Alteration of Bone Status with Ascorbic Acid Deficiency in ODS (Osteogenic Disorder Shionogi) Rats

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ABSTRACT—Rats with hereditary defects in ascorbic acid (AsA) synthesis (ODS rats) subjected to AsA-deficiency for 3 weeks showed reductions of plasma alkaline phosphatase and dry and ash weights of the tibia, but no body weight alteration. In accordance with the chemical changes, bone loss and decrease of bone formation by AsA deficiency but not by malnutrition were observed in contact microradiographs of the tibia and by a tetracycline double labeling technique, respectively. The mechanical properties of femora measured by a three point-bending procedure were also altered by AsA deficiency for 3 weeks and showed decreases of 59% in toughness, 32% in strength, 32% in ductility and 22% in stiffness. The biomechanical changes by AsA deficiency were greater than the chemical changes in bone, indicating the usefulness of measuring mechanical properties as a sensitive method for the evaluation of the bone status. The second moment of the area of the femur was not changed by AsA deficiency. These results suggest that AsA deficiency in ODS rats causes marked bone loss and reduction in bone formation, which is accompanied by a greater reduction in biomechanics of the femur without causing macroarchitectural changes.

Keywords: ODS (osteogenic disorder Shionogi) rat, Ascorbic acid, Bone, Mechanical property

It is well known that ascorbic acid (AsA) is required for the normal synthesis and secretion of collagen (1), which is a main constituent of the bone organic matrix. Thus, the physiological importance of AsA in bone formation has been thought to be due mostly to its effects on collagen production and, consequently, matrix formation.

In an earlier study, a colony of Wistar rats with a defect in L-gulonolactone oxidase, named the osteogenic disorder Shionogi (ODS), was established (2). Recently, the cause of hereditary osteopenia found in ODS rats has been demonstrated to be an imbalance between total bone resorption and formation due to AsA deficiency (3, 4). This finding indicates that ODS rats are a valuable and convenient model system for investigating the physiological significance of AsA in vivo.

Fractures are by far the most frequent abnormality of the skeletal system (5), and they occur with decreases in bone strength. This weakness is due largely to a reduction in bone mass caused by an uncoupling of bone formation and resorption. In pharmacological and pathophysiological studies of bone disorders using experimental animals, determination of bone status is usually confined to histological evaluation and/or measurement of bone density and its composition, dry weight, bone ash or mineral content. Recently, to gain a better understanding of the structural stability and resistance to bone fracture, the quality of bone has been assessed by measurements of stiffness, strength, toughness and ductility in rat femora.

In the present study, bone status with AsA deficiency was examined by chemical, morphological and biomechanical analyses to determine the significance of AsA in bone quality and quantity, using ODS rats placed on an AsA deficient or sufficient diet.

MATERIALS AND METHODS

Fifty male ODS/Shi Jcl strain rats (homozygotes, od/od), aged 7 weeks, were purchased from Nihon Clea Co., Tokyo; they were housed in individual cages at constant room temperature (23±1°C) and humidity (50±10%) with a 12-hr light-dark cycle. All rats were given a standard laboratory rat food containing no AsA (CL-2, Nihon Clea Co.) and 0.2% AsA solution prepared every morning as drinking water ad libitum until they were 13-weeks-old. To investigate the physiological role of AsA in bone, 13-week-old ODS rats were treated with AsA (0.2%) (control group, n=24) or without AsA (−AsA group, n=26) in drinking water for 2, 3, 4 or 5
weeks. In some experiments, animals received intra-peritoneal injections of tetracycline (10 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) twice at 10-day intervals to measure the appositional rate of bone minerals. After the treatment periods of 2, 3, 4 or 5 weeks, control (n=6) and -AsA (n=6-7) rats were sacrificed by drawing whole blood from the abdominal aorta by a heparinized syringe under ether anesthesia. The collected blood was centrifuged and the supernatant was used as plasma. The freshly prepared plasma was used for the determination of biochemical parameters in plasma. The body was hermetically stored at −40°C until use.

The AsA level in plasma was determined by reversed phase high performance liquid chromatography with electrochemical detection (6). Calcium and phosphorus concentrations in plasma were also determined using an atomic absorption spectrophotometer (Type 180-60; Hitachi Co., Tokyo) and the method of Chen et al. (7), respectively. Plasma alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were determined with p-nitrophenyl phosphate (10 mM) as the substrate in 50 mM glycine buffer at pH 10.5 and in 50 mM acetate buffer at pH 4.8, respectively (8, 9). Tartarate-resistant acid phosphatase (TRACP) activity was determined in the presence of 10 mM sodium tartrate (9). Reaction mixtures were incubated at 37°C for 30 min. Enzyme activity was calculated as nanomoles of p-nitrophenol liberated per min per ml of plasma.

The femora and tibiae were removed from rats thawed at 4°C. Right femora were immersed in 0.9% NaCl solution and used immediately for the breaking test. Other long bones were stored in 10% formalin saturated with magnesium carbonate for one week and used for chemical and histomorphological analyses. The mechanical properties of the femur were measured using an Instron Universal Testing Machine (Model 1122; Instron Co., Canton, MA, USA). After the length of the femur from the greater trochanter to the medial condyle was measured with a caliper, the bone was positioned on the two metal supports, 3 mm in diameter, with extensor (anterior) surface side up. The distance between the supports (fulcra points) was 15 mm. The breaking force was applied perpendicularly to the long axis of the femur at the mid-shaft with a crosshead speed of 10 mm/min, and the femur was broken from the anterior to the posterior plane. The response to loading was obtained in the form of force deflection traces.

Four parameters for the mechanical properties of bone, strength, stiffness, toughness and ductility, were assessed from the force-deflection traces, according to the previously established procedure (10). Strength was indicated by the maximum force on the force-deflection trace; stiffness was monitored by the initial slope of the force-deflection diagram; toughness was determined from the area under the maximum force-maximum deflection at maximum load.

The second moment of the area, reflecting the cross-sectional bone area, and the stress, which describes tissue strength, was also calculated by the procedure reported by Kiebzak et al. (11). Thus, cross-sectional measurements were made of the transverse surface of the bone at the breakline. The inside and outside diameters of the bone, both parallel and perpendicular to the line of force, were measured with calipers. The second moment of the area (cm⁴) was calculated according to the formula: \[ \pi[(BD^3)−(bd^3)]/64, \] where B=outside diameter perpendicular to the point of loading, D=outside diameter parallel to the point of loading, b=inside diameter perpendicular to the point of loading and d=inside diameter parallel to the point of loading. The stress (MPa) was calculated according to the formula: \[ FLC/(4 \times \text{second moment of the area})/10^2, \] where F=ultimate force (N), L=the distance between the two fulcra supporting the bone (cm), C=one-half of the bone diameter parallel to the direction of applied force (cm). The cortical thickness index of the femora was also calculated according to the formula: \[ (D−d)/D, \] as described previously (12).

Right tibiae were defatted, dried and reduced to ashes according to the procedure described in our previous study (13). The ash was dissolved in 6 N HCl, and calcium and phosphorus concentrations were determined in the same way as those in the plasma. From the left tibiae and femora, undecalcified frontal sections of proximal tibiae and transverse sections of the femora at the mid-point, all 50-μm-thick, were prepared (13). Fluorescent micrographs and contact microradiographs (CMR) of the ground sections were taken with a fluorescence microscope and a soft X-ray apparatus (Sofron, Soken Co., Tokyo), respectively. The appositional rate at the periosteal surface of the femora was calculated by dividing the average distance between tetracycline markers by the time interval between administration of the markers, as described previously (13).

Student's t-test or the Cochrane-Cox test was used to calculate the significance of differences between the control and −AsA groups.

RESULTS

Changes in body weight and plasma biochemical parameters with AsA deficiency are shown in Table 1. On switching to drinking water without AsA, a marked decrease in plasma AsA was observed, and the AsA concentration was below 1 nmol/ml in the −AsA group. Although there was no significant difference in plasma ALP activity between the control and the −AsA groups.
at 2 weeks, a decrease in ALP activity was observed in the 
-AsA group at 3 weeks. The mean body weight was not 
altered by AsA deficiency for 2 or 3 weeks, whereas the 
body weight in the -AsA group at 4 or 5 weeks was sig-
ificantly less than that in the control group. There were 
no significant differences in the other biochemical 
parameters of the plasma between the control and -AsA 
groups.

The chemical characteristics of bone are shown in 
Table 2. In the -AsA group at 3 weeks, the dry weight,
Fig. 1. Contact microradiographs of the proximal tibia. ODS rats were placed on drinking water with (control) or without AsA (−AsA) for 2 (A) or 3 weeks (B). Bar = 1 mm (×8).

Fig. 2. Contact microradiographs of the mid-shaft of the femur. ODS rats were placed on drinking water with (control) or without AsA (−AsA) for 2 (A) or 3 weeks (B). Bar = 1 mm (×8).
ash weight, calcium and phosphorus contents of the tibiae were significantly reduced relative to those of the controls. These changes in bone were not observed in the \(-\text{AsA}\) group at 2 weeks. Ash contents per dry weight and the Ca/P ratio of the tibiae showed no significant differences between the control and \(-\text{AsA}\) groups at 2 or 3 weeks. Thus, AsA deficiency for 3 weeks caused decreases in the bone mass without changing the ash content per dry weight or Ca/P ratio of the bone hydroxyapatite in ODS rats.

CMR of the proximal tibiae and the mid-shaft of the femora in the control and \(-\text{AsA}\) groups are shown in Figs. 1 and 2, respectively. The radiographs show that trabecular bones in the proximal tibia from the \(-\text{AsA}\) group at 3 weeks were much less than those in the control group (Fig. 1B). Also, the cortical thickness of the femoral cross-sections in the \(-\text{AsA}\) group at 3 weeks was obviously thinner than that in the control group (Fig. 2B). According to morphometrical measurement, the cortical thickness index (mean±S.E.M.) of the femora in the control and \(-\text{AsA}\) groups at 2 weeks were 0.459±0.013 (n=6) and 0.431±0.039 (n=6), respectively, and 0.437±0.021 (n=6) and 0.343±0.033 (n=7), respectively, at 3 weeks. The data showed a significant difference between the control and \(-\text{AsA}\) groups at 3 weeks (P<0.05). These changes in bone were not observed in the \(-\text{AsA}\) group at 2 weeks (Figs. 1A and 2A).

Representative fluorescent micrographs in the periosteal surface of the mid-shaft of the femora are shown in Fig. 3. In the control group, two fluorescent lines of tetracycline with high intensity were clearly observed at a distance of about 66 \(\mu\)m. Although two fluorescent lines of tetracycline were observed in the \(-\text{AsA}\) group at 2 weeks, the distance between them was significantly less than that in the control group. In the \(-\text{AsA}\) group at 3 weeks, only a faint single line of tetracycline was observed. Appositional rates of bone minerals assessed by tetracycline labeling (mean±S.E.M., \(\mu\)m/day) were 6.5±0.9 (n=6) in the control and 4.2±0.8 (n=6) in the \(-\text{AsA}\) groups at 2 weeks. At 3 weeks, the rate was 6.7±0.8 (n=6) in the control group, but was undetectable in the \(-\text{AsA}\) group. The data showed a significant difference between the control and \(-\text{AsA}\) groups at 2 weeks (P<0.05). In the endosteal surface of

![Fig. 3. Fluorescent micrographs in the periosteal surface of the mid-shaft of the femur. ODS rats were placed on drinking water with (control) or without AsA (\(-\text{AsA}\)) for 2 (A) or 3 weeks (B). These rats received two intraperitoneal injections of tetracycline (10 mg/kg) 13 and 3 days before death. Numbers on the fluorescent lines with high intensity indicated the order of injections of tetracycline. Bar=0.1 mm (×80).]
Parameters involved in the mechanical properties of the femora are shown in Table 3. The length (mean±S.E.M., mm) of the femora in the control and −AsA groups at 2 weeks were 32.3±0.72 (n=6) and 32.8±0.65 (n=6), respectively, and 32.8±0.60 (n=6) and 33.1±0.44 (n=7), respectively, for 3 weeks. The strength, stiffness, toughness, ductility and stress of the femora from the −AsA group at 3 weeks were significantly less than those from the control group. The percentages of these parameters in the −AsA group relative to the control group were 41% in toughness, 68% in strength, 68% in ductility and 78% in stiffness. No significant differences were observed in the second moment of the area between the control and −AsA groups. The ultimate stress in the −AsA group was reduced to 66% of that in the control group. On the other hand, no significant differences were observed in the parameters of bone mechanical properties between the control and −AsA groups for 2 weeks.

DISCUSSION

In the present study, we examined the effects of AsA deficiency on bone metabolism and mechanical properties in ODS rats, which provided a useful experimental model of osteopenia caused by AsA deficiency (3, 4). ODS rats placed on drinking water without AsA for 4 or 5 weeks showed a decrease in body weight, which is one of the prominent features of AsA deficiency and has been postulated to be due to malnutrition. It has been demonstrated that the physiological changes during the stage at which animals begin to lose body weight by AsA deficiency are almost identical to those during starvation (1). To minimize the involvement of the starvation factor in the action of AsA deficiency on bone, the effects on bone metabolism and mechanical strength were investigated in ODS rats placed on drinking water without AsA for 2 or 3 weeks, by which the body weight was not significantly altered.

Biochemical parameters in the plasma related to bone metabolism such as calcium and phosphorus concentrations, ALP activity as a marker of osteoblastic activity and TRACP activity as a marker of osteoclastic activity (9) were not changed by AsA deficiency for 2 weeks. However, plasma ALP activity in the −AsA group for 3 weeks was significantly less than that in the control group, indicating the reduction of bone formation by AsA deficiency. In fact, marked bone loss and a reduction in bone formation were observed by CMR of the proximal tibiae and by the measurement of the appositional rate of bone mineral in the femora, respectively. As shown in Fig. 3, in the −AsA group at 2 weeks, two labeled lines were observed on the fluorescent micrographs in the mid-shaft of the femora, although the distance between these lines was slightly smaller than that in the control group. The micrographical observation was confirmed by the morphometrical analysis, showing that the appositional rate of bone minerals was significantly less in the −AsA group than in the control group. However, in the −AsA group at 3 weeks, only a faint single line was observed around the periosteal surface. So, the appositional rate was not detected in the −AsA group at 3 weeks. These results indicate that the inhibition of bone formation became severe with AsA deficiency for 2 weeks.

In accordance with histological findings of bone loss in the −AsA group, dry weight and ash weight of the tibiae decreased significantly, whereas ash content per dry weight and Ca/P ratio of the tibiae were not different from those in the control group. These results suggest that the mineral composition of the bone was not affected by AsA deficiency.

In the present study, parameters of femoral mechanical properties were significantly less in the −AsA group at 3 weeks than those in the control group. These changes in mechanical properties were greater than those in dry
weight or mineral content. The magnitude of the change in the total bone mineral content induced by AsA deficiency for 3 weeks was only 12%, whereas the strength, stiffness, toughness and ductility were reduced by 32, 22, 59 and 32%, respectively. Our results are consistent with those of Currey (14) who demonstrated that a slight difference of ash content could explain a great difference in the elastic modulus used as an indicator of stiffness. Thus, the biomechanical changes induced by AsA deficiency were greater than the chemical changes in bone, indicating the usefulness of measuring mechanical properties as a sensitive method for the evaluation of the bone status.

Not only bone mineral content but also bone macro- and microarchitecture are involved in the mechanical strength (15, 16). Macroarchitectural changes are reflected by morphometric measurements of length, cortical and medullary area, and second moment of the area. Kiebzak et al. (11) proposed that the development of cortical porosities with age in rats may be an adjustment of its architecture in order to maintain bone strength. However, AsA deficiency-induced osteopenia did not change the second moment of the area at the mid-shaft of the femora.

In conclusion, AsA deficiency but not malnutrition caused marked bone loss and reduction of bone formation in ODS rats, accompanied by large reductions in toughness, stiffness, strength and ductility of the femur without macroarchitectural changes.

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