Laboratory Investigations

Bone Histomorphometric Analysis for the Cause of Osteopenia in Vitamin C-Deficient Rat (ODS Rat)

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Summary. A particular strain of rat, the osteogenic disorder rat (ODS rat), was established in 1973. Phenotypic expression of od/od in ODS rat develops signs characteristic of a vitamin C-deficient animal, with bleeding tendencies and limb fractures. We investigated the bone histomorphometry to clarify the pathogenesis of osteopathy found in ODS rat. Bone histomorphometry revealed that static parameters reflecting bone formation were found to be remarkably decreased in od/od rats. These observations were more prominent in the metaphysis of distal femurs of od/od rats than those in the tail vertebrae. Parameters reflecting bone resorption in od/od rats were reduced in the distal femoral metaphysis, but were similar to those of controls in the tail vertebrae. These parameters were restored to control levels after ascorbic acid supplementation to pair-fed od/od rats. The mineral appositional rate in od/od rats was not significantly different from that in controls. Although body weight gain in pair-fed controls was significantly reduced compared to those fed ad libitum, histomorphometric parameters, on the contrary, were unaltered between these groups. Our present study provides evidence that the cause of osteopenia found in od/od rat is attributable to an imbalance between the total amounts of resorption and formation, and the pathogenesis of osteopathy could be due to ascorbic acid deficiency itself rather than malnutrition.

Key words: osteogenic disorder rat, ascorbic acid deficiency, bone histomorphometry, decreased bone formation

In 1973, a rat with a gait disturbance was found in a subline of Wistar rats maintained in Shionogi Aburahi Laboratories (Shiga, Japan). A rat strain expressing such characteristics spontaneously was established by backcross mating and full sib-mating between phenotypically normal rats [1]. Approximately 5 weeks after birth, some animals in this strain, named the osteogenic disorder rat (ODS rat), developed bleeding tendencies and fractures of the limbs and ribs, signs characteristic of vitamin C deficiency. This phenotypic expression was discovered to be inherited as a single autosomal recessive gene (gene symbol od) [1]. Although rats can fully synthesize ascorbic acid from D-glucose unlike primates, guinea pig, and some birds, ODS rats lack L-gulonolactone oxidase in the pathway of L-ascorbic acid synthesis [1]. Indeed, it has been demonstrated that in ODS rats, the ascorbic acid contents in liver, kidney, and adrenal glands are extremely reduced [1, 2]. Ascorbic acid is known to be required for the synthesis of collagen which is the main component of bone matrix. Furthermore, as the weight of ODS rats has been reported to be lower than that of controls [1], it is possible that malnutrition may also affect the bone metabolism in these animals because malnutrition is considered to be a factor causing osteopenia [6]. However, there have been no precise reports in terms of bone lesions in neither vitamin C-deficient guinea pig nor in ODS rat. Therefore, in the present study, we investigated the bone histomorphometry to clarify the pathogenesis of osteopathy in ODS rats compared to controls which had been pair-fed or fed ad libitum.
Materials and Methods

Ten male ODS rats (homozygotes, od/od rats), five male +/+ rats which were the phenotypically normal littermates of ODS rats, and five male Wistar Shionogi (Wistar/Shi) rats were kindly provided by Shionogi Aburahi Laboratories (Shiga, Japan). All rats were 3 weeks old. These animals were pair-fed on a normal diet for 20 days from 3 to 6 weeks of age. Ten od/od rats were separated into two groups. One group (five od/od rats) received L-ascorbic acid (100 mg/100 ml) in drinking water. The other group received water with no ascorbic acid. Daily water intake was 15-25 ml/rat. Another group of five Wistar/Shi rats, five +/+ rats, five od/od rats, and five ascorbic acid-supplemented od/od rats (all 3-week-old males) were fed ad libitum for 20 days.

Double fluorescent labeling of bone was performed by the intraperitoneal injection of oxytetracycline (20 mg/kg body wt) and calcein (30 mg/kg body wt) at 6 and 2 days before sacrifice, respectively. One day before sacrifice, rats were housed in separate metabolic cages, and urine was collected for 24 hours. Then each of rats was killed under ether anesthesia at 6 weeks of age after blood was drawn for biochemical analysis. Serum calcium (Ca), phosphorus (P), and alkaline phosphatase (A1-P) levels were measured using an o-cresolphthalein complexone (OCPC) method [3], modified method of Lowry and Lopez [4], and the method of Bessy et al. [5], respectively. Hydroxyproline in the urine was separated by HPLC and analyzed colorimetrically [7].

Specimens for bone histomorphometry were prepared as follows. The fourth tail bone and right femur and tibia were dissected and fixed in 10% buffered formalin for 24 hours, then dehydrated through graded ethanol (70%, 95%, and absolute alcohol). The specimens were embedded in methymethacrylate and cut longitudinally using a Jung Model K microtome. Five-micrometer sections were stained with modified Masson method or toluidine blue O for histomorphometry. Unstained 10 μm thick sections were prepared for fluorescent microscopy.

All quantitative histologic measurements of tail bone were performed on the trabecular bone approximately 1 mm apart from the epiphysial growth plates. The measurements in the femur were made in the secondary spongiosa of the distal metaphysis.

The following histomorphometric parameters of bone were measured at ×290 magnification using a semiautomatic image analyzer (Model G-2, Mutoh Kogyo, Japan). Fifteen to 20 fields were counted for each tail bone or the metaphysis of the femur: trabecular bone volume (TBV), indicating the percentage of bone marrow space occupied by mineralized and unmineralized bone tissue; mean trabecular thickness (MTT), a derived value obtained by dividing the trabecular bone volume by the total trabecular surface; fractional formation surface (FrFS), the percentage of the trabecular bone surface covered by osteoid; relative osteoid volume (ROV), the percentage of trabecular bone tissue that was unmineralized, i.e., osteoid; active formation surface (AFS), the percentage of the osteoid surface lined by cuboidal osteoblasts; mean osteoid seam thickness (MMT), a derived value obtained by dividing the total osteoid volume by its absolute linear extent of osteoid; fractional resorption surface (FrRS), the percentage of trabecular bone surface occupied by Howship’s lacunae with or without osteoclasts; active resorption surface (ARS), the percentage of trabecular bone surface with Howship’s lacunae containing osteoclasts, and mean osteoclast number (MCN, N/mm), the number of osteoclasts per millimeter of trabecular bone surface.

Tetracycline labeling ratio (%TC), or the percentage of osteoid surface double and half single-labeled with tetracycline is calculated as follows: \( \%TC = \frac{\text{DLS} + \frac{1}{2} \text{SLS}}{\text{TTS}} \times 100 \), where DLS indicates double-labeled surface, SLS, single-labeled surface, and TOS, total osteoid surface.

The mineral appositional rate (Mo) was measured at ×550 magnification and calculated by dividing the mean distance between double labels by the interval time between tetracycline and calcein injection. Mineralization lag time (MLT) indicates a derived value obtained by dividing the mean osteoid seam thickness by the mineral appositional rate. Bone formation rate (sVf, mm²/m²/day) at tissue level, surface referent, is calculated as follows: \( sVf = \frac{\text{DLS} + \frac{1}{2} \text{SLS}}{\text{TTS}} \). Therefore, bone formation rate shows the amount of new bone mineralized per micrometer of trabecular bone surface area per day.

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Table 1. The rate of body weight gain in animals during the experimental period

<table>
<thead>
<tr>
<th>Experimental Period</th>
<th>n</th>
<th>Pair-fed</th>
<th>Fed ad libitum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g/day</td>
<td>g/day</td>
</tr>
<tr>
<td>Wistar/Shi</td>
<td>5</td>
<td>3.8 ± 0.1</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>+/+</td>
<td>5</td>
<td>3.8 ± 0.2</td>
<td>6.8 ± 0.6</td>
</tr>
<tr>
<td>od/od</td>
<td>5</td>
<td>3.7 ± 0.2</td>
<td>2.9 ± 0.5*</td>
</tr>
<tr>
<td>od/od + AsA</td>
<td>5</td>
<td>4.0 ± 0.1</td>
<td>6.8 ± 0.5</td>
</tr>
</tbody>
</table>

* Significantly different from other groups fed ad libitum; \( p < 0.01 \)
Values are means ± SEM. AsA = ascorbic acid