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Structural skeletal impairment induced by immunosuppressive therapy in rats: cyclosporine A vs tacrolimus

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Abstract The exact role of immunosuppressive drugs in the development of osteoporosis and pathologic fractures frequently reported in patients following organ transplantation is still not known. In two experiments, the effects of immunosuppressive drugs were studied on growing rats allocated randomly into five groups of eight rats each which received either FK506 (1.5 mg/kg or 3 mg/kg) or cyclosporine A (15 mg/kg or 30 mg/kg) for 28 days by daily oral gavage. In experiment I ($n=40$), bone mineral content (BMC) of the femur by dual energy X-ray absorptiometry (DXA), and bone ash weight were measured. In experiment II ($n=40$), stereologic measurements of decalcified tibiae were carried out. The BMC and ash weight values of the whole femur were significantly lower both in the low- and high-dose FK506 groups as well as in the high-dose CsA group. Decalcified sections showed lower volume density of trabecular bone of the tibial metaphysis in both CsA-treated groups and in the high-dose FK506 group. Furthermore, the volume density of the hypertrophic zone volume of the growth plate was higher in high-dose CsA-treated rats. Our data demonstrate that both CsA and FK506 have adverse effects on bone and that high doses of CsA or FK506 alter both cortical and trabecular bone with subsequent osteopenia. In addition, CsA-treated groups showed histological changes in some aspect resembling rickets/osteomalacia.

Keywords Bone density · Cyclosporine · Tacrolimus · Densitometry · X-ray · Growth plate · Rats

Introduction

The immunosuppressant drugs cyclosporine A (CsA) and tacrolimus (FK506) have dramatically improved graft survival after transplantation. A frequent complication reported in patients following kidney, liver, and heart transplantation is the development of osteopenia and pathologic fractures [8, 16, 22], and it has been suggested that the immunosuppressive therapy may be an important factor in the development of post-transplant bone disease [1, 2, 18]. However, the independent effects of CsA or FK506 on bone have been difficult to characterize in clinical studies, since transplant patients receive combined therapy with CsA or FK506 together with corticosteroids.

Both CsA and FK506 are potent immunosuppressive agents that exert their effects through inhibition of IL-2 release as well as T lymphocyte activation [8]. In experimental studies, the effect of CsA on bone metabolism has been studied extensively and revealed contra-

dictory results. In vitro, CsA has been shown to inhibit bone resorption mediated by parathyroid hormone, prostaglandin E, and 1, 25 dihydroxyvitamin D in fetal rat bone cultures [23]. Furthermore, some in vivo studies using CsA indicate regression of bone loss in adjuvant arthritic rats [6] and decreased bone resorption with increased bone formation [15]. Other studies have reported an increased bone turnover and development of severe osteopenia [13, 14]. Similar results of high turnover osteopenia have been reported in rats treated with FK506 [5, 10]. At the organ level, CsA therapy has been reported not to affect cortical bone [11].

The purpose of the present study was to examine and compare the effects of the immunosuppressants cyclosporine and FK506 on bone at the organ level by DXA of the femur and at the tissue level by stereologic analyses of the proximal metaphysis of the tibia.

Material and methods

Animals

Seven-week-old male Sprague-Dawley rats (40 rats for each experiment), each weighing approximately 350 g, were used in this study. All animals were housed under similar conditions at 21°C in a 12-h light/12-h dark cycle and maintained on a standard rat diet of 0.98% calcium, 0.75% phosphorus, 1500 IU/kg vitamin D3, and tap water ad libitum.

Drugs

Tacrolimus (FK506) in powder form, 20% w/w, kindly provided by Fujisawa, Munich, Germany, was dissolved in polyethylene glycol and diluted in distilled water to obtain a concentration of 1.5 mg/ml for oral administration.

The CsA (Sandimmun neoral, Novartis, Basel, Switzerland) in oral solution (100 mg/ml), kindly provided by Novartis AB Sweden, was diluted in distilled water to obtain a concentration of 15 mg/ml.

Experimental protocol

After acclimatization for 2 weeks, the rats were randomly allocated into five groups of eight rats each, and they received either FK506 (1.5 mg/kg or 3 mg/kg) or CsA (15 mg/kg or 30 mg/kg) daily by gavage for 28 days according to the following protocol: group A normal controls received distilled water (1 ml/kg), group B received FK506 (1.5 mg/kg), group C received FK506 (3.0 mg/kg), group D received CsA (15 mg/kg), and group E received CsA (30 mg/kg). On the day of killing, the animals were weighed and given a neuroleptic analgesia (Hypnorm, Leo, Helsingborg, Sweden). In experiment I, the anesthetized rats were killed by decapitation and both hindlimbs were disarticulated from the hip joint and preserved for subsequent densitometric analysis. In experiment II, the rats were anesthetized and tissues were fixed by vascular perfusion with 3% paraformaldehyde in 0.1 M sodium phosphate buffers, pH 7.4, for 5 min at 37°C. After fixation, the right tibial metaphyseal bone was dissected free and further fixed in the same fixative for 24 h and prepared for subsequent light microscopic stereology as detailed below.

Experimental procedures were reviewed and approved by the Regional Animal Research Ethics Committee.

Blood collection and analysis

Blood was collected at the time of sacrifice and serum was frozen and stored at –70°C until assayed. Serum calcium, phosphate, creatinine, urea, and alkaline phosphatase were determined by a photometric procedure using an automatic multichannel analyzer (Hitachi 917/ BM-regents, Hitachi, Naka, Japan). Serum immunoreactive parathyroid hormone (PTH) was measured by immunoradiometric assay (IRMA) using a commercially available kit specific for the rat (Nichols Institute Diagnostics, San Juan Capistrano, Calif., USA). This assay uses two different goat antibodies to the N-terminal region (1–34) of the rat PTH purified by affinity chromatography. Intra- and interassay coefficients of variation were 4.5% and 6.7%, respectively.

Densitometric analysis

The bone mineral content (BMC) and bone mineral density (BMD) of the intact left femur of each rat in experiment I was measured using a commercially available standard dual-energy X-ray densitometer (DXA, Lunar, Madison, Wis., USA). The femur, with all of its soft tissue, was positioned perpendicularly to the scan field in the anteroposterior position. The scan was started a few lines above the proximal end of the femur, and the end point of the scan was below the distal end of the femur. Bone scans and analyses were performed using the manufacturer's software version 1.3 for small animal measurements. The scan resolution (pixel size) was 0.3×0.6 mm, with a collimator diameter of 0.84 mm. The scan width was 40 mm and the length 45 mm. The scanning time per femur was 8–9 min. Additional to the whole femur, two regions of the femur (midshaft and distal metaphysis) were assigned for analysis to assess any regional differences in bone characteristics. For the femoral midshaft, a region of 18 lines (5.0 mm) at the midpoint of the length of the femur and for the distal metaphysis, a region of the same size located 0.5 cm proximally to the distal end of the femur, were analyzed (Fig. 1). The coefficients of variation (CV) for measurements of BMC and BMD of the femur were 2.6% and 1.2%, respectively, based on repeated measurements of six femurs with ten scans each.

Bone ash weight

After DXA measurements, each femur was dissected free of soft tissue, the bones were ashed in a muffle oven at 700°C for 24 h, and the ash weight per bone was determined.

Stereology

One tibia from each animal in experiment II was cut longitudinally and demineralized in a 4% EDTA solution, pH 7.4, for 4 weeks. Following demineralization, specimens were briefly rinsed in distilled water, dehydrated in ethanol, followed by acetone, and embedded in LX-112 (Ladd, Vt., USA). Semithin (approx. 0.8-μm) longitudinal sections were cut with a diamond knife and stained with toluidine blue, and the sections were examined by light microscopy. Micrographs were made at final magnifications of ×110 and ×275. The quantitative analyses were performed on printed copies by point counting [24] using a square lattice (with 2 cm or 4 cm between test lines) or by a semiautomatic interactive image analyzer (Videoplan, Zeiss, Oberkochen, Germany).

One overview micrograph (magnification ×110) covering the epiphyseal growth plate and the proximal metaphysis of one tibia in each animal, one micrograph (magnification ×275) covering the epiphyseal growth plate, and 2–3 micrographs (magnification ×275) covering the metaphysis were analyzed. The overview prints were

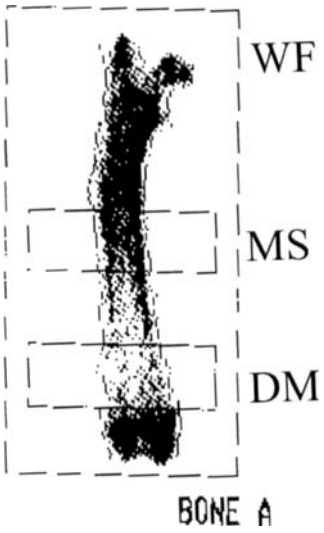

Fig. 1. Image of rat femur scanned with dual-energy X-ray densitometer (DXA) and indicating the regions selected for BMC analysis showing whole femur (*WF*), midshaft of the femur (*MS*), and distal metaphysis of the femur (*DM*)

used to estimate the volume density (Vv) of trabecular bone (relative volume of bone trabecula) in the proximal metaphysis. The compartment of interest was defined as the region between the distal end of the growth plate and 1.5 mm into the metaphysis proper (Fig. 2). The measurements were made by point counting according to stereological principles [24]. The higher power micrographs of the epiphyseal growth plate were used to measure the height of the plate and the Vv of the hypertrophic zone. The former was made by three random ruler measurements on one micrograph per animal, the latter by point counting.

The higher power micrographs of the metaphysis were used to estimate the Vv of osteoclasts and relate the results to the Vv of trabecular bone in a compartment defined as the region between the distal end of the growth plate and 0.5 mm into the metaphysis. In this way, a measure of the amount of osteoclasts relative to the amount of bone trabecula was obtained. This narrower region of the metaphysis ("primary spongiosa") was chosen for this correlative study because in some of the experimental groups the distal metaphysis was partly lost during the experimental period (Fig. 2). An osteoclast was defined as a multinucleated cell attached to a trabecular bone surface. Estimation of osteoclast volume density was performed by measuring the relative area of all osteoclast profiles on the micrographs of the metaphysis using the image analyzer (Videoplan). The measurements were performed in duplicate and the mean value calculated for each animal. Final results are given as mean values for all animals in the different groups.

For estimation of cortical bone volume, transverse sections were cut from the midtibial shaft. Sections were mounted on glass slides and micrographs were taken at ×20 magnification. The total tissue and marrow areas were measured with the Videoplan, and the percentage cortical bone was calculated by dividing the difference between total tissue volume and marrow volume with the total tissue volume.

All densitometric and stereologic measurements were performed on coded samples.

Statistical analysis

Data were expressed as means ± SEM. Nonparametric Mann–Whitney U test and analysis of variance (ANOVA) were used to test differences between normal controls and treated animals. Linear regression was used to correlate body weight and bone mineral content with other variables.

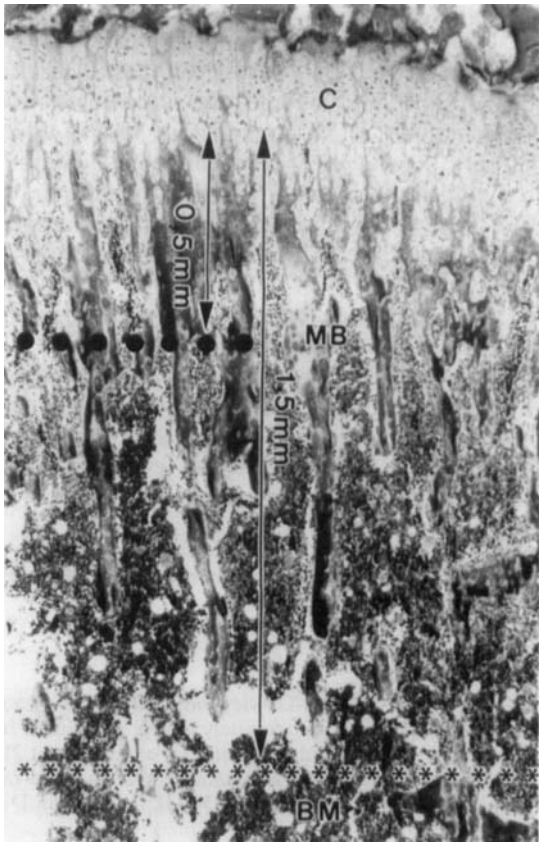

Fig. 2. Micrograph (magnification ×110) of a vertical section covering the epiphyseal growth plate and the proximal metaphysis of the tibia illustrating the two compartments of interest for calculation of trabecular bone volume density 0.5 mm and 1.5 mm from the distal end of the growth plate. *C* chondrocytes, *MB* metaphyseal bone, *BM* bone marrow

Results

Body weight

Body mass changes during the whole period of the study in relation to their baseline values for both experiments are depicted in Fig. 3. The weight gain in the control groups was 20% in experiment I and 28% in experiment II. A slower rate of weight gain was noted in rats treated with low-dose FK506 (6%, 22%) and low-dose CsA (5%, 14%). In rats treated with high-dose FK506, body weight was unchanged or slightly decreased in experiment I (–0.4%) and modestly increased in experiment II (14%). The weight decrease in the high-dose CsA groups was (–15%) in experiment 1 and (–2%) in the second experiment.

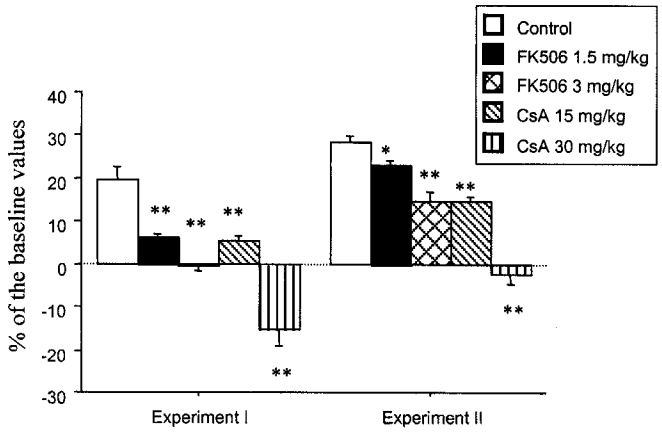

Fig. 3. Body mass changes in rats treated with FK506 (1.5 mg/kg, 3 mg/kg) and CsA (15 mg/kg, 30 mg/kg) compared to normal controls at the end of the study in percentage of their baseline values. $*P<0.05$, $**P<0.01$

Experiment I

Bone density

The changes in BMC of the whole femur, midshaft, and distal metaphysis in each group of rats were similar to those observed in BMD. Therefore, we report here only the changes in BMC. Compared to normal controls, rats treated with low and high doses of FK506 showed significantly lower BMC values of the whole femur (–11.1%, $P<0.05$ and –14.2%, $P<0.01$, respectively), midshaft (–12.9%, $P<0.05$ and –24.2%, $P<0.01$) (Fig. 4), and no changes were observed in the distal metaphysis. In rats treated with CsA, only BMC values of whole femur were lower in high-dose CsA (–11.5%, $P<0.05$) (Fig. 4). Ash weight of the femur showed a pattern similar to that observed for BMC. A positive correlation was found between femur BMC and femur ash weight ($r=0.822$, $P<0.0001$).

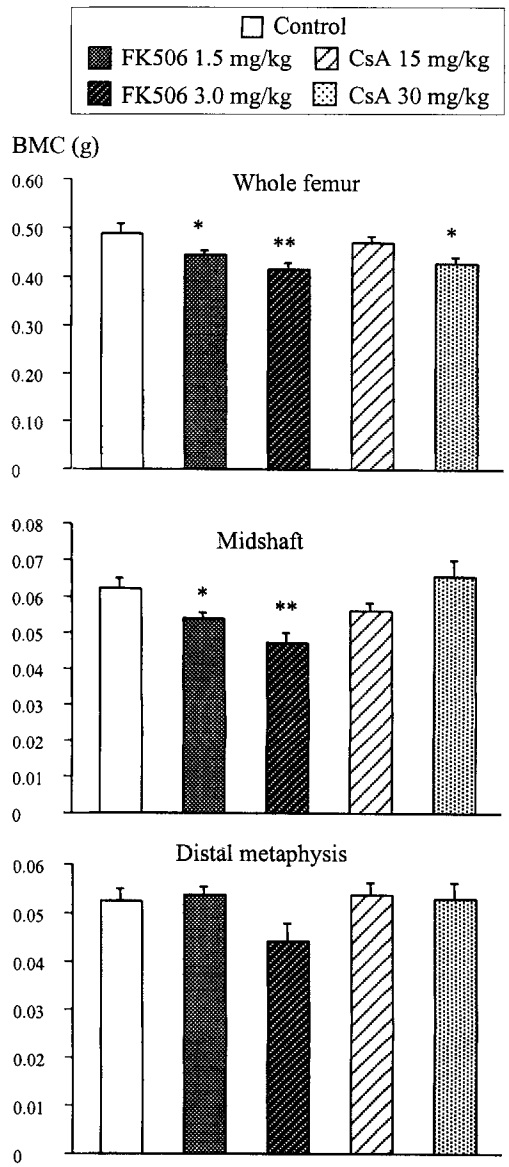

Fig. 4. Bone mineral content (*BMC*) of the whole femur, midshaft, and distal metaphysis of the femur measured by DXA in rats treated with FK506 (1.5 mg/kg, 3 mg/kg) and CsA (15 mg/kg, 30 mg/kg) compared to normal controls. $*P<0.05$, $**P<0.01$

Experiment II

Biochemical analysis

The results of blood analyses at the end of the study are shown in Table 1. Serum calcium, phosphate, and PTH levels were not significantly different in CsA- or FK506-treated groups than in controls. Blood urea was increased significantly in all four immunosuppressed groups, whereas serum creatinine was increased only in rats treated with cyclosporine. Serum alkaline phosphates (ALP) were normal or slightly increased in rats treated with FK506 but significantly increased by low-dose CsA ($P<0.01$).

Morphologic and stereologic studies

Volume density of trabecular bone in the proximal 1.5 mm of the metaphysis was significantly lower in low and high-dose CsA-treated groups (–39.1%, $P<0.01$ and –44.9%, $P<0.01$, respectively) and in the high dose FK506 group (–20.9%, $P<0.05$) (Table 2). In the primary spongiosa, the volume density of trabecular bone was lower only in CsA low- and high-dose groups (–17.7%, $P<0.01$ and –26.6%, $P<0.01$, respectively), but it was unchanged in the low- and high-dose FK506 groups in comparison with the controls (Table 2,

Table 1. Blood biochemistry in rats treated with FK506 and cyclosporine compared to normal control rats. Means ± SEM

	Control ($n=7$)	FK506 1.5 mg/kg ($n=8$)	FK506 3.0 mg/kg ($n=7$)	Cyclosporine 15 mg/kg ($n=8$)	Cyclosporine 30 mg/kg ($n=8$)
Serum PTH (ng/L)	108 ± 18	119 ± 23	60 ± 5	123 ± 24	84 ± 8
Serum calcium (mmol/L)	2.55 ± 0.06	2.64 ± 0.07	2.65 ± 0.09	2.77 ± 0.08*	2.43 ± 0.05
Serum phosphate (mmol/L)	2.8 ± 0.3	3.1 ± 0.5	2.8 ± 0.2	3.2 ± 0.4	2.7 ± 0.2
Serum urea (mmol/L)	5.1 ± 0.3	6.7 ± 0.3**	7.0 ± 0.6**	11.0 ± 0.55***	10.5 ± 1.0**
Serum creatinine (mmol/L)	60 ± 2	63 ± 3	55 ± 3	80 ± 7*	74 ± 4*
Serum alkaline phosphates (mkat/L)	3.60 ± 0.19	4.06 ± 0.17	5.09 ± 0.65	5.54 ± 0.57**	4.30 ± 0.69

*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control

Table 2. Stereologic analyses of decalcified tibial bone in rats treated with FK506 and CsA compared to normal controls. Means ± SEM. *Vv* volume density

	Control ($n=7$)	FK506 1.5 mg/kg ($n=8$)	FK506 3 mg/kg ($n=7$)	Cyclosporine 15 mg/kg ($n=8$)	Cyclosporine 30 mg/kg ($n=8$)
Growth plate height (mm)	283.39 ± 8.01	303.65 ± 6.48	273.78 ± 6.31	265.93 ± 8.75	291.61 ± 9.97
Hypertrophic zone Vv (%)	36.97 ± 1.32	38.16 ± 1.19	36.69 ± 1.73	38.68 ± 0.65	42.47 ± 1.22*
Trabecular bone Vv (%)[a]	54.00 ± 1.96	57.17 ± 1.70	56.22 ± 2.53	44.42 ± 2.58**	39.62 ± 2.62**
Trabecular bone Vv (%)[b]	49.33 ± 2.93	45.70 ± 2.92	39.00 ± 2.59*	29.51 ± 2.28**	27.13 ± 3.21**
Vv of osteoclasts on trabecular bone (%)	3.19 ± 0.48	2.48 ± 0.48	4.43 ± 0.48	3.80 ± 0.70	7.09 ± 0.92**
Cortical bone Vv (%)	64.32 ± 1.25	63.81 ± 2.38	65.26 ± 1.83	67.04 ± 1.31	64.51 ± 3.39

Trabecular bone volume density in the primary spongiosa defined as the region between the distal end of the growth plate and 0.5 mm[a] or 1.5 mm[b] into the metaphysis, *$P<0.05$, **$P<0.01$ vs control

Fig. 5). Cortical bone volume of the tibial shaft in rats treated with CsA or FK506 was not significantly different than in normal controls. Growth plate height was similar for control animals and CsA- or FK506-treated animals. High-dose CsA was associated with increased volume density of the hypertrophic zone ($P<0.05$) and volume density of osteoclasts on trabecular bone compared to control animals ($P<0.01$) (Table 2).

Discussion

In the present study, we investigated and compared the effects of two different doses of the immunosuppressive drugs CsA and FK506 on bone mass and structure as evaluated by densitometry and stereology.

Bone densitometric analysis of the whole femur demonstrated that FK506 as well as CsA caused reduced bone density, the effect being most pronounced in rats treated with FK506. In line with these results were the observations of decreasing bone ash weight. The whole femur contains approximately 80% cortical bone and 20% trabecular bone [11]. The DXA results of femur bone density therefore illustrate mainly cortical bone mass changes. Furthermore, BMC values of the femoral midshaft, a region composed mainly of cortical bone, were only lower in FK506-treated animals. To our knowledge, there is no previous densitometric report on the effect of FK506 on rat bone. Here we report a dose-dependent effect of FK506 on cortical bone at the organ level (whole and midshaft of the femur). Our findings of a less negative effect of CsA on cortical bone are in agreement with Klein et al. [11], who reported only a limited effect of CsA on cortical bone resorption. Del Pozo et al. [7] have measured total skeletal BMC with DXA in young and adult rats receiving CsA doses of 5 mg/kg to 30 mg/kg. They reported that total body BMC was not affected by a CsA dose up to 15 mg/kg, while a CsA dose of 30 mg/kg resulted in marked bone resorption and subsequent osteopenia. This is in accordance with our study, in which we also found a decreased BMC in high-dose CsA-treated animals. We found no significant differences in BMC values between treated rats and controls at the distal femoral metaphysis. This could be explained by the mixed structure of cortical and trabecular bone composed in this region, whereas DXA

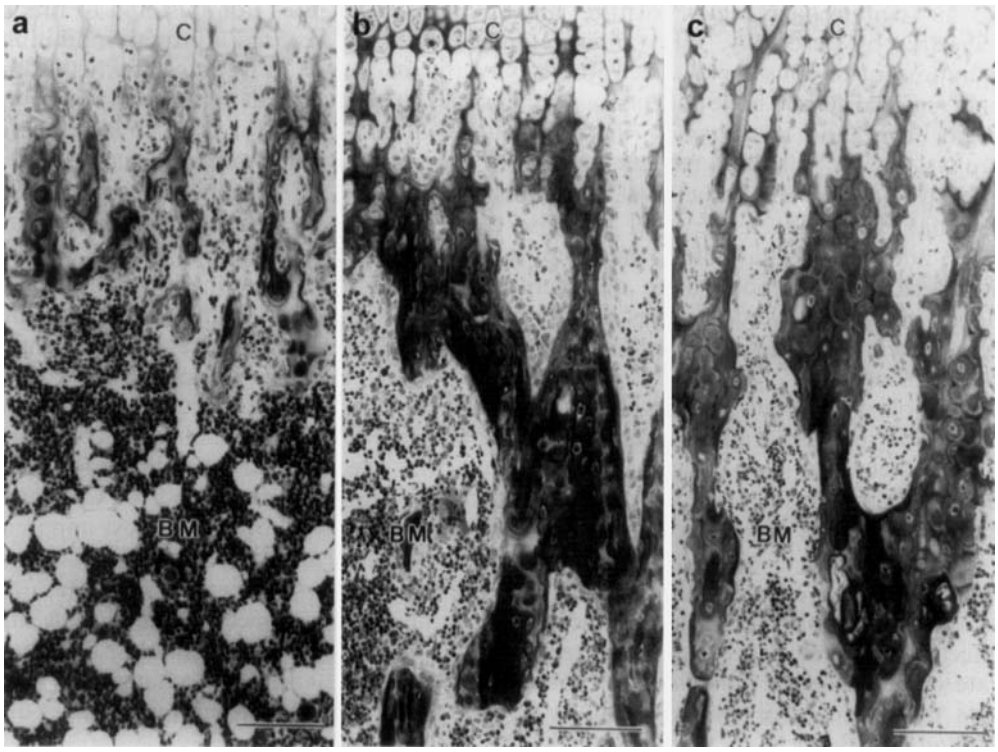

Fig. 5a–c. Micrographs (magnification ×275) of vertical sections covering the proximal tibial metaphysis in rat treated with (**a**) CsA 30 mg/kg, (**b**) FK506 3 mg/kg, and (**c**) distilled water (control rat). *C* chondrocytes, *BM* bone marrow. *Bar* = 100 μm

is an integrated measure that includes some cortical bone [20]. Furthermore, the lack of internal bone landmarks at this region precluded equivalent regional analysis between groups [21]. Unfortunately, we did not measure bone density at the lumbar spine, a region enriched with trabecular bone, and consequently no new hypotheses were formulated regarding a possible preferential effect of FK506 or CsA on cortical and trabecular bone, respectively. Such issues may be elucidated by densitometric analysis of both the femur (predominantly cortical bone) and the lumbar spine (predominantly trabecular bone) from the same animal.

One interesting finding in our study was that the growth plates of the rats treated with high dose CsA exhibited expanded hypertrophic zones, to some extent mimicking rickets. We did not measure vitamin D metabolites, but previous studies have shown increased or unaltered vitamin D metabolism in rats treated with CsA [13]. The current histologic changes were not associated with significant differences in serum calcium, phosphate, and PTH values, but with a slight elevation of serum alkaline phosphates in low-dose CsA-treated rats. It has been observed that rachitic changes induced by strontium occur without altered serum phosphate levels [17]. Thus, our observation could raise the question whether CsA exerts a direct toxic action on chondrocytes interfering with the local mineral metabolism.

Furthermore, the stereologic analyses of the tibia showed that administration of CsA to rats disturbs local skeletal metabolism at the epiphyseal/metaphyseal border, as observed with the markedly lower trabecular bone volume density of the primary spongiosa along with increased volume density of osteoclasts in rats treated with CsA than in controls. When extending our measurements distally into the secondary spongiosa of tibial metaphysis, the tendency was the same, with lower trabecular bone volume density in CsA-treated rats. These findings are in line with recent studies which have shown that CsA in doses between 7.5 mg/kg and 15 mg/kg induces a high bone turnover osteopenia of the proximal tibial metaphysis [4, 12, 19]. Erben et al. [9] also reported a high turnover osteopenia in the axial skeleton of aged rats treated with low-dose CsA. On the other hand, FK506 in doses of 3 mg/kg showed a modest effect on trabecular bone resorption, and these results differ from those describing marked decreased trabecular bone volume in comparison to CsA [5]. In a study performed by Katz et al. [10] using the same dosage and route of drugs administration as we did (1.5 mg/kg FK506 and 15 mg/kg cyclosporine), these authors reported a similar effect of both drugs on trabecular bone volume. The discrepancies between our findings and the reported marked effect of FK506 on trabecular bone could probably be related to the higher dose of FK506 (4–5 mg/kg) used by others [5, 19].

It is somewhat surprising that the lower cortical bone mineral density detected by DXA measurement of the femur shaft in FK506-treated rats was not observed

at the counterpart region of the tibial shaft when measured by stereology. When interpreting our data, it is important to consider that densitometry of the whole and regional femur bone reflects mainly changes at the organ level, and histology of the proximal tibial metaphysis and tibial shaft reflects changes in bone mass and structure at the tissue level. Attempts to extrapolate data from the tissue level to those at the organ level and vice versa could therefore be misleading. Furthermore, metabolic bone disorders are heterogeneous conditions, and changes at one specific site or type of bone may not reflect changes occurring in other sites or bones.

The observed bone changes in our study are unlikely to be due to altered renal function, since serum creatinine was elevated only in cyclosporine-treated rats. The high levels of serum urea observed in rats treated with cyclosporine or FK506 may be of prerenal origin and could be related in part to the catabolic state (muscle wasting) generated by these drugs. One of the possible factors that could be related to the growth inhibitory effect of immunosuppressive drugs relates to the slower rate of body weight gain that were observed in treated rats than in control animals. This is in line with the findings of Bennett et al. [3], who demonstrated that CsA administered to rats in doses higher than 5 mg/kg exerted a growth-inhibiting effect.

It is not clear whether the lower bone density and ash weight observed in immunosuppressed rats are the results of bone loss and/or a failure to gain bone at the same rate as the controls. In our protocol, measurements of baseline controls were not included, although such information may have contributed to the understanding of tissue dynamics. However, since the rate of bone formation in growing rats is greater than the rate of bone resorption, we assume that the net deficit in bone mass in treated rats compared with the control animals at the end of the experiment is due to the general effects of these drugs on growth and development [11].

In conclusion, combining the results of bone densitometry and histology provide evidence of the dose-dependent effect of CsA and FK506 on bone. Although osteopenia is most apparent in rats treated with FK506, both osteopenia and disturbed growth cartilage maturation were noted after administration of CsA. The exact mechanism of CsA and FK506 action on bone is, however, not clear, but the low rates of body weight gain and bone changes in treated rats indicate a general growth inhibitory effect of these drugs.

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