Effects of the calcineurin inhibitors cyclosporine and tacrolimus on bone metabolism in rats

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ABSTRACT

Immunosuppressive therapy is considered as one of the factors inducing to the onset of osteoporosis after organ transplantation. Chronic immunosuppressive therapy after transplantation is required for organ transplant patients, and it is important to prevent the occurrence of osteoporotic fractures to maintain the quality of life in patients. In this study, we examined the effects of cyclosporine and tacrolimus on bone metabolism in rats. Five-week-old male Wistar rats were treated orally with 15 mg/kg cyclosporine or 1.5 mg/kg tacrolimus daily for 4 weeks. Each of cyclosporine and tacrolimus significantly reduced the bone strength of the femoral mid-diaphysis and bone mineral density of the tibia and femur. Bone histomorphometry showed that the administration of both drugs resulted in a decrease in bone volume, number and thickness of trabeculae, and an increase in trabecular separation. Bone formation parameters such as osteoid volume, osteoblast surface, mineralizing surface, mineral apposition rate, and bone formation rate significantly increased in the cyclosporine-treated group. Bone resorption parameters such as eroded surface, osteoclast surface, and osteoclast number significantly increased in both the cyclosporine- and the tacrolimus-treated groups. These results showed that cyclosporine increases both bone formation and bone resorption, leading to a high-turnover bone loss, and that tacrolimus increases bone resorption without affecting bone formation, leading to bone loss.

As osteoporotic fractures decline quality of life (QOL) (26) and increase mortality (25) in elderly patients, the prevention of these bone fractures has become a major concern. It is well-known that therapeutic drugs can cause bone fragility, as evidenced in glucocorticoid-induced osteoporosis. Recently, several other drugs were also clearly shown to cause bone fragility. We have previously reported the effects of antidiabetic (11) and antiepileptic (12, 13) agents on bone metabolism; these led to enhanced bone fragility. The present study investigates the effects of calcineurin inhibitors, which are immunosuppressants that have greatly contributed to the advancement of transplant therapy in recent years, on bone metabolism. Calcineurin inhibitors, such as cyclosporine and tacrolimus, are used as immunosuppressants, which inhibit the differentiation and proliferation of T-cells and show potent immunosuppressive actions. These drugs inhibit the synthesis of cytokines such as interleukin-2 by binding to immu-
nophilin and suppressing the activity of calmodulin-dependent protein phosphatase calcineurin (14, 19). Long-term immunosuppressive treatment is essential for transplant recipients in order to prevent rejection after organ transplants. Few of the recipients can subsequently discontinue immunosuppressive therapy (23). Serious complications after organ transplants, such as onset of osteoporosis and an increase in risk of bone fracture, have been reported (3). Immunosuppressive therapy is considered as one of the factors inducing to the onset of osteoporosis after organ transplantation (32). Several studies have been conducted to elucidate the onset mechanism of bone loss induced by calcineurin inhibitors. It has been reported that cyclosporine induces the reduction of bone mineral density (BMD) by promoting bone formation and bone resorption (6). Meanwhile, other studies have also indicated that cyclosporine suppresses bone resorption (28), inhibits osteoclast activity, and promotes osteoblast activity (20). As with cyclosporine therapy, tacrolimus therapy has been reported to significantly reduce the BMD in transplant recipients (21, 27). However, tacrolimus has been reported to significantly decrease osteoclast number and increase bone volume (1). Because combined therapy of steroids, which cause severe osteoporosis, and calcineurin inhibitor is common in clinical practice, the effect of monotherapy with either cyclosporine or tacrolimus on bone metabolism has not been accurately evaluated. Thus, the aim of this study was to examine the effects of cyclosporine and tacrolimus on bone metabolism in rats.

MATERIALS AND METHODS

Animals. Five week-old male Wistar rats (120–130 g) were purchased from CLEA Japan Inc. (Tokyo, Japan). The rats were housed at 22 ± 2°C and 55 ± 5% humidity on a 12 h light-dark cycle with ad libitum access to a standard chow (MF; Oriental Yeast Co., Tokyo, Japan) and water. All procedures were approved by the Animal Research Committee of Niigata University of Pharmacy and Applied Life Sciences in accordance with the Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals.

Drugs. Commercially available agents of cyclosporine (Novartis Pharma K.K., Tokyo, Japan) and tacrolimus (Astellas Pharma Inc., Tokyo, Japan) were obtained, and suspended in 0.2% carboxymethylcellulose sodium (CMC-Na; Sigma-Aldrich, St. Louis, MO, USA).

Experimental procedure. Animals were divided into three groups (10 rats/group): [1] control group treated with vehicle (0.2% CMC-Na), [2] 15 mg/kg cyclosporine-treated group, and [3] 1.5 mg/kg tacrolimus-treated group. Drugs were administered via oral gavage in a volume of 0.1 mL/100 g body weight once daily for 4 weeks. Whole blood was collected under CO2 anesthesia 24 h after the final drug was administered. The blood was centrifuged at 1500×g for 10 min at 4°C, and the serum was stored at −80°C until needed. The femur and tibia were dissected, and soft tissue was removed.

Bone strength analysis. Bone strength of the femoral mid-diaphysis was evaluated via a three-point bending method using a mechanical testing machine (EZ-S; Shimadzu, Tokyo, Japan). The femur was positioned on two supports placed 12 mm apart. The bending load was vertically applied to the mid-diaphysis with a crosshead speed of 1.0 mm/min until bone fracture. The load deformation curves were calculated using operation software (Trapezium X; Shimadzu), and the maximum load, breaking energy, and stiffness were directly calculated from the load deformation curve.

BMD measurements. Total BMD, trabecular BMD, and cortical BMD of whole femur and tibia were measured using quantitative computed tomography (QCT: LaTheta LCT-100; Aloka, Tokyo, Japan) with a pixel size of 250 × 250 μm and slice thickness of 1 mm. The values of BMD were calculated using LaTheta software (ver. 1.31; Aloka).

Serum biochemical analysis. Serum calcium levels were measured with a commercial reagent (Denka Seiken, Tokyo, Japan) and an automatic analyzer (7180; Hitachi High-Technologies, Tokyo, Japan). Serum osteocalcin levels were measured with the rat Osteocalcin ELISA System (General Electric Healthcare Japan, Tokyo, Japan). Serum tartrate-resistant acid phosphatase-5b (TRAP) levels were measured with the Rat TRAP assay (SBA-Sciences, Oulu, Finland).

Bone histomorphometry. We prepared non-decalcified specimens from the proximal tibia metaphysis according to the following method. Briefly, the tibia was fixed with 70% ethanol for 7 days, stained with Villanueva Bone Stain (basic fuchsin, fast green, orange G, and azure II; Merck, Darmstadt, Germany).
in 70% methanol for 7 days, and embedded in methyl methacrylate resin. The resin blocks were then sliced to 5-μm thickness with a microtome (Leica RM2255; Leica Inc., Nussloch, Germany). All bone histomorphometric parameters were measured at the secondary spongiosa region. To exclude the primary spongiosa, the measurement region was 0.8–1.3 mm distal to the lowest point of the growth plate and 0.2 mm from the lateral cortex.

Bone histomorphometric measurements were performed using a semiautomatic image analyzing system (Histometry RT CAMERA; System Supply, Nagano, Japan) with ×320 magnification. Bone structural parameters obtained included bone volume per tissue volume, thickness, number of trabeculae, and trabecular separation. Bone formation parameters obtained included osteoid volume per bone volume and osteoblast surface per bone surface. Bone resorption parameters included the eroded surface, osteoclast surface, and osteoclast number per bone surface.

The dynamic parameter was measured using a double fluorescent labeling technique. For labeling, all rats were injected subcutaneously with 25 mg/kg of tetracycline (Sigma-Aldrich) and 10 mg/kg of calcinein (Wako Pure Chemical Industries, Osaka, Japan) 5 and 2 days before they were euthanized, respectively. The labeled surface that reflected the calcification front at the time of tetracycline and calcinein administration was visualized using a fluorescent microscope (Olympus BX50; Olympus America Inc., Center Valley, PA, USA). The parameters of single- and double-labeled surface (sLS and dLS) and inter-labels thickness and times (Ir.L.Th and Ir.L.t) were used in the calculation of mineralizing surface per bone surface [(dLS+sLS/2)/bone surface], mineral apposition rate (Ir.L.Th/Ir.L.t), and bone formation rate per bone surface ([mineral apposition rate×mineralizing surface/osteoid surface]/bone surface). Fig. 1 shows the scheme of the primary parameters (29). Standard bone histomorphometrical nomenclature, symbols, and units were based on those described in the report of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (5).

**Statistical analysis.** Data are presented as the mean ± standard error (SE). Differences between groups were analyzed with one-way ANOVA followed by Tukey-Kramer multiple comparisons. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Bone strength properties**

The following parameters of the femoral mid-diaphysis significantly decreased in the cyclosporine- and tacrolimus-treated groups compared with that in the control group: maximum load (32%, 17%), breaking energy (38%, 30%), and stiffness (34%, 18%) (Table 1).
cant difference in serum calcium levels among all groups. However, in the cyclosporine-treated group, serum osteocalcin levels and TRAP levels significantly increased (92%, 60%, respectively) compared with the control values. Serum TRAP levels significantly increased (31%) in the tacrolimus-treated group compared with control values. These results suggested that cyclosporine promotes both bone formation and bone resorption, while tacrolimus accelerates bone resorption without affecting bone formation.

**Bone histomorphometric evaluation**
For the cancellous bone structural parameters, cyclosporine or tacrolimus treatment significantly reduced bone volume per tissue volume (BV/TV) (54%, 33%), trabecular thickness (Tb.Th) (20%, 12%), and trabecular number (Tb.N) (38%, 25%), and increased trabecular separation (Tb.Sp) (68%, 45%) relative to that in the control group (Fig. 2). Additionally, cyclosporine-treated group exhibited significantly decreased total BMD (15%, 13%), trabecular BMD (16%, 20%), and cortical BMD (8.3%, 5.7%) of the whole femur and tibia, respectively, compared with that in the control group (Table 2). Additionally, the tacrolimus-treated group showed significantly decreased total BMD (9.3%, 8.7%), trabecular BMD (8.8%, 13%), and cortical BMD (4.9%, 3.9%) of the whole femur and tibia, respectively, compared with that in the control group. Furthermore, total BMD, trabecular BMD, and cortical BMD of the femur in the cyclosporine-treated group were significantly lower than that in the tacrolimus-treated group. These results indicated that cyclosporine induces more severe decreases of BMD than tacrolimus.

**Serum bone metabolism markers**
The measurements of serum bone metabolism markers are summarized in Table 3. There was no significant difference in serum calcium levels among all groups. However, in the cyclosporine-treated group, serum osteocalcin levels and TRAP levels significantly increased (92%, 60%, respectively) compared with the control values. Serum TRAP levels significantly increased (31%) in the tacrolimus-treated group compared with control values. These results suggested that cyclosporine promotes both bone formation and bone resorption, while tacrolimus accelerates bone resorption without affecting bone formation.

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### Table 1 Bone strength properties

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cyclosporine 1.5 mg/kg</th>
<th>Tacrolimus 15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum load (N)</td>
<td>98.1 ± 6.04</td>
<td>66.8 ± 3.15**</td>
<td>81.2 ± 3.66*</td>
</tr>
<tr>
<td>Braking energy (N.mm)</td>
<td>40.2 ± 2.39</td>
<td>25.1 ± 1.59**</td>
<td>32.1 ± 1.81*</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>154 ± 7.25</td>
<td>102 ± 6.23**</td>
<td>125 ± 5.79*</td>
</tr>
</tbody>
</table>

Data represents the mean ± SE (n = 10). *P < 0.05, **P < 0.01 vs Control

### Table 2 BMD of whole femur and tibia

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cyclosporine 1.5 mg/kg</th>
<th>Tacrolimus 15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole femur Total BMD (mg/cm³)</td>
<td>612 ± 5.60</td>
<td>519 ± 4.14**</td>
<td>561 ± 9.44*</td>
</tr>
<tr>
<td>Trabecular BMD (mg/cm³)</td>
<td>425 ± 3.42</td>
<td>357 ± 3.41**</td>
<td>387 ± 12.7*</td>
</tr>
<tr>
<td>Cortical BMD (mg/cm³)</td>
<td>927 ± 3.15</td>
<td>849 ± 4.28**</td>
<td>881 ± 8.25*</td>
</tr>
<tr>
<td>Whole tibia Total BMD (mg/cm³)</td>
<td>622 ± 16.7</td>
<td>541 ± 5.36*</td>
<td>568 ± 17.7*</td>
</tr>
<tr>
<td>Trabecular BMD (mg/cm³)</td>
<td>396 ± 13.8</td>
<td>318 ± 6.59*</td>
<td>343 ± 9.62*</td>
</tr>
<tr>
<td>Cortical BMD (mg/cm³)</td>
<td>947 ± 8.94</td>
<td>893 ± 5.79*</td>
<td>911 ± 5.53*</td>
</tr>
</tbody>
</table>

Data represents the mean ± SE (n = 10). *P < 0.01 vs Control, **P < 0.05 vs Tacrolimus

### Table 3 Serum bone metabolism markers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cyclosporine 1.5 mg/kg</th>
<th>Tacrolimus 15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dL)</td>
<td>12.3 ± 0.20</td>
<td>12.2 ± 0.10</td>
<td>12.2 ± 0.15</td>
</tr>
<tr>
<td>Osteocalcin (ng/dL)</td>
<td>63.8 ± 6.41</td>
<td>122.6 ± 1.57*</td>
<td>70.4 ± 3.55</td>
</tr>
<tr>
<td>TRAP (U/L)</td>
<td>9.90 ± 0.75</td>
<td>15.8 ± 0.88**</td>
<td>12.9 ± 0.81*</td>
</tr>
</tbody>
</table>

Data represents the mean ± SE (n = 10). *P < 0.05, **P < 0.01 vs Control

TRAP: tartrate-resistant acid phosphatase-5b
Effect of calcineurin inhibitor on bone

Fig. 2  Effects of cyclosporine and tacrolimus on cancellous bone structural parameters (A: bone volume/tissue volume (BV/TV), B: trabecular thickness (Tb.Th), C: trabecular number (Tb.N), D: trabecular separation (Tb.Sp)) according to bone histomorphometry of the proximal tibia metaphysis. Cyclosporine (Cyc; 15 mg/kg) or tacrolimus (Tac; 1.5 mg/kg) was orally administered once per day for 4 weeks. The control group (Cont) was treated with vehicle (0.2% carboxymethylcellulose sodium solution). Data represent the mean ± SE of 10 rats. *P < 0.05, **P < 0.01 vs Control.

Cyclosporine treatment significantly increased the bone formation parameters: osteoid volume per bone volume (OV/BV, 35%) and osteoblast surface (Ob.S/BS, 21%), and bone resorption parameters: eroded surface (ES/BS, 63%), osteoclast surface (Oc.S/BS, 77%), and osteoclast number per bone surface (N.Oc/BS, 83%) compared with that in the control group (Fig. 3). Although tacrolimus treatment did not affect bone formation parameters, it significantly increased the bone resorption parameters: ES/BS (35%), Oc.S/BS (41%), and N.Oc/BS (28%) compared with that in the control group. Furthermore, assessment of dynamic parameters using a bone labeling technique showed significant increases in mineralizing surface per bone surface (MS/BS, 26%), mineral apposition rate (MAR, 15%), and bone formation rate per bone surface (BFR/BS, 41%) in the cyclosporine-treated groups.

Fig. 4 shows typical microphotographs of the control group, cyclosporine-, and tacrolimus-treated groups using Villanueva Bone Stain. These images confirmed the marked increases of osteoid volume, eroded surface, and inter-label thickness in the cyclosporine-treated group compared with that in the control group.
DISCUSSION

Immunosuppressive therapy with calcineurin inhibitors after organ transplantation is one of the factors that induce bone loss and increase risk of bone fracture in transplant recipients (3). Cyclosporine has been reported to increase both bone resorption and bone formation, leading to a high-turnover osteoporosis (6). In contrast, there was no significant decrease in the BMD of renal transplant recipients who received cyclosporine monotherapy (22). Although cyclosporine plus steroid therapy significantly reduced BMD in renal transplant recipients, cyclosporine monotherapy significantly increased BMD of the lumbar spine (2). In addition, cyclosporine has been reported to inhibit bone resorption (28), sig-

Fig. 3  Effects of cyclosporine and tacrolimus on bone formation parameters (A: osteoid volume per bone volume (OV/BV), B: osteoblast surface per bone surface (Ob.S/BS), C: mineralizing surface per bone surface (MS/BS), D: mineral apposition rate (MAR), E: bone formation rate per bone surface (BFR/BS)) and bone resorption parameters (F: eroded surface per bone surface (ES/BS), G: osteoclast surface per bone surface (Oc.S/BS), H: osteoclast number per bone surface (N.Oc/BS)) according to bone histomorphometry of the proximal tibia metaphysis. Cyclosporine (Cyc; 15 mg/kg) or tacrolimus (Tac; 1.5 mg/kg) was orally administered once per day for 4 weeks. The control group (Cont) was treated with vehicle (0.2% carboxymethylcellulose sodium solution). Data represent the mean ± SE of 10 rats. *P < 0.05, **P < 0.01 vs Control.
Effect of calcineurin inhibitor on bone

Bone strength is defined by BMD and bone quality (18). To maintain BMD, it is essential that bone formation by osteoblasts and bone resorption by osteoclasts maintain a certain balance and function (15). The term “bone quality” refers collectively to elements other than BMD that affect bone fractures and is mainly defined by bone microstructure, bone turnover, and bone mineralization (18). To accurately evaluate the effects of cyclosporine and tacrolimus on bone metabolism, evaluation of both BMD and bone quality is necessary. This study used bone histomorphometry to evaluate the effects of cyclosporine and tacrolimus on bone quality. Cyclosporine and tacrolimus significantly reduced bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N), and significantly increased trabecular separation (Tb.Sp), showing that each calcineurin inhibitor causes rarefaction of the cancellous bone in the proximal tibia metaphysis. Cyclosporine significantly elevated both bone formation parameters and bone resorption parameters. Additionally, kinetic parameters were assessed by bone histomorphometry in this study. As a result, mineralizing surface per bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS) significantly increased in the cyclosporine-treated group. These kinetic parameters are considered to represent the mineralization, differentiation, and proliferation of osteoblasts (24). Likewise, the evaluation of serum bone metabolism markers showed a significant elevate in osteocalcin levels, a bone formation marker, and TRAP levels, a bone resorption marker, in the cyclosporine-treated group.
rine-treated group. Consistent with Goodman et al. (6), cyclosporine has been found to enhance both bone formation and bone resorption, leading to a high-turnover bone loss in which bone resorption exceeds bone formation. However, tacrolimus had no significant effect on bone formation abilities, as assessed by bone histomorphometry and evaluation of serum bone metabolism markers. Previous studies have shown that tacrolimus stimulates bone resorption by causing an imbalance of the receptor activator of nuclear factor kappa B (RANK)/RANK ligand/osteoprotegerin system (31) and inhibiting the extracellular signal-regulated kinases 1/2 pathway (8). Consistent with these findings, our study showed that bone resorption parameters in the tacrolimus-treated group significantly increased. Similarly, evaluation of serum bone metabolism markers showed that tacrolimus significantly elevated serum TRAP levels. These results showed that tacrolimus significantly promoted bone resorption without affecting bone formation, leading to bone loss. Our study showed significantly lower BMD of whole femur in the cyclosporine-treated group than in the tacrolimus-treated group, indicating that cyclosporine had more severe effects than tacrolimus did. In line with the results of this study, a previous prospective study has reported that cyclosporine induces more severe bone loss than tacrolimus does (16).

The limitations of this study are that the effects of each calcineurin inhibitor on bone metabolism have not been examined in a dose-dependent manner. However, previous studies have already shown that cyclosporine-induced bone loss depends on its dose and administration period (17).

In conclusion, this study demonstrated that daily administration of 15 mg/kg cyclosporine to rats for 4 weeks caused a high-turnover bone loss and reduced bone strength. In contrast, 1.5 mg/kg tacrolimus was demonstrated to increase bone resorption without significantly affecting bone formation, leading to decreased BMD and bone strength. Organ transplant patients usually require chronic immunosuppressive therapy after transplantation, and it is important to prevent the occurrence of osteoporotic fractures to maintain QOL in patients. Therefore, patients taking calcineurin inhibitors should be monitored for changes in BMD and bone metabolism markers to prevent the risk of bone fractures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

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