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Glucocorticoid-Induced Bone Loss in Mice Can Be Reversed by the Actions of Parathyroid Hormone and Risedronate on Different Pathways for Bone Formation and Mineralization

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Objective. **Glucocorticoid excess decreases bone mineralization and microarchitecture and leads to reduced bone strength. Both anabolic (parathyroid hormone [PTH]) and antiresorptive agents are used to prevent and treat glucocorticoid-induced bone loss, yet these bone-active agents alter bone turnover by very different mechanisms. This study was undertaken to determine how PTH and risedronate alter bone quality following glucocorticoid excess.**

Methods. **Five-month-old male Swiss-Webster mice were treated with the glucocorticoid prednisolone (5 mg/kg in a 60-day slow-release pellet) or placebo. From day 28 to day 56, 2 groups of glucocorticoid-treated animals received either PTH (5 μg/kg) or risedronate (5 μg/kg) 5 times per week. Bone quality and quantity were measured using x-ray tomography for the degree of bone mineralization, microfocal computed tomography for bone microarchitecture, compression testing for trabecular bone strength, and biochemistry and histomorphometry for bone turnover. In addition, real-time polymerase chain reaction (PCR) and immunohistochemistry were performed to monitor the expression of several key genes regulating Wnt signaling (bone formation) and mineralization.**

Results. **Compared with placebo, glucocorticoid treatment decreased trabecular bone volume (bone volume/total volume [BV/TV]) and serum osteocalcin, but increased serum CTX and osteoclast surface, with a peak at day 28. Glucocorticoids plus PTH increased BV/TV, and glucocorticoids plus risedronate restored BV/TV to placebo levels after 28 days. The average degree of bone mineralization was decreased after glucocorticoid treatment (−27%), but was restored to placebo levels after treatment with glucocorticoids plus risedronate or glucocorticoids plus PTH. On day 56, RT-PCR revealed that expression of genes that inhibit bone mineralization (Dmp1 and Phex) was increased by continuous exposure to glucocorticoids and glucocorticoids plus PTH and decreased by glucocorticoids plus risedronate, compared with placebo. Wnt signaling antagonists Dkk-1, Sost, and Wif1 were up-regulated by glucocorticoid treatment but down-regulated after glucocorticoid plus PTH treatment. Immunohistochemistry of bone sections showed that glucocorticoids increased N-terminal Dmp-1 staining while PTH treatment increased both N- and C-terminal Dmp-1 staining around osteocytes.**

Conclusion. **Our findings indicate that both PTH and risedronate improve bone mass, degree of bone mineralization, and bone strength in glucocorticoid-treated mice, and that PTH increases bone formation while risedronate reverses the deterioration of bone mineralization.**

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Glucocorticoids are effective antiinflammatory agents, but prolonged use results in many adverse effects, with bone loss and fractures being the most devastating (1–3). The pathogenesis of glucocorticoid-induced osteoporosis is complex and not completely clear. However, there appears to be an early activation of osteoclast maturation and activity followed by prolonged suppression of osteoblast maturation and activity resulting in rapid and sustained bone loss (4–11). The changes in bone metabolism with glucocorticoid exposure result in a rapid loss of trabecular bone followed by a later and slower loss of cortical bone.

Over the past 10 years, randomized placebo-controlled clinical trials have demonstrated that the aminobisphosphonates risedronate and alendronate, both potent antiresorptive agents, can prevent and treat glucocorticoid-induced osteoporosis, with a reduction in incident vertebral fractures in the bisphosphonate-treated group compared with the placebo-treated group (12–15). The increase in bone strength in glucocorticoid-treated patients who were treated with bisphosphonates was believed to be secondary to a reduction in bone turnover, which prevents the loss of trabecular bone mass and architecture and increases bone mineralization.

In addition, randomized controlled clinical trials have demonstrated that the stimulation of bone formation with human PTH 1–34 (hPTH[1–34]) can override the suppressive effects of glucocorticoids on bone formation and increase bone mass (16). Recently, Saag et al (15) reported that in glucocorticoid-treated patients, 18 months of treatment with recombinant hPTH(1–34) significantly increased both lumbar spine and hip bone mass and reduced new incident vertebral fractures compared with alendronate (70 mg/week). These studies suggest that both antiresorptive agents that reduce osteoclast activity and an anabolic agent that increases bone formation are effective in improving bone strength in the presence of glucocorticoids; however, the mechanisms that lead to the increase in bone strength may differ.

Bone strength is a combination of the amount of bone, bone structure, and other aspects of bone quality, which include localized material properties, non-mineralized matrix proteins, and bone turnover (17,18). Glucocorticoids are reported to affect many aspects of bone quality, including bone turnover, bone mineralization, and localized material properties (1,2,4,19–25), such that individuals taking glucocorticoids experience fractures at a higher bone mineral density (BMD) than do postmenopausal women (26).

The addition of bone active agents, such as PTH and bisphosphonates, to glucocorticoid treatment improves bone strength through a combination of an increase in bone mass and changes in bone quality. Bisphosphonates improve bone quality by increasing trabecular bone mineralization (17,23,25,27). PTH can also improve bone quality by changing the trabecular bone microarchitecture, e.g., trabecular thickness and spacing, which improves bone strength. In a previous study, we found that glucocorticoid excess in a mouse model decreased bone mineralization, bone formation, and osteoblast and osteocyte lifespan and altered the localized material properties within the trabecular bone around the osteocyte lacunae (23). Also, a microarray analysis of mouse bone exposed to glucocorticoids demonstrated an increase in gene transcripts in the Wnt/β-catenin signaling pathway that inhibit osteoblast maturation and mineralization gene transcripts (28,29).

Based on these findings, we hypothesized that antiresorptive agents and anabolic agents improve bone strength in the presence of glucocorticoids through different effects on bone quality, including osteoblast maturation and activity. We determined that the addition of PTH or risedronate to glucocorticoid treatment restored trabecular bone volume and bone strength in mice; however, PTH stimulated bone formation through the inhibition of Wnt/β-catenin antagonist genes while risedronate reduced the expression of mineralization-inhibiting genes. Both treatments resulted in nearly complete restoration of trabecular bone mass and strength, while higher mineralization occurred in the mice treated with risedronate than in the mice treated with PTH. These data suggest that both agents improved bone strength in the presence of glucocorticoids, but the mechanisms by which they improved bone quality were different.

MATERIALS AND METHODS

Animals and experimental procedures. Five-month-old male Swiss-Webster mice were obtained from Charles River (San Jose, CA). Mice were housed in a room that was maintained at 21°C with a 12-hour light/dark cycle. Commercial rodent chow (22/5 Rodent Diet; Teklad, Madison, WI) containing 0.95% calcium and 0.67% phosphate was available ad libitum. The mice were randomized by body weight into 4 groups of 8–15 animals each. Group 1 mice (n = 15) were given slow-release pellets (Innovative Research of America, Sarasota, FL) containing placebo, and group 2 mice (n = 15) were given 60-day slow-release pellets containing 5 mg/kg prednisolone, by subcutaneous implantation. Group 3 mice (n = 8) received 5 μg/kg/day hPTH(1–34) (Bachem, Torrance, CA) 5 days per week, and group 4 mice (n = 8) received 5 μg/kg/day risedronate 5 days per week, in addition to pred-

nisolone. All animals were treated according to the United States Department of Agriculture animal care guidelines with the approval of the Committee on Animal Research at the University of California, Davis.

Xylenol orange (90 mg/kg) was injected subcutaneously into all study animals 28 days prior to the intervention (treatment with PTH or risedronate). Calcein (10 mg/kg) and alizarin red (20 mg/kg) were injected subcutaneously 7 and 2 days before mice were killed, respectively, to access the bone formation surface. Serum samples were obtained during autopsy, and urine and serum samples were stored at −80°C until used for assessment of biochemical markers of bone turnover. At autopsy, the mice were exsanguinated by cardiac puncture. After mice were killed, L5 and right femurs were placed in 10% phosphate buffered formalin for 24 hours and then transferred to 70% ethanol for x-ray tomography, microfocal computed tomography (micro-CT), and bone histomorphometry. L4 was used for biomechanical compression tests, and L3 was decalcified and used for immunohistochemistry. The tibiae were used for RNA extraction.

Biochemical markers of bone turnover. Serum levels of type 5b tartrate-resistant acid phosphatase (TRAP5b), C-telopeptide of type I collagen (CTX), and osteocalcin were measured using mouse sandwich enzyme-linked immunosorbent assay kits according to the recommendations of the manufacturers (SBA Sciences [IDC, Fountain Hills, AZ], Nordic Bioscience [Chesapeake, Virginia], and Biomedical Technologies [Stoughton, MA], respectively). All samples were assayed in duplicate. A standard curve was generated from each kit, and the absolute concentrations were extrapolated from the standard curve. The coefficients of variation for interassay and intraassay measurements were $<10\%$ for all assays and were similar to the manufacturers' reference values (20,30,31).

Micro-CT. L5 and the distal femur from each animal were scanned and measured by micro-CT (VivaCT 40; Scanco Medical, Bassersdorf, Switzerland), with an isotropic resolution of 10 μm for the repeated in vivo distal femur and ex vivo lumbar vertebral body scans in all 3 spatial dimensions (18). Scans were initiated in the sagittal plane of the vertebral body, covering the entire cortical and trabecular bone of the lumbar vertebral body. For the distal femur, scanning was initiated at the growth plate and continued proximally for 200 mm. Three-dimensional (3-D) trabecular structural parameters were measured directly, as previously described (23,27). Mineralized bone was separated from bone marrow with a matching cube 3-D segmentation algorithm. Bone volume (BV) was calculated using tetrahedrons corresponding to the enclosed volume of the triangulated surface. Total volume (TV) was the volume of the sample that was examined. A normalized index, bone volume (BV/TV), was used to compare samples of varying size. The methods used for calculating trabecular thickness (TbTh), trabecular separation, and trabecular number (TbN) have been described previously (23,27).

Bone histomorphometry. L5 dehydrated in ethanol, embedded undecalcified in methylmethacrylate, and sectioned longitudinally with a Leica/Jung 2255 microtome into 4-μm– and 8-μm–thick sections. Bone histomorphometry was performed using a semiautomatic image analysis Bioquant system (Bioquant Image Analysis Corporation, Nashville, TN) linked to a microscope equipped with transmitted and fluorescent light (23).

A counting window, allowing measurement of the entire trabecular bone and bone marrow within the growth plate and cortex, was created for the histomorphometric analysis. Static measurements included total tissue area, bone area, and bone perimeter. Dynamic measurements included single- and double-labeled perimeter and interlabel width. These indices were used to calculate 2-D bone volume (BV/TV), TbN, TbTh, mineralized surface (mineralized surface/bone surface [MS/BS]), percentage of osteoclast surface (osteoclast surface/bone surface [OcS/BS]), and mineral apposition rate (MAR). Surface-based bone formation rate (BFR/BS) was calculated by multiplying the mineralized surface (single-labeled surface/2 + double-labeled surface) by the MAR according to the method described by Parfitt et al (32). We have used similar methodology in previous studies (23,25,27,30).

Determination of biomechanical properties. Mouse lumbar vertebrae were subjected to a lumbar vertebral compression test. L4 was identified by counting down from the last thoracic vertebra. The top and bottom of the vertebrae were polished with an 800-grit silicon carbide paper to create 2 parallel planar surfaces. The height and a 2-point average of the diameter and length were measured using digital calipers. The average cross-sectional area was approximated as an ellipse. The vertebrae were then soaked in Hanks' balanced salt solution for at least 12 hours prior to testing. Each vertebral specimen was then loaded in compression to failure using a servo-hydraulic testing machine (MTS Model 810; MTS Systems, Eden Prairie, MN); tests were performed at room temperature under displacement-control at a displacement rate of 0.001 mm/second, and the applied loads were measured with a precision, low-capacity load cell (MTS Model 461-19002, PCB Model 1401-03A). The elastic (compression) modulus was determined by multiplying the slope of the linear region of the load-displacement curve by the height of the sample and dividing by its cross-sectional area. The compressive yield strength was defined as the load at which the slope begins to deviate from linearity divided by the average cross-sectional area, and the maximum compressive strength was determined by dividing the first maximum peak load after the yield point by the specimen average cross-sectional area (33).

Real-time polymerase chain reaction (PCR). Total RNA was extracted from the tibiae using a Polytron (Kinematica, Luzern, Switzerland) and TRIzol reagent according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA). Reverse transcription was carried out with the Reverse Transcription System (Promega, Madison, WI). Primer sets for real-time PCR were purchased from SuperArray (Frederick, MD). Real-time PCR was carried out using an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA) in a 25-μl reaction that consisted of 12.5 μl of 2× SYBR Green mix (SuperArray), 0.2 μl of complementary DNA, 1 μl of primer pair mix, and 11.3 μl of H_2O. Expression of all of the test genes was normalized to a control gene, GAPDH. The results were expressed as the fold change compared with the placebo-treated group, where fold change $= 2^{-\Delta\Delta C_t}$ (34).

Immunohistochemistry. L3 was decalcified in 10% EDTA for 2 weeks and embedded in paraffin. Four-micrometer sections were prepared for immunohistochemistry using primary antibodies against Dmp1 N-terminus and

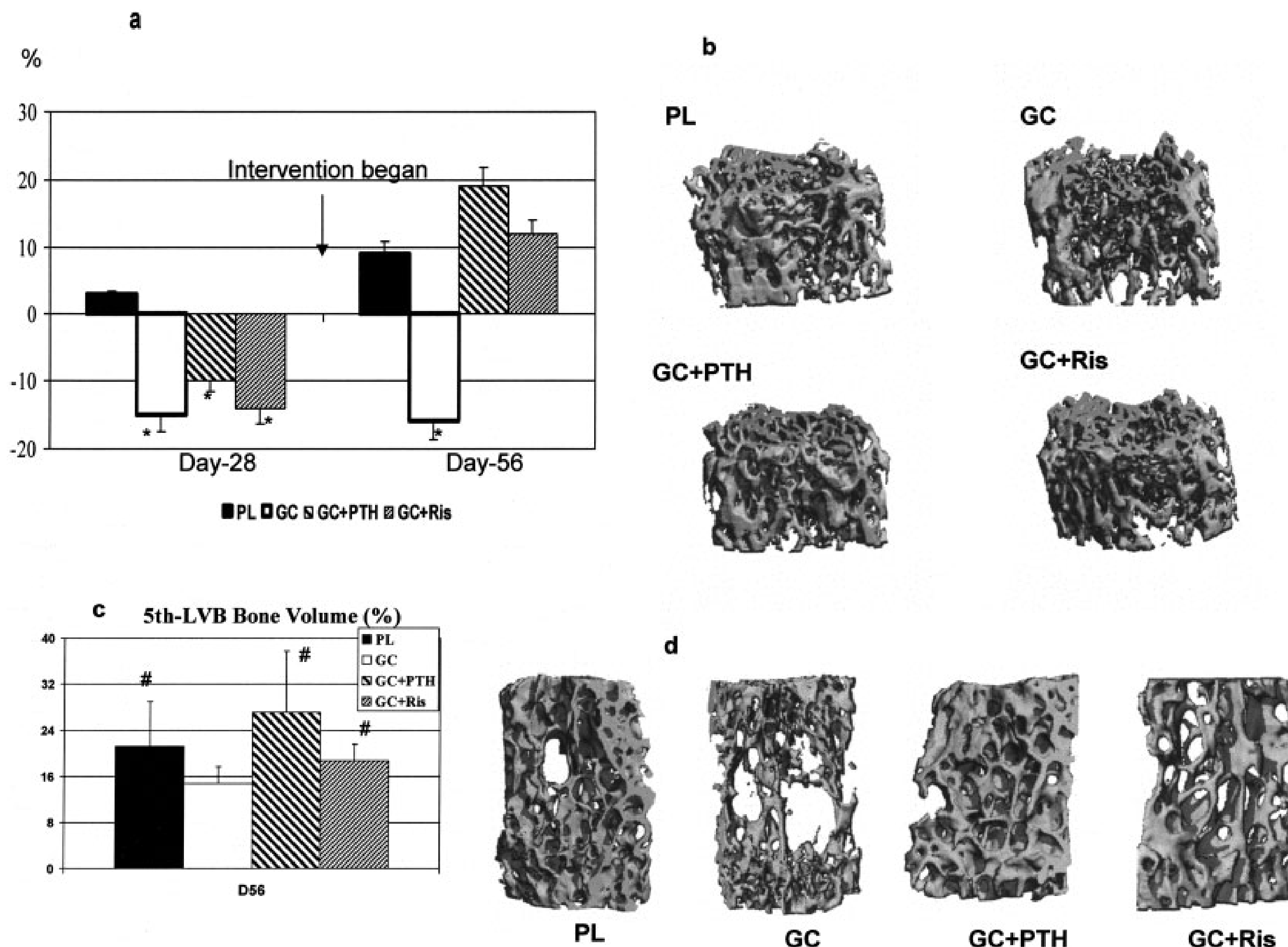

Figure 1. Time-dependent changes in mouse trabecular bone volume in the distal femur metaphysis and in L5 after 28 or 56 days of treatment with placebo (PL), 28 or 56 days of treatment with glucocorticoids (GCs) alone, and 28 days of glucocorticoid treatment followed by 28 days of treatment with glucocorticoids plus parathyroid hormone 1–34 (GC+PTH) or glucocorticoids plus risedronate (GC+Ris). **a** and **c,** Percentage change in bone volume from day 0 in the distal femur metaphysis (**a**) and the fifth lumbar vertebral body (LVB), or L5 (**c**). Glucocorticoid excess caused trabecular bone loss in both the distal femur metaphysis and L5. Bars show the mean and SD. $* = P < 0.05$ versus placebo; $\# = P < 0.05$ versus glucocorticoids alone. D56 = day 56. **b** and **d**, Representative 3-dimensional microfocal computed tomography images of the distal femur metaphysis (**b**) and L5 (**d**) obtained on day 56 from each treatment group. Glucocorticoids decreased trabecular bone mass, trabecular number, and trabecular thickness. PTH(1–34) increased trabecular bone mass and trabecular thickness compared with placebo or glucocorticoids alone. Animals treated with risedronate had bone mass similar to that in animals treated with placebo.

C-terminus (Santa Cruz Biotechnology, Santa Cruz, CA). Detections were performed with the HRP-DAB Cell and Tissue Staining kit (R&D Systems, Minneapolis, MN). Sections were briefly counterstained with hematoxylin. Control slides were included for both Dmp1 C-terminus and Dmp1 N-terminus using nonimmune IgG as a replacement for the primary antibodies. Positive staining yielded a brown precipitate. Results were presented as the percentage of the positive staining in the vertebral total trabecular area using the Bioquant imaging analyzing system as described above for bone histomorphometry (35).

Statistical analysis. The mean ± SD was calculated for all outcome variables. Statistical differences between the group treated with glucocorticoids alone, the group treated with glucocorticoids plus risedronate, the group treated with glucocorticoids plus PTH, and the control group were analyzed using the Kruskal-Wallis nonparametric test with post hoc comparisons (SPSS, version 10; SPSS, Chicago, IL). *P* values less than 0.05 were considered significant.

RESULTS

Effects of glucocorticoid excess on bone loss. Micro-CT evaluation of the glucocorticoid-treated mice demonstrated significantly lower trabecular bone vol-

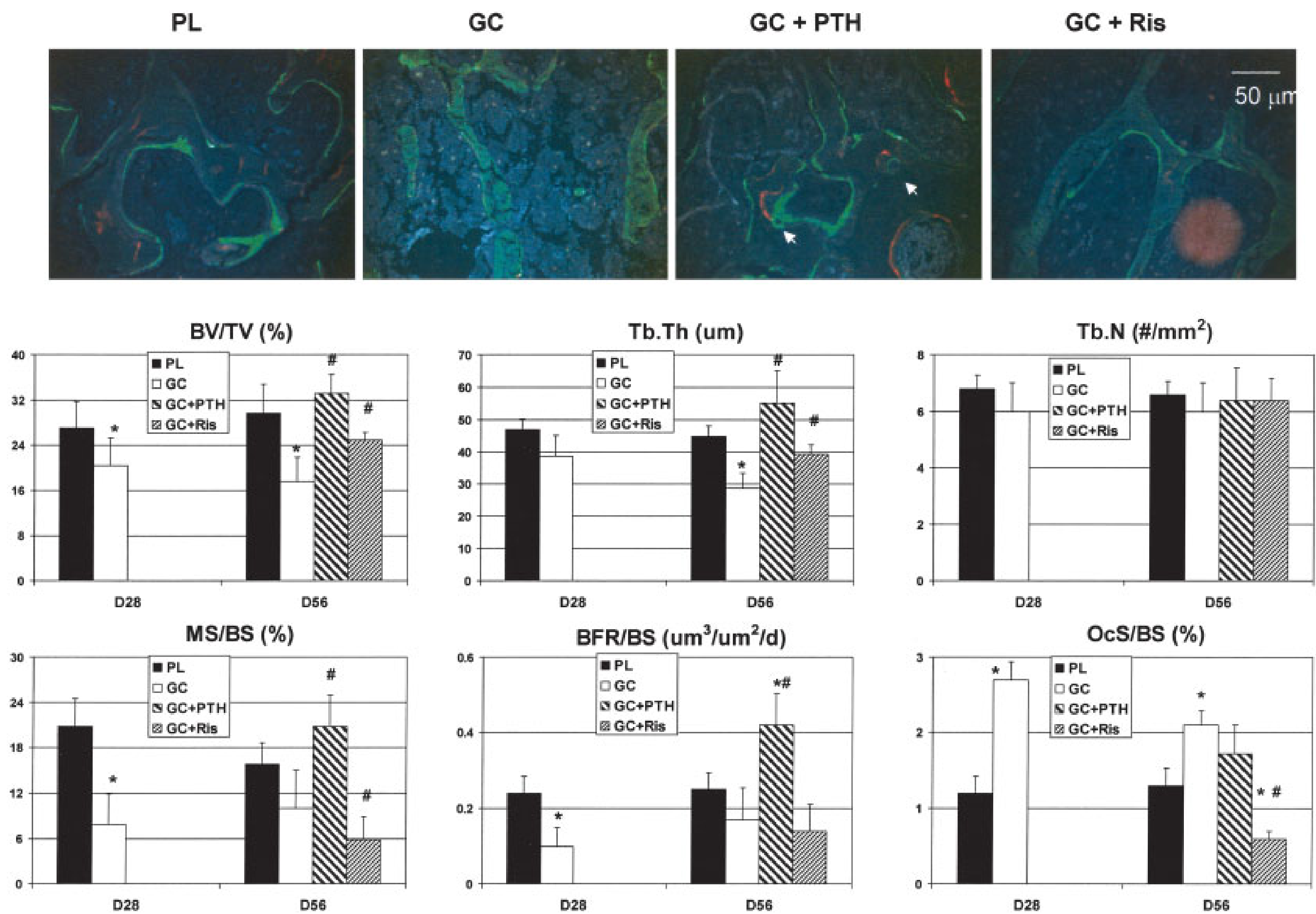

Figure 2. Trabecular bone architecture changes in mice after 28 or 56 days of treatment with placebo, 28 or 56 days of treatment with glucocorticoids alone, and 28 days of glucocorticoid treatment followed by 28 days of treatment with glucocorticoids plus PTH or glucocorticoids plus risedronate. **Top**, Unstained L5 sections obtained on day 56 from each treatment group. Fluorescent labeling shows the mineralized surface. The section from a mouse treated with glucocorticoids plus PTH showed a double-labeled surface surrounding some osteocytes just below the base of the remodeling cavity (**arrows**). **Bottom**, Measurements determined by histomorphometry in the trabecular bone regions of L5. Glucocorticoids alone decreased bone mass (bone volume/total volume [BV/TV]), trabecular thickness (TbTh), and mineralized surface (mineralized surface/bone surface [MS/BS]), but increased osteoclast surface (osteoclast surface/bone surface [OcS/BS]), compared with placebo. Mice treated with glucocorticoids plus PTH had higher BV/TV, higher TbTh, higher MS/BS, and a higher bone formation rate (BFR/BS) than did mice treated with placebo or glucocorticoids alone. Mice treated with glucocorticoids plus risedronate had higher BV/TV and TbTh compared with mice treated with glucocorticoids alone, but lower BV/TV and TbTh compared with mice treated with placebo, and lower BFR/BS and OcS/Bs compared with mice treated with glucocorticoids alone or mice treated with placebo. Bars show the mean and SD. * = $P < 0.05$ versus placebo; # = $P < 0.05$ versus glucocorticoids alone. TbN = trabecular number (see Figure 1 for other definitions).

ume (BV/TV) in the distal femurs compared with placebo-treated controls on day 28 (−18%; $P < 0.05$) and on day 56 (−19%; $P < 0.05$) (Figures 1a and b). Also, the BV/TV in L5 in glucocorticoid-treated mice was 30% lower than in placebo-treated mice, as confirmed by histomorphometry (Figures 1c, 1d, and 2). Similarly, TbTh was significantly lower in glucocorticoid-treated mice than in placebo-treated controls on day 56 (Figures 1 and 2). However, TbN did not differ significantly between glucocorticoid-treated mice and placebo-treated controls throughout the 56-day period ($P > 0.20$).

Glucocorticoid-induced trabecular bone loss was associated with increases in the bone resorption markers TRAP5b (14%) and CTX (26%; $P < 0.05$) and a decrease in the bone formation marker osteocalcin (−22%; $P < 0.05$) (Figure 3) on day 28, as compared with the placebo group. Assessment of bone turnover by

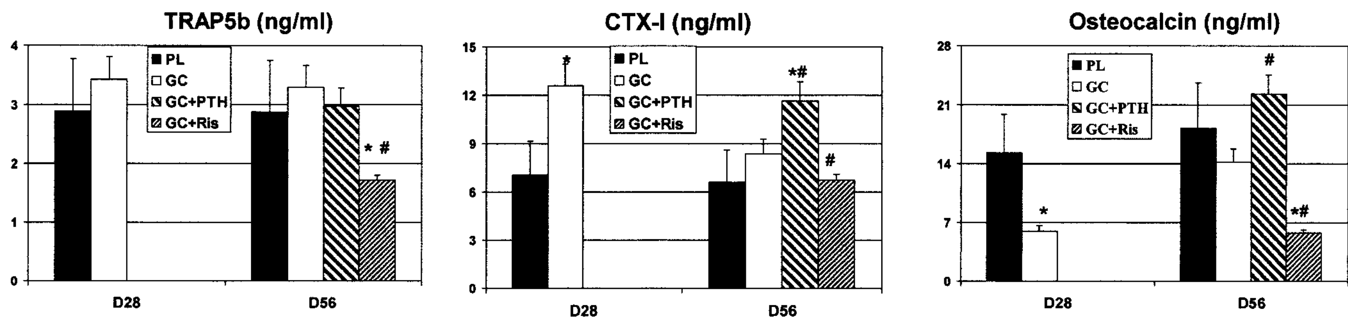

Figure 3. Bone turnover, measured by levels of the bone markers type 5b tartrate-resistant acid phosphatase (TRAP5b), C-telopeptide of type I collagen (CTX-I), and osteocalcin, in mice after 28 or 56 days of treatment with placebo, 28 or 56 days of treatment with glucocorticoids alone, and 28 days of glucocorticoid treatment followed by 28 days of treatment with glucocorticoids plus PTH or glucocorticoids plus risedronate. Glucocorticoid excess increased osteoclast formation (TRAP5b levels) and activity (CTX-I levels), while it decreased osteoblast function (osteocalcin levels). On day 56, mice treated with PTH(1–34) had increased CTX-I and osteocalcin levels, while mice treated with risedronate had decreased CTX-I and osteocalcin levels, compared with mice treated with glucocorticoids alone. Bars show the mean and SD. * = $P < 0.05$ versus placebo; # = $P < 0.05$ versus glucocorticoids alone. See Figure 1 for other definitions.

histomorphometry in the glucocorticoid-treated group revealed a decrease in the MS/BS (−37% on day 28) and surface-based BFR/BS (−30% on day 28) and an increase in the OcS/BS (200% on day 28 and 61% on day 56) (Figure 2) compared with the placebo group.

Effect of PTH treatment on glucocorticoid-treated mice. On day 56, after 28 days of treatment with both glucocorticoids and PTH, distal femoral trabecular bone volume (BV/TV) was 10% higher than in placebo-treated animals ($P < 0.05$) and 31% higher than in animals treated with glucocorticoids alone ($P < 0.05$) (Figure 1). Treatment with both glucocorticoids and PTH also increased trabecular bone volume in the lumbar vertebral body, with a significant increase in TbTh ($P < 0.05$) (Figures 1 and 2). Also, increases in MS/BS and BFR/BS were observed on day 56 in mice treated with glucocorticoids and PTH, as compared with animals treated with glucocorticoids alone ($P < 0.05$) (Figure 2). Multiple fluorochrome-labeled osteocytes within the trabeculae were observed in mice treated with both glucocorticoids and PTH but not in any of the other groups (Figure 2). OcS/BS in mice treated with glucocorticoids and PTH was similar to that in mice treated with glucocorticoids alone.

Serum TRAP5b levels in mice treated with glucocorticoids and PTH were slightly elevated after 28 days of treatment, but were not significantly different from those in animals treated with glucocorticoids alone. However, mice treated with glucocorticoids and PTH showed significant increases in serum CTX compared with all other groups ($P < 0.05$) (Figure 3). Serum osteocalcin levels were also significantly higher on day 56 in mice treated with glucocorticoids and PTH than in mice treated with glucocorticoids alone (Figure 3), but did not differ significantly from levels in the placebo group.

Effect of risedronate treatment on glucocorticoid-treated mice. After 28 days of treatment, trabecular BV/TV was increased by 18% in mice treated with both glucocorticoids and risedronate compared with animals treated with glucocorticoids alone ($P < 0.05$) (Figures 1 and 2) and was similar to that found in the placebo-treated animals. Treatment with both glucocorticoids and risedronate significantly increased TbTh compared with treatment with glucocorticoids alone ($P < 0.05$) (Figure 2). On day 56, MS/BS and BFR/BS were not significantly different in mice treated with risedronate and glucocorticoids than in mice treated with glucocorticoids alone. However, OcS/BS was significantly lower in animals in the glucocorticoids and risedronate group than in animals in the placebo and glucocorticoid only groups ($P < 0.05$) (Figure 2). Serum TRAP5b, CTX, and osteocalcin levels in animals treated with glucocorticoids and risedronate were all significantly decreased on day 56 compared with animals treated with glucocorticoids alone ($P < 0.05$ for all) (Figure 3).

Effect of glucocorticoids, PTH, and risedronate on bone mineralization and strength. The global degree of mineralization in the lumbar vertebral trabecular bone was lowered by 27% in the glucocorticoid-treated animals compared with the placebo-treated animals. However, mice treated with glucocorticoids plus PTH and mice treated with glucocorticoids plus risedronate both had a total degree of mineralization and a surface

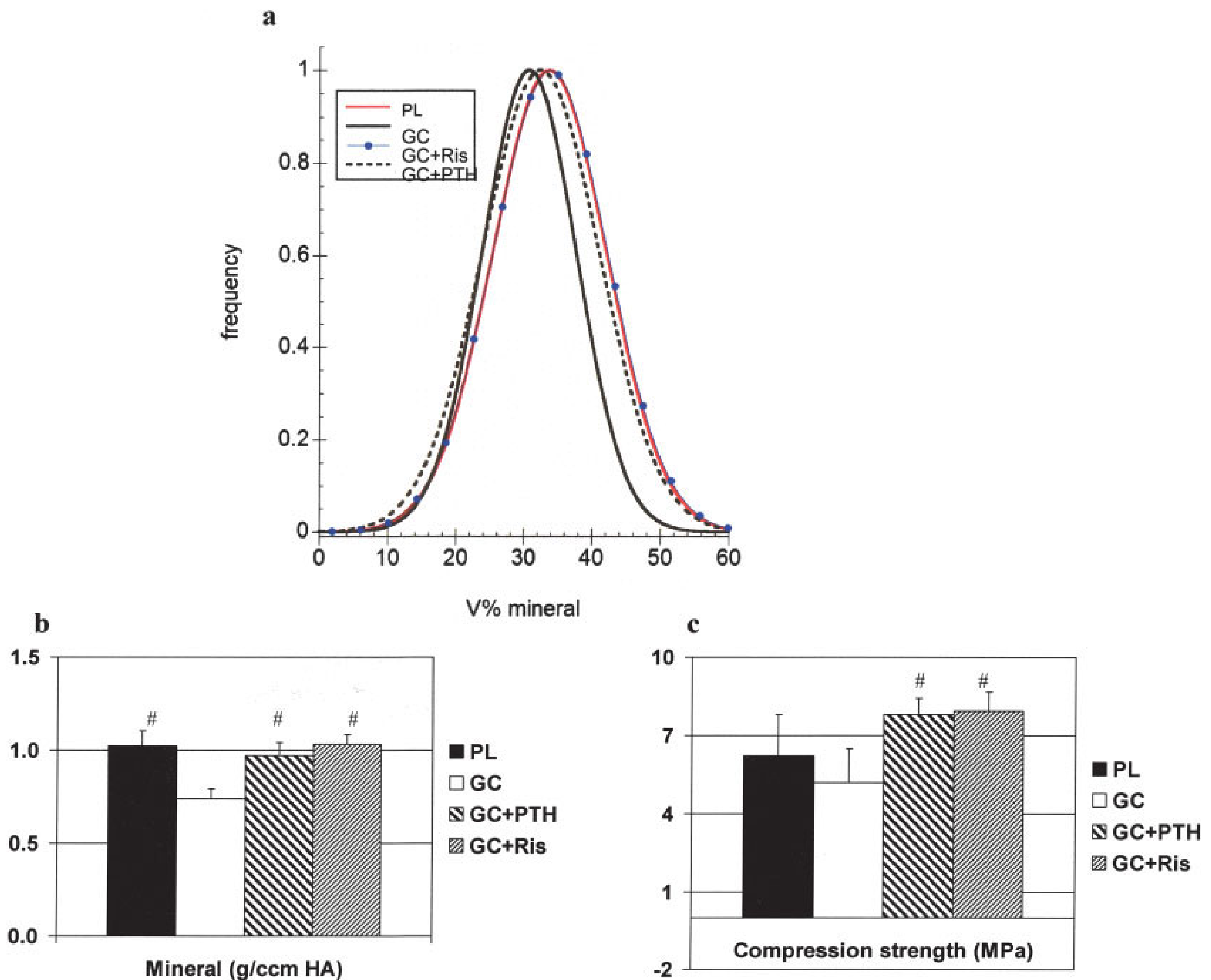

Figure 4. Degree of bone mineralization and lumbar compression strength in lumbar vertebral bodies from mice after 56 days of treatment with placebo, 56 days of treatment with glucocorticoids alone, and 28 days of glucocorticoid treatment followed by 28 days of treatment with glucocorticoids plus PTH or glucocorticoids plus risedronate. **a,** Glucocorticoid excess decreased the average degree of mineralization and shifted the curve to the left, with lower percentages of minerals. V = bone volume. **b,** Quantitation of bone mineralization, determined by x-ray tomography. Both glucocorticoids plus PTH and glucocorticoids plus risedronate restored the degree of bone mineralization to the level in placebo-treated mice. Bars show the mean and SD. HA = hydroxyapatite. **c**, Lumbar compression yield strength. Glucocorticoid excess decreased lumbar compression yield strength. Both glucocorticoids plus PTH and glucocorticoids plus risedronate restored lumbar compression yield strength to the level in placebo-treated mice. Bars show the mean and SD. # = $P < 0.05$ versus glucocorticoids alone. See Figure 1 for definitions.

distribution of the mineral similar to that of mice in the placebo group (Figures 4a and b). The lumbar compression yield strength was 19% lower in glucocorticoid-treated animals than in placebo-treated animals, and animals treated with glucocorticoids plus PTH and animals treated with glucocorticoids plus risedronate had lumbar compression yield strength similar to that in animals in the placebo group (Figure 4c).

Glucocorticoid, glucocorticoid plus PTH, and glucocorticoid plus risedronate regulation of expression of genes critical for bone formation and mineralization. In a previous study, we demonstrated the time-dependent gene profiling of glucocorticoid excess in tibiae that were excised from experimental animals after 7, 28, or 56 days of glucocorticoid treatment (29). From the microarray data of bone exposed to long-term

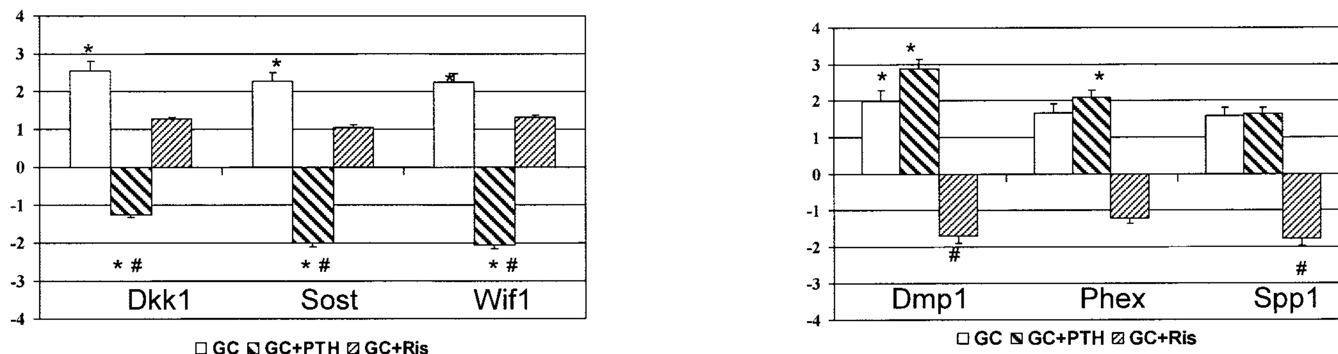

Figure 5. Expression of genes inhibiting Wnt signaling (**left**) and mineralization (**right**) in whole bone RNA extracted from mice after 56 days of treatment with glucocorticoids alone and after 28 days of glucocorticoid treatment followed by 28 days of treatment with glucocorticoids plus PTH or glucocorticoids plus risedronate. Expression was normalized to that in mice treated with placebo for 56 days (set at 1). Glucocorticoid excess increased mRNA expression of the Wnt inhibitors Dkk-1, Sost, and Wif1. Mice treated with glucocorticoids plus PTH had decreased Dkk-1, Sost, and Wif1 expression, and mice treated with glucocorticoids plus risedronate showed expression equal to that in placebo-treated mice. Glucocorticoid excess also increased expression of the mineralization inhibitors Dmp1 and Phex. Mice treated with glucocorticoids plus PTH had increased expression of these genes, and mice treated with glucocorticoids plus risedronate had decreased expression of these genes. Bars show the mean and SD. $* = P < 0.05$ versus placebo; $\# = P < 0.05$ versus glucocorticoids alone. See Figure 1 for definitions.

treatment with glucocorticoids, we derived a list of genes that were significantly changed after exposure to glucocorticoid excess in vivo. Among these genes were Wnt-signaling inhibitors (Dkk-1, Sost, and Wif1) and mineralization inhibitors (Dmp1, Phex, and Spp1).

In order to verify the results obtained from the iterative microarray analysis, messenger RNA (mRNA) levels of these genes were analyzed using real-time PCR (Figure 5) on days 7, 28, and 56. Real-time PCR showed that glucocorticoid excess increased the expression of Dkk-1, Sost, and Wif1 on day 56 (Figure 5), while glucocorticoids plus PTH down-regulated these gene transcripts. Expression of these genes was not altered from that in the placebo group after treatment with glucocorticoids and risedronate (Figure 5). Bone RNA samples from mice treated with glucocorticoids alone had increased levels of mRNA for Dmp1 and Phex on day 56 (Figure 5) compared with placebo-treated mice. These genes were up-regulated 1.5–2.8-fold after combination glucocorticoid and PTH treatment, but were down-regulated more than 1-fold on day 56 after treatment with glucocorticoids plus risedronate, compared with placebo (Figure 5).

Since our previous study had shown increased osteocyte lacunae size and local perilacunar demineralization and reduced elastic modulus after treatment with glucocorticoids (23), in the present study we evaluated whether expression of an osteocyte mineralization–regulating gene, Dmp1, might be altered by glucocorticoid excess and bone active agents (36). Tissue levels of Dmp1 were assessed by quantitative immunohistochemistry (Figure 6) of lumbar vertebral body samples obtained on day 56. The 37-kd N-terminus fragment of Dmp1 was up-regulated 7-fold by treatment with glucocorticoids alone and 9-fold by treatment with glucocorticoids plus PTH, and was localized to the area of the bone matrix around the osteocytes and at the bone remodeling surface (Figure 6). Interestingly, the 57-kd C-terminus fragment of Dmp1 was also up-regulated by treatment with glucocorticoids plus PTH (14-fold) and by treatment with glucocorticoids plus risedronate (3.2-fold). The C-terminus of Dmp1 was predominantly localized in bone-remodeling pockets and around the osteocytes (Figure 6).

DISCUSSION

Glucocorticoid treatment for 56 days reduced trabecular bone volume, mineralization, turnover, and compression yield strength and was associated with increased expression of genes that inhibit Wnt signaling and mineralization. Intervention with PTH restored lost trabecular bone volume, increased bone formation, and reversed the glucocorticoid-induced inhibition of Wnt signaling. An intervention with risedronate also restored lost trabecular bone volume and mineralization through a reduction in bone turnover and reversed the glucocorticoid-induced inhibition of mineralization. The differential effect of these 2 compounds on gene transcription may explain the different bone material changes and bone architecture changes observed with concurrent glucocorticoid use in vivo.

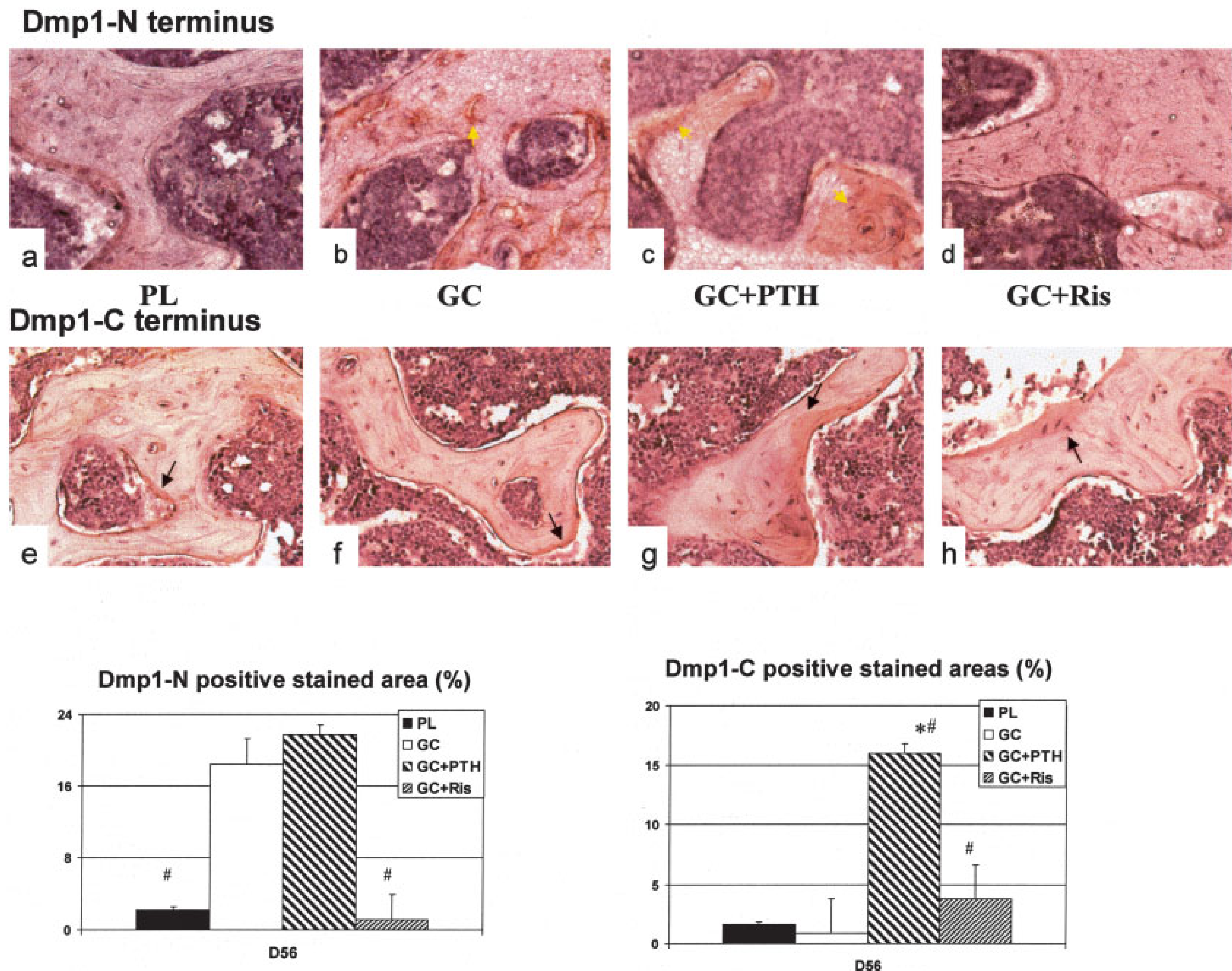

Figure 6. Top, Immunohistochemical staining for Dmp1 N-terminus (**a–d**) and Dmp1 C-terminus (**e–h**) expression in lumbar vertebral body sections obtained from mice after 56 days of treatment with placebo (**a** and **e**), 56 days of treatment with glucocorticoids alone (**b** and **f**), and 28 days of glucocorticoid treatment followed by 28 days of treatment with glucocorticoids plus PTH (**c** and **g**) or glucocorticoids plus risedronate (**d** and **h**). Treatment with glucocorticoids alone up-regulated Dmp1 N-terminus (**yellow arrows**) but not Dmp1 C-terminus (**black arrows**), which was diffusely distributed in the bone matrix. Treatment with glucocorticoids plus PTH up-regulated both the N-terminus and the C-terminus of Dmp1, which were seen around the osteocytes or in the remodeling pockets. Treatment with risedronate up-regulated the C-terminus of Dmp1, especially around the osteocytes. (Original magnification × 10.) **Bottom**, Percentage of area that showed positive staining for Dmp1 N-terminus (**left**) and Dmp1 C-terminus (**right**) in each treatment group. Bars show the mean and SD. $* = P < 0.05$ versus placebo; $\# = P < 0.05$ versus glucocorticoids alone. See Figure 1 for definitions.

We selected the Swiss-Webster mouse strain since it has a high percentage of trabecular bone in its distal femoral metaphysis and since other studies have shown that this strain experiences significant loss of both cancellous and cortical bone within 21 days following glucocorticoid excess (19,23,37). Using this model, we have consistently observed trabecular bone loss associated with rapid increases in osteoclast activation and function after 7 days of glucocorticoid excess (19,23,37). In the present study, we observed increased osteoclast and decreased osteoblast activities on the bone surface, which resulted in marked trabecular bone loss after 28 days of glucocorticoid excess. Histomorphometric assessment showed that osteoclast surface and activity were increased over the baseline value on day 28 but then declined by day 56. However, suppression of bone

formation was present on day 28 and continued to day 56 with continued glucocorticoid exposure (23). Overall, the Swiss-Webster mouse model of glucocorticoid-induced osteoporosis has changes in bone mass and metabolism that are similar to those found in humans taking glucocorticoids (4–6,22).

Treatment with PTH increased trabecular mass and thickness in this model of glucocorticoid-induced bone loss. We also observed double-labeled osteoid surface consistent with new bone formation around osteocyte lacunae adjacent to the remodeling surface. However, risedronate treatment in addition to glucocorticoid treatment restored bone mass in this model by suppressing bone resorption. Coupling of bone turnover was restored, such that trabecular bone mass was recovered at a level equivalent to that seen in placebo-treated animals.

Glucocorticoid-induced bone loss is rapid for the first 6 months and then slows, but is continual (3). Despite a slower loss of bone mass with long-term glucocorticoid use, bone quality appears to continue to deteriorate, since patients seem to experience fractures at a higher BMD than do postmenopausal patients (11). In one clinical trial of glucocorticoid-treated patients who were receiving hormone replacement therapy, spine BMD increased nearly 11% after 12 months of treatment with hPTH(1–34) at 40 μg/day, with very little gain at the hip (16). However, the full effects of PTH on cortical bone sites, femoral neck, and total hip were not fully appreciated until 12 months after PTH was discontinued (38). Saag et al (12) reported that glucocorticoid-treated patients randomized to receive recombinant hPTH(1–34) exhibited a significant reduction in incident vertebral fractures, compared with patients randomized to receive alendronate, after 18 months.

Based on these results, we hypothesize that PTH increases bone strength in glucocorticoid-treated subjects by improving bone material properties in addition to or independent of its effects on bone mass. In support of this hypothesis, our study found that PTH improved microarchitecture by increasing TbTh, the degree of bone mineralization, and compressive bone strength. In addition, PTH treatment increased bone formation by osteocytes, which resulted in a reduction in the osteocyte lacunae size. O'Brien et al (7) reported that glucocorticoid excess changed the canaliculi–lacunar network by allowing deformation of canaliculi that might be associated with the demineralization observed locally around the osteocytes in our previous studies (7,23). Concurrent treatment with glucocorticoids and PTH might alter osteocyte size and the perilacunar space and allow for any shear force to be more evenly distributed within the bone matrix so that tissue strains are maintained at a level below the fracture threshold (39,40). To further elucidate this observation, additional studies of the effects of glucocorticoid excess or PTH treatment on the relationship between canaliculi space and lacunae size and localized and whole bone strength will need to be performed.

Risedronate is a bisphosphonate that is approved for the prevention and treatment of glucocorticoid-induced osteoporosis. Risedronate in addition to glucocorticoids maintains bone homeostasis by inhibiting bone resorption, while simultaneously preventing osteoblast and osteocyte apoptosis induced by glucocorticoid excess (37). Moreover, it increases or prevents the change in bone mineralization following estrogen deficiency (41–45). If risedronate treatment is initiated concurrently with glucocorticoid treatment, risedronate may prevent osteocyte death.

When risedronate treatment was started 28 days after glucocorticoid treatment was initiated in the present study, it restored bone strength by increasing bone mineralization. However, we observed enlarged empty lacunae on the trabeculae and cortical bone surfaces. The accumulated empty lacunae may affect the localized shear force distribution within the bone, which may reduce both localized and whole bone strength. The findings of a recent study demonstrating recombinant hPTH(1–34) to be more effective than alendronate in reducing incident vertebral fractures in subjects taking glucocorticoids long term (15) support our preclinical in vivo findings that PTH may alter the localized material properties of bone and improve bone strength in the presence of glucocorticoids more effectively than a bisphosphonate.

In a previous study, transcription profiling of whole bone exposed to long-term glucocorticoid excess was used to identify important regulatory transcription factors (29). Glucocorticoid excess altered gene expression in 2 important pathways, genes that are primarily expressed by osteocytes and affect bone mineralization and genes in the Wnt signaling pathway, which affect bone formation. The genes expressed by osteocytes included Dmp1, Phex, and osteopontin (Spp1). These gene products, together with other small, integrin-binding ligand, *N*-linked glycoprotein (SIBLING) family members (bone sialoprotein, dentin sialophosphoprotein, and matrix extracellular matrix protein) are critical mineralization mediators in bone (46,47). These SIBLING proteins are highly phosphorylated integrin-

binding proteins and are rich in acidic amino acids (46,47).

The most extensively studied protein within the family is Dmp1. Nonphosphorylated Dmp1 is targeted to the nucleus, where it activates the transcription of osteoblast-specific genes (48–50). In rodents, but not in humans, Dmp1 can be cleaved by bone morphogenetic protein 1 family proteases, generating a 37-kd N-terminus fragment and a 57-kd C-terminus fragment (51,52). The C-terminus Dmp1 fragment, in concert with type I collagen, provides a nucleation site for hydroxyapatite crystal formation (48,51,52). Dmp1 is also able to induce the activation of pro–matrix metalloproteinase 9 (proMMP-9) and displace mature MMP-9 from tissue inhibitor of metalloproteinases 1 (53) in tumor cells, which validates our observation that glucocorticoid excess was associated with increased Dmp1 and MMP-9 expression and the local demineralization observed around the osteocytes (23).

PTH treatment has been shown to increase the expression of Phex (54), matrix extracellular matrix protein (55), and osteopontin (56), which were associated with inhibition of mineralization, crystal growth, and crystal proliferation in vivo. In this study, treatment with glucocorticoids plus PTH increased the transcripts of these mineralization inhibitory genes to similar levels as treatment with glucocorticoids alone. However, risedronate reduced the expression of these mineralization inhibitors and reduced surface remodeling, which ultimately allowed for increased mineralization.

Wingless (Wnt) proteins are a family of secreted proteins that regulate many aspects of cell growth, differentiation, function, and death (57,58). The binding of Wnt proteins to the Frizzled receptor stabilizes β-catenin, which would otherwise be phosphorylated with a complex consisting of glycogen synthase kinase 3β, Axin, Frat1, and Disheveled, in the cytoplasm. If β-catenin accumulates and is translocated to the nucleus, it binds to transcription factor/lymphoid enhancer binding factor, causing displacement of transcriptional corepressors and inducing gene expression favoring bone formation (59–62).

Wnt signaling can be blocked by interactions with inhibitory factors, including Wnt inhibitory factor 1, secreted Frizzled-related protein, or the Dkk/Kremen complex (63–65). One other Wnt antagonist is sclerostin, a soluble factor, the majority of which is secreted by osteocytes (66), that binds to low-density lipoprotein receptor–related protein 5 (LRP-5) and LRP-6 and antagonizes canonical Wnt signaling (67). Increased sclerostin expression in osteocytes has been reported to reduce bone formation by promoting osteoblast apoptosis (68,69). Glucocorticoids increase the expression of Dkk-1 in primary human osteoblasts (70). In osteoblastic cell lines, glucocorticoid excess targets Wnt inhibitors, such as Dkk-1, Frizzled 2, Frizzled 7, and Wnt-induced signaling protein 1, that may contribute to glucocorticoid-induced suppression of osteoblast function (28).

Our microarray data on in vivo glucocorticoid excess suggested that Wnt antagonists, including Dkk-1, Sost, and Wif1, were up-regulated. Therefore, suppression of Wnt signaling may account for the glucocorticoid-induced suppression of bone formation. PTH, but not risedronate, in addition to glucocorticoid treatment reversed the elevations of these Wnt antagonists, suggesting that the effect of PTH on glucocorticoid excess occurred at least in part through regulation of these antagonists and revealing a possible mechanism for the efficacy of PTH in the treatment of glucocorticoid-induced osteoporosis.

In summary, glucocorticoid-induced inhibition of osteoblast maturation and function occurs in part through increasing expression of inhibitory genes for Wnt signaling (bone formation) and mineralization. The addition of either PTH or risedronate to concurrent glucocorticoid treatment improved bone architecture bone strength. Our data suggest that part of the mechanism of action of PTH in the prevention of glucocorticoid-induced bone loss may be the ability of PTH to inhibit Wnt signaling antagonists and stimulate bone formation. Risedronate may reduce the synthesis of mineralization-inhibiting proteins to stimulate bone mineralization. The different actions of these 2 medications on genes regulating mineralization and bone formation may help to explain the in vivo changes in mineralization and bone mass in the presence of glucocorticoids.

AUTHOR CONTRIBUTIONS

Dr. Lane had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Yao, Lane.

Acquisition of data. Yao, Cheng, Pham, Busse, Zimmermann, Ritchie, Lane.

Analysis and interpretation of data. Yao, Cheng, Pham, Zimmermann, Ritchie, Lane.

Manuscript preparation. Yao, Zimmermann, Ritchie, Lane.

Statistical analysis. Yao, Cheng, Lane.

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