Background—Oxidative stress is thought to play an important role in atherogenesis, suggesting that antioxidants could prevent coronary artery disease. However, the efficacy of vitamin C in reducing atherosclerosis is debatable in humans and has not been tested rigorously in animals.

Methods and Results—Gulo<sup>−/−</sup> A po e<sup>−/−</sup> mice were used to test a hypothesis that chronic vitamin C deficiency enhances the initiation and development of atherosclerosis. These mice are dependent on dietary vitamin C because of the lack of L-gulonolactone-γ-oxidase and are prone to develop atherosclerosis because of lacking apolipoprotein E. Beginning at 6 weeks of age, the Gulo<sup>−/−</sup> A po e<sup>−/−</sup> mice were fed regular chow or Western-type diets containing high fat and supplemented with either 0.033 g or 3.3 g/L of vitamin C in their drinking water. This regimen produced mice with chronically low vitamin C (average 1.5 μg/mL in plasma) or high vitamin C (average 10 to 30 μg/mL in plasma). Morphometric analysis showed that within each sex, age, and diet group, the sizes of the atherosclerotic plaques were not different between low vitamin C mice and high vitamin C mice. However, advanced plaques in the low vitamin C mice had significantly reduced amounts of Sirius red–staining collagen (36.4±2.2% versus 54.8±2.3%, P<0.0001), larger necrotic cores within the plaques, and reduced fibroproliferation and neovascularization in the aortic adventitia.

Conclusions—Chronic vitamin C deficiency does not influence the initiation or progression of atherosclerotic plaques but severely compromises collagen deposition and induces a type of plaque morphology that is potentially vulnerable to rupture. (Circulation. 2002;105:1485-1490.)

Key Words: antioxidants n atherosclerosis n collagen n plaque rupture n vitamin C

Strong evidence suggests that oxidation of low-density lipoprotein (LDL) by free radicals is one of the most important factors for the initiation of atherosclerosis<sup>1,2</sup> and provides a rationale for the use of antioxidants to prevent the early onset of atherosclerosis. Ascorbic acid (vitamin C) is a strong reducing agent that is water soluble and consequently can act directly with aqueous free radicals in the blood. It is also thought to act indirectly in the lipid compartment via its ability to react at the lipid/aqueous interphase with tocopherol (vitamin E) radicals.<sup>3</sup> Furthermore, physiological concentrations of ascorbic acid strongly inhibit LDL oxidation in cultured cells.<sup>4</sup> Thus, it is a reasonable hypothesis that vitamin C supplement helps prevent atherosclerosis.

See p 1396

However, evidence linking vitamin C to cardiovascular diseases in humans is still largely circumstantial. Some studies showed an association of low plasma vitamin C levels with high cardiovascular mortality,<sup>5,6</sup> whereas no association could be established in other studies.<sup>7,8</sup> The inconsistency of these results reflects at least in part the limitations of population-based human studies, confounded by the interaction between dietary constituent differences and differences in the genetic compositions of individuals. In addition, plasma ascorbic acid is strongly correlated with dietary ascorbic acid intake, and a high dietary intake of vitamin C might reflect a healthy diet or a healthy lifestyle in general.

Animal models allow us to minimize these confounding factors when investigating the effects of dietary modulation and its interaction with various genetic factors during the pathogenesis of complex diseases. When the endogenous redox systems of humans and animals such as mice are compared, however, there is a major difference: humans have lost the ability to synthesize ascorbic acid as a consequence of mutations in the gene coding for a key enzyme, L-gulono-γ-lactone γ oxidase (Gulo). Consequently, whereas humans depend entirely on vitamin C derived from their diet, mice synthesize ascorbic acid and are only slightly influenced by exogenous vitamin C. More importantly, latent vitamin C deficiency in humans cannot be replicated in most animal models, including mice.
To examine rigorously the importance of antioxidants for the prevention of disease, we generated Gulo⁻/⁻ mice that, like humans, are dependent on dietary vitamin C and thus can be used to model human conditions more closely. In the present work, we have crossed Gulo⁻/⁻ mice with apolipoprotein E–deficient mice (Apoe⁻/⁻), which develop advanced atherosclerotic lesions spontaneously. In this study we provide evidence that vitamin C deficiency does not alter the initiation or volume of the atherosclerotic plaques but significantly reduces their collagen content, giving them a potentially more vulnerable plaque morphology.

Methods

Mice

Gulo⁻/⁻ heterozygotes were backcrossed 5 generations to C57BL/6 before they were crossed with Apoe⁻/⁻ mice in a C57BL/6 background. The double-heterozygous Gulo⁻/⁻ Apoe⁻/⁻ mice were mated with Apoe⁻/⁻ mice to generate Gulo⁻/⁻ Apoe⁻/⁻ mice. Intercrossing the Gulo⁻/⁻ Apoe⁻/⁻ mice produced mice doubly homozgyous for the 2 loci (Gulo⁻/⁻ Apoe⁻/⁻) that were then used to generate the experimental animals. During the breeding and maintenance, mice were given vitamin C–supplemented water containing 0.33 g/L of L-ascorbic acid and 0.01 mmol/L EDTA. Water was changed twice a week. All of the protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. All mice were bred in-house.

Diet and Vitamin C Supplement

At 6 weeks of age, both male and female Gulo⁻/⁻ Apoe⁻/⁻ mice were randomly assigned to high and low vitamin C groups, supplemented with 3.3 g/L and 0.033 g/L of ascorbic acid in their drinking water, respectively. Mice were fed chow (RMH3000-5P76, PMI Feeds) or Western-type high-fat diets that contained 21% fat and 0.15% cholesterol (HFW, modified TD88137, Halan Teklad). The vitamin C content of chow was 200 mg/kg assayed by the α, α′-dipirydil method. Western-type diet was adjusted to contain either 200 or 50 mg/kg vitamin C. Daily vitamin C intakes were estimated assuming that a mouse eats 4 g/day and drinks 5 mL/day.

Plasma Analyses

Plasma levels of total cholesterol and high-density lipoprotein (HDL) cholesterol were measured using kits from Wako BioProducts. Triglyceride and glucose levels were measured with kits from Sigma. Ascorbic acid levels in plasma and in liver were measured by the α, α′-dipirydil method.

Atherosclerosis Assay

Mice were euthanized with an overdose of 2,2,2-tribromoethanol and perfused with 4% paraformaldehyde (pH 7.4) through the left ventricle at a physiological pressure. Frozen section of the heart containing the aortic root were stained with H&E or with Sudan IV (Fisher Scientific). Atherosclerotic lesion size was measured using NIH Imaging software from 4 sections chosen by strict anatomical criteria, as described elsewhere. Sirius red staining of sections was performed as described.

Statistical Analyses

Data presented are mean±SEM. Means of different groups were compared by ANOVA, and correlations were analyzed using simple linear regression with the JMP software (SAS Institute Inc.). P<0.05 was considered statistically significant.

Results

Vitamin C Deficiency in the Gulo⁻/⁻ Apoe⁻/⁻ Mice

All of the mice in this study lacked both Gulo and apolipoprotein E (Gulo⁻/⁻ Apoe⁻/⁻) but were apparently healthy and reproducively active as long as their diets were supplemented with vitamin C, except that female Gulo⁻/⁻ Apoe⁻/⁻ mice older than 7 months of age rarely became pregnant.

To alter vitamin C levels in Gulo⁻/⁻ Apoe⁻/⁻ mice on normal chow, the vitamin C content of their drinking water was adjusted to 0.033 g/L, 0.33 g/day, or 3.3 g/L. The estimated daily vitamin C intake was 0.37 mg/day vitamin C. To lower vitamin C levels additionally, we fed mice augmented diet and vitamin C supplement groups. In individual animals, vitamin C levels in plasma and liver tissue were strongly correlated (P<0.0001) in both males and females. Correlation between plasma vitamin C levels and triglyceride levels was statistically significant in females (P=0.01, r²=0.23) but not in males. Conversely, plasma vitamin C levels were correlated with glucose levels in individual males (P=0.02, r²=0.34) but not in females. Males had higher plasma total cholesterol (P<0.0001), triglycerides (P=0.003), and HDL cholesterol (P=0.002) than females, whereas females had a trend toward higher plasma and liver vitamin C levels than males (P=0.06).

Mice fed a HFW containing 21% (wt/wt) fat, 0.15% (wt/wt) cholesterol, and 200 mg/kg vitamin C had significantly higher vitamin C levels in their plasma and liver than mice fed normal chow (Table 2). The plasma and liver vitamin C levels of mice fed HFW with a daily intake of 17 mg vitamin C were 2 to 3 times those of the mice fed normal chow with the same amount of vitamin C intake. This is presumably because the HFW diet contains not only high fat but also high antioxidants, including vitamin C. Mice with 1 mg/day vitamin C that were fed HFW also had higher plasma and liver vitamin C levels than mice fed chow with 1 mg/day vitamin C. To lower vitamin C levels additionally, we fed mice a HFW containing 50 mg/kg of vitamin C and supplemented their drinking water with 0.033 mg/L vitamin C (estimated daily intake, 0.37 mg/day vitamin C). These mice survived for 2.5 months without loss of body weight (Table 2), and their plasma and liver vitamin C levels decreased to the levels in low vitamin C mice fed chow. We therefore used this regimen for the low vitamin C mice fed HFW and compared them to mice with medium (1 mg/day) and high (17 mg/day) vitamin C. The difference in vitamin C levels among animals in the 3 groups was significant (P<0.0001). Both plasma and liver vitamin C levels were significantly higher in females compared with males (P<0.0001). As in mice fed normal chow, the daily vitamin C intake did not significantly affect plasma total cholesterol, triglycerides, and HDL-cholesterol in mice fed HFW.

Thus, in summary, by adjusting the daily intake of vitamin C, we were able to maintain plasma vitamin C levels in the Gulo⁻/⁻ Apoe⁻/⁻ mice between 1.5 and 30 μg/mL. Chronic
groups, although sex (male or female), age (4 or 9 months) did not differ between the high vitamin C and low vitamin C of their dietary vitamin C levels (Figure 1). The lesion size months of age, which was 2.5 or 7.5 months after adjustment sinus of the 

The mean sizes of the atherosclerotic lesions in the aortic 

Atherosclerotic Plaque Size 

The mean sizes of the atherosclerotic lesions in the aortic sinus of the Gulo$^+/−$ Apoe$^−/−$ mice were measured at 4 or 9 months of age, which was 2.5 or 7.5 months after adjustment of their dietary vitamin C levels (Figure 1). The lesion size did not differ between the high vitamin C and low vitamin C groups, although sex (male or female), age (4 or 9 months) and diet (chow or HFW) had strong influences ($P<0.0001$ in each). The mean plaque size in individual animals was not correlated with either the plasma or the liver vitamin C level. Thus, low versus high vitamin C had no effect on the size of the atherosclerotic plaques that developed in the Gulo$^+/−$ Apoe$^−/−$ mice. The Gulo$^+/−$ Apoe$^−/−$ mice fed normal chow at 4 months of age had only a few small plaques in the aortic arch, and none had visible plaques in the abdominal aorta. In contrast, at 9 months of age, all 14 mice fed normal chow had significant

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<th>TABLE 1. Vitamin C and Plasma Lipid Levels in the Gulo$^+/−$ Apoe$^−/−$ Mice Fed Normal Chow</th>
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<td>Vitamin C Intake</td>
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<td>Males</td>
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<td>Plasma vitamin C, $\mu$g/ml</td>
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Values are mean±SEM. The number of animals is given in parentheses. HDL-C indicates high-density lipoprotein cholesterol.

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<th>TABLE 2. Vitamin C and Plasma Lipids in the Gulo$^+/−$ Apoe$^−/−$ Mice on Western-Type High-Fat Diet</th>
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<td>Vitamin C Intake</td>
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<td>Males</td>
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<tr>
<td>Body weight, g</td>
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Values are mean±SEM. Numbers of animals are given in parentheses. HDL-C indicates high density lipoprotein cholesterol.
numbers of plaques visible under the dissection microscope in the aortic arch, and 11 of 14 had visible plaques in the abdominal aorta near the renal bifurcation. However, there were no significant differences in the number of plaques in the abdominal aorta between the low vitamin C mice and high vitamin C mice (3.8±1, n=9, and 3.0±1.3, n=5, P=0.6). Thus, dietary vitamin C levels did not influence the development or distribution of atherosclerotic plaques throughout the aortic tree of the Gulo−/− Apoe−/− mice.

**Histological Examination of the Plaques**

Plaques at the aortic sinus of mice in the present study were at various stages, ranging from small accumulations of foam cells to raised lesions with cholesterol clefts and fibrous caps. The lesions in the high and low vitamin C mice were not only indistinguishable in size but were also histologically similar (Figure 2). Foam cells were equally present in early lesions of both groups, indicating that the vitamin C does not influence the recruitment of monocytes and their transformation into foam cells. In raised and advanced plaques, foam cells, cholesteryl clefts, necrotic cores, and calcification were present in both high vitamin C plaques (Figure 2A and 2E) and low vitamin C plaques (Figure 2B and 2F).

There were, however, subtle but important differences in the advanced lesions of the low and high vitamin C mice. When stained for collagen with Sirius red, the advanced lesions of the high vitamin C mice showed intensely staining collagen fibers forming intricate networks throughout the plaque (Figure 2C and 2G). In contrast, Sirius red staining of the advance plaques in low vitamin C mice was less marked (Figure 2D and 2H). Areas with large, lipid-rich necrotic core were devoid of collagen filaments. Notably, the fibrous caps of the low vitamin C plaques contained only a few thin fibers that were weak green under polarized light, contrasting with the strong yellowish red color of the fibrous caps of high vitamin C plaques (Figure 2I and 2J).

Additionally, the low vitamin C plaques tended to contain larger necrotic cores (Figure 2F) compared with the high vitamin C plaques. The adventitia adjacent to the advanced plaques in the high vitamin C animals was thickened and showed a fibroproliferative response (Figure 2A and 2E) with intense Sirius red staining of collagen (Figure 2C and 2G). Multiple small vessels in the adventitia indicate active neovascularizations (Figure 2E, arrows). In contrast, adventitial thickening and neovascularization in the low vitamin C animals was minimal (Figure 2B and 2F).

Advanced plaques of similar size and maturity were selected from at least four animals in each group, and their collagen content was measured as Sirius red-stained area in each plaque (Figure 3). The collagen-stained area in advanced low vitamin C plaques was 56% of the area in high vitamin C plaques at 4 months and 70% at 9 months in the chow-fed animals and 65% at 4 months in the HFW-fed mice. The effect of vitamin C was highly significant (P<0.0001). Additionally, the green/red color ratios under polarized light of plaques from low (1.13±0.09, n=13) and high vitamin C animals (0.68±0.08, n=9) revealed significantly higher green fiber content in the low versus high vitamin C plaques (P<0.005). Green fibers are thinner and/or contain relatively more type III collagen than red fibers. These data clearly demonstrate that vitamin C deficiency compromises deposition of collagen in the atherosclerotic plaques.

**Discussion**

Using mice that are unable to synthesize ascorbic acid and are prone to develop atherosclerosis, we tested whether altered levels of vitamin C in the diet influence the initiation or maturation of atherosclerotic plaques. Our data demonstrate that vitamin C does not alter either foam cell formation or the size of atherosclerotic plaques but significantly influences their collagen content and collagen in the adventitia surrounding vessels with plaques.

Numerous proatherogenic effects of oxidized LDL have been documented. These include processes important for foam cell formation, such as the induction of cell adhesion molecules and monocyte chemoattractant protein 1 in endothelial cells and of scavenger receptor expression in macrophages. The demonstrated ability of vitamin C to inhibit LDL oxidation in vitro additionally argues in favor of a potential role for vitamin C in protection against foam cell formation. Consequently, earlier animal studies mainly focused on the effects of vitamin C on early lesions. The results have been conflicting, ranging from increased fatty streaks in vitamin C–deficient guinea pigs to no effects of vitamin C supplementation in cholesterol-fed rabbits.

LDL oxidation in Apoe−/− mice occurs in vivo as demonstrated by increased amounts of circulating autoantibody...
against oxidized LDL, and antioxidants, such as vitamin E and N,N-diphenyl 1,4-phenylenediamine, have been shown to reduce atherosclerosis in Apoe<sup>−/−</sup> mice. In support of a role for vitamin E, Apoe<sup>−/−</sup> mice that also lack vitamin E transfer protein and have vitamin E deficiency (<10% normal in plasma) had modest but significant increases in atherosclerosis. However, not all antioxidants are protective, and supplementing the diet with either red wine polyphenol or ethanol had no effect. Furthermore, probucol paradoxically increased atherosclerosis in Apoe<sup>−/−</sup> mice, raising a caution that antioxidants can also be strong oxidants in some circumstances. Our results show that vitamin C is not a key player in the early steps of atherogenesis in vivo. There are numerous antioxidants and antioxidant enzymes in the body that coordinately defend against free radical formation. Perhaps alteration in the levels of a single nutrient has only a limited effect on total oxidative stress in the body as long as homeostatic mechanisms are intact.

Our finding that the content and networking of collagen fibers in the plaques are less in the low vitamin C animals compared with high vitamin C animals demonstrates that vitamin C plays a crucial role during the maturation of atherosclerotic plaques. Ascorbic acid is important in connective tissue metabolism, where it acts as the reducing cofactor in the reactions catalyzed by prolyl and lysyl hydroxylases. Hydroxyproline and hydroxylysine are essential for the secretion, cross-linking, and maturation of collagen, a key component of extracellular matrix important for maintenance of vascular integrity. Preliminary measurements of the hydroxyproline, hydroxylysine, and their cross-linked products in tissues isolated from the Gulo<sup>−/−</sup> mice on high or low vitamin C supplements did not reveal significant differences when the values were normalized to total proline content (data not shown). This fact suggests that only a small fraction of tissue collagen is affected or that collagen with low hydroxylation is degraded intracellularly. Further biochemical analysis of newly synthesized collagen is necessary to properly address this issue. Nevertheless, de novo synthesis of collagen in atherosclerotic plaques promotes migration and proliferation of smooth muscle cells, which transform into myofibroblasts and contribute to fibrous cap formation. Adventitial fibroblasts also migrate to the intima in response to vascular injury in vivo. These cells are fully capable of secreting collagen and additionally stabilizing the plaque. The fibroproliferative response and neovascularization of adventitial tissue ensures an adequate blood supply to the vessels and growing plaques. In the vitamin C-deficient state, this neovascularization is reduced and may limit the supply of oxygen and nutrients to the plaques and thereby promote necrosis. The marked reduction in collagen deposition is likely to additionally weaken the plaques. In humans, symptoms related to atherosclerotic heart disease develop when plaques enter an unstable phase. The features characteristic of

Figure 2. Aortic lesions at aortic sinus of the Gulo<sup>−/−</sup>-Apoe<sup>−/−</sup> mice with high vitamin C (sections A, C, E, G, and I) and low vitamin C (sections B, D, F, H, and J). Sections A, C, E, G, and I were stained for lipids with Sudan IVB and counterstained with hematoxylin. Their neighboring sections C, D, and G through J were stained for collagen with Sirius red. Sections I and J were photographed with a polarizing filter. Sections A through D are from 4-month-old females fed Western-type diet. Sections E through J are from 9-month-old males fed normal chow. Arrows in E indicate small vessels in adventitia. Bar=100 μm.

Figure 3. Collagen-stained area in advance atherosclerotic plaques. White bars represent mean±SEM in high vitamin C plaques, and black bars represent mean±SEM in low vitamin C plaques.
the unstable plaques, seen in clinically symptomatic patients, include thin fibrous caps, large lipid accumulations, large numbers of macrophages and T cells, and depletion of smooth muscle cells.\textsuperscript{27,28} Thus the morphological features in the vitamin C–deficient plaques are very similar to those that characterize high-risk plaques in humans.

A link between chronic vitamin C deficiency and unstable plaque morphology may be relevant to human atherosclerosis. Alarming studies by Levine et al\textsuperscript{29} have shown that plasma vitamin C levels (4.4 μg/mL) in healthy male volunteers given the recommended daily allowance of 60 mg/dL are below saturation (12 μg/mL). Atherosclerotic plaques begin to develop at an early age in humans. Conceivably, plaques in which collagen deposition has been compromised by subsaturation levels of vitamin C could become clinically symptomatic in later life. In addition to diet, smoking and various inflammatory conditions can lead to reduced plasma vitamin C levels. Vitamin C deficiency under these circumstances could then contribute to the vulnerability of plaques in human patients.

Whether the morphological features induced by chronic vitamin C deficiency in the Gulo\textsuperscript{-/-} ApoE\textsuperscript{-/-} mice are sufficient to cause plaque rupture is yet to be determined. Additional changes, such as hemodynamic factors, may well be necessary for even a vulnerable plaque to enter a clinically significant stage. Nevertheless, the vitamin C–deficient Gulo\textsuperscript{-/-} ApoE\textsuperscript{-/-} mice should allow us to explore genetic and environmental factors that trigger the rupture of plaques that have a high-risk phenotype.

Acknowledgments

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References

Vulnerable Atherosclerotic Plaque Morphology in Apolipoprotein E–Deficient Mice Unable to Make Ascorbic Acid
Yukiko Nakata and Nobuyo Maeda

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