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Accumulated uremic toxins attenuate bone mechanical properties in rats with chronic kidney disease

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ABSTRACT

The prevalence of hip fracture is very high among patients with chronic kidney disease (CKD); however, the reason for this is unclear. We examined the effects of accumulated uremic toxins on bone chemical composition and elastic mechanical properties. Rats underwent thyroparathyroidectomy and progressive partial nephrectomy (TPTx-Nx), and were administered with vehicle or AST-120 to reduce serum indoxyl sulfate (IS) levels. Bone mechanical properties, bone mineral density (BMD), cortical bone chemical composition, and histomorphometry were determined. Storage modulus was reduced in TPTx-Nx rats compared with rats that underwent TPTx alone. BMD and histomorphometric parameters did not differ between the groups. In terms of cortical bone chemical composition, the mineral/matrix ratio and carbonate substitution was increased, whereas crystallinity was decreased in TPTx-Nx rats. The enzymatic crosslink ratio and pentosidine:matrix ratio were increased in TPTx-Nx rats. AST-120 abolished the effects of TPTx-Nx and decreased the serum IS concentration. Stepwise multiple regression analysis revealed that the pentosidine:matrix and mineral:matrix ratios were independent contributors to the storage modulus. In conclusion, the accumulated uremic toxins, including IS, seem to play an important role in deteriorating bone mechanical properties by altering the chemical composition of bone. This mechanism may account for the increased prevalence of hip fracture among patients with CKD.

Introduction

Hip fracture critically limits activities of daily living and is associated with shorter life expectancy [1–3]. The risk of hip fracture is particularly high among patients on hemodialysis [4–7]. The prevalence of hip fracture is also increased among patients with pre-dialysis chronic kidney disease (CKD), and the risk seems to increase with progression of renal dysfunction [8]. On the other hand, it remains controversial whether the risk of bone fracture other than hip fracture also elevated in CKD patients.

Generally, the most effective predictor of the risk of fracture is bone mineral density (BMD). However, previous reports indicated that BMD was less able to predict the risk of future fracture among hemodialysis patients than in the general population [9,10]. Of course, bone mass provides the strongest assessment of bone strength, and it seems implausible that BMD is not associated with fracture risk in CKD patients [11]. Nevertheless, the earlier results suggest that factors other than bone mass play important roles in the occurrence of hip fracture among hemodialysis patients.

A high risk of falling because of muscle weakness is likely to be a major cause of hip fracture among dialysis patients [12]. However, despite the high prevalence of hip fracture, clinical studies have failed to find an elevated risk of vertebral bone fracture among dialysis patients [6]. It is also unclear why falling affects the risk of hip fracture but not of vertebral fracture. Consequently, several hitherto unknown factors may account for the specific fragility of long bones.

Severely hyperactive parathyroid function is thought to increase bone fragility among dialysis patients, but this is supported by limited clinical evidence. Nowadays, we rarely encounter hemodialysis patients associated with hyperparathyroidism-related skeletal complications. Furthermore, supporting this concept, recent clinical studies have failed to find a close relationship between the risk of hip fracture and parathyroid function among dialysis patients [6,13,14]. Now, severe hyperparathyroidism is rarely left untreated because of recent developments in medical and surgical strategies for this disease. Therefore, mild hyperparathyroidism observed at the bedside is unlikely to be a major risk factor for skeletal damage.

Despite these advances, the prevalence of hip fracture remains very high among dialysis patients. Therefore, if the mechanical properties of long bones are specifically deteriorated in CKD

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patients, it is essential to identify the possible cause, independent of hyperparathyroidism.

We previously reported that the bone storage modulus was decreased in rats with CKD without excess parathyroid function [15]. If the phenomenon is reproducible in human, it could become one of the explanations why the risk of hip fracture is elevated in CKD patients. From those results, we generated a hypothesis that uremia can influence bone mechanical properties independently of parathyroid and/or mineral metabolic abnormalities.

However, it remained unclear how decreased kidney function may deteriorate bone mechanical properties. In our previous study, we hypothesized that advanced glycation end-products (AGE) may modify non-collagenous bone matrix proteins [15]. Even if this hypothesis is correct, it may not be the only cause. In addition, if uremia affects bone metabolism through pathways independent of mineral metabolism, the accumulation of uremic toxins is likely to play an important role in this process. However, no studies have been conducted to support this hypothesis.

Therefore, we used spectrochemical approaches to assess bone composition/structure, and validated the effects of an oral uremic toxin adsorbent in a rat model of CKD that was used in our previous studies.

Materials and methods

Animal experiments

A rat model of CKD without hyperparathyroidism was established as previously described [16]. Briefly, 13-week-old male Sprague–Dawley rats weighing approximately 350 g underwent TPTx and two-stage subtotal Nx. Normal rats (n = 6) were sacrificed at the time of first Nx (week 0) to collect baseline data. Six weeks after the second Nx (i.e., week 7), TPTx rats (n = 6) and TPTx-Nx rats (n = 6) were sacrificed. The remaining TPTx-Nx rats were divided into two groups. One group (n = 10) was treated with the oral adsorbent AST-120 (4 g/kg body weight admixed in animal feed) and the other group (n = 9) was treated with vehicle alone. AST-120 was obtained from Kureha Corporation (Tokyo, Japan). Rats that underwent TPTx alone (n = 9) were included as a control group. The rats were sacrificed 12 weeks after the second Nx (i.e., week 13). All TPTx and TPTx-Nx rats were given a continuous infusion of a physiological level of 1–34 PTH (0.1 μg/kg/h; Peninsula laboratories, Talyo Way, San Carios, CA) using a subcutaneously implanted Alzet osmotic mini-pump (Model 2002; Alza Corp., Palo Alto, CA; the pumps were replaced every 2 weeks). L-thyroxin (Sigma Chemical Company, St. Louis, MO) was also subcutaneously injected at a dose of 4 μg/kg body weight three times per week, starting 2 days after TPTx. Blood and bone samples were collected from all rats. The right femur was used for BMD, mechanical properties, and chemical composition analyses. Fig. 4 shows the measurement sites. The tibia was used for bone histomorphometric studies. Urine was collected to measure creatinine clearance before sacrificing the rats. All animal experiments were approved by the Animal Care and Use Committee of the Biomedical Research Laboratories, Kureha Chemical Industry Co., Ltd.

Serum and urine biochemistry

Serum samples were stored at −70 °C and urine samples were stored at −20 °C until required for analyses. Serum calcium and phosphorus were determined by the Calcium-E and Phospha-C assays (both from Wako Pure Chemicals, Tokyo Japan). Serum and urine creatinine concentrations were determined using Wako Kit 277-1050, and urea nitrogen levels were determined using Wako Kit 279-36201 (Wako Pure Chemicals). Serum PTH levels were measured with an immunoradiometric assay for rat PTH (Immutopics, San Clemente, CA). Serum $1,25(OH)_2D_3$ levels were measured by a radioimmunoassay (RIA). Serum IS concentrations were determined by high-performance liquid chromatography (HPLC), as previously described [17], with a Shiseido Capcell Pak MF ph-1 column (size: 150 mm × 4.6 mm I.D.) and a guard column (4.0 mm × 10 mm I.D.). The mobile phase (0.1 M KH_2PO_4/tetrahydrofuran = 95/5 (v/v) adjusted to pH 6.5 with 1 M NaOH) was delivered at a flow rate of 1.0 mL/min at ambient temperature. The eluate was monitored by a fluorescence detector (excitation: 295 nm, emission: 390 nm) for analysis.

Bone histomorphometry

Histomorphometric analysis of the secondary spongiosa was performed in the tibial proximal metaphysis between 1.2 and 3.6 mm distal to the growth plate-epiphyseal junction. A semiautomated system (Osteoplan II; Carl Zeiss, Thornwood, NY) was used, and measurements were made at a magnification of ×200. Cancellous bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N,/mm), and trabecular separation (Tb.Sp, μm) were measured. Then, single-labeled surface (sLS/BS, %), double-labeled surface (dLS/BS, %), and mineralized surface adjusted for total bone surface (MS/BS, %) were calculated. Label width was determined as the mean distance between the double labels. Mineral apposition rate (MAR, μm/day) was calculated by dividing the label width by the number of days between the two calcein administrations (at 7 and 14 days before sacrifice). Bone formation rate adjusted for bone surface (BFR/BS, $\mu m^3/\mu m^2$/year) was calculated as (sLS/2 + dLS) × MAR/BS. Trabecular osteoclast surface (Oc.S/BS, %) and eroded surface (ES/BS, %) were determined as parameters of bone resorption. The nomenclature, symbols, and units used in this study are those recommended by the American Society for Bone Mineral Research Nomenclature Committee [18].

Measurement of BMD

Femoral bone samples were collected and all connective tissue was carefully removed. BMD of the right femur was determined by single-energy X-ray absorptiometry using a bone mineral analyzer (DCS-600R; Aloka Co., Tokyo, Japan). BMD was measured in the distal quarter of the femur, including the epi-metaphyseal region, and in the second–third quarters of the femur, including the diaphysis.

Measurement of storage modulus by DMA

DMA has been used to characterize the viscoelastic mechanical properties of cortical bone. This method is nondestructive and a specimen can be measured repeatedly at various frequencies using a dynamic mechanical analyzer [19]. DMA is also widely used to evaluate bone mechanical properties. After measuring BMD, we determined the viscoelastic mechanical properties of the femur using previously described method [15]. Briefly, after measuring the thickness and width at the center of each femur, the femur was placed in the DMA device (DMA 7e; PerkinElmer, Norwalk, CT) and baseline viscoelasticity was measured in 0.9% saline solution at 37 °C by an oscillatory test with three-point bending. The scan frequencies ranged from 1 to 10 Hz (0.2 Hz increments). The test was conducted under controlled displacement. Storage modulus E1 (obtained from dynamic test, equivalent to Young's modulus), loss factor, and tan delta (an indication of the amount of energy dissipated by viscous mechanisms relative to energy stored in the elastic component) were measured for each sample.

Confocal Raman spectroscopic measurements

Confocal laser Raman microspectroscopy was used to examine the composition and relative amounts of minerals and matrix in the femur. Raman spectroscopy is particularly useful for the analysis of mineral and matrix components in unprocessed bone preparations, in which these components are preserved [15,17]. A Nicolet Almega XR Dispersive Raman microscope system and OMNIC Atlus TM imaging