Effects of Methylprednisolone on Bone Formation and Resorption in Rats

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ABSTRACT—Excessive glucocorticoids induce osteoporosis. However, there is some controversy regarding the mechanism of action, and even the endpoint result. The present study was carried out to obtain further insight into the action of glucocorticoids on bone formation and resorption in rats. Growing rats were injected subcutaneously with methylprednisolone (mPSL) at doses of 0, 2.5, 5, 10 or 20 mg/kg per day for 4 weeks. Bone mineral density (BMD), enchondral and periosteal bone formation, collagen synthetic activities of osteoblasts, numbers of osteoblasts and osteoclasts, and serum markers to assess bone turnover were determined. Administration of mPSL dose-dependently increased the BMD in the tibial metaphysis, while it dose-dependently decreased the BMD in the diaphysis. Both enchondral and periosteal bone formation were decreased in a dose-dependent fashion. The incorporation and secretion of ³H-proline by osteoblasts were both decreased in trabecular and cortical bones. The number of osteoclasts, together with the number of osteoblasts, in the tibial metaphysis was drastically decreased. Serum alkaline phosphatase and osteocalcin were decreased at higher doses. These results support the recent notion that glucocorticoids inhibit both bone formation and resorption. In addition, BMD as an endpoint result might differ from site to site in bone due to a different balance between bone formation and resorption.

Keywords: Methylprednisolone, Osteoblast, Osteoclast, Bone formation, Bone resorption

It has been documented that prolonged exposure to glucocorticoids (GCs) at supraphysiological doses induces osteoporosis associated with an increased risk of bone fracture (1–3). The mechanism of action has been proposed to be decreased osteoblast differentiation and matrix-synthesis (4–7), increased bone resorption (8–12) resulting from hyperparathyroidism due to decreased intestinal calcium absorption (13–19), and decreased secretion of sex-steroids including estrogen (20, 21).

However, the results of *in vivo* studies in man and animals, and even endpoint results on bone mass and bone mineral density (BMD), are inconsistent; high dose and/or long-term GC therapy causes rapid bone resorption and results in a decrease in BMD (22, 23), whereas a recent animal study demonstrated that, in contrast to the findings in man, GCs treatment increased bone mass in rats, which might be related to a relatively greater suppression of

bone resorption than of bone formation (24–26). Thus, comparisons of the results of previous studies are complicated by differences in steroid formulation, the dose and duration of administration, and the age and strain of the animals. These discrepancies might be resolved by obtaining basic data on the dose-dependent effects of GCs on bone formation and resorption.

The purpose of the present study was to obtain further insight into the effects of methylprednisolone (mPSL), one of the most widely used corticosteroids which has almost no mineralcorticoid effects, on bone metabolism in rats. Generally, we examined the effects of a 4-week treatment with mPSL on BMD, bone mass, enchondral and periosteal bone formation, collagen synthetic and secretory activities of osteoblasts, number of osteoclasts and serum markers of bone turnover. Our present data show that sustained treatment with mPSL in the rat inhibits both bone formation and resorption by decreasing the number and function of both osteoblasts and osteoclasts. Furthermore, BMD as an endpoint effect differed among the sites observed; BMD in

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the metaphyseal trabecular bone increased, while that in the diaphysis (cortical bone) decreased. These results are discussed in the light of current knowledge regarding bone metabolism and the effects of GCs on bone.

MATERIALS AND METHODS

Experiment 1

Animals and experimental protocol

Forty-five 5-week-old male Wistar rats (Japan SLC Co., Shizuoka), with a mean body weight of 101 ± 5 g (S.D.), were divided into 5 groups. The animals in each group were given either 0 (0.9% NaCl), 2.5, 5, 10 or 20 mg/kg of 6-methylprednisolone (Sigma Chemical Co., St. Louis, MO, USA) by daily subcutaneous injection at around 1600 h for 4 weeks: control, mPSL-2.5, mPSL-5, mPSL-10 and mPSL-20 groups, respectively. Each group consisted of 10 animals, except the mPSL-20 group that consisted of 5 animals. All animals received subcutaneous injection of nitrilotriacetato lead solution (NTA-Pb, 4 mg Pb/kg) on days, 15, 22 and 27 to chronologically label the growing bones. During the experimental period, the animals were maintained on a 12 h/12 h light-dark illumination cycle at $24.0 \pm 2.5^\circ\text{C}$ and $55 \pm 5\%$ humidity, and they were allowed free access to food (Laboratory Chow F-2; Funabashi Farm Co., Chiba) and de-ionized water ad libitum. Food consumption and body weight were measured daily.

The animals were treated ethically in compliance with the regulations of Tohoku University School of Medicine and Dentistry, whose guide for the use of experimental animals is based on the Principles of Laboratory Animal Care from NIH.

Sampling of blood and bones

On day 29, the rats were anesthetized with ether, and blood samples were obtained by heart puncture. The serum was then collected and stored at -40°C until use. After the animals were sacrificed, the tibiae, mandibulae and adrenals on both sides were dissected and immediately fixed in Karnovsky solution.

Determination of bone mineral density in tibial metaphysis and diaphysis

After removing adherent soft tissues, the right tibia was used to determine BMD according to the method previously described by Shinoda and co-workers (27, 28). Briefly, soft X-ray microradiographs of the tibia were taken together with a standard step-wedge made of synthetic hydroxyapatite. All of the microradiographic images of the target area were then fed to an image analyzer through a video camera. Since there is a linear relationship between the gray level in microradiographic images of the step-

Fig. 1. Objective areas for the determination of BMD in the tibial metaphysis and diaphysis.

wedge and the logarithm of the hydroxyapatite content $/\text{mm}^2$, the BMD in the target bone area could be determined by analyzing the gray levels in microradiographic images. The objective bone areas in the tibia in the present study were the proximal metaphysis and middle diaphysis, as shown in Fig. 1.

Measurement of bone formation rate

Preparation of specimens: After taking soft X-ray microradiographs to determine BMD, right tibiae and mandibulae were decalcified for 5 days with 10% formic acid solution saturated with H_2S gas. The saturation with H_2S turns Pb that has been deposited in the bone tissue to less-soluble PbS , which stays in situ in the demineralized bone matrix. The specimens were then infiltrated sequentially with 10%, 20% and 30% gelatin and embedded in 30% gelatin. The gelatin blocks were immersed in 10% neutral formalin in a refrigerator for hardening. After washing with tap water, the blocks of tibia and mandibular bone were mounted on a cryostat (OFT/AS; Bright Instrument Co., Huntingdon, UK). Mid-sagittal longitudinal sections of the tibial metaphysis, about $15\text{-}\mu\text{m}$ -thick, were prepared, and cross-sections of mandibula were prepared from the portion between the 2nd and 3rd molars. They were dipped in 1% chloroauric acid solution until dark purple Pb bands emerged, rinsed in distilled water, and treated with 5% $\text{Na}_2\text{S}_2\text{O}_3$ to tone the bands. The sections were then stained with Carazzi's hematoxylin, rinsed, and coverslipped with glycerin gel.

Measurement of longitudinal trabecular bone formation in the tibial metaphysis and appositional bone formation in the tibial diaphysis: Longitudinal bone formation for the last 2 days in the proximal end of the tibia was determined by measuring the distance between the epiphyseal borderline of the growth cartilage and the Pb label (marked on day 27) in the metaphysis. Using transverse sections of the tibial diaphysis, the appositional bone growth for 2 weeks (from day 15 to day 29) was measured in the most frontal cortical bone by determining the distance between the most internal Pb label (injected on day 15) and the bone surface.

All of the measurements were performed using an image analyzer connected to a light microscope (Axiophoto; Carl Zeiss, Frankfurt, Germany) at an original magnification of 200.

Measurement of appositional bone formation in the mandibular cortical bone: Using transverse mandibular sections, the appositional bone formation for 2 weeks (from day 15 to day 29) was measured in the cortical bone at the lateral basis of the mandible by determining the distance between the most internal Pb label (marked on day 15) and the bone surface, since the most active cortical bone formation was observed in this region in cross-sections (Fig. 2a). The distance was determined at 3 sites that were about 50, 150 and 250 μm apart from the lateral corners, respectively (Fig. 2b). The mean of the 3 values was then calculated.

Count of osteoclast number in the tibial metaphysis

The left tibia was decalcified with 10% EDTA-2Na (pH 7.4) for 4 weeks at 4°C, dehydrated in a graded series of ethanol and xylene, and embedded in paraffin. Mid-

sagittal longitudinal sections, 5- μm -thick, were prepared from the proximal tibial metaphysis. The sections were then stained by tartrate-resistant acid phosphatase (TRAP) (29) to identify osteoclasts and counter-stained with hematoxylin or by the Azan method. The number of osteoclasts in a target metaphyseal trabecular bone area, as shown in Fig. 3 (region A), was counted under a light microscope (Axiophoto, Carl Zeiss) at an original magnification of 200. TRAP-positive cells adjacent to the bone surface that contained more than 3 nuclei were considered osteoclasts.

Determination of serum parameters

Calcium (Ca), phosphorus (Pi), alkaline-phosphatase (ALP) activity and TRAP activity in the serum were determined by the OCPC method (Calcium-C test-Wako; Wako Pure Chemical Industries, Ltd., Osaka), Chen's method (30), and the Bessey-Lowry method for alkaline and acid phosphatase (Alkaline-Phospha-B test-Wako and Acid-Phospha-B test-Wako, Wako Pure Chemical). Serum pyridinoline cross links (Pyd) and osteocalcin (OC) were measured using an EIA kit (EIA Kit for Serum Pyd; Metra Biosystems, Inc., Mountain View, CA, USA and an ELISA kit for Osteocalcin, rat ELISA system; Amersham Pharmacia Biotech, Buckinghamshire, UK), respectively.

Experiment 2

Twelve 5-week-old male Wistar rats (Japan SLC Co.), with a mean body weight of 96 ± 4 g (S.D.), were divided into 2 groups. The animals in one group were given 0.9% NaCl, and those in the other group were given 10 mg/kg of mPSL by subcutaneous injection every day for 4 weeks.

Fig. 2. Determination of appositional bone formation in the cortical bone at the lateral base of the mandible. Cross sections of mandibula were prepared from the portion between the 2nd and 3rd molars. A: The determination was performed in the lateral base of the mandible enclosed by the rectangle. B: Distance between the most internal Pb label (marked on day 15) and the bone surface was determined at 3 sites (a, b and c) that were about 50-, 150- and 250- μm apart from the lateral corners. The mean of the 3 measurements was taken.

Fig. 3. Metaphyseal bone in the proximal tibia in rats. Region A: central region ($0.3 \times 1.0 \text{ mm}^2$) between the two cortices, subjacent to the epiphyseal growth plate within the trabecular bone. The numbers of osteoclasts per bone area and per trabecular bone surface were counted in this region. Region B: central region ($0.5 \times 0.5 \text{ mm}^2$) between the two cortices and 5-mm distal from the epiphyseal borderline. The number of adipocytes was counted in this region.

During the experiment, animals were maintained under the same conditions as in Experiment 1. On day 29, all of the rats were injected intraperitoneally with 37 MBq (1.0 mCi)/kg of L-(2,3- ^3H) proline (specific activity: 1.78 TBq (48 Ci)/mmol, Amersham). One hour after the injection, the rats were anesthetized with pentobarbital, and 4% paraformaldehyde solution was perfused through the heart to fix the tissues. The tibiae and mandibulae were dissected and further fixed in the same paraformaldehyde solution.

Radioautography

The tibia and mandible were freed from adherent soft tissues and decalcified with 10% EDTA-2Na (pH 7.4) for 4 weeks. After dehydration, they were embedded in paraffin, and mid-sagittal longitudinal sections, 5- μm -thick, were prepared from the proximal tibial metaphysis. In addition, cross-sections of the mandible, with the same thickness as above, were also prepared from the portion between the 2nd and 3rd molars. They were mounted for radioautography on glass slides, coated with NR-M2 emulsion (Konica Co., Tokyo), and exposed at 4°C in a light-tight box containing silica gel. Four weeks later, the emulsion-coated sections were developed for 5 min at 20°C with Konicadol X (Konica Co.), fixed for 5 min at 20°C with Konifix (Konica Co.), and stained lightly with hematoxylin.

Using these radioautographs, we counted the number of silver grains over the osteoblasts and over the bone matrix in the tibial metaphysis or mandibular cortical bone to

quantify the incorporation and secretion of ^3H -proline by osteoblasts. Furthermore, the ratio of the number of grains over the bone matrix to the number of grains over osteoblasts (BM/OB ratio) was calculated as an index of the matrix secretory activity of osteoblasts.

Concerning the tibial metaphysis, grain counts were performed at every other 50 μm (Fig. 4) along either the frontal or posterior surface of one trabecular bone from the epiphyseal borderline (region 1) to the distal end (regions 2 to 10).

Concerning the periosteal surface of mandibular cortical bone, grain counts were performed on 200 μm of bone surface of the lateral basis of the mandible, which is the same region where appositional bone formation was determined (Fig. 2b). All of the grain counts were carried out under a light microscope at a magnification of 1,000.

Number of osteoblasts on the trabecular and cortical bone surface and the number of adipocytes in the tibial bone marrow

Using histological sections for autoradiography, the number of osteoblasts on the bone surface was counted at every other 50 μm along one trabecular bone from the epiphyseal borderline to the distal end (Fig. 4) and at 200 μm on the bone surface in the mandible, where autoradiographic grain counts were performed (Fig. 2b).

The number of adipocytes was also counted in the tibial bone marrow. The sampling area for the adipocyte count was a square (500 $\times$ 500 μm) 5-mm distal from the antero-

Fig. 4. Determination of the number of radioautographic silver grains over osteoblasts and over the bone matrix in the trabecular bone of the tibial metaphysis. Grain counts were performed at every other 50 μm along either the frontal or posterior bone surface of one trabecular bone from the epiphyseal borderline (region 1) to the distal end (regions 2–10). A: Schematic representation of the objective areas for grain counts on the trabecular bone surface. B: Schematic representation of ^3H -proline radioautograph showing silver grains over osteoblasts and over the bone matrix. OB, osteoblast.

posterior midpoint on the borderline of the epiphyseal growth plate (Fig. 3, region B).

Statistical analyses

The significance of differences between mean values was evaluated by one- or two-way ANOVA, followed by Tukey's multiple comparison test. $P < 0.05$ was considered to represent a significant difference.

RESULTS

Experiment 1

Body weight, food consumption and adrenal weight

Throughout the experimental period, no significant differences in mean body weight or food consumption were observed among the groups (two-way ANOVA, $P > 0.05$; data not shown), whereas adrenal weight decreased as the dose of mPSL increased (Table 1). This decrease was significantly lower in the mPSL-5, -10 and -20 groups than in the control group ($P < 0.05$, by one-way ANOVA followed by Tukey's multiple comparison test).

Soft X-ray microradiographic observation of tibial metaphysis and diaphysis

Figure 5 shows soft X-ray microradiographic images of the tibial metaphysis (left panels) and diaphysis (right panels) in the 5 groups. Administration of mPSL decreased the radiolucency of the metaphysis in a dose-dependent fashion, which might be due to an increase in the density of trabecular bone. In contrast to the results in the metaphysis, the radiolucency of the diaphysis increased as the dose of mPSL increased.

BMD in the tibial metaphysis and diaphysis

BMD in the tibial metaphysis increased as the dose of mPSL increased (Table 2). The mean values in the mPSL-10 and -20 groups were significantly higher than that in the control group ($P < 0.05$, one-way ANOVA followed by

Fig. 5. Soft X-ray microradiographs of tibial metaphyses and diaphyses in rats treated with mPSL at different doses. a: control group; b, c, d and e: mPSL-2.5, -5, -10 and -20 groups, respectively. The administration of mPSL dose-dependently decreased the radiolucency in the proximal metaphysis (left panels), while it dose-dependently increased the radiolucency in the diaphysis (right panels).

Table 1. Adrenal weight

	Control (n = 10)	mPSL-2.5 (n = 10)	mPSL-5 (n = 10)	mPSL-10 (n = 10)	mPSL-20 (n = 5)
Right side (mg)	16.2 ± 3.0	15.0 ± 1.5	13.6 ± 1.7**	14.3 ± 1.9*	13.2 ± 0.9**
Left side (mg)	18.2 ± 2.8	16.6 ± 1.5	15.3 ± 2.1	15.1 ± 1.9*	14.7 ± 1.3*

Mean ± S.D. * $P < 0.05$, ** $P < 0.01$ (vs control, one-way ANOVA followed by Tukey's multiple comparison test).

Table 2. BMD in tibial metaphysis and diaphysis

	Control (n = 10)	mPSL-2.5 (n = 10)	mPSL-5 (n = 10)	mPSL-10 (n = 10)	mPSL-20 (n = 5)
Metaphysis ($\mu\text{gHA}/\text{mm}^2$)	609 $\pm$ 280	637 $\pm$ 20	633 $\pm$ 23	645 $\pm$ 35*	668 $\pm$ 29*
Diaphysis ($\mu\text{gHA}/\text{mm}^2$)	551 $\pm$ 35	530 $\pm$ 26	528 $\pm$ 18*	521 $\pm$ 15*	519 $\pm$ 14*

Metaphysis: proximal metaphysis in tibia, Diaphysis: middle diaphysis in tibia, HA: hydroxyapatite. Mean $\pm$ S.D.

* $P < 0.05$ (vs control, one-way ANOVA followed by Tukey's multiple comparison test).

Table 3. Bone formation in the tibial metaphysis, tibial diaphysis and the cortex in the mandibula

	Control (n = 10)	mPSL-2.5 (n = 10)	mPSL-5 (n = 10)	mPSL-10 (n = 10)	mPSL-20 (n = 5)
Metaphysis ^a (μm)	290 $\pm$ 36	248 $\pm$ 22*	223 $\pm$ 21**	199 $\pm$ 18**	194 $\pm$ 23**
Diaphysis ^b (μm)	92 $\pm$ 15	87 $\pm$ 16	91 $\pm$ 18	84 $\pm$ 15	79 $\pm$ 10
Cortical bone ^c (μm)	118 $\pm$ 20	99 $\pm$ 12	102 $\pm$ 13	96 $\pm$ 15*	87 $\pm$ 16**

^aLongitudinal formation of trabecular bone for 2 days in the tibial metaphysis. ^bAppositional formation for 14 days in the cortical bone in the middle diaphysis in tibia. ^c Appositional formation for 14 days in the cortical bone at the lateral basis of the mandibula (see Fig. 2). Mean $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$ (vs control, one-way ANOVA followed by Tukey's multiple comparison test).

Tukey's multiple comparison test). The value in the mPSL-20 group was about 10% higher than that in the control group. In contrast to the results in the tibial metaphysis, the mean BMD in the tibial diaphysis dose-dependently decreased with the administration of mPSL. The value in the mPSL-20 group was about 6% lower than that in the control group.

Bone formation rate

The longitudinal formation of trabecular bone in the tibial metaphysis for the last 2 days and the appositional formation of cortical bone in the mandible for the last 2 weeks are shown in Table 3. In both the tibial metaphysis and cortical mandibular bone, the administration of mPSL dose-dependently inhibited bone formation. These inhibitions were about 35% for the tibial metaphysis and about 25% for the cortex of the mandible. The appositional formation of cortical bone in the tibial diaphysis tended to decrease, but there were no significant differences between the means in the different groups.

Histological observation

Figure 6 shows histological sections of the tibial metaphysis in the control group and the mPSL-20 group. The

Fig. 6. Tibial metaphyses in the control (A) and mPSL-20 (B) groups. TRAP and Azan staining. Arrows: TRAP-positive osteoclasts.

Fig. 7. Adipocytes in the bone marrow of tibial diaphyses in the control (A) and mPSL-20 (B) groups. Hematoxylin staining. Arrows: Adipocytes.

administration of mPSL induced a marked elongation of trabecular bone in the distal direction, which was thought to be due to the inhibition of bone resorption in the distal end of trabecular bone by osteoclasts. Osteoclasts were usually most densely populated around the distal ends of trabecular bone and near the epiphyseal borderline in control animals, whereas in the present mPSL-treated animals, the number of TRAP-positive osteoclasts in these areas was drastically decreased. Trabecular bones with flat and straight surfaces and with fewer resorption lacunae were also prominent. In addition to these changes, the number of multi-layered cuboidal osteoblasts, which were densely populated on the surface of primary trabeculae in control animals, appeared to have decreased in mPSL-treated animals. Furthermore, a marked increase in the number of adipocytes in the diaphyseal bone marrow was evident (Fig. 7).

Number of osteoclasts in the tibial metaphysis (Table 4)

The administration of mPSL increased the trabecular bone mass in the objective metaphyseal area in a dose-dependent fashion. On the other hand, the administration of mPSL decreased the total number of osteoclasts, as well as the number of osteoclasts both per bone surface and per bone area in the tibial metaphysis in a dose-dependent fashion. The total number of osteoclasts in the mPSL-20 group was about 47% of that in the control. The numbers of osteoclasts per bone surface and bone area in the mPSL-

20 group were about 36% and 38% of those in the control, respectively.

Serum parameters

The means of the serum parameters in the different groups are shown in Table 5. There were no statistically significant differences in the mean values of Ca, Pi, TRACP and Pyd among the groups, whereas significant decreases were observed in ALP and OC in the mPSL-10 and -20 groups compared with those in the control group.

Experiment 2

Radioautographic observation of the incorporation and secretion of ^3H -proline by osteoblasts

Tibial metaphysis (Fig. 8): Figure 8a shows the number of grains over the bone matrix secreted by osteoblasts, and the total number of silver grains within a distance of 50 μm (Fig. 8b) (number of grains over osteoblasts plus those of over the bone matrix), which reflects the incorporation of ^3H -proline by osteoblasts, was measured at every other 50 μm along the surface of a trabecular bone from the epiphyseal borderline (region 1) to the distal end (regions 2 to 10). In the control group, both the number of silver grains over the bone matrix and the total number of silver grains gradually decreased from region 1 to 4 (which might correspond to primary spongiosa), then began to increase until region 6, and finally gradually decreased thereafter.

Table 4. Bone area, total number of osteoclasts, number of osteoclasts per bone surface and number of osteoclasts per bone area in the tibial metaphysis

	Control (n = 10)	mPSL-2.5 (n = 10)	mPSL-5 (n = 10)	mPSL-10 (n = 10)	mPSL-20 (n = 5)
Total number	18.2 $\pm$ 4.9	15.0 $\pm$ 5.1	15.2 $\pm$ 3.0	8.0 $\pm$ 4.6**	7.4 $\pm$ 3.1**
OC/Bs	4.7 $\pm$ 1.8	3.2 $\pm$ 1.4	3.2 $\pm$ 0.8	1.4 $\pm$ 0.7**	1.8 $\pm$ 1.1**
OC/Ba	170 $\pm$ 33	138 $\pm$ 56	121 $\pm$ 34	64 $\pm$ 33**	65 $\pm$ 24**

All measurements were carried out in region A in Fig. 3. Total number: total number of osteoclasts, OC/Bs: number of osteoclasts per bone surface (mm), OC/Ba: number of osteoclasts per bone area (mm²). Mean $\pm$ S.D. ** P <0.01 (vs control, one-way ANOVA followed by Tukey's multiple comparison test).

Table 5. Biochemical parameters in serum

	Control (n = 10)	mPSL-2.5 (n = 10)	mPSL-5 (n = 10)	mPSL-10 (n = 10)	mPSL-20 (n = 5)
Ca (mg/dl)	10.9 $\pm$ 0.6	10.9 $\pm$ 0.3	10.9 $\pm$ 0.5	10.9 $\pm$ 0.5	10.7 $\pm$ 0.5
Pi (mg/dl)	12.5 $\pm$ 0.5	12.2 $\pm$ 1.1	12.4 $\pm$ 1.8	12.3 $\pm$ 1.1	11.6 $\pm$ 1.5
ALP (B-L) ^a	33.6 $\pm$ 4.1	31.5 $\pm$ 2.3	30.9 $\pm$ 3.9	28.2 $\pm$ 2.6*	27.0 $\pm$ 1.8*
OC (ng/ml) ^b	64.4 $\pm$ 3.1	ND	ND	59.0 $\pm$ 6.2	54.1 $\pm$ 3.8*
TRACP (B-L) ^a	2.46 $\pm$ 0.36	2.39 $\pm$ 0.24	2.53 $\pm$ 0.26	2.42 $\pm$ 0.22	2.22 $\pm$ 0.31
Pyd (nmol/L) ^b	3.81 $\pm$ 0.88	ND	ND	3.42 $\pm$ 0.44	3.64 $\pm$ 0.89

^aBessey-Lowry units, ND: not determined, ^bn = 6. Mean $\pm$ S.D. * P <0.05 (vs control, one-way ANOVA followed by Tukey's multiple comparison test).

Fig. 8. Number of radioautographic silver grains over the bone matrix and over osteoblasts on the trabecular bone from the epiphyseal borderline (region 1) to the distal end (regions 2 to 10) (see Fig. 4). a: number of grains over the bone matrix. b: total number of grains over the bone matrix and over osteoblasts. c: ratio of the number of grains over the bone matrix to the number of grains over osteoblasts. BM, bone matrix; OB, osteoblasts.

In the mPSL group, however, the increases from region 4 to 6 in both the number of silver grains over the bone matrix and the total number of silver grains were suppressed. The mean values in the mPSL group at region 6 were about 48% and 60% of the corresponding control values for the number of grains over the bone matrix and the total number, respectively.

Figure 8c shows the ratio of the number of silver grains over the bone matrix to the number of silver grains over osteoblasts (BM/OB ratio), which is an index of the collagen-secretory activity of osteoblasts during the last hour before the animal was sacrificed. The BM/OB ratio

Fig. 9. Number of radioautographic silver grains over the bone matrix and over osteoblasts on the periosteal surface of cortical bone in the lateral base of the mandibula. a: number of grains over the bone matrix. b: total number of grains over the bone matrix and over osteoblasts. c: ratio of the number of grains over the bone matrix to the number of grains over osteoblasts. BM, bone matrix; OB, osteoblasts; * $P < 0.05$, ** $P < 0.01$.

in the mPSL group was lower than that in the control group in all of the regions measured.

Cortical bone in the mandibula: Figure 9 shows the number of silver grains over the bone matrix (Fig. 9a), the total number of silver grains over osteoblasts and the bone matrix (Fig. 9b), and the BM/OB ratio within a 200 μ m distance on the surface of the lateral base of the mandibula (Fig. 9c). There were statistically significant decreases in all of the variables measured in the mPSL group compared with those in the control group. The mean number of silver grains over the bone matrix, the total number and the BM/OB ratio were about 64%, 71% and 79% of the corresponding control values, respectively.

Numbers of osteoblasts and adipocytes

Osteoblasts: The numbers of osteoblasts over 200 μ m

Fig. 10. Numbers of osteoblasts and adipocytes. a: numbers of osteoblasts on the trabecular bone surface of tibial metaphyses and on the periosteal surface of cortical bone in the lateral base of the mandibula. b: numbers of adipocytes in the tibial bone marrow (see region B in Fig. 4). ** $P < 0.01$.

of the periosteal surface of mandibular cortical bone at the lateral base of the mandibula and the trabecular bone surface in the tibial metaphysis were decreased by about 20% and 11% in the mPSL group, respectively, compared with those in the control group (Fig. 10).

Adipocytes: The number of adipocytes in the tibial bone marrow showed a marked increase: that in the mPSL group was about 55% greater than that in the control group (Figs. 7 and 10b).

DISCUSSION

It has been shown that the administration of excessive GCs induces a decrease in bone formation and an increase in bone resorption, which results in a decrease in BMD in various bones in human (22, 31). Even in animal experiments, it has been demonstrated that the administration of excessive GCs induces a decrease in the total body bone mineral content and results in a decrease in BMD (32). In the present study, however, the administration of mPSL for 4 weeks dose-dependently increased the BMD of the tibial metaphysis in rats. Although our present findings

were contrary to previous findings in humans and in certain animal species (8–12, 22, 23), they are consistent with the data from previous studies using rats that showed increased bone mass or BMD after the treatment with GCs (24, 25, 33, 34).

As observed in the present autoradiographic study using ^3H -proline, the collagen synthetic and secretory activities of osteoblasts on the trabecular bone surface in the metaphyses (Fig. 8), as well as on the periosteal bone surface in cortical mandibular bone (Fig. 9), were decreased by the administration of mPSL. The longitudinal formation of trabecular bone in the metaphyses and the appositional formation of cortical bone in the mandible were also decreased in a dose-dependent fashion by treatment with mPSL. In addition, serum markers of bone formation, OC and ALP, significantly decreased in the groups treated with higher doses of mPSL (Table 5). Furthermore, most previous histomorphometrical studies and studies using bone turnover parameters in serum have consistently shown that the administration of GCs induces the retardation and inhibition of bone formation (31, 35). Although all of the present and previous data have shown decreases in bone formation with GCs, BMD and bone volume in the tibial metaphysis unexpectedly increased in a dose-dependent fashion in the present study. These results imply that the observed increases in BMD were not due to an increase in the formation of bone, but rather to the greater inhibition of bone resorption, which would exceed the inhibition of bone formation. In fact, drastic decreases in the total number of osteoclasts and the number of osteoclasts per bone surface and bone area (Table 4) were observed in the tibial metaphysis. Trabecular bone with a rather flat surface and fewer resorption lacunae, and with an increased longitudinal length, which are features of reduced osteoclast activity, was also observed histologically (Fig. 6). Although serum TRAP and Pyd in the present study did not decrease significantly (Table 5), these histologic changes showing marked inhibition of bone resorption in the metaphysis might result in a positive balance in the bone volume there. A recent study by Okazaki et al. (26) demonstrated that the administration of prednisolone prevented a decrease in bone mass in rats with adjuvant-induced arthritis by reducing the increase in bone resorption and the decrease of bone formation at both the local and systemic levels. King et al. (24) and Turner et al. (25) also reported that treatment with dexamethasone or prednisone increased the bone mass in normal rats or ovariectomized rats, which might be due to a greater suppression of resorption rather than formation.

In contrast to the increase in BMD in the metaphysis, BMD in the tibial diaphysis decreased in a dose-dependent fashion (Fig. 5, right panels). This difference in the endpoint result of BMD between the metaphysis and diaphysis

might be explained by a difference in the balance between bone formation and resorption in these bone sites; i.e., the balance might be much in favor of formation in the modeling of diaphysis. In fact, osteoclasts can be rarely observed in the endosteal surface of diaphysis, whereas significant numbers of osteoblasts can be consistently observed in the diaphyseal periosteum. Consequently, inhibitory effects of mPSL on both bone formation and resorption would be predominantly expressed in the bone formation, which would result in a decrease in the BMD in the diaphysis. In summary, BMD as an endpoint result of mPLS treatment might differ from site to site in bone due to different balances between bone formation and resorption.

Previous studies have consistently demonstrated that the administration of excessive GCs results in a decrease in bone formation in both human and animals. The present study using a chronological labeling method with NTA-Pb also clearly showed that the administration of mPSL dose-dependently decreased both longitudinal and appositional bone formation in the metaphyseal trabeculae adjacent to the epiphyseal cartilage plate (Fig. 2) and in the cortical mandibular bone, respectively. Such inhibition of bone formation might be due to at least two different mechanisms, i.e., inhibition of osteoblast function and inhibition of the proliferation or differentiation of precursor cells to osteoblasts. GCs have also been reported to promote the apoptosis of osteoblasts and osteocytes (36). In fact, our present autoradiographic study using ^3H -proline revealed that the incorporation and secretion of ^3H -proline by osteoblasts were diminished by mPSL. The greatest inhibitory effect was seen in the secondary trabecular bone where an increase in trabecular bone formation (increase in the thickness of trabecular bone) was observed in control rats (Fig. 8). GCs are known to have various effects on osteoblast gene expression, including down-regulation of type I collagen and osteocalcin. The expression of IGF-1, which is an important stimulator of osteoblast function, is also known to be decreased by GCs (37).

The possibility that GCs inhibit the proliferation or differentiation of precursor cells to osteoblasts may be supported by the present observations that the number of osteoblasts on the bone surface decreased in mPSL-treated rats in both the trabecular bone surface and periosteal bone surface. GCs at physiological concentrations are known to induce the proliferation and differentiation of bone marrow stromal cells into cells that express a mature osteoblast phenotype, whereas GCs at higher concentrations or pharmacological doses drastically reduce the proliferation of osteoblast precursors (38) and inhibit the differentiation to mature osteoblasts (39). It is possible that the present increase in the number of adipocytes in the bone marrow in mPSL-treated rats (Fig. 10b) was related to the decrease in the number of osteoblasts on the bone surface (Fig. 10a).

Stromal mesenchymal stem cells are known to be pluripotent and to have the capacity to differentiate into osteoblasts and adipocytes (40). These two lineages are thought to be reciprocally related (41, 42). The present finding of a decrease in the number of osteoblasts and an increase in the number of adipocytes in the bone marrow are consistent with the above notion.

In conclusion, the administration of mPSL for 4 weeks inhibited both bone formation and bone resorption in rats and resulted in a site-dependent increase or decrease in BMD, which might be determined by the balance between bone formation and resorption.

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