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Effect of Etidronate on Aortic Calcification and Bone Metabolism in Calcitoriol-Treated Rats With Subtotal Nephrectomy

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Abstract. The present study was undertaken to determine the effects of etidronate (ED) on calcitriol-induced aortic calcification and bone metabolism in rats with renal failure. Severe aortic calcification was induced by treatment with calcitriol for 3 weeks in rats in which 5/6 of the kidneys were removed (SNx group). Treatment of ED (10 mg/kg) together with calcitriol after subtotal nephrectomy (SNx) significantly inhibited thoracic and abdominal aortic calcification 3 weeks after the operation; however, ED (2 mg/kg) was ineffective. The serum levels of osteocalcin and pyridinoline decreased in ED (10 mg/kg) treated-renal failure rats compared with SNx rats. Total bone mineral density (BMD) in the SNx group was lower than that in the sham group, in which animals were treated with calcitriol after a sham operation. The total BMD value in the ED (10 mg/kg)-treated group was similar to that in the SNx group, whereas the levels of cancellous BMD were low in the ED (10 mg/kg)-treated rats. Our data show that ED at a dosage that suppresses bone metabolism markedly inhibits vascular calcification in rats with renal failure.

Keywords: etidronate, calcification, aorta, nephrectomy

Introduction

More than two hundred thousand people in Japan are undergoing long term dialysis, and the number of these patients increases every year. Significant bone density loss and the appearance of ectopic calcification, which greatly influences the quality of life, are frequently observed in long term dialysis patients (1 – 3). Vascular calcification is one type of ectopic calcification that is recognized as a crucial factor leading to the progression of cardiac failure, myocardial infarction, and cerebrovascular obstruction that accounts for 50% of patient mortality (4, 5). The clinical significance of vascular calcification has been realized only recently. Arterial medial calcification (Monckeberg's type calcification) is the major type of arteriosclerosis in dialysis patients (6, 7). The medial sclerosis progresses in proportion to the period of dialysis without any relation to age since it occurs even in young adults in coronary as well as peripheral arteries (1). The formation of vascular calcification has been accepted as the terminal consequence of blood vessel disease caused by arteriosclerosis, and calcium (Ca) and phosphate (P) accumulation accompanies the death of vascular cells through passive degeneration and necrosis in the calcified region. However, a new concept has been proposed and designated as "the correlation of bone and vasculature", which means that Ca in the bone shifts into vascular cells to result in ectopic calcification (8).

The pyrophosphonate compound bisphosphonate can control bone absorption and ectopic calcification (9, 10). Clinically among these types of drugs, etidronate (ED) has been used predominantly and is generally considered the standard bisphosphonate. Since ED suppresses

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calcification when administered at high doses, it is used as a curative medicine for regulating ectopic calcification after spine damage and hip-joint formation or for improving Paget's disease (11). ED was also the first medicine administered for osteoporosis. The structure of ED does not contain nitrogen (N), and it must be metabolized to an ATP analog to exert its action (9, 10). It has high affinity for hydroxyapatite (calcium phosphate) and suppresses the functions of osteoclasts (12). Recently, we showed that ED treatment for 6 months inhibited progression of vascular calcification in hemodialysis patients (13); however, a beneficial effect of ED on patients with vascular calcification has not been generally accepted and applied clinically. Subtotal nephrectomy (SNx) provides a well-characterized model of renal failure (14), and it has been reported that calcitriol promotes artery calcification in rats who received an SNx operation (15). In the present study, we determined the effects of ED administration on vascular calcification induced by calcitriol treatment in an animal model of renal failure.

Materials and Methods

Experimental schedule

Male rats (7.5-week-old) of the Wistar-Imamichi strain (Imamichi Institute for Animal Reproduction, Ibaraki) were maintained in an air-conditioned room (temperature: 23 ± 1°C and humidity: 55 ± 5%) under controlled lighting (12-h light/day schedule) with free access to food and water. All animal-handling protocols and surgical procedures were approved by the Institutional Animal Care Committees at Tokyo University of Pharmacy & Life Science in compliance with institutional guidelines for experimental animal care. Renal failure was induced by subtotal nephrectomy (SNx) on 9-week old rats. The SNx operation was performed by excision of two thirds of the right kidney followed by left nephrectomy 1 week later (12). The survival rate after nephrectomy was approximately 95%. Calcitriol (1 μg/kg) dissolved in sesame oil was administered p.o. for 3 weeks from the day following the SNx operation. Food pellets were changed to chow containing high calcium (4%) and phosphate (1.8%). All animals were treated with calcitriol and the high calcium/phosphate diet. ED (2, 10 mg/kg; Sumitomo Pharmaceuticals Co., Ltd., Osaka) dissolved in saline was injected s.c. for 3 weeks together with calcitriol. Forty-eight hours after the final administration (approximately 3 weeks after SNx operation, 12-week-old), animals were sacrificed by bleeding from the femoral artery under ether anesthesia. Individual 7-ml blood samples were collected and allowed to clot at 4°C, and the serum was separated by centrifugation and stored frozen at −80°C until it was assayed for osteocalcin, pyridinoline, and creatinine. The thoracic and abdominal aortae were fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for preparing paraffin-embedded tissue sections.

Tissue preparation and staining by the von Kossa method

Blood vessels (the 1/4 upper part, approximately 1 cm of thoracic and abdominal aorta) that had been fixed with PFA for 6 h in room temperature were soaked in 70% ethanol at 4°C. The tissues were parraffin-embedded using a paraffin infiltration device (Tissue Tec VIP; Miles and Sankyo Co., Ltd., Tokyo), and the cross sections at 4-μm intervals were prepared on poly-L-lysine-coated slides (Matsunami, Kishiwada) (16). Ca in tissues was stained by the von Kossa method (17).

Measurements of the bone mineral density (BMD), bone metabolic markers, and creatinine

Radiographs of the femur were taken by soft X ray (model CMB-2; SOFTEX, Ebina), and the BMD of the femur was measured by dual X-ray absorptiometry (model DCS-600R; Aloka, Mitaka) as previously reported (18). The BMD was calculated by dividing the bone mineral content of the measured area by the total area. The scanned area was divided into three parts: proximal femur, midshaft, and distal femur. Serum concentrations of osteocalcin and pyridinoline were determined using a two-site immunoradiometric assay (IRMA) kit (Immutopics, Inc., San Clemente, CA, USA) and an enzyme linked immunosorbent assay (ELISA) Metra serum PYD EIA kit (Quidel Corporation, San Diego, CA, USA), respectively.

Statistical analyses

Data are represented as the means ± S.E.M. The experiments were repeated three times independently. The significance of differences was tested with a Tukey multiple test of the ANOVA test. Differences of $P<0.05$ were considered statistically significant.

Results

Effect of ED treatment on body weight in SNx rats

Changes in the body weight of SNx rats during ED treatment after SNx surgery are shown in Fig. 1. The body weight in the SNx group just before the 5/6 nephrectomy (rats with 2/3 removal of the right kidney) was lower than that in the Sham operation (0 week in Fig. 1) and gradually decreased throughout the 3 week treatment period. A decrease in body weight was also found in the ED (2 mg/kg)-treated groups, but in ED

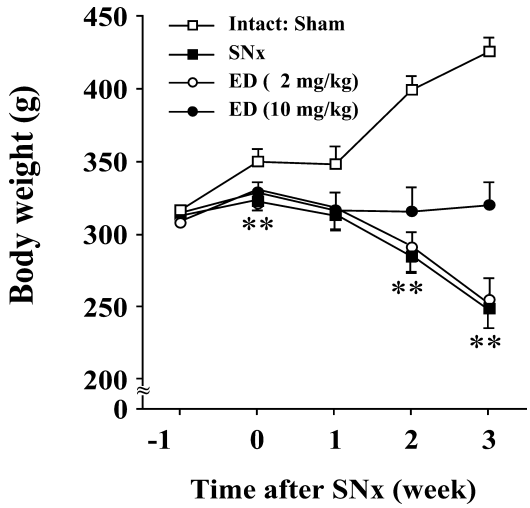

Fig. 1. Effect of etidronate (ED) on body weight in subtotally nephrectomized (SNx) rats. Eight-week-old male rats underwent subtotal nephrectomy (SNx) or sham surgery. Calcitriol (1 μg/kg) was administered p.o. for three weeks from the day following the SNx operation. Concurrently, ED (2, 10 mg/kg) was injected s.c. for 3 weeks. Sham operated animals were treated with calcitriol only after the sham operation. Body weight was measured once a week. **P<0.01 *vs* Sham.

(10 mg/kg)-treated rats, the weight on the day of 5/6 nephrectomy (0 week) was maintained for at least 3 weeks and was significantly higher (P<0.05) than that in the SNx group.

Effect of ED on induction of calcification in the aorta

Ca deposition in the thoracic and abdominal aortae was evaluated by the von Kossa method (Fig. 2). As shown in Fig. 2A, no vascular calcification was seen in the thoracic aorta of the sham group that had been administered calcitriol without the SNx operation. However, after treatment with calcitriol for 3 weeks, severe aortic calcification was induced in the rats in which 5/6 of the kidneys were removed (the SNx group). ED at 10 mg/kg suppressed calcification, while a lower dose (2 mg/kg) appeared to have little effect. To evaluate the effect of ED on aortic calcification quantitatively, the ratio of the length of calcification to the circumference of the aorta was measured. ED treatment (10 mg/kg) for 3 weeks significantly inhibited the thoracic and abdominal aortic calcification (48% and 42% decreases, respectively). However, the value in the ED (2 mg/kg)-treated groups was similar to that in the untreated SNx group.

Effect of ED on bone metabolism

As shown in Fig. 3, in the SNx group, the serum levels of osteocalcin and pyridinoline were markedly increased but could be lowered by treatment with 10 mg/kg ED. Additionally, the total BMD was significantly lower in the SNx group compared to the sham group. ED at either 2 or 10 mg/kg did not appear to affect the total BMD. Finally, an inhibitory effect of ED on cancellous BMD was seen at the 10 mg/kg dose.

Creatinine levels in SNx rats and the influence of ED treatment

The serum levels of creatinine were measured to confirm the state of renal failure in each group (Table 1). Overall, the serum creatinine values in the SNx groups were higher than that in the sham group. Creatinine levels in ED (2 mg/kg)-treated rats were similar to those in the untreated SNx group, whereas the value in the ED (10 mg/kg)-treated group was significantly lower.

Discussion

Recent clinical research has revealed that ED, one of the bisphosphonate compounds, has an inhibitory effect on vascular calcification in dialysis patients (13). The mechanism of its action is still unknown, and the evaluation of the inhibitory action of ED using an experimental model has not been performed. Therefore, the effect of ED on aortic calcification was examined using a rat model with vascular calcification similar to that seen in long-term dialysis patients. Vascular calcification is accelerated by calcitriol (15). In this study, severe vascular calcification was induced in rats with renal failure by first performing a 5/6 nephrectomy and then subsequently administering a high dose of calcitriol. Comparable to hemodialysis patients (13), vascular calcification was localized in the arterial media. Interestingly, treatment of SNx rats with a low dose of calcitriol (20 – 500 ng/kg) did not cause vascular calcification (data not shown), thus implying that a relatively high dose of active calcitriol is needed for inducing vascular calcification in this model. The present study using SNx rats demonstrates the beneficial effects of ED at a relatively high dose (10 mg/kg) in the reduction of vascular calcification and suppression of the decrease in body weight seen in animals with renal injury.

The serum levels of creatinine in ED (10 mg/kg)-treated rats were significantly lower when compared to those of the SNx group. Because administration of ED was started on the day following the SNx operation, ED might have inhibited ectopic calcification in the remaining 1/6 kidney which could have made the extent of functional impairment in the kidney gradual in comparison to the sham group. The inhibitory effect of ED on the formation of ectopic calcification in other

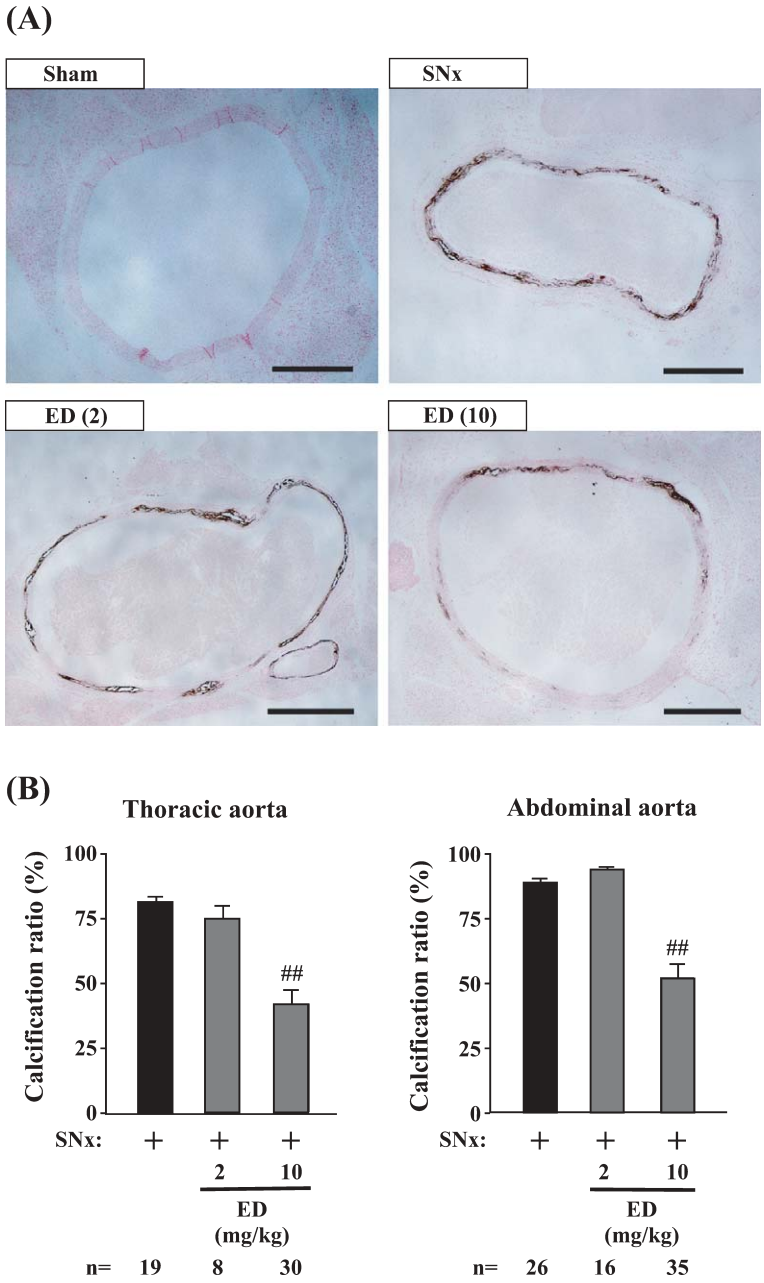

Fig. 2. Effect of etidronate (ED) on aortic calcification in subtotally nephrectomized (SNx) rats. SNx and drug treatments were performed as described in Fig. 1. The thoracic and abdominal aortae were removed 48 h after the final treatment and fixed in 4% paraformaldehyde. Calcium in the cross sections of aorta was visualized using von Kossa staining. A: Representative photographs were taken. Bars represent 250 μm. ED (2 or 10): SNx animals treated with ED (2 or 10 mg/kg). B: The degree of calcification in thoracic and abdominal aortae in ED-treated SNx animals. Aortic calcification was evaluated by measuring the aortic calcification ratio (the length of calcification/the circumference of the aortic wall). Control animals were treated with calcitriol only after a sham operation. Each column shows the mean ± S.E.M. obtained from three independent experiments. $^{\#\#}P<0.01$ *vs* SNx (SNx: +).

tissues, especially the cardiovascular system, might also contribute to the suppression of body weight loss. In order to evaluate the effect of ED on calcification during the state of complete renal failure, it will be necessary to measure and confirm a significant increase in creatinine levels several weeks following the SNx operation.

It has been reported that a high dose of ED suppresses bone formation as well as bone resorption, which is to say that bone calcification is inhibited, resulting in a decrease in bone mass (19). We observed a significant decrease in total BMD at a dose of ED effective for inhibiting aortic calcification (10 mg/kg), and a marker

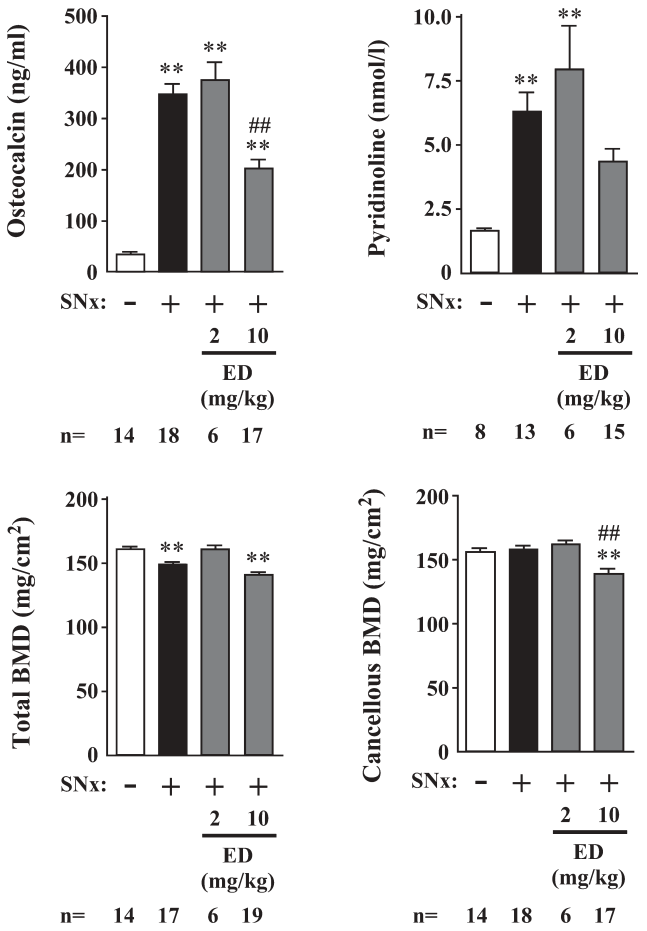

Fig. 3. Effect of etidronate (ED) on markers of bone metabolism and bone mineral densities (BMD) in subtotally nephrectomized (SNx) rats. SNx and drug treatments were performed as described in Fig. 1. Blood samples were collected 48 h after the final treatment, and the serum levels of osteocalcin and pyridinoline were determined. Right femurs were also removed 48 h after the final treatment, and total and cancellous BMD were measured using dual-energy absorptiometry X-ray analysis (DEXA). Each value represents the mean ± S.E.M. obtained from three independent experiments. $^{**}P<0.01$ *vs* Sham (SNx: –), $^{\#\#}P<0.01$ *vs* SNx (SNx: +).

of effect on osteodystrophy, osteocalcin (20), was also reduced in these treated rats. Our results are consistent with those previously reported showing ED-induced inhibition of bone calcification (19). An elevation in bone metabolic turnover and a decrease in total bone density were also observed in the untreated SNx group. This may imply that the animals were in a state of renal osteodystrophy. In dialysis patients, the maintenance of normal bone is difficult because of the appearance of renal osteodystrophy, the loss of bone density, and the elevation of Ca and P levels. All of these conditions contribute to vascular calcification that is related to the rise in mortality closely associated with cardiovascular system disorders.

Table 1. Effect of etidronate (ED) on serum creatinine levels in subtotally nephrectomized (SNx) rats

Groups	Creatinine (mg/dl)	(n)
Sham	0.63 ± 0.03	(8)
SNx	1.13 ± 0.05**	(19)
ED (2)	1.12 ± 0.10**	(8)
ED (10)	0.91 ± 0.03**,#	(30)

SNx and drug treatments were performed as described in Fig. 1. Blood samples were collected 48 h after the final treatment. Each value represents the mean ± S.E.M. obtained from 3 independent experiments. Sham: rats received sham surgery only, ED (2, 10): SNx rats treated with ED (2, 10 mg/kg). $^{**}P<0.01$ *vs* Sham, $^{\#}P<0.05$ *vs* SNx.

A clinical correlation between hyperphosphatemia and aortic or coronary calcification has been established (21, 22). High P levels induced vascular calcification in a dose-dependent manner in human vascular smooth muscle cells (VSMC) in vitro and also enhanced expression of the sodium-dependent P co-transporter (Pit-1) in calcified lesions (23). Additionally, P enhanced the gene expression of the osteoblastic differentiation markers osteocalcin and core binding factor α-1 (cbfa-1) (23, 24). Thus, intracellular P taken up by Pit-1 in VSMC appears to transform these cells into osteoblast-like cells with the potential capability of forming vascular calcification. There is a possibility that ED can modulate Pit-1 activity in VSMC to block the progression of vascular calcification because of structural similarity of ED to phosphonoformic acid that inhibits Pit-1 (25). Using an in vitro calcification model of bovine VSMC, it was reported that parathyroid hormone-related peptide (PTHrP) secretion may be involved in the inhibitory effects of ED on vascular calcification (26). However, serum Ca and P did not change significantly between SNx and ED-treated groups (data not shown), suggesting that ED does not affect circulating Ca and P levels under the present study conditions. ED has been reported to have anti-atherogenic effects in vivo (27), and ED treatment decreased atherosclerosis in type 2 diabetic patients (28). Inflammatory cells such as macrophages and T lymphocytes play an important role in calcified atherosclerotic lesions. Non-aminobisphosphonates, including ED, are metabolized in macrophages and may inhibit the inflammatory response of these cells (29); therefore, the inhibition of vascular calcification seen upon ED treatment may be related to the anti-inflammatory action of this drug in calcified atherosclerotic lesions.

In summary, the present study demonstrates that ED treatment may be useful in preventing the progression of vascular calcification, which causes cardiac and vascular

dysfunction, and may improve survival in hemodialysis patients.

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