의학석사 학위논문

The effect of ascorbic acid deficiency on bone formation in *Gulo* knock out mice

Gulo 유전자 적중 생쥐에서의 아스코르빈산 결핍이 골형성에 미치는 영향

2012년 8월

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의학과 해부학 전공

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A thesis of the Master's degree

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The effect of ascorbic acid deficiency on bone formation in *Gulo* knock out mice

by
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Professor _____ **Chairman**
Professor _____ **Vice chairman**
Professor _____

ABSTRACT

L-gulono-γ-lactone oxidase (Gulo) is an essential enzyme in the synthesis of ascorbic acid from glucose. It is known that gulo is mutated in humans and certain primates. Gulo (-/-) mice are a useful animal model of human scurvy when they are raised under an ascorbic acid-deficient condition, since Gulo (-/-) mice have a defect in the expression of the gulo gene. Based on previous findings on bone abnormalities, including multiple chondrocostal junction fractures in Gulo (-/-) mice upon ascorbic acid insufficiency, the specific factors at work are investigated on the present study. At 4 weeks after ascorbic acid withdrawal, a definite loss of weight was found in ascorbic acid-insufficient Gulo (-/-) mice. Interestingly, the plasma level of osteocalcin, which is secreted solely by osteoblasts and is thought to play a role in the metabolic regulation of bone, was dramatically decreased in ascorbic acid-insufficient Gulo (-/-) mice 3 weeks after ascorbic acid withdrawal. In addition, it was no longer detected at 4 weeks after ascorbic acid withdrawal. The tibia weight of the ascorbic acid-sufficient Gulo (-/-) mice was significantly higher than the other three groups. The trabecular bone volume was decreased near the growth plate. Moreover, we found a significant decrease in the trabecular bone attachment to the growth plate in ascorbic acid-insufficient Gulo (-/-) mice at 3 or 4 weeks after ascorbic acid withdrawal. Taken together, there are severe defects in normal bone formation as the result of ascorbic acid insufficiency, and this effect is closely related to a decrease in the osteocalcin level.

Keywords: ascorbic acid, vitamin C, Gulo, osteocalcin, bone formation

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LIST OF ABBREVIATIONS

3w *Gulo (-/-)* : 3 week ascorbic acid-insufficient *Gulo (-/-)* mice

4w *Gulo (-/-)* : 4 week ascorbic acid-insufficient *Gulo (-/-)* mice

ALP: alkaline phosphatase

AsA: ascorbic acid

BMD: bone mineral density

Gulo: L-gulono-γ-lactone oxidase

Gulo (-/-) + AsA: ascorbic acid-sufficient *Gulo (-/-)* mice

H&E: hematoxylin and eosin

HDL: high density lipoprotein

LDL: low density lipoprotein

mRNA: messenger ribonucleic acid

ODS rat: osteogenic disorder shionogi rat

rER: rough endoplasmic reticulum

SD: standard deviation

ND: not detected

WT: wild type

INTRODUCTION

Scurvy is associated with certain connective tissue symptoms, such as vascular purpura, bleeding and gum abnormalities (1). It is caused by severe ascorbic acid-deficiency, because ascorbic acid is essential for the normal synthesis of collagen (2). When it is considered that the organic bone matrix is mainly composed of Type I collagen (3), ascorbic acid-deficiency might have a role in bone abnormalities. An inhibition of new bone formation after bone grafting and a delay in bone regeneration after injury have been observed in scorbutic guinea pigs (4, 5). In human beings, ascorbic acid-deficiency also causes bone abnormalities such as microfracture, osteolysis and osteoporosis (6-8). However, not only deficiency, but also supplementation with mega-dose ascorbic acid might exert an effect on bone. Indeed, epidemiologic studies were recently reported that suggested a supplementation with ascorbic acid may enhance bone formation and overall bone health (9, 10).

Since ascorbic acid is synthesized from glucose in most animals, they do not have to take in ascorbic acid by food. However, human beings cannot synthesize ascorbic acid (11). This defect is due to the lack of L-gulono-γ-lactone oxidase that is an essential enzyme for the synthesis of ascorbic acid that converts L-gulono-γ-lactone to L-ascorbic acid (12, 13). Therefore, ascorbic acid must be obtained in food in humans. For this reason, it is believed that ascorbic acid is a clinically important nutrient. Most

experimental animals are able to synthesize large amounts of ascorbic acid by themselves. This is a major impediment to the study of the *in vivo* effect of ascorbic acid. Therefore, the *Gulo (-/-)* mice used in this experiment are considered a useful animal model for the investigation of the *in vivo* effect of ascorbic acid. Because these mice have a defect in the *gulo* gene, they are unable to synthesize ascorbic acid.

Gulo (-/-) mice were originally generated for a study of the effect of ascorbic acid on the prevention of atherosclerotic plaque formation upon ascorbic acid insufficiency (14). Therefore, *Gulo (-/-)* mice are a very useful model for studying the *in vivo* consequences of supplementation with ascorbic acid.

In the course of a previous investigation of the general characteristics of *Gulo (-/-)* mice (15), skeletal changes such as chondrocostal junction thickening and multiple fractures at 5 weeks after withdrawal of ascorbic acid were clearly evident. However, the specific mechanisms have remained largely undetermined. Therefore, we investigated the effect of ascorbic acid insufficiency on factors related to bone metabolism in *Gulo (-/-)* mice.

MATERIALS AND METHODS

Mice

Gulo (+/-) breeding pairs were obtained from the Mutant Mouse Regional Resource Center, MMRRC (University of California, Davis, CA, USA). We determined the genotypes of the offspring by PCR as recommended in the literature (14). *Gulo (-/-)* and C57BL/6 wild type (WT) mice were maintained in specific pathogen free conditions in the animal facility at the Seoul National University College of Medicine. Twelve week old mice were used in this experiment and the protocol was reviewed and approved by Ethics Committee of the Seoul National University.

Supplementation and withdrawal of ascorbic acid

Gulo (-/-) mice were supplemented with ascorbic acid (3.3g/L, Sigma, St. Louis, MO, USA) in water to maintain the general health of the mice for 12 weeks. The mice were divided into three groups. One received continuous ascorbic acid supplementation untill the end of the experiment, referred to as "ascorbic acid-sufficient *Gulo (-/-)* mice". Another underwent a stoppage of ascorbic acid supplementation for the last 3 weeks of the experiment, referred to as "3 week ascorbic acid-insufficient *Gulo (-/-)* mice". The last underwent a stoppage of ascorbic acid supplementation for the last 4 weeks of the experiment, referred to as "4 week ascorbic acid-insufficient

Gulo (-/-) mice". To examine the general physiological changes in *Gulo (-/-)* mice upon ascorbic acid insufficiency, the body weight of each of the mice was measured at the beginning and end of the experiment.

Analysis of the biochemical components

At the end of the experiment, blood was collected from the intra-orbital plexus with a heparinized capillary tube. Plasma was obtained from each collected blood sample by centrifugation at 14,000 rpm for 30 min. The plasma levels of calcium, phosphorus and alkaline phosphatase (ALP) were measured with a chemistry analyzer (Hitachi 7070, HITACHI Science Systems, Ltd., Hitachinaka-shi, Japan). The plasma level of osteocalcin was measured with a mouse specific enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (Biomedical Technologies, INC., Stoughton, MA, USA).

Determination of the tibia dry weight

Mice were sacrificed by cervical dislocation under ether anesthesia. The left was dissected and the surrounding soft tissues were removed. After de-fatting with a mixed solution of chloroform/ethanol (2:1) for 24 hours, the tibia was flushed out with saline to remove the remnant marrow element. The fat free tibia was then dried in an oven at 80℃ over 48 hours. The tibia dry weight was measured with a microbalance (AEX-200G, SHIMADZU Corporation, Kyoto, Japan)

Histologic examination

The dissected right tibia was fixed in 4% paraformaldehyde for 24 hours after removal of the surrounding soft tissues. Decalcification was performed with 10% EDTA solution for 5 days. The fixed and decalcified tibia was embedded in paraffin and sectioned at 4 ㎛. Paraffin bone sections were stained with hematoxylin and eosin (H&E).

Statistical analysis

Results are presented as the mean ± SD. Differences among the groups were tested using the Kruskal–Wallis test and the subsequent comparisons of each group were performed with the Mann-Whitney test. Data were analyzed using PASW for Windows (Version 18.0, SPSS Inc., Chicago, IL, USA). All the statistical outcomes were based on a two-sided test, and a P-value < 0.05 was regarded as statistically significant.

RESULTS

The weight of *Gulo(-/-)* mice was decreased 4 weeks after ascorbic acid withdrawal

To establish that all of the mice fit the same experimental conditions, we first determined the weight of each animal before starting the withdrawal of ascorbic acid. As shown in Figure 1A, there was no significant difference in body weight. The drinking water for the ascorbic acid-insufficient *Gulo (-/-)* mice was replaced with water that did not contain ascorbic acid. Interestingly, we did not find a significant change at 3 weeks after ascorbic acid withdrawal, but a definite change was observed at 4 weeks in the ascorbic acid-insufficient *Gulo (-/-)* mice. This suggests that ascorbic acid insufficiency does affect the loss of weight. However, it remains to be clarified whether it is accompanied by changes in bone formation and/or bone density.

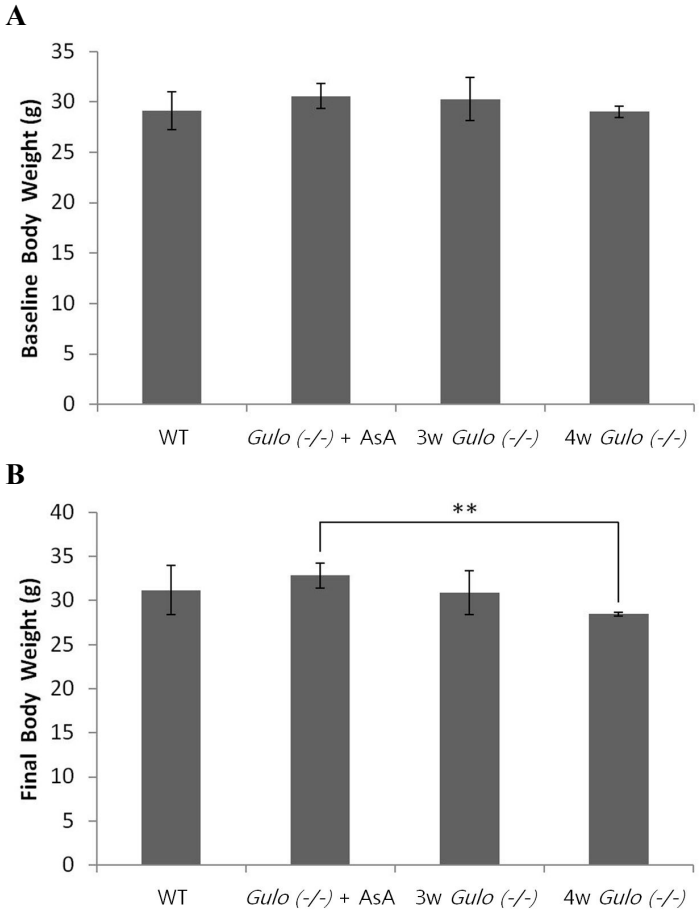

Figure 1. The weight of mice at baseline (A) and after ascorbic acid withdrawal (B)

The weight of the mice was measured at baseline and after ascorbic acid withdrawal. The weight did not exhibit any significant difference among the groups at baseline (A). After ascorbic acid withdrawal in the *Gulo (-/-)* mice, the weight was significantly decreased at 4 weeks compared with that of the ascorbic acid-sufficient *Gulo (-/-)* mice (B). Data are presented as the means ± SD, and each group included 5 animals. **$p < 0.01$.

The plasma levels of osteocalcin in *Gulo(-/-)* mice was decreased from 3 weeks after ascorbic acid withdrawal

To examine the effect of ascorbic acid insufficiency on bone metabolism, we measured the plasma levels of calcium, phosphorus, alkaline phosphatase and osteocalcin. At the end of the experiment, the plasma levels of calcium (Figure 2A) and phosphorus (Figure 2B) did not exhibit any significant difference among the groups. The plasma level of alkaline phosphatase, known as a non-specific marker of bone formation, was relatively higher in the ascorbic acid-sufficient *Gulo (-/-)* mice, but this was not statistically significant (Figure 3A). To examine the effect of ascorbic acid on bone formation more precisely, the plasma level of osteocalcin, which originates solely from bone, was measured. The plasma level of osteocalcin was significantly lower in the 3 week ascorbic acid-insufficient *Gulo (-/-)* mice than the WT and ascorbic acid-sufficient *Gulo (-/-)* mice (Figure 3B). In the 4-week ascorbic acid-insufficient *Gulo (-/-)* mice, osteocalcin was not detected in the plasma except in one mouse (7.8 ng/mL).

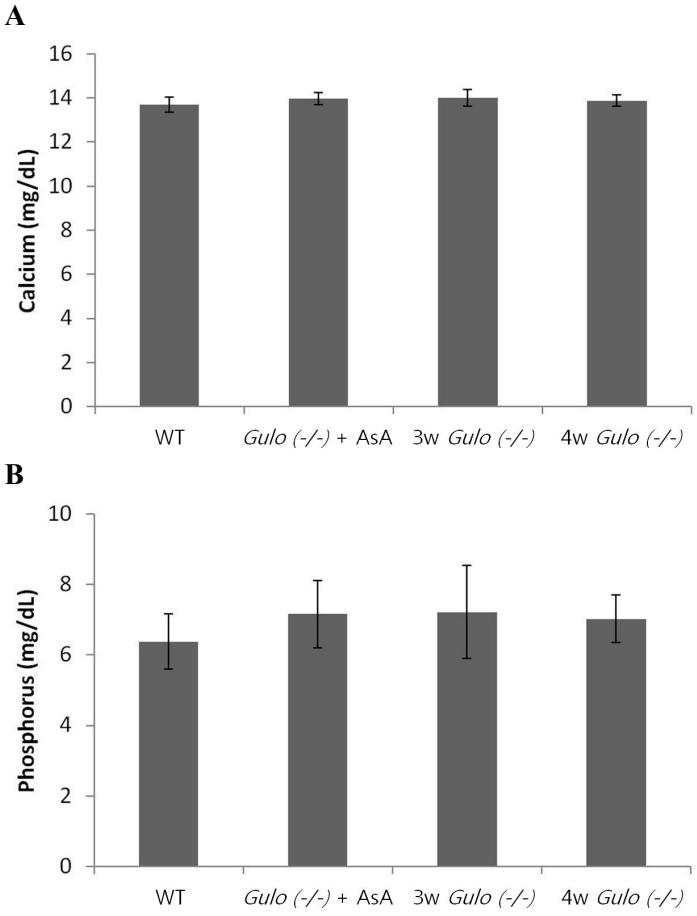

Figure 2. The plasma levels of calcium (A) and phosphorus (B) after ascorbic acid withdrawal

After ascorbic acid withdrawal in *Gulo (-/-)* mice for the last 3 or 4 weeks, the plasma levels of cacium (A) and phosphorus (B) were measured. The plasma levels of cacium and phosphorus did not exhibit any significant difference among the groups. The data are presented as the means ± SD, and each group included 5 animals.

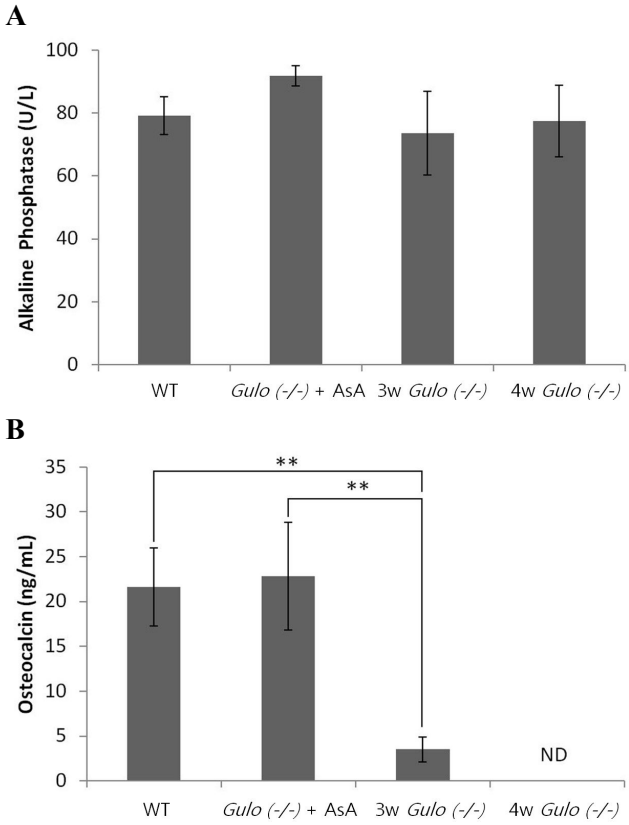

Figure 3. The plasma levels of alkaline phosphatase (A) and osteocalcin (B) after ascorbic acid withdrawal

After ascorbic acid withdrawal in *Gulo (-/-)* mice for 3 or 4 weeks, the plasma levels of alkaline phosphatase (A) and osteocalcin (B) were measured. The plasma level of alkaline phosphatase was not significantly different among the groups (A). The plasma level of osteocalcin was significantly lower in the 3-week ascorbic acid-insufficient *Gulo (-/-)* mice than those in the WT and ascorbic acid-sufficient *Gulo (-/-)* mice (B). In the 4-week ascorbic acid-insufficient *Gulo (-/-)* mice, osteocalcin was not detected in the plasma. Data are presented as the means ± SD, ND = not detected, Each group included 5 animals. **$p < 0.01$.

The tibia dry weight of the ascorbic acid-sufficient *Gulo (-/-)* mice was higher than in the other groups

To examine the total net effect of ascorbic acid-deficiency on bone metabolism during the experiment, tibia dry weight was measured after ascorbic acid withdrawal in the *Gulo (-/-)* mice for 3 or 4 weeks (Figure 4A). The tibia dry weight of the ascorbic acid-sufficient *Gulo (-/-)* mice was significantly higher than that in the other three groups. However, significant differences were not observed among the WT, 3-week and 4-week ascorbic acid-insufficient *Gulo (-/-)* mice. To adjust for the effect of general growth difference, the ratio of the tibia weight to final body weight was compared between the WT and ascorbic acid-sufficient *Gulo (-/-)* mice. The ratio was significantly higher in the ascorbic acid-sufficient *Gulo (-/-)* mice than the WT (Figure 4B).

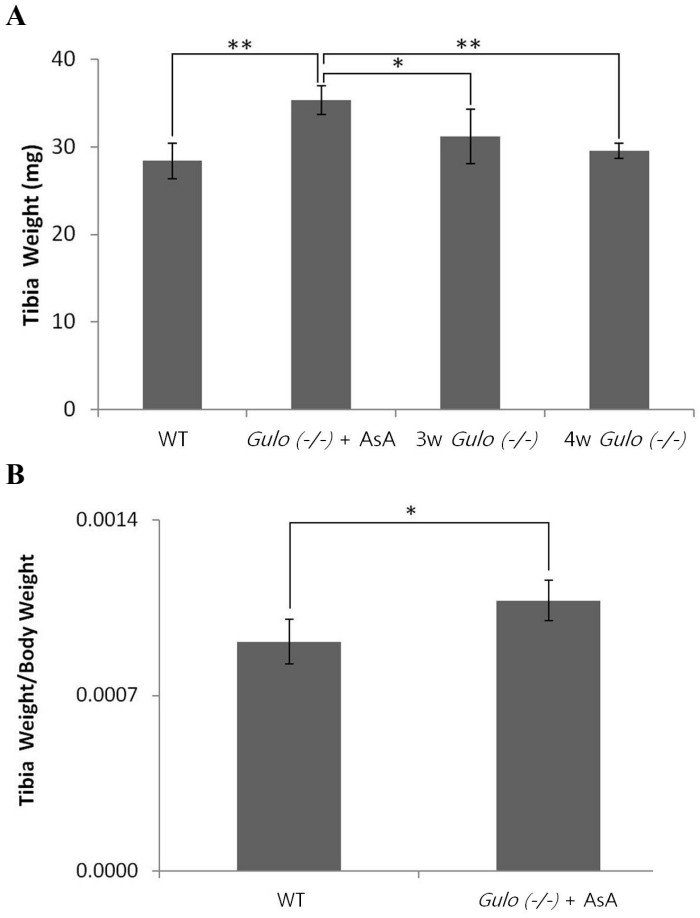

Figure 4. Tibia dry weight and the ratio of the tibia weight to the final body weight at the end of the experiment

After ascorbic acid withdrawal in the *Gulo (-/-)* mice for the last 3 or 4 weeks, the tibia dry weight was measured (A). The tibia dry weight in the ascorbic acid-sufficient *Gulo (-/-)* mice was significantly higher than the other three groups. The ratio of the tibia weight to the final body weight was significantly higher in the ascorbic acid-sufficient *Gulo (-/-)* mice than the WT (B). Data are shown as the means ± SD, Each group included 5 animals. *$p < 0.05$, **$p < 0.01$.

The trabecular bone volume in the *Gulo (-/-)* mice was decreased near the growth plate starting at 3 weeks after ascorbic acid withdrawal

To examine the alteration in the bone architecture after ascorbic acid withdrawal, histologic examination was performed. The growth plate of the tibia was observed in all four groups, which shows that bony growth was not completed at that age (Figure 5). In the metaphysis, the trabecular bone was well organized in both the WT and ascorbic acid-sufficient *Gulo (-/-)* mice (Figure 5 A, B). In these two groups, trabecular bone was well attached to the growth plate (Figure 5 E, F). In the 3 and 4 week ascorbic acid-insufficient *Gulo (-/-)* mice, the trabecular bone volume was decreased near the growth plate (Figure 5 E, F), and there was a significant decrease in the trabecular bone attachment to the growth plate (Figure 5 G, H). These findings show that new bone formation was disturbed by ascorbic acid insufficiency.

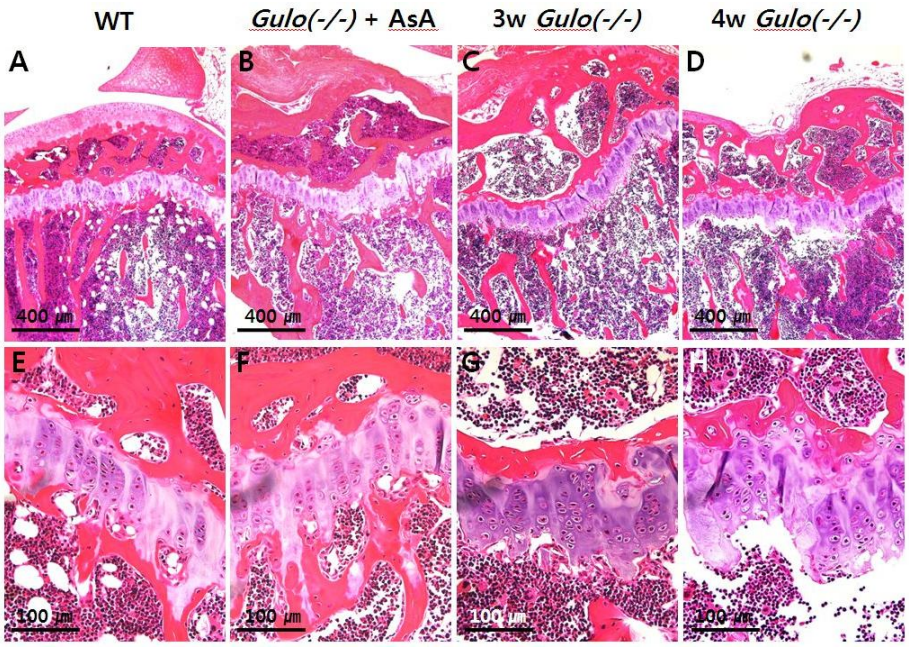

Figure 5. The proximal metaphysis and growth plate of the proximal tibia after ascorbic acid withdrawal

After ascorbic acid withdrawal in *Gulo (-/-)* mice for 3 or 4 weeks, the mouse tibia was harvested, fixed, decalcified, sectioned at 4 ㎛ and stained with H&E. Compared with the WT (A, E) and ascorbic acid-sufficient *Gulo (-/-)* mice (B, F), in the 3 week (C, G) and 4 week (D, H) ascorbic acid-insufficient *Gulo (-/-)* mice the trabecular bone volume was decreased near the growth plate, and there was a significant decrease in trabecular bone attachment to the growth plate.

DISCUSSION

It is well-known that ascorbic acid is a major co-factor for collagen synthesis. It is reported that various abnormalities in supporting tissues, such as scurvy and chondrocostal fracture, occur under a condition of ascorbic acid insufficiency (1, 16). The extensive physiological changes that occur in humans with ascorbic acid insufficiency are also reported in *Gulo (-/-)* mice. For example, decreased total cholesterol and high density lipoproteins (HDL), but increased low density lipoproteins (LDL) are found in both these mice and humans. A prominent decrease of catecholamine production is also reported. All of these developments are accompanied by a loss of weight (14). There are several reports on the role of ascorbic acid in the generation and maintenance of bone in animals under ascorbic acid-insufficient conditions. The osteogenic disorder shionogi (ODS) rat is unable to synthesize ascorbic acid due to the lack of L-gulono-γ-lactone oxidase, like humans and *Gulo (-/-)* mice. When ascorbic acid is insufficiently supplied to these rats, there is a detachment of osteoblasts, characterized by a round-shaped rough endoplasmic reticulum (rER), from bone surfaces due to the accumulation of malformed collagens inside the rER (17). In the bone of ascorbic acid insufficient guinea pigs, there was a remarkable reduction of hydroxyproline content and osteocalcin mRNA expression (18, 19). Moreover, decreases in the enzymatic activity of alkaline phosphatase in the bone and serum were also observed in ascorbic acid insufficient guinea pigs (19). It is generally known that spontaneous fracture occurs in sfx mice, because they cannot

synthesize ascorbic acid due to the deletion of the L-gulono-γ-lactone oxidase gene, the same as *Gulo (-/-)* mice. Interestingly, it is reported that there is a severe impairment of the differentiation of osteoblasts in sfx mice (20). Based on the several reports described above, ascorbic acid is evidently one of the important factors for maintaining the structure and function of bone, but the factors and mechanisms which are involved in this process are largely unknown. Therefore, we investigated the consequences of ascorbic acid insufficiency in *Gulo (-/-)* mice focusing on bone metabolism.

As shown in Figure 1, a decrease in body weight in ascorbic acid-insufficient *Gulo (-/-)* mice occurred at 4 weeks after ascorbic acid withdrawal. However, there was no evident weight change in ascorbic acid-insufficient *Gulo (-/-)* mice at 3 weeks after ascorbic acid withdrawal. This is in agreement with our previous report on the time- and organ-specific changes in the *in vivo* ascorbic acid concentration in *Gulo (-/-)* mice (15). Interestingly, there was a remarkable change in the trabecular bone volume near the growth plate in ascorbic acid-insufficient *Gulo (-/-)* mice at 3 weeks after ascorbic acid withdrawal, although there was no significant difference in weight. In accordance with our findings, similar results were found in ascorbic acid-deficient guinea pigs (19, 21). There was a definite change in bone volume without any evident physiological change observed in ascorbic acid-insufficient *Gulo (-/-)* mice at 3 weeks after ascorbic acid withdrawal, so it seems that ascorbic acid-deficiency exerts its initial effect on bone metabolism.

Alkaline phosphatase (ALP) and osteocalcin are representative markers of bone formation (22). Since both of these factors are increased by the activation of osteoblasts, the plasma levels of ALP and osteocalcin directly reflect the changes in bone formation *in vivo* (22, 23). As expected, we found a definite decrease of osteocalcin in the plasma of ascorbic acid-insufficient *Gulo (-/-)* mice upon ascorbic acid withdrawal for 3 and 4 weeks (Figure 3B), but the plasma concentration of alkaline phosphatase did not exhibit any significant changes in *Gulo (-/-)* mice at either of these timepoints after ascorbic acid withdrawal (Figure 3A). This may be due to the presence of a liver isoform of ALP in the circulation, since the liver is one of the major sources of blood ALP (24). Since ascorbic acid plays an important role in the production of various kinds of essential enzymes, especially in the liver, the increased ALP levels in the ascorbic acid-supplemented *Gulo (-/-)* mice appears to be the result of the continuous supplementation with ascorbic acid. Taken together, an abnormality of bone formation was developed in *Gulo (-/-)* mice upon ascorbic acid insufficiency. However, the underlying mechanisms related to the decrease in osteocalcin require further investigation.

Like the increase in the plasma ALP level that resulted from ascorbic acid supplementation, the tibia weight in ascorbic acid-sufficient *Gulo (-/-)* mice was higher than in the WT mice and ascorbic acid-insufficient *Gulo (-/-)* mice (Figure 4A). In particular, the relative ratio of the gross weight of the tibia against the final body weight was also significantly higher in ascorbic acid-sufficient *Gulo (-/-)* mice than in the WT (Figure 4B). Although this was not statistically significant, there was a trend to an in increase in the plasma

osteocalcin level in the *Gulo (-/-)* mice by the supplementation of ascorbic acid. The amount of ascorbic acid used in this experiment was 3.3 g/L. Even though this is relatively higher than that spontaneously produced by mice, it suggests that sufficient amount or mega-dose uptake of ascorbic acid could enhance bone formation and effectively maintain bone health.

There have been reports that mega-doses of ascorbic acid have an enhancing effect on bone health in epidemiologic studies. According to a report by Hall et al., a positive association was found in postmenopausal estrogen/progestin trials between ascorbic acid intake and the BMD (bone mineral density) of the spine and hip (9). Interestingly, it is also reported that higher total ascorbic acid intake was associated with a reduction of femoral neck and trochanter BMD loss in men who had low calcium or low vitamin E intake (10). In addition, the femoral neck BMD in male nonsmokers is reportedly positively correlated with total ascorbic acid intake (10). However, there are other reports that did not find any positive correlation between ascorbic acid and bone health (25, 26). Therefore, further studies are required. Moreover, the evaluation of ascorbic acid levels in bone after either a sufficient amount or mega-dose is also needed.

Well organized trabecular bone was observed in WT mice and ascorbic acid-sufficient *Gulo (-/-)* mice (Figure 5 A, B). In addition, there was intact attachment of trabecular bone to the growth plate (Figure 5 E, F). The most striking findings in the ascorbic acid-insufficient *Gulo (-/-)* mice were the reduction of trabecular bone in the proximal metaphysis near the growth plate as well as just a small amount of trabecular bone attachment to the

growth plate (Figure 5 C, D, G, H). Since the area just distal of the growth plate is the most active site for new bone formation, it is thought that this is the reason that the reduction in the trabecular bone in the proximal metaphysis near the growth plate was more definitely observed than at any other site. Based on the previous reports of the trabecular bone reduction in ascorbic acid insufficient animal models (16, 21, 27), it is expected that other bony abnormalities, such as cortical bone thinning, growth plate reduction and malalignment would occur after a longer period of ascorbic acid withdrawal than was employed in this experiment.

In the present study, ascorbic acid-insufficient *Gulo(-/-)* mice exhibited low plasma osteocalcin levels and a definite reduction of trabecular bone in the proximal metaphysis near the growth plate. Moreover, all of these changes in bone metabolism and structure were not observed upon supplementation with a sufficient amount of ascorbic acid. Therefore, these findings suggest that the bone formation defect which occurs in cases of ascorbic acid-deficiency can be prevented with ascorbic acid supplementation. In addition, an enhancement of bone formation as well as the maintenance of bone health may be feasible with ascorbic acid supplementation. Further studies are needed.

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국문 초록

L-gulono-γ-lactone 산화효소(Gulo)는 포도당으로부터 아스코르빈산을 합성하는데 있어서 필수적인 효소이다. 사람과 몇몇 유인원에서는 *gulo* 유전자의 변이가 있는데, 그로 인해 이들은 아스코르빈산을 체내에서 합성하지 못한다. *Gulo (-/-)* 생쥐는 사람과 같이 *gulo* 유전자의 발현에 결핍이 있기 때문에, 아스코르빈산을 결핍시킨 상태에서 키울 경우에 사람 괴혈병에 대해 연구하는데 유용한 동물 모델로 이용된다. 본 연구팀은 이전 연구에서 다발성 늑골연골 접합부 골절과 같은 뼈의 이상이 아스코르빈산을 결핍시킨 *Gulo (-/-)* 생쥐에서 발생하는 것을 관찰하였다. 이를 바탕으로 본 연구에서는 아스코르빈산의 결핍이 *Gulo (-/-)* 생쥐의 뼈 대사에 미치는 영향 및 관련 인자에 대해 분석하였다. 아스코르빈산의 공급을 중단하고 4 주 째가 되자 아스코르빈산을 결핍시킨 *Gulo (-/-)* 생쥐에서 분명한 체중의 감소가 관찰되었다. 흥미롭게도 뼈의 대사를 조절하고 골모세포에 의해서만 분비되는 오스테오칼신의 혈장내 농도가 아스코르빈산의 공급을 중단한지 3 주 째에 *Gulo (-/-)* 생쥐에서 급격히 감소하였고, 4 주 째에는 오스테오칼신이 검출되지 않았다. 경골의 무게는 아스코르빈산을

충분히 공급하여준 *Gulo (-/-)* 생쥐에서 다른 3 군보다 더 나갔다. 아스코르빈산을 결핍시킨 후 3 주와 4 주 차의 *Gulo (-/-)* 생쥐에서는 성장판 근처에서 해면골의 양이 감소되어 있었고 해면골과 성장판과의 부착이 감소되어 있었다. 이상의 소견은 아스코르빈산의 결핍은 정상적인 뼈의 생성에 심각한 장애를 일으키며, 이는 오스테오칼신 농도의 감소와 밀접하게 연관되어있음을 시사한다.

주요단어: 아스코르빈산, 비타민 C, Gulo, 오스테오칼신, 뼈 생성
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