraldehyde followed by 0.1% OsO₄ in 0.1 mol/L phosphate buffer for 1 hour at room temperature, dehydrated through a graded ethanol series, and embedded in epoxy resin for electron microscopy observation.

**Immunohistochemical Analysis of Proteoglycans**

For immunohistochemical observation, the indirect immunohistochemistry method was performed using monoclonal anti–chondroitin sulfate proteoglycan (Seikagaku Kogyo Co), monoclonal anti–human dermatan sulfate proteoglycan (Seikagaku Kogyo Co), and monoclonal anti–heparan sulfate proteoglycan (Seikagaku Kogyo Co) antibodies on mirror-image sections. The deparaffinized sections were treated as follows. Endogenous peroxidase was inhibited by treatment with 0.3% H₂O₂ in 100% methanol. For enhancement of antigen selectivity, the sections were treated with protease. The reaction with primary antibody was performed using the following dilutions for 1 hour: monoclonal anti–chondroitin sulfate proteoglycan 1/100, monoclonal anti–human dermatan sulfate proteoglycan 1/1000, and monoclonal anti–heparan sulfate proteoglycan 1/100. After incubation with the primary antibody, the sections were treated with a histofine SAB-PO (streptavidin-biotin) kit (Nichirei Co Ltd). For control specimens, appropriately diluted anti-rat immunoglobulin G was applied instead of the type-specific monoclonal anti–chondroitin sulfate proteoglycan, monoclonal anti–human dermatan sulfate proteoglycan, or monoclonal anti–heparan sulfate proteoglycan.

**Histochemical Analysis With Ruthenium Red Stain**

The vascular segments were fixed for 1 hour with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) mixed with an equal volume of ruthenium red solution (3000 ppm) and washed with 0.1 mol/L cacodylate buffer. The specimens were then postfixed in 4% OsO₄ in 0.2 mol/L cacodylate buffer (pH 7.4) mixed.
Measurement and Statistics

The samples were postfixed in 1% OsO\textsubscript{4} in 0.1 mol/L phosphate buffer for 1 hour, dehydrated through a graded ethanol series, and embedded in epoxy resin. The treated samples for ultrastructural observation were cut into 1-μm-thick semithin sections and stained with toluidine blue for observation by light microscopy. Ultrathin sections were cut on a Sorvall MT-5000 ultramicrotome, stained with uranyl acetate or combined uranyl acetate–lead citrate, and observed with a Hitachi H-7000 transmission electron microscope.

Vascular Permeability Measurement Using HRP

To investigate endothelial permeability, the vascular segments were fixed for 1 hour with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) and washed with 0.1 mol/L cacodylate buffer. The specimens were then treated for 15 minutes with diaminobenzidine-4HCl 25 mg in 0.05 mol/L tris-buffer. The tissues were finally dehydrated through a graded ethanol series and embedded in epoxy resin.

The specimens were then treated for 1 hour with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) and washed with 0.1 mol/L cacodylate buffer. The specimens were then treated for 15 minutes with diaminobenzidine-4HCl 25 mg in 0.05 mol/L tris-(hydroxymethyl)aminomethane-HCl buffer (pH 7.4), 50 mL, and allowed to react for 30 minutes in the same solution containing 0.01% H\textsubscript{2}O\textsubscript{2} at room temperature. The samples were postfixed in 1% OsO\textsubscript{4} in 0.1 mol/L phosphate buffer for 1 hour, dehydrated through a graded ethanol series, and embedded in epoxy resin.

The treated samples for ultrastructural observation were cut into 1-μm-thick semithin sections and stained with toluidine blue for observation by light microscopy. Ultrathin sections were cut on a Sorvall MT-5000 ultramicrotome, stained with uranyl acetate or combined uranyl acetate–lead citrate, and observed with a Hitachi H-7000 transmission electron microscope.

Measurement and Statistics

With an image analyzer (Nireco Co, Ltd), measurements were made as follows. The areas of aortic fatty streaks stained by Daddi’s method\textsuperscript{8} were measured using printed photographs enlarged 10 times, and the ratio of fatty streak area to normal tissue area was calculated.

The degree of intimal thickening from the superior border of the internal elastic lamina to the endothelial surface was measured on 72 photographs enlarged 400 times. For measuring the reaction products of proteoglycans in the intima, five sections from each of four thoracic aortas at intervals of 4 mm were used. At the light-microscopic level (enlarged 400 times), the reaction products of proteoglycans showed a brownish color, and the area with the greatest degree of hypertrophy was chosen as the area of measurement.

The thickness of the glyocalyx on the endothelium stained with ruthenium red was measured in 36 segments from the thoracic aorta, and 108 photomicrographs printed at ×7000 magnification were used.

For evaluation of vascular permeability, endothelial vesicles labeled with HRP were analyzed on the photographs. In each series of cross-sections through the endothelial cell, labeled vesicles in the entire area of the cytoplasm were measured on 108 photomicrographs printed at ×10 000 magnification.

Statistical comparisons among samples were made using Student’s t test and two-way ANOVA.

Results

Increases in the levels of blood serum cholesterol, lipoprotein, and TBARS were observed in the cholesterol group compared with the other two groups. However, the triglyceride level in the cholesterol group did not differ from the control value (Table 1). Typical fatty streaks stained with Sudan III were localized mainly on the orifices of vascular branches in the aortic arch and thoracic and abdominal aortas of the cholesterol group. The cholesterol group showed a greater percentage of fat-stained area than the vitamin E group (Fig 1). Histological examination of the aorta revealed the accumulation of lipid in the aortic intima. However, lipid deposition in the vitamin E group was significantly decreased compared with the cholesterol group. Fatty streaks were characterized by intimal aggregates of foam cells containing numerous lipid droplets. A few spindle-shaped cells were interspersed among the round foam cells.

Histologically, the area of the aortic intima was 212±20 μm\textsuperscript{2} in the control group. In the cholesterol group, the intimal area increased to 634±65 μm\textsuperscript{2}, but that of the vitamin E group was only 419±40 μm\textsuperscript{2} after 3 months (Fig 2).

Immunohistochemical observation showed that proteoglycans were present in intimal lesions. In the control group after 3 months, the reaction product of chondroitin sulfate was uniformly distributed in the extracellular matrix of the vascular wall. Sparse reaction product was observed in the intima, mainly in the subendothelial space. Dermatan sulfate showed almost the same pattern as chondroitin sulfate, but there was only a small amount of the reaction product of the former scattered in the vascular wall. The reaction product of heparan sulfate was distributed in the cytoplasm of foam cells in the vascular wall. In the cholesterol group after 3 months, the reaction products of chondroitin and dermapan sulfates were concentrated in the extracellular matrix of fatty streaks and the subintimal region. However, the reaction product of dermapan sulfate showed a different distribution pattern. The foam cells in fatty streaks contained the most reaction product, but it was not prominently demonstrated in the subendothelial space. In the vitamin E group, the distributions of chondroitin, dermatan, and heparan sulfates were decreased after 3 months compared with the cholesterol group (Fig 3).
Fig 3. Photomicrographs showing that the amounts of reaction products of chondroitin sulfate (left panels) and dermatan sulfate (middle panels) are increased in the intercellular space of aortas in cholesterol-fed guinea pigs. Heparan sulfate proteoglycan (right panels) was increased in the foam cells of animals in the cholesterol group, but in the vitamin E group the distribution of proteoglycans except for heparan sulfate was decreased to nearly that of the control group. IEL indicates internal elastic lamina and SM, smooth muscle cell. Enzyme immunohistochemistry by the indirect method. Bar=10 μm.

Fig 4. Image analysis of dark-field photomicrographs shows localization of reaction products (immunohistochemical proteoglycans) in the thoracic aorta of guinea pigs after 3 months. Positive areas of chondroitin, dermatan, and heparan sulfates are increased in the cholesterol group, but in the vitamin E group the distribution of proteoglycans except for heparan sulfate is decreased to nearly that of the control group. IEL indicates internal elastic lamina and SM, smooth muscle cell. Enzyme immunohistochemistry by the indirect method. Bar=10 μm.
According to morphometric evaluation, in the control group the positive areas of chondroitin sulfate and dermatan sulfate were 20±1.6 μm² and 10±1.0 μm², respectively, but the area of heparan sulfate was 125±12 μm². The positive areas of chondroitin sulfate and dermatan sulfate were 160±10 μm² and 90±15 μm², respectively, in the cholesterol group, and the area of heparan sulfate was also increased to 250±25 μm². In the vitamin E group, the reaction products of chondroitin, dermatan, and heparan sulfates were measured at 37±5.7 μm², 15±1.0 μm², and 175±15 μm², respectively, after 3 months (Figs 4 and 5).

Ultrastructurally, after 3 months, increased numbers of pinocytotic vesicles and lipid droplets were demonstrated in the endothelium of the cholesterol group, and the subendothelial smooth muscle cells contained many lipid droplets. On the other hand, after 3 months fewer lipid droplets and less high-density granular material were seen in the cytoplasm of endothelial cells in the vitamin E group, but subendothelial smooth muscle cells revealed no significant changes (Fig 6). Histochemically the surface coat of endothelial cells in the intima was made visible by ruthenium red staining. The luminal coat appeared as a continuous band of densely stained granular material. Thin filaments containing densely staining fine granules were demonstrated under the basal lamina of endothelial cells. However, in the cholesterol group, large amounts of ruthenium red reaction products were increased in the subendothelial space, and a decreased distribution of ruthenium red reaction products was demonstrated on the surface and in the cytoplasm of endothelial cells (Fig 7A). In contrast, aortas from animals in the vitamin E group had a regular localization of ruthenium red reaction products on the surface of the aortic endothelium and decreasing amounts of the reaction product in the subendothelial space (Fig 7B). Morphometrically, the thickness of the glycocalyx after 3 months in the cholesterol group was 16.8±2.0 nm on average, compared with 28.6±2.0 nm in the vitamin E group (Fig 8).

In the cholesterol group, animals injected with HRP showed evidence of the tracer at the ultrastructural level in pinocytotic vesicles, including intracellular vesicles, dilated intercellular spaces, foam cells, and subendothelial spaces after 3 months (Fig 9A). However, a decreased permeability of the endothelium to HRP was found in the vitamin E group (Fig 9B). The HRP permeability rate was evaluated morphometrically, and the area of HRP-positive vesicles in sections was
12.0±0.4 nm$^2$/μm$^2$ in the control group and 25.0±9.0 nm$^2$/μm$^2$ in the cholesterol group, but in the vitamin E group it was 14.0±7.0 nm$^2$/μm$^2$ after 3 months (Fig 10).

Discussion

In the present study, fatty streaks developed mainly in the areas of turbulence in the aortas of guinea pigs in the cholesterol group compared with the control and vitamin E groups. The fatty streaks were composed of foam cells, with irregularly distributed proteoglycans in the foam cells and adjacent matrix of the thoracic aorta in the animals with hypercholesterolemia. Many factors may contribute to the changes in the proteoglycan distribution in the vascular wall in disease states.9-11 Proteoglycans are high-molecular-weight protein/poly-saccharides consisting of carbohydrate polymers and glycosaminoglycans covalently linked to a protein backbone or core, and they may contribute to the viscoelasticity and selective permeability of the aorta.24 In the thickened intima, there was a greater proportion of extracellular space that was occupied by proteoglycan, which was present in higher concentration in the intima than the media. It is possible that the accumulation of proteoglycan as heparan sulfate in the thickened intima increased the susceptibility of the intima to accumulate lipids after imposition of an additional stimulus, such as hyperlipemia, in the initial stages of atherosclerosis.12 The alterations in arterial glycosaminoglycans, therefore, seem to be related to two processes, ie, the normal maturation of the vessels and atherogenesis, both of which involve increased formation of connective tissue components by arterial smooth muscle cells.13 The
endothelial basement membrane contains small proteoglycan granules (heparan sulfate), which decrease in concentration in older animals and increase in disease conditions. The glyocalyx, stainable with ruthenium red, is responsible for the surface properties of normal endothelial cells. The luminal endothelial cell surface is made up of microdomains of different charge densities that are generated by the preferential distribution of sialoconjugates, proteoglycans, and glycoproteins. An increase in the serum cholesterol concentration is accompanied by a loss or an irregular distribution of the surface glyocalyx, resulting in an initial reduction of anionic charge on the endothelial surface. A decrease in the amount of sulfated glycosaminoglycans on the surface of the aorta may be related to its anticoagulant activity and thus may promote vascular permeability. In addition to these surface changes, deeper-lying carbohydrates and proteoglycans provide new binding sites that facilitate the adhesion of LDL to the vascular wall in hypercholesterolemia. Proteoglycans influence arterial wall properties, such as vascular elasticity, permeability, lipid metabolism, hemo- tasis, and thrombosis. To elucidate whether or not a vitamin E–supplemented diet affect the extracellular matrix of the guinea pig aorta, the effect on proteoglycans, as one component of the extracellular matrix of the aorta, was examined. Vitamin E administration inhibited an increase in ruthenium red–positive material in the subendothelial space and a decrease of glyocalyx on the endothelium. Vascular endothelial cells synthesize and secrete both heparan sulfate– and dermatan sulfate–containing proteoglycans, which are stained by ruthenium red. These results may suggest that cellular superoxide ion may change the cellular structure and influence the synthesis of sulfate proteoglycans. Some investigators believe that the first event in atherogenesis may be focal damage to the endothelium, resulting in the entry of platelets and other blood components that stimulate the migration and growth of the underlying medial smooth muscle cells. Oxidatively modified lipoprotein and superoxide may have a direct toxic effect on the endothelial cell, resulting in increased permeability of the endothelial barrier to macromolecules, including lipids. HRP has been used to study the transport processes of a wide spectrum of macromolecules, including LDL, across the vascular endothelium. Accumulation of HRP was demonstrated in the vascular wall as a result of dysfunction of the endothelial cell layer due to loss of the macromolecular barrier function. The presence of HRP may be related to its retention space, probably produced by
regenerating and hyperactive arterial cells that are present in hypercholesterolemia. The vitamin E content of LDL in nonsmokers is significantly higher than that in smokers. Cigarette smoke may have an effect on the endothelium, and vitamin E decreases the level of lipid peroxides in serum. Exposure to superoxide leads to gradual depletion of α-tocopherol and contributes to the oxidation of LDL by lowering the antioxidant content. These results suggest that endogenous α-tocopherol has a role in the protection of LDL against oxidative stress and also against atherogenesis. LDL has the potential to damage endothelial cells through oxidation, and the aortic intima may regulate the function and metabolism of proteoglycan-LDL complexes. In this study, vitamin E inhibited endothelial changes, abnormal vascular permeability, and lipid deposition in the intima. The nonselective transfer of LDL may be inhibited by α-tocopherol, and oxidative modification may be protected by vitamin E administration, since significant differences between the vitamin E and non-vitamin E groups were morphologically and histochromatically demonstrated in the aortas. In addition, serum samples from cholesterol-fed guinea pigs showed a higher concentration of TBARS than those from the group given vitamin E. Superoxide production in hypercholesterolemia may contribute to the endothelial damage that is associated with increased permeability of the vascular wall to plasma proteins and lipids. Vitamin E inhibited these alterations and preserved the morphological and functional integrity of the vascular wall.

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References
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