Strain-Dependent Differences in Vertebral Bone Mass, Serum Osteocalcin, and Calcitonin in Calcium-Replete and -Deficient Mice (43574)

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Abstract. **The effects of dietary calcium intake on vertebral bone mass, composition, and turnover (calcium deposition and resorption) were determined in 10- and 14-week-old C57BL/6 (small) and SENCAR (large) mice. Total vertebral mass, percent ash, calcium, magnesium, and phosphorus were higher in SENCAR mice than in C57BL/6 mice at 10 weeks of age and after being fed 0.02% or 0.6% dietary calcium for 4 additional weeks. Relative calcium deposition was higher in C57BL/6 mice than in SENCAR mice, while relative calcium resorption was similar in both strains. The rate of resorption was higher in mice fed 0.02% dietary calcium than in those fed 0.6% dietary calcium. Thus, C57BL/6 mice gained vertebral calcium, while it remained unchanged or declined in SENCAR mice under conditions of both calcium depletion and calcium repletion. Serum osteocalcin (an index of bone formation) was higher in C57BL/6 mice than in SENCAR mice. Mathematically significant correlations between osteocalcin levels and vertebral calcium resorption and the net vertebral calcium loss were observed only in SENCAR mice. The serum calcitonin concentration was correlated with the amount of vertebral calcium resorbed in SENCAR mice, but not in C57BL/6 mice. Thus, vertebral resorption and formation are more tightly coupled in 10- to 14-week-old SENCAR mice than in C57BL/6 mice. In addition, remodeling appears to dominate vertebral calcium dynamics in SENCAR mice, while growth dominates in C57BL/6 mice during this period. Rodents have frequently been dismissed as potential models of bone aging based on the expectation that continued growth, rather than remodeling, dominates skeletal dynamics. These data clearly demonstrate that increases in body mass ("growth") are not invariably associated with continued vertebral growth. In this murine model, both heredity and dietary calcium intake modulate vertebral bone mass, turnover dynamics, and composition at sexual maturity. These differences in the development and regulation of vertebral bone mass in small C57BL/6 and large SENCAR mice suggest that animal, as well as clinical, models provide useful insights into the cellular and hormonal mechanisms of somatotype-dependent vertebral growth.** [P.S.E.B.M. 1993, Vol 203]

In the absence of malnutrition and metabolic abnormalities, genetic factors, such as gender, ethnicity, and somatotype, are a major determinant of peak bone mass (1). Bone mass has been found to be low in the daughters of women with osteoporosis (2) and in relatives of osteoporotic patients (3). Bone mass is greater in males than in females, and age-related bone loss is more rapid at all sites in women than in men (4). Bone density is greater in black and Polynesian adults than in white (5, 6) and Asian subjects (7). Population and individual differences in the kinetics of site-specific bone development have been reported for the lumbar spine, femoral neck, and midfemoral shaft of both sexes during late childhood, puberty, and adolescence (8, 9). Pocock *et al.* (1) suggested that a single

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gene or set of genes may determine peak bone mass potential at all skeletal sites.

Bell *et al.* (10, 11) proposed that differences in calcitropic hormone status and calcium metabolism account for high or low peak bone mass in at least one distinct population. Immunoreactive parathyroid hormone and 1,25-dihydroxyvitamin D_3 levels are higher, while osteocalcin (an index of bone formation) and 25-hydroxyvitamin D_3 levels are lower in black than in white subjects (10, 11). Serum total and ionized calcium levels are the same in both groups. However, nephrogenous cAMP levels are higher, and urinary calcium is lower in black than in white subjects (10). In contrast, differences in calcitropic hormone levels do not account for the higher bone mass in Polynesians (6) or the lower bone mass in Asians, Eskimos, or Mexican-Americans relative to that in individuals of European ancestry (5). Cortical and cancellous bone architectures are not significantly different in black and white subjects (12). However, the rate of bone formation is lower in black adults than in white subjects, and reduced rates of skeletal remodeling or closer coupling between formation and resorption have been proposed to account for the greater bone mass of black adults (12).

While the roles of humoral factors and the relative rates of bone resorption and formation have been examined in clinical models of low and high bone mass, the local levels of autocrine and paracrine factors in bone and the intrinsic functional characteristics of osteoprogeniter cells, osteoblasts, and osteocytes have not been compared in detail. Progress has been hampered by the absence of a readily available animal model of the robust and gracile extremes in somatotype. The present study was conducted in large (SENCAR) and small (C57BL/6) mouse strains fed a calcium-sufficient (0.6% Ca) or calcium-deficient (0.02% Ca) diet during a critical period of the lifespan in which major skeletal differences evolve (8, 9). Vertebral bone mass, composition, and turnover (calcium deposition and resorption) were quantitated in order to determine the effects of heredity on vertebral development. Serum osteocalcin and calcitonin levels were also measured in order to validate the indirect pharmacokinetic estimation of the rate of bone formation and examine the underlying mechanisms that may account for strain-specific differences in bone mass.

Materials and Methods

Animals. Two groups (I and II) of 30 5-week-old male SENCAR (large) and C57BL/6 (small) mice were obtained from the National Institutes of Health/National Cancer Institute Mammalian Genetics and Animal Products Section, Cancer Research Facility (Frederick, MD). After a mandatory 1-week evaluation, quarantine, and conditioning program at the Sepulveda Veterans Affairs Medical Center Animal Research Facility, the mice were released to the investigator. Group I mice were labeled *in vivo* with [^{3}H]tetracycline, and calcium deposition and resorption were indirectly estimated pharmacokinetically, as outlined below. Isotope-free serum was obtained from the mice in Group II and used in the radioimmunoassay of calcitonin and osteocalcin.

SENCAR and C57BL/6 mice were used in all experiments because they are readily available commercially, and their origins have been documented (13, 14). They represent extremes in body size that are apparent at birth and persist throughout life (13, 14). All mice were fed certified rodent chow containing 0.8% calcium, 0.6% phosphorus, and 4.4 IU vitamin D/g (No. 5002; Ralston-Purina, St. Louis, MO) during the period of rapid growth from 6 to 10 weeks of age. Purified synthetic test diets containing 0.6% Ca or 0.02% Ca (certified rodent diets 5755 and 5855, respectively; Ralston-Purina), 0.4% phosphorus, and 2.2 IU vitamin D_3/g were fed during the experimental period from 10 to 14 weeks of age. Test diets 5755 and 5855 differ in calcium content only and provide all other essential nutrients required to support the normal gestation, growth, development, lactation, and maintenance of laboratory mice. All mice were given deionized water *ad libutum*. The housing conditions were 20–23°C, 50–60% relative humidity, with a 12-hr light/12-hr dark cycle. Body weights were checked at the beginning of the study (6 weeks of age) and biweekly or more often thereafter. Animal use was conducted with institutional approval and conformed to the "Guide for the Care and Use of Laboratory Animals" (DHEW Publication 86–23).

Materials. [7-(*N*)-^{3}H]Tetracycline ([^{3}H]tetracycline; 18.5–37.0 GBq/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). The commercial test kits and reagents used to measure mouse serum osteocalcin and calcitonin were purchased from Biomedical Technologies (Stoughton, MA) and Incstar (Stillwater, MN), respectively. The osteocalcin radioimmunoassay employs an antibody specific for highly purified (≥99.9% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reverse phase high performance liquid chromatography) murine osteocalcin isolated from mouse bone by gel filtration and ion exchange chromatography. The calcitonin radioimmunoassay uses an antibody to synthetic human calcitonin (1–32), a highly conserved mammalian sequence, which exhibits excellent cross-reactivity (≥99%) with rat calcitonin. Calcium, magnesium, and phosphorus reference standards were obtained from Fisher Scientific (Tustin, CA).

Isotopic Labeling and Termination Schedule (Group I). Thirty SENCAR and C57BL/6 mice were injected subcutaneously with [^{3}H]tetracycline at a rate of 13 kBq/10 g body wt·week in 12 injections over the

4-week period from 6 to 10 weeks of age to radiolabel bone mineral (15–17). Bone turnover in [^{3}H]tetracycline-labeled mice was then determined by the indirect pharmacokinetic method of Klein and Jackman (15). Briefly, this approach is based on the following well documented principles. (i) [^{3}H]Tetracycline binds to calcium (18) and is deposited with it in bone mineral during periods of bone formation (19, 20). After resorption of labeled bone, [^{3}H]tetracycline is rapidly and irreversibly eliminated by urinary excretion (15, 16, 21). (ii) Repeatedly labeling animals with [^{3}H]tetracycline during periods of rapid growth (15) results in even distribution of [^{3}H]tetracycline throughout the bones of the skeleton (19, 21). (iii) The tritium in [^{3}H]tetracycline does not undergo metabolic transformation *in vivo* or chemical alteration *in vitro* (22, 23).

Ten Time 0 control SENCAR and C57BL/6 mice were sacrificed at the end of the radiolabeling period at 10 weeks of age, while the remaining 20 [^{3}H]tetracycline-labeled animals of each strain were randomly assigned to either the calcium-sufficient (0.6% dietary Ca) or the calcium-deficient (0.02% dietary Ca) experimental feeding group for 28 days, as outlined above, and sacrificed at 14 weeks of age. All mice were sacrificed by carbon dioxide inhalation overdose, followed by exsanguination. At autopsy, the first six lumbar vertebrae were removed intact and cleaned of soft tissue.

Chemical and Isotopic Measurements. The vertebrae were defatted with ethanol:diethylether (1:1) for 48 hr, dried at 105°C for 16–18 hr, and held desiccated until dry fat-free weights were obtained. Half of the vertebrae were then extracted with 6 *M* HCl for 48 hr, and the [^{3}H]tetracycline content of the digest was determined by liquid scintillation spectrophotometry (model 1410 LS counter; Wallac, Turku, Finland) after corrections for background and color quenching (24).

The remaining vertebrae were ashed at 550°C for 16 hr. Ash weights were recorded, and the bones were ground to a fine powder. Preweighed portions (4–6 mg) of ash were hydrolyzed to dryness in 1.0 *M* HCl, resuspended in 0.1 *M* HCl, and made to 0.25% lanthanum for calcium and magnesium analyses by atomic absorption spectrophotometry (model 2380; Perkin Elmer, Norwalk, CT) (24). Phosphorus was determined colorimetrically by the method of Fiske and Subbarow (25) using a DU-68 spectrophotometer (Beckman, Fullerton, CA). The calcium content is expressed as total calcium/vertebra (mg Ca/vertebra).

Data Computation. *Bone resorption.* The computational method of Li and Klein (17) was employed. The net change in the calcium content of the vertebrae was calculated for each of the mice in the two experimental groups (14-week-old calcium-sufficient and calcium-deficient) using the appropriate Time 0 control values for 10-week old mice for each of the two strains (SENCAR and C57BL/6). The percentage of loss of the radioactive mass of [^{3}H]tetracycline within each group during the 4-week experimental period was also calculated, representing the rate of bone resorption (15–17). The absolute amount of calcium (expressed as mg Ca) resorbed from each bone (bone resorption) was calculated by multiplying the amount of calcium at Time 0 by the rate of bone resorption for each of the two strains (15–17).

Bone formation. The total amount of calcium (expressed as mg of Ca) deposited in each bone (bone formation) was determined by adding the amount of calcium resorbed to the net change in the calcium content of the vertebrae for each of the two strains (15–17). In this context, bone resorption was independent of bone formation, and bone formation was the sum of newly added and resorbed calcium (15–17).

Computation of bone formation and bone resorption. The relative amount (percentage) of calcium deposited into (formation) and resorbed from (resorption) each vertebra was calculated by dividing each absolute value by the corresponding baseline value (10-week-old Time 0 controls) for each of the two strains.

Serum Osteocalcin and Calcitonin (Group II). Calcitonin and osteocalcin were measured in triplicate by radioimmunoassay in multiple dilutions of serum obtained from Group II mice reared to 10 or 14 weeks of age without *in vivo* [^{3}H]tetracycline radiolabeling, as outlined above, and in standardized control sera containing high or low levels of osteocalcin and calcitonin. Assay results were calculated using the four-parameter spline fit option of RIACALC software (Wallac) run on a 386-series computer.

Statistical Analyses. Data are expressed as the mean ± SE (number in parentheses) for all groups. All data were subjected to two-way multiple analysis of variance (ANOVA), using dietary calcium intake and strain as the main effects, and testing for interaction using Statview 512$^+$ software (Brainpower Software, Agoura Hills, CA). In addition, data obtained for each of the individual strains (SENCAR and C57BL/6) were analyzed by two-tailed unpaired Student's *t* test. The absolute amounts of calcium resorbed or deposited and the net change in vertebral calcium content were plotted against serum osteocalcin or calcitonin concentrations, and simple linear regression analysis was performed. The F-ratio for the regression was calculated by ANOVA, and the probability was determined using Statview 512$^+$ software. Where statistically significant results were obtained, the equation of the regression line, r^2 (coefficient of determination), F-ratio (ratio of mean squares), and *P*-values are noted. The level accepted for significance was 5%.

Results

Throughout the radiolabeling and experimental feeding periods, SENCAR mice were consistently heav-

ier than C57BL/6 mice (Fig. 1). Weekly food consumption was higher in SENCAR mice than in C57BL/6 mice fed the same diet during the entire experimental period from 10 to 14 weeks of age (Fig. 2). Low calcium-fed SENCAR and C57BL/6 mice consumed less food than mice of the same strain fed a calcium-sufficient diet, but the absolute differences in food consumption did not produce a significant difference in body weights within either strain over the 4-week test period.

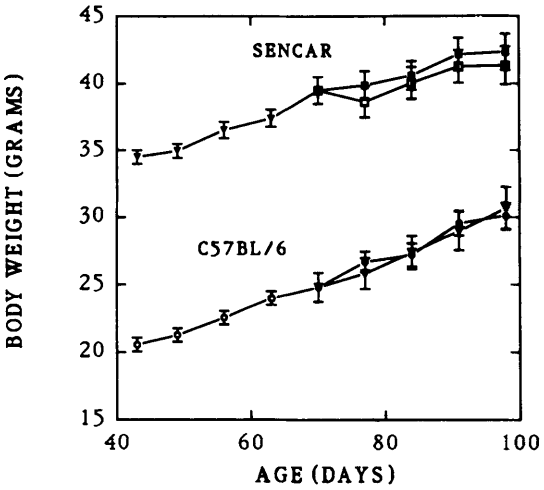

Figure 1. Body mass of SENCAR (▼, ■, and □) and C57BL/6 (○, ●, and ▽) mice. SENCAR (▼) and C57BL/6 (○) mice were fed a standard diet containing 0.8% Ca and 0.6% P from 42 to 70 days of age. High calcium (0.6% Ca; ■ and ▽) or low calcium (0.02% Ca; □ and ●) test diets were fed from 71 to 98 days of age. The values are reported as the mean ± SE (n = 30 from 42 to 70 days of age, and n = 10 from 71 to 98 days of age) in grams and were subjected to ANOVA, using strain and dietary calcium as the main effects. Body weights of SENCAR and C57BL/6 mice were significantly different ($P \leq 0.0001$) at all time points. Dietary calcium intake and the interaction between dietary calcium intake and strain had no significant effects ($P \geq 0.05$) by ANOVA.

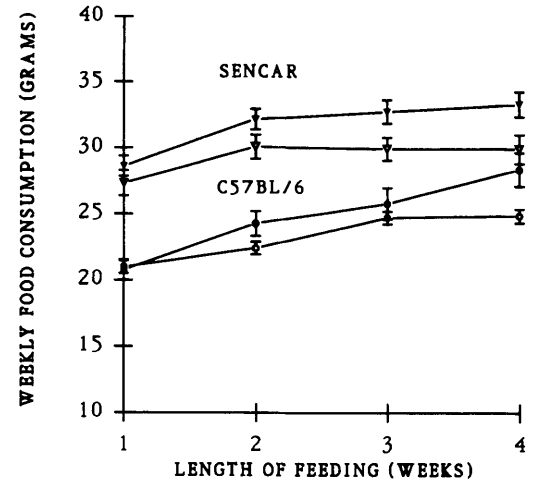

Figure 2. Weekly food consumption of SENCAR (▼ and ▽) and C57BL/6 (● and ○) mice during the 4-week experimental period. Mice were placed on high calcium (0.6% Ca; ▼ and ●) or low calcium (0.02% Ca; ▽ and ○) test diets at 10 weeks of age and maintained for 4 weeks. The values are reported as the mean ± SE (n = 10) in grams and were subjected to ANOVA, using dietary calcium intake and strain as the main effects and testing for interaction. Food consumptions by SENCAR and C57BL/6 mice fed comparable diets were significantly different ($P \leq 0.0001$) over the 4-week course.

Vertebral Bone Mass. Bone mass, expressed as dry, fat-free, or ash weight (mg/vertebra), was 2.2- to 2.3-fold greater in SENCAR mice than in C57BL/6 mice at 10 weeks of age, and 1.7- to 2.0-fold greater in SENCAR mice than in C57BL/6 mice at 14 weeks of age (Table I). When subjected to analysis of variance, strain had a significant effect on bone mass, but dietary calcium intake and the interaction between strain and dietary calcium intake had no effect. Thus, by analysis of variance, 4 weeks of 0.02% or 0.6% dietary calcium feeding had no independent or interactive effects on vertebral bone mass in these two mouse strains. When the data for each strain were analyzed independently by the two-tailed Student's t test, however, 14-week-old SENCAR mice fed 0.02% calcium diets were found to have significantly lower vertebral ash weights than 10-week-old SENCAR mice. Dietary calcium deficiency reduced vertebral ash content in both strains, and SENCAR mice had a higher ash content than C57BL/6 mice (Table I).

Total Ca, Mg, and P Contents. The total calcium, magnesium, and phosphorus contents of the vertebrae are shown in Table II. SENCAR mice, with their heavier vertebrae (Table I), had greater total vertebral calcium, magnesium, and phosphorus contents than C57BL/6 mice by ANOVA (Table II). When the data

Table I. Vertebral Bone Mass in C57BL/6 and SENCAR Mice[a]

Strain[b]	Group	Variable		
		Dry, fat free wt (mg)	Ash wt (mg)	Ash content[c] (%)
C57BL/6	t_0	5.2 ± 0.5	3.3 ± 0.3	62.7 ± 0.5
	0.02% Ca	6.1 ± 0.4	3.6 ± 0.2	58.2 ± 0.5
	0.6% Ca	6.1 ± 0.6	3.8 ± 0.4	62.1 ± 0.5
SENCAR	t_0	11.4 ± 0.4	7.6 ± 0.3*	66.6 ± 0.7
	0.02% Ca	10.5 ± 0.4	6.7 ± 0.3*	63.5 ± 0.4
	0.6% Ca	11.3 ± 0.7	7.6 ± 0.5	67.4 ± 0.5

[a] All mice received diet containing 0.8% Ca and 0.6% P during the 4-week radiolabeling period from 6 to 10 weeks of age. At 10 weeks of age, the time 0 (t_0) control group was sacrificed, and the remaining mice were randomly assigned to one of two experimental feeding groups (0.02% Ca or 0.6% Ca) for an additional 4 weeks and sacrificed at 14 weeks of age. All data are expressed as the mean ± SE (n = 10). Data for both strains were pooled and subjected to analysis of variance (ANOVA) using strain and dietary calcium intake as the main effects and testing for interaction. Results for each strain were also analyzed independently using the Student's unpaired t test. Groups with same symbol (*) were significantly different at the P = 0.03 level by the two-tailed Student's t test.

[b] Strains (C57BL/6 and SENCAR) were significantly different at the P = 0.0001 level by ANOVA for all variables.

[c] Dietary calcium intake (0.02% Ca vs 0.6% Ca) had a significant effect at the P = 0.0001 level by ANOVA. Percentage ash = (ash wt/dry, fat-free wt) × 100.

Table II. Vertebral Ca, Mg, and P in C57BL/6 and SENCAR Mice[a]

Strain[b]	Group	Variable		
		Calcium (mg/ vertebra)	Magnesium (μg/ vertebra)	Phosphorus[c] (mg/ vertebra)
C57BL/6	t_0	1.2 ± 0.1 (10)	25.2 ± 2.6 (10)	0.63 ± 0.06 (10)
	0.02% Ca	1.5 ± 0.2 (10)	28.2 ± 2.2 (10)	0.73 ± 0.02 (8)
	0.6% Ca	1.5 ± 0.2 (10)	29.5 ± 3.3 (10)	0.76 ± 0.06 (9)
SENCAR	t_0	2.9 ± 0.1* (10)	58.2 ± 2.3† (10)	1.68 ± 0.06 (10)
	0.02% Ca	2.5 ± 0.1* (10)	48.3 ± 2.1† (10)	1.32 ± 0.04 (9)
	0.6% Ca	2.8 ± 0.2 (10)	53.4 ± 3.5 (10)	1.63 ± 0.10 (10)

[a] All mice received diet containing 0.8% Ca and 0.6% P during the 4-week radiolabeling period from 6 to 10 weeks of age. At 10 weeks of age, the Time 0 (t_0) control group was sacrificed, and the remaining mice were randomly assigned to one of two experimental feeding groups (0.02% Ca or 0.6% Ca) for an additional 4 weeks and sacrificed at 14 weeks of age. All data are expressed as the mean ± SE (n). Data for both strains were pooled and subjected to analysis of variance (ANOVA) using strain and dietary calcium intake as the main effects and testing for interaction. Results for each strain were also analyzed independently using the Student's unpaired t test. Groups with the same symbols (*,†) are significantly different at the $P = 0.03$ (*) or $P = 0.01$ (†) level by the two-tailed Student's t test.
[b] Strains (C57BL/6 and SENCAR) were significantly different at the $P = 0.0001$ level by ANOVA for all variables.
[c] Dietary calcium intake (0.02% Ca vs. 0.6% Ca) had a significant effect at the $P = 0.027$ level by ANOVA.

were analyzed separately for both strains using Student's t test, it was found that the total calcium content of the vertebrae increased in calcium-replete and calcium-deplete 14-week-old C57BL/6 mice compared to 10-week-old controls of the same strain. In contrast, the vertebral calcium content of 14-week-old SENCAR mice declined significantly with 0.02% Ca feeding and remained unchanged with 0.6% Ca feeding compared to that of 10-week old SENCAR time 0 control mice. However, when the data for both strains were pooled for ANOVA, and all mice receiving the 0.02% Ca test diet were compared to all mice receiving the 0.6% Ca test diet, no significant effect of dietary calcium intake on total vertebral calcium content was found (Table II). Likewise, dietary calcium intake and the interaction between strain and dietary calcium intake were not found to have a significant effect on total vertebral magnesium content by ANOVA (Table II). However, when the data for each strain were analyzed separately using Student's unpaired t test, the vertebral magnesium content was found to be significantly lower in 14-week-old SENCAR mice that were fed 0.02% Ca diets than in 10-week-old SENCAR mice (Table II). Calcium-deficient mice of both strains had lower total vertebral phosphorus contents than calcium-replete mice (Table II).

Relative Formation and Resorption. Relative bone formation and resorption, expressed as the amount of calcium deposited or resorbed divided by the baseline (Time 0) values, are estimated in Figure 3, A (0.02% dietary Ca) and B (0.6% dietary Ca). Relative formation (calcium deposition) was similar within each of the two strains at 0.02% Ca and 0.6% Ca. When the two strains were contrasted, however, the relative amount of vertebral calcium deposited was greater in C57BL/6 mice than in SENCAR mice (Fig. 3, A and B). Dietary calcium deficiency increased the relative resorption rate by 28% in C57BL/6 mice and by 31% in SENCAR mice compared to that in calcium-replete mice of the same strain (Fig. 3, A and B). However, strain alone and the interaction between strain and dietary calcium intake had no significant effect on the relative resorption rate (Fig. 3, A and B).

Absolute Calcium Deposition and Resorption and Net Change in Vertebral Calcium Content. The amount of calcium deposited or resorbed, expressed as milligrams of Ca/vertebra, is estimated in Figure 4, A (0.02% dietary Ca) and B (0.6% dietary Ca). No significant differences in the absolute amounts of calcium deposited (open bars) due to strain, dietary calcium intake, or the interaction between these two factors were found. The absolute mass of calcium resorbed was significantly greater in SENCAR mice than in C57BL/6 mice and greater in all mice receiving the 0.02% Ca test diet than in mice receiving the 0.6% Ca test diet (Fig. 4, A and B). In addition, the interaction between strain and dietary calcium intake had a significant effect on the absolute amount of calcium resorbed (Fig. 4, A and B). The net change in vertebral calcium content (deposition − resorption) was significantly affected by strain, but not by dietary calcium intake or interactive effects, when the data for both strains were pooled and analyzed by ANOVA. However, the net loss of vertebral calcium was found to be significantly greater in SENCAR mice fed the 0.02% Ca diet than in SENCAR mice fed the 0.6% Ca diet when the data for each of the strains were analyzed separately by two-tailed Student's t test.

Serum Osteocalcin and Calcitonin Levels. Serum osteocalcin levels are summarized in Figure 5. At 10 and 14 weeks of age and at 0.02% or 0.6% dietary calcium intake, C57BL/6 mice had higher osteocalcin levels than SENCAR mice. Serum osteocalcin levels were significantly affected by both strain and dietary calcium intakes as well as the interaction between strain and calcium intake when analyzed by ANOVA. By 14 weeks of age, SENCAR mice fed either 0.02% or 0.6% dietary calcium had lower serum osteocalcin levels than 10-week-old SENCAR control mice. In contrast, significant declines in serum osteocalcin levels were observed

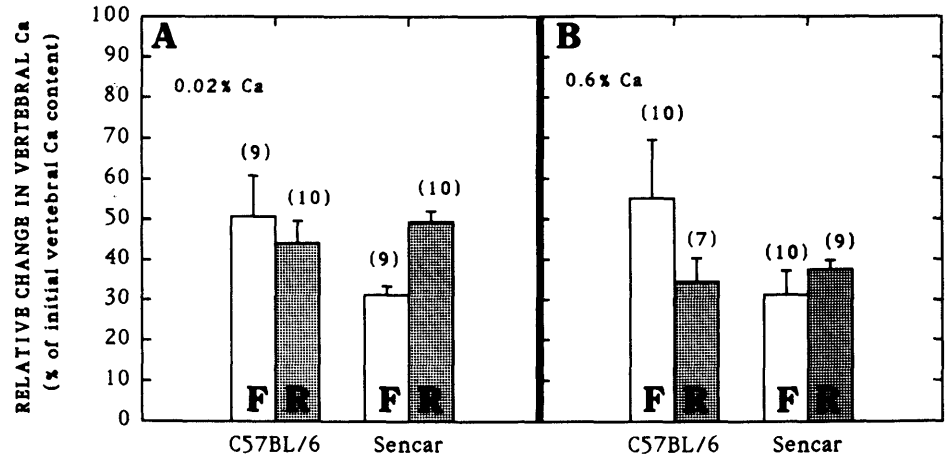

Figure 3. Relative bone formation (F) and resorption (R) in 14-week-old C57BL/6 and SENCAR mice fed 0.02% Ca (A) or 0.6% Ca (B) diets. The values were obtained by dividing the amount of calcium deposited or resorbed by the initial calcium content of the vertebra and are reported as a percentage. All data are expressed as the mean ± SE (n) and were subjected to ANOVA, using strain and dietary calcium intake as the main effects and testing for interaction. The relative amount of calcium deposition was higher in C57BL/6 mice than in SENCAR mice ($P = 0.03$). The relative amount of resorption was significantly higher in mice fed 0.02% Ca diet than in mice fed 0.6% Ca diet ($P = 0.014$).

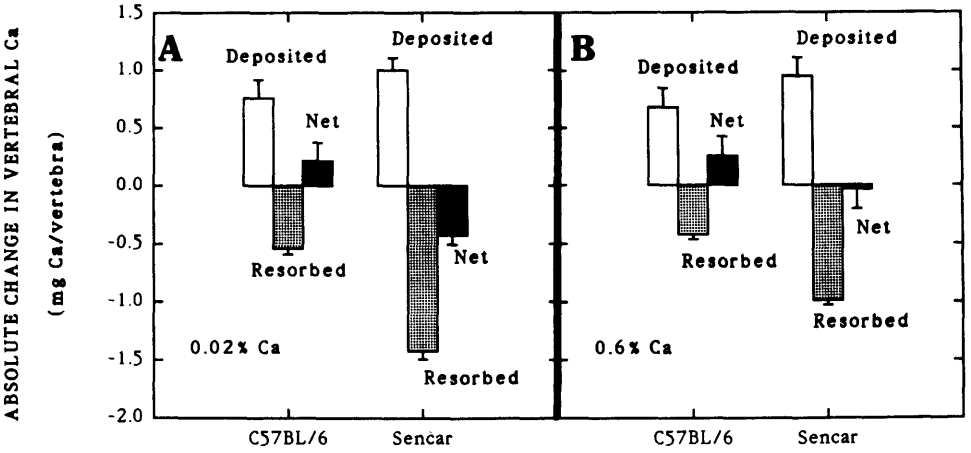

Figure 4. Vertebral bone formation (open bars) and resorption (stippled bars) and the net change in mass (heavily stippled bars), expressed as milligrams of Ca/vertebra, in 14-week-old C57BL/6 and SENCAR mice after 4 weeks of low calcium (0.02% Ca; A) or high calcium (0.6% Ca; B) feeding. All data are expressed as the mean ± SE ($n = 10$) and were subjected to ANOVA, using strain and dietary calcium intake as the main effects and testing for interaction. The amount of calcium resorbed/vertebra was significantly affected by strain ($P = 0.0001$), dietary calcium intake ($P = 0.0001$), and the interaction between the two variables ($P = 0.0023$). The net change in vertebral calcium content was significantly affected by strain ($P = 0.002$), but not by dietary calcium intake or the interaction between strain and calcium ($P = 0.05$) by ANOVA. However, the net loss of vertebral calcium was significantly greater in SENCAR mice receiving the 0.02% Ca diet than in SENCAR mice receiving the 0.6% Ca diet ($P = 0.02$) by two-tailed Student's t test.

in 14-week-old C57BL/6 mice fed 0.6% dietary calcium, but not in those fed the 0.02% calcium diet.

Osteocalcin levels in SENCAR mice were negatively correlated with the net change in the vertebral calcium content (previously shown to be ≤0 in Fig. 4, A and B), with a regression line of $y = -1021.5x + 2028.0$ (y = [BGP] in ng/ml, x = net change in vertebral calcium content in mg Ca/vertebra, F = 5.9, $P = 0.03$, and $r^2 = 0.247$). No similar statistically significant correlation between serum osteocalcin levels and vertebral calcium loss was found in C57BL/6 mice. Serum osteocalcin levels in SENCAR mice were also negatively correlated with the mass of vertebral calcium resorbed, with a regression line of $y = -1739.5x + 713.3$ (y = [BGP] in ng/ml, x = mg Ca resorbed/vertebra, F = 63.3, $P = 0.0001$, and $r^2 = 0.791$). Thus, high serum osteocalcin levels were correlated with high amounts of vertebral calcium resorption in SENCAR mice, but not in C57BL/6 mice.

Serum calcitonin levels in SENCAR mice were significantly greater than those in C57BL/6 mice at 14 weeks of age (Fig. 6). Dietary calcium intake and interactive effects between strain and dietary calcium had no significant effect on serum calcitonin levels when analyzed by ANOVA. In SENCAR mice, serum calcitonin concentration was positively correlated with the

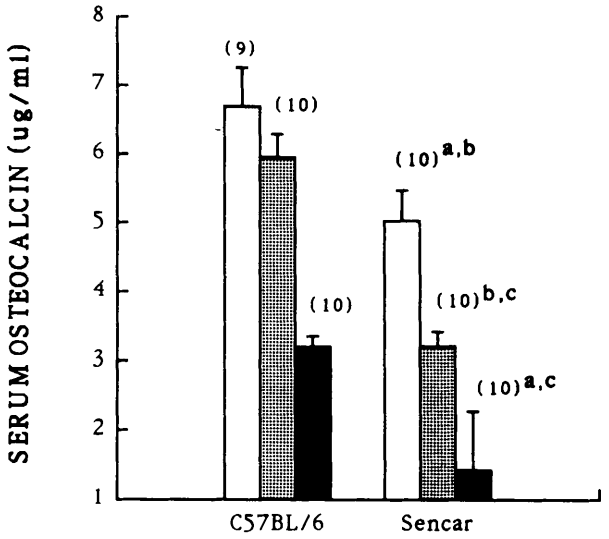

Figure 5. Serum osteocalcin levels (micrograms/ml) in 10-week-old (open bars) and 14-week-old 0.02% Ca-fed (stippled bars) and 0.6% Ca-fed (heavily stippled bars) C57BL6 and SENCAR mice. Values are expressed as the mean ± SEM (n) and were subjected to ANOVA, using strain and dietary calcium intake as the major variables and testing for interaction. Strain and diet were both significant (P = 0.0001), and the interaction was significant (P = 0.04) by ANOVA. The net change (loss) in vertebral calcium was negatively correlated with serum osteocalcin levels in SENCAR mice (P = 0.03, r^2 = 0.247) when subjected to simple linear regression followed by ANOVA. Similarly, the amount of vertebral calcium resorbed was negatively correlated with serum osteocalcin levels in SENCAR mice (P = 0.0001, r^2 = 0.791). Groups indicated by lowercase superscripts are significantly different (a and c, P = 0.0001; b, P = 0.003) by two-tailed Student's t test.

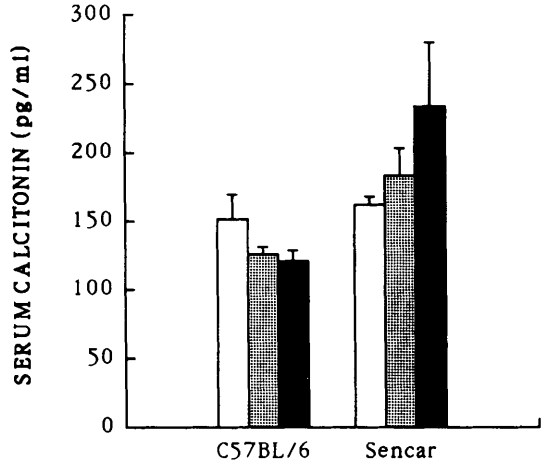

Figure 6. Serum calcitonin levels (picograms/ml) in 10-week-old (open bars) and 14-week-old 0.02% Ca-fed (stippled bars) and 0.6% Ca-fed (heavily stippled bars) C57BL/6 and SENCAR mice. Values are expressed as the mean ± SEM (n = 8) and were subjected to ANOVA, using strain and dietary calcium intake as the major variables and testing for interaction. Strain was significant (P = 0.0025) by ANOVA. Serum calcitonin levels were positively correlated with the net vertebral calcium change (loss) in SENCAR mice (P = 0.012, r^2 = 0.375) by simple linear regression and ANOVA, with the highest calcitonin levels in 14-week-old mice observed in SENCAR mice with stable vertebral calcium contents.

net change in vertebral calcium content, with a regression line of $y = 137.8x + 263.7$ (y = [calcitonin] in pg/ml, x = net change in vertebral calcium content in mg Ca/vertebra, F = 8.4, P = 0.012, and r^2 = 0.375). As noted previously, the net changes in vertebral calcium contents were 0 or less in SENCAR mice (Fig. 4, A and B). Thus, low serum calcitonin levels were associated with large declines in vertebral calcium content in SENCAR mice, but not in C57BL/6 mice.

Discussion

The patterns of vertebral bone composition and turnover in SENCAR and C57BL/6 mice, which differ in body mass, food consumption, as well as serum osteocalcin and calcitonin levels, are significantly different at sexual maturity, as summarized in Table III. The relative amount of formation is primarily strain dependent (genetically predetermined) in C57BL/6 and SENCAR mice, while the relative rate of resorption is modulated by dietary calcium intake. In contrast to rats, in which the rate of bone formation is consistently higher than bone resorption during periods of growth (26), these data revealed unequal relative rates of vertebral bone formation and resorption that resulted in distinct patterns of vertebral bone mass gain (C57BL/6 mice fed 0.02% or 0.6% calcium diets), loss (SENCAR mice fed 0.02% calcium diet), or stability (SENCAR mice fed 0.6% calcium diet; Table III), which occurred concurrently with increases in total body mass in both strains.

Dietary calcium intake appeared to have no effect

Table III. Summary of Strain-Dependent Differences in Vertebral Bone and Serum Markers in C57BL/6 (Small) and SENCAR (Large) Mice[a]

Variable	Strain[b]			
	C57BL/6		SENCAR	
	0.02%	0.6%	0.02%	0.6%
Vertebral mass	+	+	++	++
Relative formation	++	++	+	+
Relative resorption	++	+	++	+
Absolute change in vertebral Ca (mg)	↑↑	↑↑	↓↓↓↓	θ
Serum osteocalcin (μg/ml)	+++++	+	++	+
Serum calcitonin (pg/ml)	++	++	+++	++++

[a] The relative values of each individual variable are represented in arbitrary weighted units (+), with the exception of absolute changes in vertebral Ca mass, where increases (↑), decreases (↓), or no net change (θ) are indicated. All variables, except the relative resorption rate, were significantly different for each strain at $P \leq 0.03$ or better by analysis of variance.

[b] The relative resorption rate was significantly higher in mice fed 0.02% Ca diet than in those fed 0.6% Ca diet at the P = 0.014 level; strain had no significant effects on this variable.

on vertebral bone ash weight or calcium and magnesium content by ANOVA, in which all mice receiving 0.02% dietary calcium are compared to all mice receiving 0.6% dietary calcium. These results may be compared to several major clinical studies that have failed to document beneficial skeletal effects of dietary calcium supplementation (27–30) despite epidemiological evidence which suggests that high bone mass is positively correlated with high lifetime dietary calcium intake (31). However, when additional statistical analysis was conducted for each of the strains independently, dietary calcium intake was shown to have a significant effect on vertebral ash weights and calcium and magnesium contents in SENCAR mice, but not in C57BL/6 mice. Beneficial clinical effects of dietary calcium supplementation have previously been reported (32–34). The data presented here (Tables I and II) suggest that population heterogeneity may contribute to the apparent absence of beneficial effects of therapeutic modalities, such as dietary calcium supplementation, aimed at increasing skeletal mass when the results are averaged over the entire population. With few exceptions (5–8, 10–12), clinical populations are not readily distinguishable based on somatotype or predictable differences in bone mass. Therefore, with the exception of twins, clinical studies tend to deal with genetically heterogeneous populations. Interpretation of clinical studies in general populations focuses on mean outcomes and may predispose to type II statistical errors if discrete subpopulations with unique response characteristics exist. The results presented here highlight the potential utility of animal models of skeletal growth, development, and aging, in which the genetic composition of the subjects can be carefully documented and controlled.

The mechanisms responsible for the differential rates of calcium deposition and resorption in C57BL/6 and SENCAR mice have not been elucidated by the indirect pharmokinetic methods emplyoed, but may include differences in bone cell biology or calcitropic hormone status. Additional studies, such as quantiative dynamic histomorphometric measurements in double fluorochrome-labeled mouse bone, would provide useful insights into the number, relative distribution, and biological capabilities of osteoblasts and osteoclasts in SENCAR and C57BL/6 mice. Differences in bone formation or resorption rates, longitudinal bone growth, or mineralization lag time may account for the strain-dependent differences observed in these mice.

The data presented here suggest that serum calcitonin levels are positively correlated with net vertebral calcium conservation in sexually mature SENCAR mice, whereas they are not in C57BL/6 mice. Previous investigators have demonstrated that blacks have higher bone mineral density at skeletal maturity than whites, even when dietary calcium levels are low (35–37). Greater bone mineral density in the radius, spine, and hip develops in black Americans during childhood (38–40) and puberty (8). In addition, calcitonin and katacalcin (its flanking peptide) are higher in blacks than in age- and sex-matched whites (41, 42). The biological effects of calcitonin include inhibition of bone mineral and matrix resorption (mediated primarily by inhibition of osteoclastic bone resorption and osteocytic osteolysis) and are maximal under conditions characterized by high rates of remodeling or after parathyroid hormone or vitamin D stimulation (43). In total, the data suggest that remodeling processes tend to dominate vertebral bone dynamics in SENCAR mice from 10 to 14 weeks of age, while growth-related processes dominate in C57BL/6 mice.

Serum osteocalcin levels tend to be higher in C57BL/6 mice than in SENCAR mice at 10 and 14 weeks of age. There was an inverse relationship between serum osteocalcin levels and net vertebral calcium change in SENCAR mice, but not in C57BL/6 mice. Lower serum osteocalcin levels (Fig. 5) were observed in calcium-replete (0.6% calcium-fed) SENCAR mice that had relatively stable vertebral calcium contents (Fig. 4B) than in SENCAR mice that lost significant amounts of vertebral calcium after 4 weeks of 0.02% dietary calcium feeding (Fig. 4A). In SENCAR mice, when resorption is high, formation (estimated as serum osteocalcin) is also high, and there appears to be close coupling between vertebral formation and resorption. These patterns were not observed in C57BL/6 mice, in which no significant mathematical relationship was found between serum calcitonin or osteocalcin levels and vertebral bone mass or calcium changes. The data should be interpreted cautiously. Serum osteocalcin levels are thought to reflect overall rates of new bone formation in man and other animals (44, 45), but differential rates of secretion or metabolism may underlie the strain-dependent differences in serum osteocalcin levels observed in 14-week-old SENCAR and C57BL/6 mice. In addition, calcitonin is a systemic hormone and may be regulated differently in these two mouse strains.

Saito *et al.* (46) have reported that conditioned medium obtained from intermittent pressure-treated MC3T3-E1 osteoblastic cells (which were originally derived from the calvaria of neonatal C57BL/6 mice) is incapable of stimulating bone resorption in calvaria culture or osteoclastic cell formation and suggested that these cells exhibit defective coupling of resorption and formation. SENCAR and C57BL/6 mice exhibit marked differences in sensitivity to phorbol esters (47), induction of 8-lipoxygenase (47), and arachidonic acid synthesis (48), with high levels observed *in vivo* and *in vitro* in the epidermal cells (49), keratinocytes (50), macrophages (48), and peripheral mononuclear leukocytes (51) of SENCAR mice. The molecular biological

bases for the differences in vertebral bone mass in C57BL/6 and SENCAR mice have not been determined, but research at the cellular level may provide useful insights into the evolution of somatotype-dependent differences in bone mass. In conclusion, vertebral bone mass and regulation vary depending upon the diet and genetic composition of the animal. To avoid misinterpretation of data and to elucidate specific mechanisms of skeletal mass regulation across the lifespan, careful assessment of bone formation and turnover, hormonal status, and bone cell biology should be conducted in a wide spectrum of strains for each of the species under consideration. Preclinical trials of various pharmacological agents or dietary modifications, such as calcium supplementation, should be conducted in more than one strain of each species to more accurately estimate their potential effects.

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