Ascorbic acid-independent synthesis of collagen in mice

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Ascorbic acid-independent synthesis of collagen in mice. Am J Physiol Endocrinol Metab 290: E1131–E1139, 2006. First published December 13, 2005; doi:10.1152/ajpendo.00339.2005.—The mouse has become the most important model organism for the study of human physiology and disease. However, until the recent generation of mice lacking the enzyme gulonolactone oxidase (Gulo), the final enzyme in the ascorbic acid biosynthesis pathway, examination of the role of ascorbic acid in various biochemical processes using this model organism has not been possible. In the mouse, similar to most mammals but unlike humans who carry a mutant copy of this gene, Gulo produces ascorbic acid from glucose. We report here that, although ascorbic acid is essential for survival, its absence does not lead to measurable changes in proline hydroxylation. Vitamin C deficiency had no significant effect on the hydroxylation of proline and collagen production during tumor growth or in angiogenesis associated with tumor or mammary gland growth. This suggests that factors other than ascorbic acid can support proline hydroxylation and collagen synthesis in vivo. Furthermore, the failure of Gulo−/− mice to thrive on a vitamin C-deficient diet therefore suggests that ascorbic acid plays a critical role in survival other than the maintenance of the vasculature.

ascorbic acid; collagen; angiogenesis; prolyl hydroxylase

ASCORBIC ACID, ALSO KNOWN AS VITAMIN C, was first discovered in the mid-1700s when a Scottish naval surgeon determined that he could cure scurvy with a diet containing citrus fruits. Scurvy is a condition characterized in humans by bleeding gums, tooth loss, weakening of bones, swelling of joints, and anemia. Ascorbic acid is a cofactor for the enzyme prolyl hydroxylase, which catalyzes the hydroxylation of peptidyl proline in a posttranslational process (39). In this reaction, it is thought that ascorbic acid reacts with oxidized iron bound to prolyl hydroxylase, reducing the iron and facilitating the release of the enzyme (26). Hydroxyproline is an important amino acid in collagen, a prominent structural protein found in connective tissues such as bones, tendons, ligaments, teeth, skin, cartilage, and blood vessels. Inadequate maintenance of these connective tissues leads to the symptoms associated with scurvy and eventually death in humans deficient in vitamin C. Collagen is formed by the association of three collagen monomers to form a triple helix (32). At least 35% of the prolines in a collagen monomer must be hydroxylated to assure thermal stability (4), and underhydroxylated molecules are usually degraded within the cell without being excreted in the extracellular matrix (3).

The theory that ascorbic acid is required for proline hydroxylation was established based on results of experiments in cell-free systems (35) and in cells and tissues in culture (3, 37). However, it has not been supported by experiments in animal models, in part, because of the lack of a mouse model with a demonstrated dependence on dietary vitamin C. Virtually all animals other than primates and guinea pigs are able to synthesize ascorbic acid in the liver and transport it to other parts of the body. Studies in guinea pigs, animals that are naturally unable to synthesize ascorbic acid, have produced conflicting results concerning the requirement for ascorbic acid in hydroxyproline synthesis in collagen. To determine whether ascorbic acid is essential for collagen formation in vivo, we used a mouse lacking an enzyme in the ascorbic acid synthesis pathway, making it dependent on exogenous vitamin C for survival.

The process of creating new blood vessels, known as angiogenesis, involves the deposition of an extracellular matrix that is rich in collagen. Given its role in the production of collagen, ascorbic acid is likely to be important in the formation of new blood vessels. Results of previous in vitro experiments have supported this theory. Angiogenesis assays using a rat aortic ring model showed that microvessels grown in the absence of ascorbic acid were large and dilated, whereas vessels grown in the presence of ascorbic acid resembled normal capillaries. Presumably, collagen serves as a scaffold to maintain the shape of the newly formed vessels. In the same study, cells incubated with ascorbic acid increased their synthesis and extracellular deposition of collagen, measured by the incorporation of [14C]proline in collagen monomers (29).

Angiogenesis is rare in normal tissue, occurring mostly during embryogenesis and in adults in the female reproductive system. In pathological tissues, angiogenesis occurs during wound healing and during tumor growth (13). Angiogenesis takes place in the normal mammary gland during puberty, pregnancy, and lactation and also during the growth of mammary tumors (9). Because proliferating mammary glands and tumors both depend on the generation of blood vessels, and therefore the generation of collagen, we chose to investigate the effect of ascorbic acid deficiency on the growth of mammary glands and mammary tumors in mice. Mammary glands were induced to proliferate by the pituitary implant model, and tumors were induced by the injection of tumor cells in the mammary fat pad. Both mammary glands and tumors proliferated in the presence and absence of ascorbic acid. Tumors from vitamin C-deficient animals contained an abundance of hydroxyproline and collagen despite a lack of ascorbic acid within the tumors. Collagen extracted from the skin of young mice raised in the absence of ascorbic acid contained normal amounts of ascorbic acid.
amounts of hydroxyproline, indicating that proline hydroxylation also occurred in other tissues. These results led to the conclusion that ascorbic acid is not required for the development of blood vessels in mice.

METHODS

Animal care. A mouse deficient in gulonolactone oxidase (Gulo−/−) and on a mixed genetic background was obtained from Maeda and backcrossed to BALB/c mice from Jackson Laboratories for 10 generations. All Gulo−/− mice were supplied with water containing 330 mg L-ascorbic acid/l and 0.01 mM EDTA. Westerm-type high-fat diet was modified to contain no ascorbic acid (TD.04073, modified version of TD.88137; Harlan Tekla, Indianapolis, IN).

Pituitary implants. Female mice (7 wk old) received one pituitary isograft under the right renal capsule from Balb/c donors, as previously described (25). Gulo−/− mice were withdrawn from vitamin C supplementation 1 wk before pituitary implantation. Animals were killed 4 wk after surgery; mammary glands were saved for whole mount and hydroxyproline analysis. Serum was collected for prolactin measurement. Tissue from nonisografted animals served as controls.

Tumor injection. All Gulo−/− mice were removed from vitamin C supplementation 2 wk before initiation of the experiment unless otherwise noted. For tumor cell injections, the mouse mammary tumor cell line 4T1 was obtained from ATCC and grown in RPMI 1640 medium with 2 mM L-glutamine, 10% FBS, 10 mM HEPEs, pH 7.2, 1 mM sodium pyruvate, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate. At 90% confluence, cells were harvested, and 10^6 cells were injected in 0.1 ml in the right mammary fat pad of each experimental and control mouse. Mice were monitored daily until tumor onset, and tumors were measured one time a week. Animals were killed at 3 wk, and tumors were collected and frozen in Eppendorf tubes for RNA analysis and hydroxyproline measurements and in OCT compound for immunohistochemistry and Sirius red staining.

Hydroxyproline assay. For skin analysis, hair was removed from the back of the neck with Nair, and a 1-cm² piece of skin was removed for analysis. Hydroxyproline was measured in dried tissue samples as previously described (18). Hydroxyproline concentrations were determined based on a generated standard curve.

Sirius red staining. Frozen tissue sections were fixed for 10 min in Omni-fix (FR Chemicals, Mount Vernon, NY) and stained for 30 min in saturated picric acid containing 0.1% Sirius red and 0.1% Fast green. Slides were dipped in 70% ethanol, rinsed in water, and mounted with Permount. Staining was quantitated using Meta-Morph (Universal Imaging, Downingtown, PA).

Immunohistochemistry. Frozen tissue sections were fixed for 10 min in Omni-fix (FR Chemicals) and air-dried for 30 min. Sections were quenched with 0.1% H₂O₂ for 10 min, rinsed three times in PBS for 5 min, blocked in 2% goat serum for 10 min, and rinsed three times in PBS for 5 min. Samples were incubated with CD34 primary antibody (PharMingen, San Jose, CA) diluted 1:10 in block for 1 h at room temperature in a humidified chamber and rinsed three times in PBS. Samples were incubated with Multi Adsorbed Biotin-Conjugated Goat anti-Rat secondary antibody (PharMingen) diluted 1:200 in block for 1 h at room temperature in a humidified chamber, rinsed three times in PBS, and incubated with avidin-biotin complex (Vector, Burlingame, CA) for 1 h at room temperature in a humidity chamber. Slides were rinsed three times in PBS and stained with diamonobenzidine reagent for 8 min, followed by hematoxylin counterstaining. Slides were rinsed with tap water, air-dried, and mounted with Permount. Staining was quantitated using Meta-Morph.

In vivo collagen labeling. Mice were injected intravenously in the tail vein with 100 μCi [³H]proline in sterile PBS. After 18 h, animals were killed by CO₂ asphyxiation, and tissues were harvested and frozen at −80°C. Tissues were analyzed for hydroxyproline content as previously described, using a toluene extraction to remove proline and other factors that may result in false positives (22).

Collagen extraction. Tumor tissue (400 mg) was homogenized in 0.5 M acetic acid, and collagen was extracted by incubating homogenized tissue in 20 ml 0.5 acetic acid containing 0.005% pepsin (Sigma-Aldrich, St. Louis, MO) at 4°C with shaking. After 24 h, samples were sedimented, and lipids were removed from the supernatant fluid by chloroform/methanol extraction. The acid phase was filtered using Centriplus 50-kDa cutoff filters (Millipore, Billerica, MA) and lyophilized, and protein was resuspended in HNTG buffer (50 mM HEPEs, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) for SDS-PAGE. Tail collagen was isolated from tail tendon and treated in a similar manner. For HPLC analysis, dried collagen samples were hydrolyzed with 6 N HCl at 105°C overnight, dried, dissolved in distilled water, and filtered. An aliquot of the hydrolysate was analyzed with the Varian HPLC system 9050/9012 (Varian, Walnut Creek, CA) on a cation-exchange column (AA-911; Transgenomic) to determine hydroxyproline content as described previously (43).

Ascorbic acid measurement. Tissue ascorbic acid levels were determined as previously described (31).

RESULTS

Vitamin C deficiency resulted in significant weight loss. A phenotype associated with scurvy in guinea pigs is the onset of weight loss ~2 wk after the removal of vitamin C from the diet (2, 36). To determine whether this phenotype is apparent in the Gulo−/− mice and, if so, how long the these mice can remain healthy without vitamin C, female Gulo−/− mice were weighed at intervals after removal of vitamin C supplementation. Gulo−/− littermates remaining on vitamin C supplementation served as controls. No significant weight loss was observed until the 4th wk without vitamin C supplementation. By this time, unsupplemented mice had lost nearly 20% of their body weight (Fig. 1A), and the experiment was discontinued.

Ascorbic acid levels varied in different organs after vitamin C withdrawal. Ascorbic acid levels were measured at weekly time points after withdrawal of vitamin C supplementation in tissues known to store high levels of ascorbic acid. Figure 1B shows the ascorbic acid levels in Gulo−/− mice liver, brain, and adrenal glands, expressed as the percentage of wild-type ascorbic acid levels. Ascorbic acid was undetectable in the liver after 2 wk without supplementation, and levels were reduced to 20 and 10% of wild type in the brain and adrenal glands. Because ascorbic acid levels were extremely low after 2 wk and continued to decrease in the brain and adrenal glands, the 2-wk time point was selected as an appropriate starting point for future experiments.

Skin collagen levels were normal in vitamin C-deficient mice. Skin is a tissue that is abundant in collagen. To determine the effect of vitamin C deficiency on the collagen content of mouse skin, we measured the amount of hydroxyproline in the skin from wild-type and Gulo−/− mice that had been depleted of vitamin C. Surprisingly, hydroxyproline measurements with a colorimetric biochemical assay revealed an ~80% increase in hydroxyproline in a 1-cm² area of skin from vitamin C-deficient mice compared with wild-type mice (Fig. 2). However, this elevation may be artifactual, reflecting an increase in the density of collagen in the skin resulting from shrinkage caused by decreased fluid intake observed after removal of vitamin C from the diet.

Collagen levels in stimulated mammary glands of vitamin C-deficient mice were significantly higher than levels in than nonstimulated glands. Mammary gland proliferation was stimulated in wild-type and Gulo−/− mice by implantation of a
impaired during vitamin C deficiency and that prolactin deficiency, rather than impaired collagen synthesis, most likely caused the decreased hydroxyproline levels in Gulo<sup>−/−</sup> isografted animals. The lower serum prolactin levels of the Gulo<sup>−/−</sup> mice could also explain the difference in hydroxyproline between the two control nonisografted groups. The importance of prolactin in the normal maturation of the mouse mammary gland during puberty has been well documented. Studies in prolactin receptor knockout mice have shown that, in the absence of prolactin signaling, mammary glands fail to develop normally during puberty (5). The isograft surgeries were performed at 7 wk of age, during puberty and mammary gland development; therefore, some angiogenesis would be expected to occur in these animals, even those not obtaining grafts.

Vitamin C-deficient mice developed tumors at the same rate as wild-type controls. To determine whether vitamin C deficiency affects the production of collagen in the blood vessels of mammary tumors, 4T1 mouse mammary tumor cells were injected in the mammary fat pads of 6-wk-old wild-type and Gulo<sup>−/−</sup> mice after 2 wk of vitamin C withdrawal. The 4T1 tumor cell line was chosen because it was shown to be responsive to anti-angiogenic therapy and therefore should be highly dependent on the formation of blood vessels for growth (16). All 14 experimental and 15 wild-type mice developed tumors, with an average onset of 3.9 and 4.4 days, respectively. Tumors were measured weekly, and at the time of death 3 wk after injection, the average tumor size was not statistically different between the two groups of mice (data not shown).

Therefore, vitamin C deficiency did not affect the incidence, onset, or growth of mammary tumors in mice.

Tumors in vitamin C-deficient mice had lower levels of hydroxyproline but similar amounts of collagen and blood vessels as those in wild-type controls. After animals were killed, tumor tissue was collected for histological and biochemical analysis. An initial experiment analyzing tumors from 14 wild-type and 14 Gulo<sup>−/−</sup> mice suggested that ascorbic acid could enhance collagen synthesis in these tumors. In this setting, a 20% decrease in hydroxyproline was observed in tumors obtained from vitamin C-deficient mice (P = 0.04). However, expansion of the analysis to include data from all harvested tumors (n = 20 wild type, n = 22 Gulo<sup>−/−</sup>) failed to support this conclusion. Although the combined data continued to show a small decrease in collagen levels in the tumors from donor pituitary gland under the renal capsule. Collagen content of the mammary glands was measured by hydroxyproline analysis. Total hydroxyproline per dried gland increased significantly, 46% in wild-type mice and 24% vitamin C-deficient mice, compared with controls (P < 0.05; Fig. 3A). However, a significant difference was also detected between wild-type and vitamin C-deficient controls (P < 0.005) and between wild-type and vitamin C-deficient isografts (P < 0.001). The possibility existed that the difference in collagen content between the mammary glands from isografted wild-type and vitamin C-deficient mice was not directly because of an impaired ability to synthesize collagen but indirectly because of insufficient prolactin production. Therefore, serum prolactin levels were measured by ELISA. Average serum prolactin levels in Gulo<sup>−/−</sup> mice that were vitamin C deprived but did not receive an isograft were 5.4 ng/ml. Pituitary isografting increased average serum prolactin levels in Gulo<sup>−/−</sup> mice to 18.7 ng/ml (Fig. 3B). Serum prolactin levels in wild-type mice were 22.1 ng/ml for control mice and 40.6 ng/ml for isografted mice. Although pituitary isografting significantly increased serum prolactin levels in both wild-type and Gulo<sup>−/−</sup> animals, levels in vitamin C-deficient mice were significantly lower than those in wild-type controls. In addition, baseline measurements were significantly decreased in knockout animals compared with the wild type. These data parallels the differences in hydroxyproline seen in the mammary glands of these animals. These results indicate that prolactin production or secretion was
vitamin C-deficient animals (19% decrease), the difference failed to achieve statistical significance (P = 0.1).

The failure to observe a biochemical difference in hydroxyproline levels was consistent with histological analysis of the tumors. Sections of tumors from both wild-type and Gulo−/− mice were stained for collagen using Sirius red and for blood vessels using an antibody to the endothelial cell marker CD34. Tumors from both vitamin C-deficient and wild-type mice contained an abundance of collagen (Fig. 4, A and C). Quantitation of collagen staining showed no difference between the two groups (Fig. 4D). Sections of tumors that were immunostained for vasculature showed similar staining in both groups of mice (Fig. 5, A and B), and quantitation showed no difference in the amount of staining between the vitamin C-deficient and wild-type controls (Fig. 5C). Thus collagen and blood vessels were produced in tumors despite vitamin C deficiency.

Proline incorporation and hydroxylation occurred at the same rate in wild-type and vitamin C-deficient mice. L-[5-3H]proline was injected intravenously in wild-type and vitamin C-deficient animals with mammary tumors to determine proline incorporation and hydroxylation. Radioactive counts in total tissue hydrolysates were comparable in wild-type and vitamin C-deficient animals (Fig. 6A), indicating that protein synthesis was equivalent in the two groups. Radioactive counts in hydroxyproline fractions separated by toluene extraction were also equivalent (Fig. 6B); therefore, the conversion of proline to hydroxyproline occurred at the same rate in wild-type and vitamin C-deficient mice.

The proline-to-hydroxyproline ratio was equivalent in tail and tumors from wild-type and vitamin C-deficient mice. Collagen was extracted by acid hydrolysis and examined by SDS-PAGE and HPLC. Collagen extracted from vitamin C-deficient tumors (Fig. 7A, lanes 6–8) did not appear different from those of wild-type mice (lanes 3–5) when visualized on an SDS-PAGE gel. Total amino acid content of acid-hydrolyzed collagen samples revealed that the ratio of proline to hydroxyproline was not significantly different in samples from Gulo−/− mice (Fig. 7B). However, the observed ratios (15:1) were higher than the expected proline-to-hydroxyproline ratio of 1.5:1, indicating that the samples contained large amounts of noncollagen protein. Because the dilution of collagen by noncollagen proteins could impair our ability to observe small but significant changes in hydroxyproline, we chose to verify these results using collagen extracted from mouse tail. Because collagen is a major constituent of tail protein, small differences in hydroxylation are more likely to be observed. Collagen was extracted from the tails of wild-type and 2-wk unsupplemented mice at age 4 wk. Between the ages of 3 and 4 wk, body weight increases by 40%, and a proportional increase in tail weight is observed. Tail collagen was subjected to HPLC analysis, and the resulting proline-to-hydroxyproline ratios were close to the expected 1.5:1 ratio. No significant difference in proline-to-hydroxyproline ratios between vitamin C-deficient and wild-type animals was detected (Fig. 7C). These results again fail to support a dependence of hydroxylation on vitamin C in the mouse.

Ascorbic acid uptake does not differ between tumors in Gulo−/− and wild-type mice. Ascorbic acid can be transported in cells through glucose transporters in addition to the two sodium-dependent ascorbic acid transporters (40), and glucose transporters tend to be upregulated in tumor cells (1). Therefore, we investigated whether the tumors increased their ascorbic acid concentrations by absorbing any ascorbic acid that may have been circulating or stored in neighboring tissues. Ascorbic acid measurements in tumor samples revealed very low levels of ascorbic acid in tumors of vitamin C-deficient mice compared with the wild type (Fig. 8A). The difference correlates with the disparity of ascorbic acid levels in the livers of wild-type and Gulo−/− unsupplemented mice (Fig. 8B), indicating that the tumors did not concentrate ascorbic acid against the gradient. Pretreatment of samples with ascorbate oxidase before ascorbic acid measurement showed that the small amount of activity seen in the Gulo−/− samples could be attributed to background likely from other reducing agents. To show that ascorbate oxidase was actually destroying ascorbate in this complex system, purified ascorbic acid was added to liver homogenates before ascorbate oxidase treatment and ascorbate measurement. Ascorbate oxidase degraded the ascorbate in the samples to the same irreducible amount regardless of the quantity of purified ascorbate added (Fig. 8C).
DISCUSSION

Ascorbic acid is involved in numerous biological processes, including scavenging free radicals to prevent oxidative damage, acting as a cofactor for reactions such as the hydroxylation of proline necessary for collagen needed for blood vessels and wound healing, hormone synthesis, cholesterol metabolism, neurotransmitter synthesis, iron absorption, and immune system function, among others. Its role in the formation of collagen led us to investigate whether ascorbic acid is required for collagen synthesis in mouse skin and the development of blood vessels during mouse mammary gland proliferation and tumorigenesis.

The use of animal models for the study of the roles of ascorbic acid has been limited by the fact that all animals other than primates and guinea pigs are able to synthesize ascorbic acid, rather than having to rely on a dietary source of this nutrient. Recently, this problem has been addressed by the generation of a mouse line in which the gene for L-gulono-γ-lactone oxidase, the final enzyme in the ascorbic acid synthesis pathway, was mutated by homologous recombination (23). Because this mutation leads to a dependence on dietary vitamin C, we used these mice to determine the role of ascorbic acid in the production of collagen in various organs.

The collagen content of skin from adult mice did not decrease in Gulo<sup>−/−</sup> mice after vitamin C supplementation was removed. Because this result might reflect low turnover of collagen in this tissue, we also examined tissues that were actively proliferating and thus developing new blood vessels in vitamin C-deficient, growing animals. Angiogenesis, the development of new blood vessels, is rare in normal adult tissue but does occur in the mammary gland during puberty, pregnancy, and lactation. Examination of pregnant mammary glands in vitamin C-deficient mice proved to be difficult because of the fact that these mice do not often maintain pregnancies. Therefore, we chose to induce mammary gland proliferation by performing pituitary implant surgeries. When the pituitary gland from a donor mouse is placed under the renal capsule, prolactin is released continuously in the bloodstream, stimulating mammary gland growth. Pituitary isografting led to budding of the epithelium in wild-type glands and in Gulo<sup>−/−</sup> glands in spite of vitamin C deficiency. Collagen content of proliferating glands, measured by hydroxyproline analysis, increased in both groups, although to a lesser extent in the glands from vitamin C-deficient mice. From these data, we conclude that collagen production is reduced but not abolished in the mammary gland as a result of vitamin C deficiency. However, a significant decrease in serum prolactin concentration in vitamin C-deficient mice compared with wild-

Fig. 4. Hydroxyproline content but not collagen content of 4T1 tumors was reduced in tumors from vitamin C-deficient mice. A: Collagen content of tumors was analyzed indirectly through hydroxyproline measurement. Hydroxyproline amounts are represented as average mg of hydroxyproline/mg of dry tissue ± SE. No significant difference was detected in samples from wild-type (n = 20) and Gulo<sup>−/−</sup> (n = 22) mice by Student’s t-test. Frozen tumor sections were immunostained with Sirius red and counterstained with Fast green. Staining was quantitated using Meta-Morph and is presented as average %staining ± SE (D). No significant difference in staining was seen between wild-type (n = 14) and Gulo<sup>−/−</sup> tumors (n = 15) by Student’s t-test. Representative samples are shown from wild-type (B) and Gulo<sup>−/−</sup> (C) mice.
type controls suggests that this reduction in collagen is the result of decreased prolactin production rather than reduced proline hydroxylation. These results suggest that the Gulo<sup>-/-</sup> mouse may serve as a model for studying the role of ascorbic acid in the regulation of prolactin expression.

Angiogenesis is common in pathological tissues such as tumor development. Using the injection of a tumor cell line in the mammary gland fat pad as a model, we have shown that tumors grow at the same frequency and rate in vitamin C-deficient mice as in wild-type controls. Also, tumors in the experimental and control groups contain equivalent amounts of hydroxyproline, collagen, and blood vessels. Because underhydroxylated collagen is more sensitive to intracellular degradation (3), a significant decrease in hydroxylation should be reflected by a reduction in extracellular collagen deposition. The observation that amounts of extracellular matrix collagen in tumors from wild-type mice did not differ significantly from Gulo<sup>-/-</sup> levels suggests that collagen is not severely underhydroxylated despite the state of vitamin C deficiency. Further analysis of these tumors revealed that hydroxyproline is actively synthesized in vitamin C-deficient animals, even after 4 wk without vitamin C supplementation. The ratio of hydroxyproline to proline was not measurably affected by the absence of tissue ascorbic acid. Theses studies suggest that the production of collagen, presumably required for stable blood vessel development, is not compromised even during severe ascorbic acid deficiency.

In humans, plasma ascorbic acid levels measuring <11% of normal are indicative of scurvy (15). Therefore, connective tissue damage occurs in humans even when ascorbic acid is detectable. In contrast, Gulo<sup>-/-</sup> mice display no evidence of connective tissue injury even when ascorbic acid was undetectable in tissue. This observation, along with our results showing normal levels of proline hydroxylation in the tail collagen, mammary gland, and tumors of vitamin C-deprived Gulo<sup>-/-</sup> mice, suggests that ascorbic acid is not required for proline hydroxylation in the mouse. However, it is possible that small amounts of ascorbic acid, below the level of detection,
are sufficient to catalyze the hydroxylation reaction in mice. In cell-free systems in vitro, only 1/9262 mol of ascorbic acid is required to catalyze proline hydroxylation (19). In addition, ascorbic acid is not consumed during the reaction and can therefore be recycled for additional hydroxylations (41). If this hypothesis is correct, our results would suggest that mice are better able than humans to concentrate ascorbic acid at the sites of collagen synthesis.

Studies in guinea pigs, which are unable to synthesize ascorbic acid, have not proven a direct connection between vitamin C deficiency and defects in proline hydroxylation. Experiments separating collagen production from proline hydroxylation in guinea pigs have shown that vitamin C-deficient animals have a 50% reduction in collagen synthesis but only a 30% decrease in proline hydroxylation. Furthermore, vitamin C-supplemented animals that were acutely starved showed the same reduction in collagen synthesis as the vitamin C-deficient animals (7, 33, 38). These data suggest that, although ascorbic acid does play a role in proline hydroxylation, the substantial decreases in collagen seen during scurvy in guinea pigs are the indirect consequence of weight loss accompanying vitamin C deficiency and may not reflect a defect in proline hydroxylation. In this same study, weight loss was shown to affect collagen production through inhibition of insulin-like growth factor I, a molecule known to stimulate production of collagen. Our data demonstrating a moderate decrease in hydroxyproline supports the idea that mice, similar to guinea pigs, utilize ascorbic acid as a cofactor for the production of hydroxyproline but do not rely solely on ascorbic acid for this function.

**Fig. 7.** Ratio of proline to hydroxyproline was not increased in tumors from vitamin C-deficient mice. Collagen was extracted from tumors, visualized by SDS-PAGE, and analyzed by HPLC for total amino acid content. A: lane 1, ladder; lane 2, collagen control extracted from mouse tail; lanes 3–5, collagen from wild-type tumors; lanes 6–8, collagen from Gulo−/− tumors. B: average ratio of hydroxyproline to proline in mouse tumors, determined by HPLC, ±SE. No significant difference was detected between the wild type (n = 3) and Gulo−/− (n = 3) by Student’s t-test. C: average ratio of hydroxyproline to proline in mouse tail collagen, determined by HPLC, ±SE. No significant difference was detected between the wild type (n = 3) and Gulo−/− (n = 3) by Student’s t-test.

**Fig. 8.** Tumors in vitamin C-deficient mice did not concentrate ascorbic acid. To eliminate the possibility that tumors in Gulo−/− are able to accumulate ascorbic acid, ascorbate content was measured in tumors (A) and liver (B) and expressed as μg of ascorbic acid/g of tissue. Wild-type samples contain high levels of ascorbate (n = 3), whereas ascorbic acid levels in tumor and liver of Gulo−/− mice (n = 3) are not significantly different from samples treated with ascorbate oxidase. *Significantly different from untreated Gulo−/− samples and ascorbate oxidase-treated samples (P < 0.005) by Student’s t-test. C: to show that ascorbate oxidase was actually destroying ascorbate, ascorbic acid was measured in liver samples that had been supplemented with purified ascorbic acid before ascorbate oxidase treatment.

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Although most cell lines tested in culture require the addition of ascorbic acid for proline hydroxylation, several cell lines, such as L-929 and virally transformed 3T3 cells, efficiently synthesize hydroxyproline in the absence of ascorbic acid (12, 34). The microsomal fractions of these cell lines contain a protein with a cysteinyl-cysteine active sequence that is able to function as a reducing agent in place of ascorbic acid in the proline hydroxylation reaction (6, 24). Additionally, in hydroxylation assays in cell-free systems, other reductants such as dithiothreitol, L-cysteine, tetrahydrofolate, and tetrahydroxylation assays in cell-free systems, other reductants in the proline hydroxylation reaction (6, 24). Interestingly, the initial characterization of *Gulo*−/− mice revealed hemorrhages and disrupted aortic vasculature after vitamin C withdrawal that were not observed in our studies (23). In addition, atherosclerotic plaques in *Gulo*−/− *Apoe*−/− mice contained significantly less collagen, measured by Sirius red staining (28). These apparently conflicting results could be the result of the difference in genetic background of the *Gulo*−/− in our study (BALB/c) and those in previous studies (B6/129 mixed and C57BL/6, respectively). A second possible explanation is that these phenotypes are secondary effects of weight loss and are not directly caused by vitamin C deficiency, similar to the results in guinea pigs. Unpublished preliminary data in the study of atherosclerotic plaques indicating that hydroxyproline measurements were equivalent in tissues from *Gulo*−/− mice receiving high and low vitamin C supplementation when these values were normalized to total proline content supports this hypothesis (28). The decreased collagen content in the plaques may be because of a down-regulation of collagen production during starvation rather than impaired proline hydroxylation.

The observations that proline hydroxylation can still occur and connective tissues remain intact in vitamin C-deficient mice indicate that the eventual death that results in these mice is not because of scurvy. The only obvious phenotype that develops in the *Gulo*−/− mice after the removal of vitamin C supplementation is weight loss, a phenotype that has also been reported in studies on scurviatic guinea pigs. Several studies have reported that weight loss in vitamin C-deficient guinea pigs is the result of a decrease in food intake (2, 36), indicating that ascorbic acid plays some role in appetite regulation. Ascorbic acid is known to be required for the synthesis of neurotransmitters such as norepinephrine (11), norepinephrine (27), serotonin, and dopamine (20). Depression and changes in eating behaviors are often associated with deficiencies in these biogenic amines. In addition, depression is often a symptom of scurvy in humans (14, 21, 42). The anti-obesity drug fenfluramine, which acts through the serotonin pathway, has been shown in guinea pigs to reduce the levels of ascorbic acid in the brain. Also, vitamin C supplementation reduces the weight loss associated with fenfluramine administration, indicating that ascorbic acid and serotonin may work together in appetite regulation (30).

In guinea pigs, control animals that were fed an amount equivalent to the food consumed by the vitamin C-deficient animals lost less weight than the vitamin C-deficient animals (36). These results point toward inefficient digestion or metabolism during vitamin C deficiency. The possibility of ascorbic acid aiding in digestion is supported by experiments with guinea pigs that have demonstrated reduced absorption of certain amino acids by the small intestine of vitamin C-deficient animals (10). Because ascorbic acid is involved in so many different pathways, it is possible that the weight loss could be the result of breakdowns in multiple physiological pathways. The *Gulo*−/− mouse should provide a valuable model for distinguishing between these various pathways.

**GRANTS**

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