1,25(OH)₂D₃ acts as a bone-forming agent in the hormone-independent senescence-accelerated mouse (SAM-P/6)

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Duque, Gustavo, Michael Macoritto, Natalie Dion, Louis-Georges Ste-Marie, and Richard Kremer. 1,25(OH)₂D₃ acts as a bone-forming agent in the hormone-independent senescence-accelerated mouse (SAM-P/6). Am J Physiol Endocrinol Metab 288: E723–E730, 2005. First published November 30, 2004; doi:10.1152/ajpendo.00180.2004.—Recent studies suggest that vitamin D signaling regulates bone formation. However, the overall effect of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] on bone turnover in vivo is still unclear. In this study, our aim was to examine the effect of 1,25(OH)₂D₃ on bone turnover in SAM-P/6, a hormone-independent mouse model of senile osteoporosis characterized by a decrease in bone formation. Male and female 4-mo-old SAM-P/6 mice were treated with 1,25(OH)₂D₃ (18 pmol/24 h) or vehicle for a period of 6 wk, and a group of age- and sex-matched nonosteoporotic animals was used as control. Bone mineral density (BMD) at the lumbar spine increased rapidly by >30 ± 5% (P < 0.001) in 1,25(OH)₂D₃-treated SAM-P/6 animals, whereas BMD decreased significantly by 18 ± 2% (P < 0.01) in vehicle-treated SAM-P/6 animals and remained stable in control animals during the same period. Static and dynamic bone histomorphometry indicated that 1,25(OH)₂D₃ significantly increased bone volume and other parameters of bone quality as well as subperiosteal bone formation rate compared with vehicle-treated SAM-P/6 mice. However, no effect on trabecular bone formation was observed. This was accompanied by a marked decrease in the number of osteoclasts and eroded surfaces. A significant increase in circulating bone formation markers and a decrease in bone resorption markers was also observed. Finally, bone marrow cells, obtained from 1,25(OH)₂D₃-treated animals and cultured in the absence of 1,25(OH)₂D₃, differentiated more intensely into osteoblasts compared with those derived from vehicle-treated mice cultured in the same conditions. Taken together, these findings demonstrate that 1,25(OH)₂D₃ acts simultaneously on bone formation and resorption to prevent the development of senile osteoporosis.

1,25-dihydroxyvitamin D₃; osteoporosis; bone turnover; histomorphometry; bone markers

THE EFFECT OF VITAMIN D ON BONE and mineral homeostasis is complex, and many studies conducted both in vitro and in vivo have brought conflicting results. In vitro, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] induces bone resorption (30) indirectly by stimulating the expression of RANK ligand at the surface of the osteoblast, which in turn activates the osteoclast (36). In contrast, in vivo, 1,25(OH)₂D₃ inhibits osteoclastogenesis by decreasing the pool of osteoclast precursors in the bone marrow (31). The effect of 1,25(OH)₂D₃ on bone formation is also unresolved. Earlier studies indicate that the effect of 1,25(OH)₂D₃ on osteoblasts in vitro depends on the timing of administration. Treatment of rat calvaria osteoblasts during the proliferative phase with 1,25(OH)₂D₃ inhibits osteoblast proliferation, collagen synthesis, and, subsequently, osteoblast differentiation (26). In contrast, addition of 1,25(OH)₂D₃ to cultures of mature osteoblasts results in an upregulation of osteoblast-associated genes and subsequent osteoblast differentiation (23). 1,25(OH)₂D₃ has been reported to stimulate osteocalcin and osteopontin production (28, 29) as well as the expression of the osteoblast differentiation factor Osf2/Cbfa1 (6). Recent observations suggest that vitamin D signaling regulates bone formation. Specific overexpression of the vitamin D receptor (VDR) in transgenic mouse results in enhanced periosteal bone formation and increased bone mass (13). The possibility that 1,25(OH)₂D₃ regulates bone formation in vivo is attractive from both mechanistic and therapeutic standpoints. A number of studies indicate that vitamin D metabolism and action change with age. Circulating 1,25(OH)₂D₃ levels decrease with age (8, 21) as well as the level of 1,25(OH)₂D₃ precursor 25(OH)D₃ (2). Furthermore, VDR number or sensitivity may be reduced with age (9, 15). Consequently, these alterations may result in bone loss in aging individuals. This hypothesis on the role of vitamin D in the pathogenesis of osteoporosis is further supported by clinical studies in the elderly showing a significant improvement in bone density and fracture risk following administration of vitamin D₃ and calcium in double-blind, randomized clinical trials (21, 33). As well, treatment of postmenopausal women with calcitriol results in moderate improvement in bone mass (38). Despite these promising observations, vitamin D is still considered a supplement to the currently approved treatment of osteoporosis.

In the present study, we used a hormonally independent model of osteoporosis, the senescence-accelerated mouse (SAM-P/6), in which ovariectomy does not lead to an increase in osteoclastic activity or a further decrease in bone mass (24). This strain of mice is characterized by a decrease in osteoblastogenesis and an increase in adipogenesis within the bone marrow, leading to bone loss and fractures, mimicking the human form of senile osteoporosis (16, 17, 20, 24, 32, 37, 39). SAM-P/6 mice start losing bone at 4 mo of age and have a mean life span of 8 mo compared with a bone loss starting only at 12 mo and a mean life span of 24 mo for their normal counterparts, SAM-R/1TA mice (24). At 7 mo, spontaneous fractures start to occur in SAM-P/6 mice as a consequence of...
severe osteoporosis (24, 32). In this study, we demonstrate that 1,25(OH)2D3 acts simultaneously on bone formation and resorption to increase bone mass and prevent the development of osteoporosis.

EXPERIMENTAL PROCEDURES

Animals. SAM-P/6 and SAM-R/1TA mice were kindly provided by Dr. Toshio Takeda (Kyoto University, Kyoto, Japan). Male and female mice were housed in cages in a limited-access room restricted to aging mice (12:12-h light-dark cycle). Bedding, food, and water were given as previously described (10). Animal husbandry adhered to Canadian Council on Animal Care standards, and all protocols were approved by the McGill University Health Center Animal Care Utilization Committee. The colony was free from any parasitic, bacterial, or viral pathogens, as determined by a sentinel program. At 4 mo of age, mice (n = 12 per group) in a 50:50 ratio of male and female SAM-P/6 were implanted with Alzet osmotic minipumps (Alzet, Cupertino, CA) containing 1,25(OH)2D3 (LEO Pharmaceuticals, Ballerup, Denmark) prepared as previously described (10) and delivering a constant infusion of either 18 pmol/24 h of 1,25(OH)2D3 or vehicle alone. A group of nonosteoporotic control animals (SAM-R/1TA) was treated with either 1,25(OH)2D3 or vehicle alone, and its parameters were analyzed simultaneously. Osmotic minipumps were replaced after 3 wk for a total of 6 wk of treatment. Blood calcium levels were monitored at timed intervals by microchemistry (Kodak Ektachrome, Mississauga, ON, Canada) and corrected for albumin concentrations according to the formula: plasma total calcium – plasma albumin × 0.02. After 6 wk of treatment, animals were killed and femora isolated. One femur was used for histomorphomet-

Fig. 1. A–I: histological and histomorphometric analysis of bone. Goldner trichrome staining of proximal tibiae is shown in senescence-accelerated mouse (SAM)-R/1TA (A and D), vehicle-treated SAM-P/6 (B and E), and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]-treated SAM-P/6 (C and F) mice (A–C, ×40; D–F, ×100). G–I: fluorescent staining with demeclocycline in bone specimens from SAM-R/1TA (G), vehicle-treated (H), and 1,25(OH)2D3-treated (I) animals (G–I, ×80). Quantification of dynamic and static parameters is shown in Table 1. a–d: micro-computed tomography analysis of bone from SAM-P/6 mice treated with 1,25(OH)2D3 or vehicle. Shown are differences in coronal sections in vehicle-treated (a) vs. 1,25(OH)2D3-treated (c) mice and in 3-dimensional digitally extracted segments for vehicle-treated (b) and 1,25(OH)2D3-treated (d) mice. Note higher trabecular thickness and number (Tb) as well as higher cortical thickness (Ct) in 1,25(OH)2D3-treated vs. vehicle-treated mice. Measurements correlate with those obtained by histomorphometry (data not shown).
meric analysis; the other femur was cut longitudinally, and trabecular cells were scraped, separated from red blood cells, and suspended in 1 ml of conservation buffer Easy-Kit mini prep (Qiagen, Valencia, CA) for further RNA extraction. In addition, one side tibiae was 1 ml of conservation buffer Easy-Kit mini prep (Qiagen, Valencia, CA) for further RNA extraction. In addition, one side tibiae was analyzed with micro-CT and the other flushed for ex vivo cultures, as described in Micro-CT of bone samples.

Histological and histomorphometric analysis of bone. The details of these methods were described previously (10). At the time the animals were killed, one femur from each animal in each treatment group and in SAM-R/ITA controls was removed, fixed, dehydrated in 70% ethanol, and embedded undecalified in methyl methacrylate (J-T Baker, Phillipsburg, NJ). At 50-μm intervals, longitudinal sections of 5 and 8 μm thick using a polycut-E microtome (Reichert-Jung, Leica, Heerbrugg, Switzerland) were mounted on gelatin-coated glass slides. Five-micrometer-thick sections were stained with Goldner’s trichrome method and used for the structural and static parameters of bone remodeling. For the purpose of dynamic bone formation parameters, mice were injected with 20 mg/kg demeclocycline (Sigma-Aldrich, St. Louis, MO) 7 and 2 days before they were killed. Sections of 8 μm were left unstained for observation, under UV light, of the epifluorescence of tetracycline labeling. Bone parameters were quantified in cancellous bone tissue at distances greater than 0.4 mm from the growth plate metaphyseal junction to exclude the primary spongiosa. Histomorphometry was done with a semiautomatic image-analyzing system combining a microscope equipped with a camera lucida and digitizing tablet linked to a computer using the OsteoMeasure Software (Osteometrics, Decatur, GA). Nomenclature and abbreviations of histomorphometric parameters follow the recommendations of the American Society for Bone and Mineral Research (Ref. 27; see also Table 1 legend).

Histochemistry. Detection of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity was carried out on 5-μm-thick sections according to the method of Liu et al. (22). Naphthol-AS-TR was used as substrate for both enzymes, and Fast Blue BB salt (Sigma-Aldrich) and pararosaniline were used as couplers for ALP and TRAP, respectively.

Micro-CT of bone samples. Tibiae were fixed in 10% formalin, scanned by micro-CT at ×40 magnification with a SkyScan 1072, and analyzed with bone analysis software (v. 2.2.2; Skyscan, Aartselaar, Belgium). During scanning, the samples were enclosed in a tightly fitting, rigid plastic tube to prevent movement. Analyses of the trabecular bone were carried out in a 2.3-mm-thick region of the tibia, distal to the growth plate of the knee joint. The software was used to separate regions of trabecular bone from cortical bone in the sections. Thresholding was then applied to the images to segment the bone from the background, and the same threshold setting was used for all the samples. The following three-dimensional (3-D) parameters were measured: volume, surface, and surface-to-volume ratio. To calculate these, the software builds a 3-D cuboidal voxel model of the bone. The following bone architectural measurements were then made: trabecular thickness and separation. In addition, cortical width and diameter were measured in transversal sections of specific locations. Details of these measurements have been published elsewhere (3).

Dual-energy X-ray absorptiometry analysis. Dual-energy X-ray absorptiometry (DEXA) analysis was performed at time 0 (4 mo) and at 3 and 6 wk after Alzet osmotic minipump implantation. Hip and spine BMD was measured using a PIXIMUS bone densitometer (PIXIMUS, GE Medical Systems). A phantom (PIXIMUS) was used to calibrate the densitometer before each experiment.

Ex vivo cultures of bone marrow cells. When the animals were killed, one side tibiae was flushed using Dulbecco’s α-modified Eagle’s medium ( Gibco; Grand Island, NY) containing 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin placed in 150-cm² dishes and incubated at 37°C for 24 h. The bone marrow cells were filtered through a cell strainer with 70-μm nylon mesh (BD Bioscience, Bedford, MA) and plated in 10-cm² tissue culture dishes. The cells were incubated in mesenchymal stem cell (MSC) growth medium (MSCGM; BioWhittaker) at 37°C with 5% humidified CO₂ and isolated by their adherence to tissue culture plastic. Medium was aspirated and replaced with fresh medium to remove nonadherent cells every 2–3 d. The adherent MSC were grown to confluence for ~7 days defined as MSC at passage 0, harvested with 0.25% trypsin and 1 ml EDTA for 5 min at 37°C, diluted 1:3 in MSCGM, plated, and grown to confluence for further expansion. After second and third passages, MSC were used for subsequent experiments.

To induce differentiation, a total of 10⁵ cells were diluted in osteoblastogenesis induction medium (OIM, BioWhittaker) and plated in 4-cm² dishes. Medium was aspirated and replaced with fresh OIM every 3 d. At 21 d, medium was removed, and cultures were fixed in 10% vol/vol formol-saline solution for 5 min. Mineralizing bone nodules were detected by Alizarin red (40 mM, pH 4.2; Sigma-Aldrich) staining. The total number of bone nodules per dish was counted macroscopically. There were six animals per group, and bone marrow cells from each animal were divided in four aliquots and plated separately. There were 24 dishes per group.

Biochemical assays. After 6 wk of treatment, mice were killed by cervical dislocation, and blood was removed by cardiac puncture. Serum was stored at −80°C for further analysis. Osteocalcin was measured in 200 μl of serum with the mouse Osteocalcin-IRMA kit (Immutopics, San Clemente, CA) to assess bone formation. N-telopeptide was measured in 20 μl of serum with the R A T telopeptide kit (Osteometer Biotech; Herlev, Hovedgade, Denmark) to assess osteoclastic activity. Parathyroid hormone (PTH) was measured in 200 μl of serum and analyzed by using the mouse intact PTH immunometric assay kit (Immutopics). A normal range was established by measuring serum levels of bone markers and PTH in SAM-R/ITA control mice (n = 12) of the same age.

Table 1. Histomorphometric analysis of bone in 1,25(OH)₂D₃-treated vs. nontreated SAM-P/6 mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SAM-R/ITA (n = 5)</th>
<th>SAM-P/6 Control (n = 7)</th>
<th>SAM-P/6 (n = 7)</th>
<th>ANOVA P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV, %</td>
<td>6.0 ± 1.2</td>
<td>7.4 ± 1.6</td>
<td>15.7 ± 0.7</td>
<td>&lt;0.001ab</td>
</tr>
<tr>
<td>Tb.Th, μm</td>
<td>31.5 ± 1.3</td>
<td>26.4 ± 2.9</td>
<td>47.6 ± 2.4</td>
<td>&lt;0.001ab</td>
</tr>
<tr>
<td>Tb.Sp, μm</td>
<td>719 ± 298</td>
<td>384 ± 54</td>
<td>256 ± 8.8</td>
<td>0.007*</td>
</tr>
<tr>
<td>Tb.N/mm</td>
<td>1.9 ± 0.4</td>
<td>2.6 ± 0.3</td>
<td>3.3 ± 0.1</td>
<td>0.005*</td>
</tr>
<tr>
<td>Ct.Wi, μm</td>
<td>182 ± 113</td>
<td>167 ± 6.7</td>
<td>202 ± 7.5</td>
<td>0.019b</td>
</tr>
<tr>
<td>OV/BV, %</td>
<td>0.014 ± 0.005</td>
<td>0.14 ± 0.08</td>
<td>0.012 ± 0.007</td>
<td>NS</td>
</tr>
<tr>
<td>Os/BS, %</td>
<td>0.18 ± 0.09</td>
<td>0.93 ± 0.43</td>
<td>0.13 ± 0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Ob/S, BS, %</td>
<td>0.08 ± 0.07</td>
<td>0.44 ± 0.21</td>
<td>0.03 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>O.Th, μm</td>
<td>1.48 ± 0.22</td>
<td>1.5 ± 0.08</td>
<td>1.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>ES/BS, %</td>
<td>12.6 ± 3.1</td>
<td>8.3 ± 1.4</td>
<td>1.3 ± 0.3</td>
<td>0.001ab</td>
</tr>
<tr>
<td>N.Oc/B.Pm/mm</td>
<td>4.2 ± 0.7</td>
<td>3.1 ± 0.3</td>
<td>0.5 ± 0.08</td>
<td>&lt;0.001ab</td>
</tr>
<tr>
<td>Dynamic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS/BS, %</td>
<td>29.3 ± 3</td>
<td>17.2 ± 1.3*</td>
<td>15.4 ± 6.8</td>
<td>NS</td>
</tr>
<tr>
<td>MAR, μm/day</td>
<td>1.14 ± 0.13</td>
<td>0.82 ± 0.06*</td>
<td>0.84 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>BFR/BS, μm²/year</td>
<td>12.75 ± 28</td>
<td>52.5 ± 6*</td>
<td>61.7 ± 33</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE for all groups. 1,25(OH)₂D₃, 1,25-dihydroxvitamin D₃; SAM-R/ITA and SAM-P/6, strains of senescent-accelerated mice; NS, not significant; BV/TV, bone volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular no.; Ct.Wi, cortical width; OV/BV, osteoblast volume; BS, osteoblast; Ob/S, BS, osteoblast surface; O.Th, osteoid thickness; ES/BS, eroded surface; N.Oc/B.Pm, osteon; Ct.Wi, cortical no.; BS, MS, mineralizing surface; MAR, mineral apposition rate; BFR/BS, bone formation rate per bone surface. Significant differences between means were evaluated by one-way ANOVA and individual between-group contrasts determined using Tukey’s test, P < 0.05 SAM-P/6 treated vs. *SAM-R/ITA or #SAM-P/6. *Unpaired t-test between SAM-R/ITA and SAM-P/6 mice was considered significantly different, P < 0.05.
SE. Cell culture experiments were performed in triplicate and repeated three times. Differences between treated and nontreated groups were determined by statistical analysis using Levene’s test for homogeneity of variances and unpaired t-test for equality of means for histomorphometry measurements. All other variables were compared using one-way analysis of variance. A value of \( P < 0.05 \) was considered significant.

**RESULTS**

**Bone histomorphometry.** Figure 1 shows Goldner’s trichrome staining of tibiae from vehicle-treated SAM-R/1TA (Fig. 1, A and D), vehicle-treated SAM-P/6 (Fig. 1, B and E) and 1,25(OH)2D3-treated SAM-P/6 mice (Fig. 1, C and F). Figure 1, G–I, show fluorescence staining with demeclocycline in bone specimens from vehicle-treated SAM-R/1TA (Fig. 1, G) as well as vehicle-treated SAM-P/6 (Fig. 1, H) and 1,25(OH)2D3-treated SAM-P/6 mice (Fig. 1, I). Table 1 shows the complete set of histomorphometric measurements in both static and dynamic parameters as well as its statistical differences. Static histomorphometric analysis shows a significant increase in bone volume (BV/TV, \( P < 0.001 \)) and trabecular thickness (Tb.Th, \( P < 0.001 \)) as well as cortical width (Ct.Wi, \( P = 0.019 \)) in the 1,25(OH)2D3 treated SAM-P/6 compared with vehicle-treated SAM-P/6 and SAM-R/1TA animals (Table 1). Furthermore, a significant decrease in bone resorption parameters (ES/BS, and N.Oc/B.Pm) was observed in 1,25(OH)2D3-treated SAM-P/6 compared with vehicle-treated SAM-P/6 and SAM-R/1TA groups (Table 1). Dynamic parameters of bone formation were significantly higher in SAM-R/1TA than in vehicle-treated SAM-P/6 mice, as indicated by measures of trabecular bone formation indexes (Table 1). No significant increase in trabecular bone formation indexes were observed at 6 wk in 1,25(OH)2D3-treated SAM-P/6 compared with vehicle-treated animals (Table 1). However, the bone formation rate was significantly increased in the subperiosteal areas of 1,25(OH)2D3-treated compared with vehicle-treated mice (Fig. 2, A–D). In addition, Fig. 2, E–G, shows ALP and TRAP staining in bone sections from SAM-R/1TA (Fig. 2, E), vehicle-treated (Fig. 2, F), and 1,25(OH)2D3-treated (Fig. 2, G) SAM-P/6 mice. Qualitatively, we observed a stronger staining intensity of ALP and a less intense staining intensity of TRAP.
in 1,25(OH)2D3-treated mice compared with vehicle-treated mice. Furthermore, microarchitectural changes assessed by 3-D micro-CT (Fig. 1, a–d), demonstrated morphological changes similar to those seen by histomorphometry (data not shown).

**Differentiation of bone marrow cells into osteoblasts in ex vivo cultures.** The mean number of bone nodules per dish increased significantly (Fig. 3, A and B) in 1,25(OH)2D3-treated mice (72 ± 4) compared with vehicle-treated mice (41 ± 3) in bone marrow cells induced to differentiate (P < 0.05).

**Changes in bone mass in SAM-P/6 treated with 1,25(OH)2D3.** DEXA was performed on SAM-P/6 mice at 4 mo of age (peak bone mass) and after treatment with 1,25(OH)2D3 or vehicle as well as in their SAM-R/1TA counterparts. A significant increase in bone mineral density (BMD) was observed in both lumbar spine (0.096 ± 0.005 vs. 0.052 ± 0.006, P < 0.01; Fig. 3C) and hip (0.085 ± 0.007 vs. 0.066 ± 0.003, P < 0.01; Fig. 3D), at 6 wk in 1,25(OH)2D3-treated compared with vehicle-treated SAM-P/6 animals. These absolute values correspond to an increase of 30 ± 5% at the spine and 15 ± 2% at the hip (P < 0.001) in 1,25(OH)2D3-treated
mice compared with a decline of \(-18 \pm 2\%\) at the spine and \(-6 \pm 1.5\%\) at the hip (\(P < 0.01\)) in vehicle-treated animals. In control SAM-R/ITA mice, BMD increased slightly over the time course of the experiment in both 1,25(OH)_{2}D_{3}- and vehicle-treated animals. At the lumbar spine, no difference between 1,25(OH)_{2}D_{3}- and vehicle-treated mice was observed at any time point. At the hip, a small increase in BMD was observed at the 6-wk time point in 1,25(OH)_{2}D_{3}-treated compared with vehicle-treated mice. When BMD was compared with SAM-R/ITA mice at the same age points, a significant increase in bone mass was found in 1,25(OH)_{2}D_{3}-treated SAM-P/6 compared with vehicle-treated SAM-R/ITA mice at the end of the study period (\(P < 0.05\)). Finally, BMD in SAM-R/ITA mice was significantly higher than in vehicle-treated SAM-P/6 mice at all age points (\(P < 0.001\)).

**Biochemical markers of bone formation and resorption.** Both 1,25(OH)_{2}D_{3}- and vehicle-treated animals remained normocalcemic for the duration of the experiment (Table 2). Serum osteocalcin concentrations increased significantly (\(P < 0.05\)), whereas serum N-telopeptide concentrations decreased significantly (\(P < 0.001\)) in 1,25(OH)_{2}D_{3}-treated SAM-P/6 compared with vehicle-treated SAM-P/6 and SAM-R/ITA animals (Table 2). In addition, serum concentrations of PTH decreased significantly in 1,25(OH)_{2}D_{3}-treated SAM-P/6 compared with vehicle-treated SAM-P/6 and SAM-R/ITA mice (\(P < 0.001;\) Table 2).

**DISCUSSION**

In this study, we used the SAM-P/6 mouse, developed by Matsushita et al. (24) and Takeda et al. (37), a widely accepted model for senile osteoporosis (16–19, 34, 35) due to their particular characteristics that correlate with those seen in senile osteoporosis in humans. The peak bone mass of SAM-P/6 occurs earlier (4 mo) than that of SAM-R/ITA, their normal nonosteoporotic counterpart. After attaining their peak bone mass, SAM-P/6 mice lose bone rapidly due to a premature decrease in the ability of mesenchymal progenitors of bone marrow to differentiate toward the osteoblastic lineage (16) and a concomitant increase in bone marrow adipogenesis (17, 19). Bone loss in SAM-P/6 mice is hormone independent, as shown by a lack of accelerated bone loss after oophorectomy or orchietomy (24, 37). Furthermore, in this model, bone mass does not change following estrogen therapy (34, 35). Our goal here was to assess the effect of 1,25(OH)_{2}D_{3} on bone loss that occurs during the aging process, a phenomenon that starts around age 35 yr in humans and is characterized by a slow but continuous decrease in bone formation (5). This aging process is superimposed by accelerated bone resorption characteristic of estrogen deficiency after the menopause (4). SAM-P/6 mice may therefore be extremely useful in assessing factors influencing bone formation independent of the changes in turnover occurring after hormonal deprivation. We noted a relatively rapid bone loss in nontreated SAM-P/6 mice during the 6-wk study period following peak bone mass. Bone loss was seen in both hip and spine. Dynamic histomorphometric analysis of bone sections in vehicle-treated SAM-P/6 mice indicated that parameters of bone formation were significantly lower than the dynamic parameters observed in nonosteoporotic control SAM-R/ITA animals, indicating a decrease in bone formation, a hallmark of senile osteoporosis characteristic of this mouse model. In contrast, indexes of bone resorption were not statistically different between vehicle-treated SAM-P/6 and SAM-R/ITA mice (Table 1), indicating that the primary mechanism of bone loss in this model is a decrease in bone formation. Biochemically, the SAM-P/6 mice did not differ significantly from the control SAM-R/ITA mouse strain with respect to their circulating concentrations of calcium, markers of bone formation (osteocalcin) and resorption (N-telopeptide) concentrations. However, we noted a moderate but significant increase in their circulating PTH concentrations, indicative of a secondary hyperparathyroidism. Previous reports in SAM-P/6 mice had suggested that secondary hyperparathyroidism in this model is due to the decreased calcium absorption that is frequently observed in these animals (24, 37), also reported in type II senile osteoporosis (21). Because aging is characterized by decreased bone formation, it is tempting to speculate that osteoblastogenesis deficiency during age-related bone loss might be the culprit of the bone aging process. The molecular events that underlie the changes in bone formation with aging have not yet been determined. Furthermore, these changes may be confounded by other homeostatic imbalances that occur frequently in aging and are noted in SAM-P/6 mice. Indeed, aging is associated with a deficiency in absorption of nutrients such as calcium and vitamin D (7). Furthermore, vitamin D synthesis and sunlight exposure decrease with age and lead to vitamin D deficiency, a commonly occurrence in this age group (14). As a result, several studies conducted in elderly individuals given vitamin D_{3} supplementation in combination with calcium have proved beneficial in increasing bone mass and subsequent fracture prevention (12, 21, 25, 33). In contrast, major trials with the active form of vitamin D, 1,25(OH)_{2}D_{3}, or its analogs have not yet been conducted. Such trials will require careful examination of important clinical parameters, such as dosage, characteristics of the study population, vitamin D status, and the obvious confounding effect of hormonal deprivation in the subjects chosen for these studies.

In this study, we tried to address some of these issues by using an estrogen-independent model of senile osteoporosis and by administering the drug at a rate that would not induce hypercalcemia. The overall effect of 1,25(OH)_{2}D_{3} on bone turnover in vivo was determined by careful histomorphometric and biochemical analysis. Administration of 1,25(OH)_{2}D_{3} significantly increased bone mass without affecting the animal’s weight and well-being. There was a trend toward higher circulating calcium levels in 1,25(OH)_{2}D_{3}-treated animals;
however, no significant increase in serum calcium was observed compared with vehicle-treated animals. The positive effect on bone mass was the result of a concomitant increase in bone formation and decrease in bone resorption, as indicated by histomorphometric analysis of distal femur (Figs. 1 and 2 and Table 1). We noted a significant increase in indexes in bone mass (BV/TV and Cr.Wi) and a marked reduction in indexes of bone resorption (ES/BS, NOc/Tar, and NOc/BPm) after 1,25(OH)_{2}\text{D}_3 administration in SAMP/6 (Table 1). Interestingly, bone volume (BV/TV) was not significantly different between vehicle-treated SAMP/6 and SAM-R/ITA mice despite a significant difference in BMD (Fig. 3) and bone formation parameters (Table 1). This apparent discrepancy may be explained by the fact that BMD integrates both cortical and trabecular measurements, whereas BV/TV assesses only secondary spongiosa at the distal femur. Furthermore, small differences in BMD at the hip level may not be observed by use of the less precise measurement of BV/TV. Similar observations were made previously by Jilka et al. (16) in the same mouse models. Additionally, this discrepancy may have been accentuated by different patterns of secondary mineralization between the two strains of mice. Although we did not observe significant changes in dynamic parameters of bone formation at the trabecular level (Table 1), the anabolic effect of 1,25(OH)_{2}\text{D}_3 was evident at the periosteal envelope at 6 wk and was characterized by an increase in the active mineralizing surfaces. In trabecular bone (Table 1), we did not observe any change in MS/BS, MAR, and BFR/MS in 1,25(OH)_{2}\text{D}_3-treated animals despite a qualitative increase in ALP expression in cells of the osteoblast lineage (Fig. 2). It was previously reported that 1,25(OH)_{2}\text{D}_3 can enhance ALP expression in osteoblast precursors; however, osteoblast activation characterized by an increase in ALP does not always result in an increase in bone formation indexes (1). Consequently, the enhancement of ALP expression observed here may have come from activation of osteoblast precursors but did not translate into an increased bone formation. The mechanism underlying this differential effect on cortical vs. trabecular bone is still elusive. In a recent study, Gardiner et al. (13) observed a similar phenomenon exclusively in mature cells of the osteoblastic lineage by use of transgenic mice overexpressing the VDR. The anabolic effect was observed exclusively in cortical bone. Trabecular bone volume was also increased secondarily to a sharp decrease in bone resorption. It suggests that this VDR-mediated osteoclastic inhibition in trabecular bone is indirectly mediated via mature osteoclasts counterbalancing the osteoclastogenic signals expressed by immature osteoclasts and osteoblastic stromal cells. This anabolic effect of 1,25(OH)_{2}\text{D}_3 on cortical bone was in sharp contrast with the low levels of bone formation observed in vehicle-treated SAM-P/6 and SAM-R/ITA mice (Fig. 2). Biochemically, this anabolic effect was reflected by an increase in circulating osteocalcin levels. The in vivo antiresorptive effect of 1,25(OH)_{2}\text{D}_3 was characterized histochemically by a lower number of TRAP^{+} cells, i.e., osteoclasts (Fig. 2, G and F), and biochemically by a decrease in circulating levels of N-telopeptide (Table 2), supporting a recent report that in vivo administration of 1,25(OH)_{2}\text{D}_3 inhibits osteoclast formation (11). We noted a relatively rapid bone loss in nontreated SAM-P/6 during the 6-wk study period following peak bone mass. This bone loss was reversed by the administration of 1,25(OH)_{2}\text{D}_3 compared with vehicle-treated SAM-P/6 mice. Furthermore, bone mass continued to increase throughout the study period in 1,25(OH)_{2}\text{D}_3-treated SAM-P/6 and became significantly higher than nonosteoporotic SAM-R/ITA control mice by the end of the treatment period (Fig. 3). These results therefore indicate that 1,25(OH)_{2}\text{D}_3 not only prevents bone loss normally occurring in this SAM-P/6 model but also results in additional bone gain compared with nonosteoporotic control mice. The overall effect of 1,25(OH)_{2}\text{D}_3 on bone turnover observed in this study may therefore be both direct on osteoblastic bone formation and indirect on osteoclastic bone resorption. Administration of 1,25(OH)_{2}\text{D}_3 corrects secondary hyperparathyroidism and is likely to improve calcium absorption, further augmenting the effect on PTH suppression.

However, nontreated SAM-P/6 mice were not characterized by elevation of bone resorption indexes, suggesting that secondary hyperparathyroidism did not play the significant role in bone loss characteristic of the SAM-P/6 model. The observed suppression of osteoclastic resorption indexes in 1,25(OH)_{2}\text{D}_3-treated mice may therefore be secondary to suppression of PTH levels or a direct or indirect effect of 1,25(OH)_{2}\text{D}_3 on the osteoclast, as reported previously (31). In any event, the anabolic effect of 1,25(OH)_{2}\text{D}_3 on bone is clearly independent of 1,25(OH)_{2}\text{D}_3-induced PTH inhibition and is further evidenced by the significant gain in bone mass observed in 1,25(OH)_{2}\text{D}_3-treated SAM-P/6 mice compared with their nonosteoporotic SAM-R/ITA controls. In summary, our data indicate that, in this model of age-related osteoporosis, 1,25(OH)_{2}\text{D}_3 simultaneously stimulates bone formation while inhibiting bone resorption with an overall beneficial effect on bone mass.

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REFERENCES

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