Effects of Age on Plasma Levels of Calcium-Regulating Hormones and Bone Status in Male SAMP8 Mice

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Abstract

This study was undertaken to examine whether the plasma levels of calcium-regulating hormones and bone status alter with age in male senescence accelerated mice (SAM), SAMP8. Age-matched senescence-resistant mice, SAMR1, were used as controls. The blood and femur samples were collected at 2.5 months of age (M) and then monthly from 3 to 12 M for physicochemical analyses, biochemical analyses, and the determination of hormones by radioimmunoassay. With advancing age, the plasma calcitonin (CT) levels decreased progressively, and the plasma parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) levels increased in both SAMR1 and SAMP8. The plasma calcium concentrations were maintained within a narrow range throughout the experimental period, while the plasma phosphorus (P) concentrations decreased with age in both strains. In contrast to SAMR1, the curves of age-related changes in the plasma CT levels and P concentrations were lower, and those in the plasma PTH levels were higher in SAMP8. The femoral bone densities and calcium contents increased gradually with age from the beginning of the experiment and peaked at 6 M in both strains, then declined. Those peaks were lower in SAMP8 than in SAMR1. These results indicate that the male SAMP8 develops osteoporotic signs earlier than SAMR1, and is proved to be a satisfactory animal model for longitudinal studies related to osteoporosis for men.

Key Words: mice, SAM, age, calcium, phosphorus, calcium-regulating hormones, bone

Introduction

Osteoporosis is characterized by low bone mass and enhanced fragility of the bones, making the bones susceptible to fractures from minor trauma (18), and it is often associated with aging. Bone loss occurs universally with aging, and is accelerated in women coinciding temporally with menopause, though the reduction in bone mass proceeds gradually (24). Until recently, the focus of age-related bone loss has been on postmenopausal women mainly because women start loosing bone mass earlier than men and bone loss proceeds more rapidly in women than in men (3). However, men do not undergo the equivalent of menopause, the skeletons of men also suffer predictable age-related changes in bone mass (25). Similarly, bone mineral loss tends to increase with age in rats (13, 15, 29). Much evidence has shown that the activation of bone remodeling increases with age. In terms of rats, rapid bone formation and mineralization occur during the first few months of life, and peak bone mass and mineral contents are attained during the first half of life, while progressive bone loss is apparent during the second half of the in lives, especially during senescence (13, 15, 31). Bone remodeling is considered to be responsible for bone gain or loss during normal growth and adult life in humans and rats, since the longitudinal skeletal growth is not continuous

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Received: August 20, 2003; Revised: October 23, 2003; Accepted: October 25, 2003.

through life (9).

Age-related bone mineral loss may be attributed to the combined effects of a variety of hormones, which regulate calcium homeostasis. Age-associated physiological changes of calcium-regulating hormones have been investigated previously in both humans and rats. For example, the levels of serum parathyroid hormone (PTH) increase (5-7, 21, 23), and the levels of plasma calcitonin (CT) decrease with age in humans (4). In rats, the alterations of serum PTH levels (12, 40, 42) are similar to those in humans, but the levels of serum CT do not decrease and are significantly higher in the older rats (12, 19, 22, 38), unlike in humans. Nevertheless, it is less known whether the impact of the above-mentioned bone status and endocrine changes with age (from youth to senescence) also affect mice.

Animal models have contributed significantly to the understanding of, and development of therapies for bone loss in humans. An animal model is not necessary an exact replica of the human disease, but a good animal model should have many similarities with the disease in question (2). Although rats are often used as animal models for the study of age-related bone loss, the alteration in plasma CT, one of the main calcium-regulating hormones (12, 19, 22, 38), is contradictory to that of humans (4); besides, their life spans are too long. The senescence-accelerated mouse (SAM), a murine model of accelerated senescence, has been bred and offered by Prof. Dr. Toshio Takeda (Kyoto University, Japan) for the research of aging (36). The SAM consists of two series, SAMP (senescence-prone) and SAMR (senescence-resistant). The characteristics common to all SAMP and SAMR mice are accelerated senescence and normal aging, respectively (33). In general, aging is accelerated in the SAMP series, determined by the shortened life span and early manifestation of various signs of senescence, including changes in physical activity, skin, hair, eyes, and spinal curvature, as compared with the SAMR series used as the controls (10, 36). The mean life span of SAMP8 is 10.0 months of age (M), but that of the SAMR1, a normal counterpart, is 18.9 M, when the animals are kept under conventional conditions (34, 35).

With a shortened life span, the male SAMP8 mice were used in this study to investigate the effect of aging on calcium homeostasis, the possible roles of CT, PTH and 1,25-dihydroxy-cholecalciferol (1,25 $(OH)_2D_3$) in age-related bone loss, and to evaluate if the male SAMP8 can serve as a good animal model for the study of age-related bone loss in men.

Materials and Methods

Animals

Only male mice were used in this study. The SAMP8 and SAMR1 mice were housed in a temperature-controlled room (22±2°C) with 14 h of artificial illumination daily (0600-2000). During the experimental period, they were provided with water and a commercial chow (Fushou, Ltd., Taiwan) containing 0.95% calcium, 0.75% phosphorus and 2.60 U vitamin D/g, *ad libitum*. Six mice were decapitated at 2.5 M and then monthly from 3 to 12 M. The blood samples were collected and the plasma samples were separated and stored at -20°C for biochemical and hormonal analyses. The femurs were dissected out and cleaned off all soft tissues and also stored at -20°C for analysis.

RIAs of Hormones

CT The concentrations of plasma CT were measured by heterogenous RIA with human CT RIA kits purchased from BioSource International (Camarillo, CA, USA). The binding curves of mouse plasma, rat plasma and human plasma were parallel to the standard curve of human CT (Fig. 1, top). The antisera for CT showed virtually no cross-reactivity against salmon-CT, up to 100 ng/ml, CGRP (calcitonin gene-related peptide), PDN 21 (katacalcin, the 21-aminoacid carboxyl-terminal flanking peptide), and procalcitonin N terminal. The sensitivity of the RIA was 4.0 pg/ml. The cold recovery rate of CT from mouse plasma pools was 94.55 ± 2.00% (Mean ± SEM). The intra- and interassay coefficients of variation were 3.95% (n = 6) and 6.93% (n = 4), respectively.

PTH The concentrations of plasma PTH were measured with the intact human PTH-specific RIA kits from Nichols Institute (San Juan Capistrano, CA, USA). The binding curves of mouse plasma, rat plasma and human plasma were parallel to the standard curve of human PTH (Fig. 1, middle). The antisera for intact human PTH showed virtually no cross-reactivity against human PTH-(1-34) at a concentration of 300 pg/ml, and human PTH fragments 39-68, 53-84, 44-68 and 39-84 each at concentrations of 100 ng/ml. The sensitivity of the RIA was 2.0 pg/ml. The cold recovery rate of PTH from mouse plasma pools was 96.20 ± 1.23%. The intra- and interassay coefficients of variation were 3.59% (n = 6) and 7.00% (n = 4), respectively.

$1,25(OH)_2D_3$ The concentrations of plasma $1,25(OH)_2D_3$ were measured by human $1,25(OH)_2D_3$ RIA kits purchased from BioSource International (Camarillo, CA, USA). The competitive inhibition curves of mouse plasma and human plasma were parallel to the $1,25(OH)_2D_3$ standard curve (Fig. 1, bottom). The percentage of cross-reactivity estimated

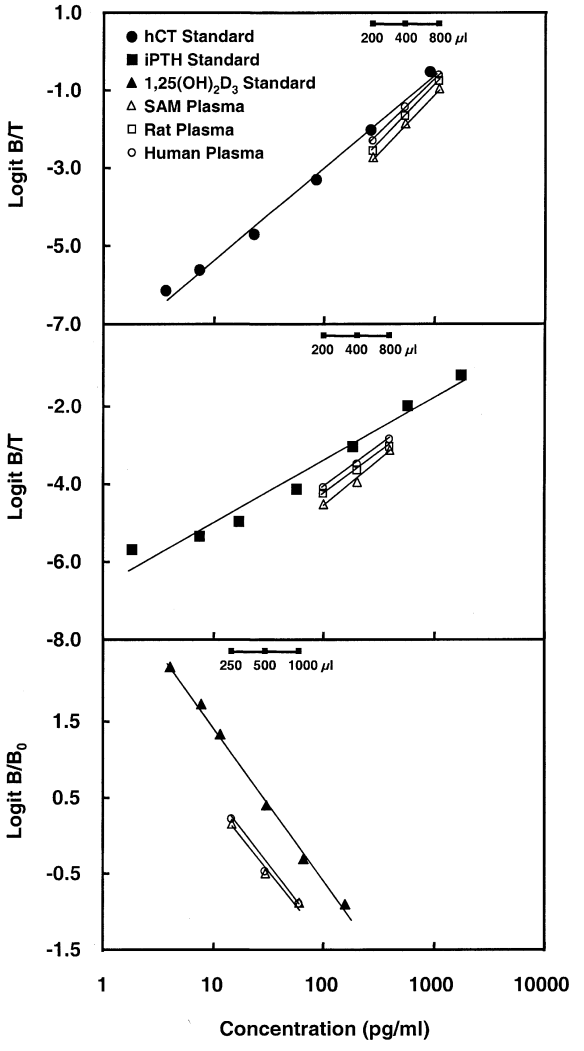

Fig. 1. The standard curves of synthetic human CT (top, ●), human intact PTH (middle, ■) and 1,25$(OH)_2D_3$ (bottom, ▲). Dose-response curves for human (○), rat (□) and mouse (△) samples after a log-logit transformation in radioimmunoassay system.

by comparison of the concentration yielding a 50% inhibition were 1,25(OH)2D3 100%, 1,25$(OH)_2D_2$ 70%, 25$(OH)D_3$ < 0.01%, 24,25$(OH)_2D_3$ < 0.01%, and 25,26$(OH)_2D_3$ < 0.01%, respectively. The sensitivity of the RIA was 5.0 pg/ml. The cold recovery rate of 1,25$(OH)_2D_3$ from mouse plasma pools was 92.63 ± 1.72%. The intra- and interassay coefficients of variation were 7.33% (n = 4) and 13.33% (n = 3), respectively.

Biochemical Analyses of Plasma

The plasma concentrations of calcium (Ca), phosphorus (P) and activities of alkaline phosphatase (ALP) were measured by commercial kits with Kodak Ektachem DT Chemistry System (Johnson & Johnson Clinical Diagnostics, Inc., NY, USA).

Physicochemical and Chemical Analyses of Femurs

Bone density The bone densities of the femurs were measured by Archimedes' principle (28). Each femur was placed in an unstoppered vial filled with deionized water, and was agitated periodically to ensure that all trapped air diffused out of the bone. The vial was put in a desiccator connected to a vacuum for 3 h. Then the femur was cleaned up, weighed, and returned to the vial containing deionized water. To calculate the density, the dry weight was divided into the excluded volume.

Calcium content The femurs were dried for 24 h at 100°C, and the dry weights were recorded. The dry femurs were then ashed in a muffle furnace at 550-600°C for 6 h, and the ash was weighed. The ash was pulverized and hydrolyzed with 6 M HCl. The hydrolysate was diluted with 0.1% lanthanum solution, and calcium content was determined by atomic absorption spectrophotometry (Hitachi, Model No. Z-6100).

ALP The cleaned femurs were weighed and subsequently homogenized in 1.5 ml sodium bicarbonate buffer (0.15 M NaCl and 3 mM $NaHCO_3$, pH 7.2) using a Polytron homogenizer (Kinematica, Model No. PT-MR 2100) for 2 min at 0°C. The alkaline phosphatase activities of homogenized samples were determined by commercial kits (Sigma, St, Louis, MO, USA) and colorimeter (Bio-Tek, Model No. ELx-800).

Statistical Analysis

All values are presented as the mean ± SEM. The differences among means were determined by a two-way factorial analysis of variance (ANOVA) with equal replication. The differences between 2.5 months of age and other ages in each strain were determined with Duncan's multiple range test when the ANOVA indicated significant differences among means. The differences between SAMR1 and SAMP8 at the same age were analyzed with Student's *t*-test. (27). A difference between means was considered significant when $P < 0.05$.

Results

Hormones of Plasma

At 2.5 M (the beginning of the experiment), there were no significant differences in the plasma PTH and 1,25$(OH)_2D_3$ levels between SAMP8 and

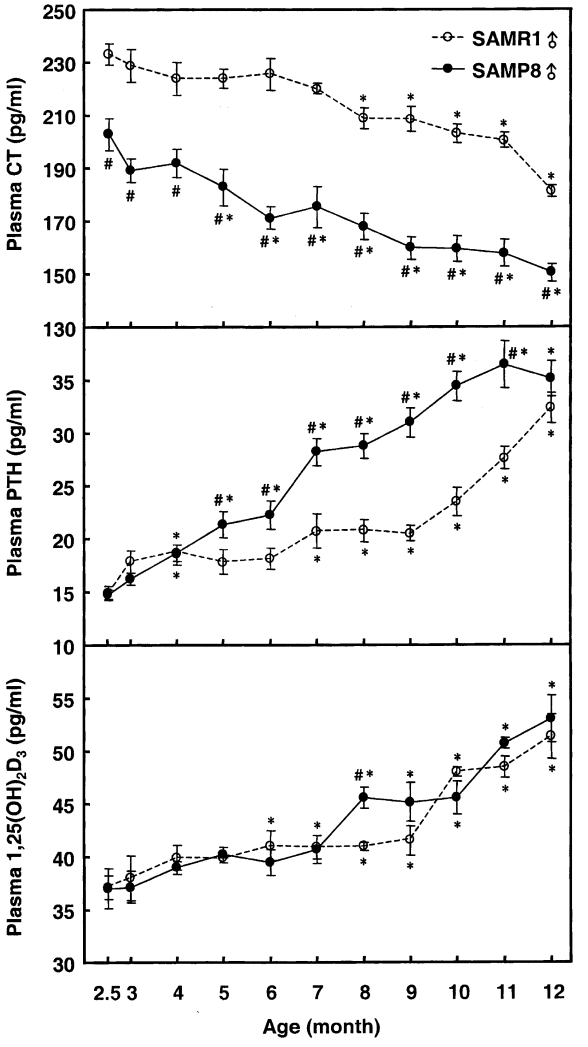

Fig. 2. Effects of age on plasma calcitonin (top), parathyroid hormone (middle) and 1,25$(OH)_2D_3$ (bottom) levels in SAMP8 (●) and SAMR1 (○). Each point represents the mean ± SEM (n = 4). Each sample consists of a pool of plasma from two animals. *, Significantly different from the mean at 2.5 months of age within each strain ($P < 0.05$). #, Significantly different between SAMP8 and SAMR1 at the same age ($P < 0.05$).

SAMR1 ($P > 0.05$), but the plasma CT level in SAMP8 (202.7 ± 5.9 pg/ml) was significantly lower than that in SAMR1 (233.1 ± 3.9 pg/ml) ($P < 0.05$).

With advancing age, the plasma PTH and 1,25 $(OH)_2D_3$ levels increased progressively, and the plasma CT levels decreased in both strains (Fig. 2). Compared with the age-matched SAMR1, the plasma PTH levels were higher, and the population mean in SAMP8 was significantly higher than that in SAMR1 ($P < 0.05$) in the multiple comparison testing. The plasma CT levels were significantly lower in SAMP8 throughout the experimental period ($P < 0.05$) as compared with SAMR1. However, the plasma 1,25 $(OH)_2D_3$ levels were not significantly different between SAMP8 and SAMR1 ($P > 0.05$) except at 8 M. After 4, 8 and 5 M, respectively, the plasma PTH, 1,25$(OH)_2D_3$ and CT levels were significantly different from those at 2.5 M in SAMP8 ($P < 0.05$), while these were not significantly different from those at 2.5 M until 7, 6 and 8 M in SAMR1 ($P < 0.05$), respectively.

Biochemistry of Plasma

At 2.5 M, there were significant differences in plasma Ca, P, concentrations and ALP activity between SAMP8 and SAMR1 ($P < 0.05$).

The plasma Ca concentrations were maintained within a narrow range in both SAMP8 and SAMR1 (6.7-7.7 mg/dl) (Fig. 3, top). However, the concentrations in SAMP8 were significantly higher than in SAMR1 at 2.5-4 M ($P < 0.05$).

The plasma P concentrations decreased with age, but fluctuated during 8-10 M in SAMR1. The concentrations decreased from 5 M, but fluctuated during 8-9 M in SAMP8 (Fig. 3, middle). Compared with SAMR1, the concentrations in SAMP8 were lower throughout the experimental period, and the population mean in SAMP8 was significantly lower than that in SAMR1 ($P < 0.05$) in the multiple comparison testing. After 5 M, the concentrations were significantly lower than those at 2.5 M in SAMR1 ($P < 0.05$), while these were not significantly lower than those at 2.5 M in SAMP8 ($P > 0.05$).

The plasma ALP activities decreased during the early stage of the experiment, and then the activities fluctuated in both strains (Fig. 3, bottom). The population mean in SAMP8 was not significantly different from that in SAMR1 ($P > 0.05$) in the multiple comparison testing. After 4 M, the activities were significantly lower than those at 2.5 M in both strains ($P < 0.05$).

Physicochemistry and Chemistry of Femurs

There was no significant difference in bone Ca content between SAMP8 and SAMR1 at 2.5 M ($P > 0.05$), but the bone density and ALP activity in SAMP8 were significantly different from those in SAMR1 ($P < 0.05$).

The femoral bone densities increased gradually with age from 2.5 M in both strains, and peaked at 6-7 M in SAMR1 and at 6 M in SAMP8, then followed by a decline (Fig. 4, top). The changes in the bone calcium contents also increased with age during the earlier stage of the experiment and peaked at 6 M in both SAMR1 and SAMP8, then revealed a gradual reduction (Fig. 4, middle). The bone ALP activities tended to decrease during the early stage of the experiment in both strains, although the activities were maintained within a narrow range from 5 to 12

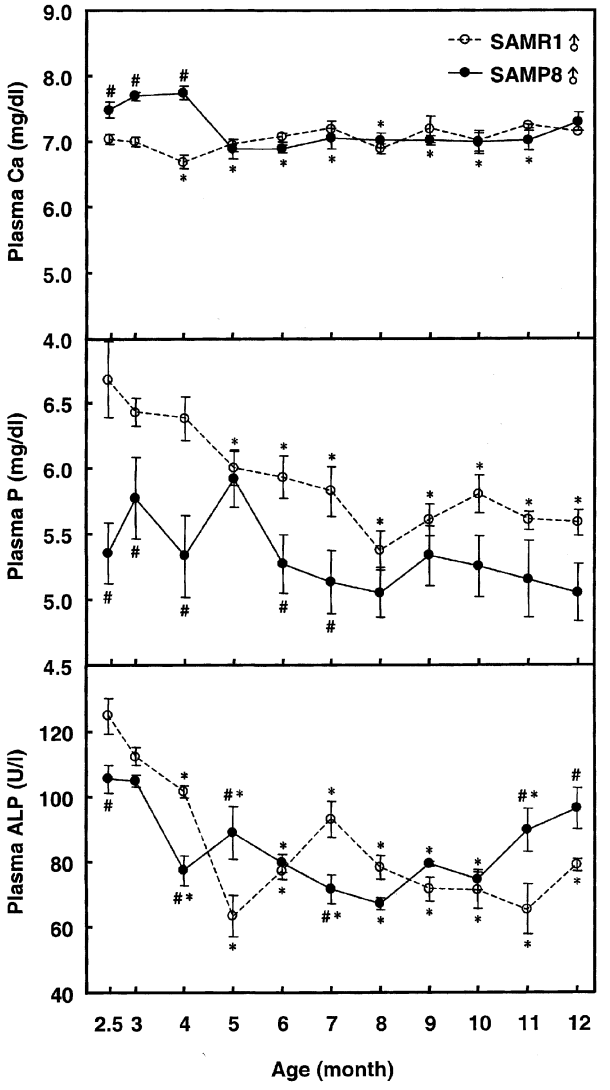

Fig. 3. Effects of age on plasma calcium (top), phosphorus (middle) concentrations and alkaline phosphatase activities (bottom) in SAMP8 (●) and SAMR1 (○). Each point represents the mean ± SEM (n = 6). *, Significantly different from the mean at 2.5 months of age within each strain ($P < 0.05$). #, Significantly different between SAMP8 and SAMR1 at the same age ($P < 0.05$).

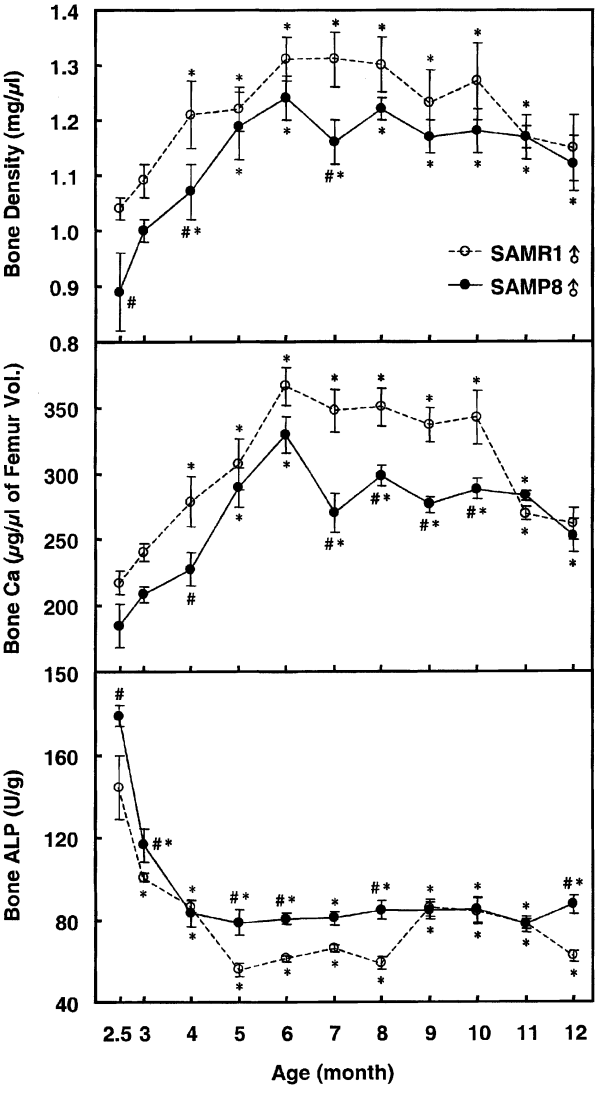

Fig. 4. Effects of age on femoral bone densities (top), calcium contents (middle) and alkaline phosphatase activities (bottom) in SAMP8 (●) and SAMR1 (○). Each point represents the mean ± SEM (n = 6). *, Significantly different from the mean at 2.5 months of age within each strain ($P < 0.05$). #, Significantly different between SAMP8 and SAMR1 at the same age ($P < 0.05$).

M in both SAMP8 and SAMR1 (Fig. 4, bottom). Besides, the population means of bone densities and calcium contents in SAMP8 were significantly lower than those in SAMR1 ($P < 0.05$), but the population mean of ALP activities in SAMP8 was significantly higher than that in SAMR1 ($P < 0.05$) in the multiple comparison testing.

Discussion

The plasma P concentrations decreased with age in both strains in our observations, and a similar tendency was also found in men (23, 30, 37) and rats (12, 13). The decreased plasma P concentrations in the senescent rats are regarded as a result of the decreased renal conservation of phosphate (1). This finding is consistent with our observation (unpublished data). It indicates that much more P than Ca was lost in the urine, and mobilization of P from bone might not keep pace with the renal loss (1, 11, 13); hence, senescent animals became slightly but significantly hypophosphatemia.

Plasma Ca concentrations in the living animals are normally maintained in a narrow range. Plasma Ca is derived from intestinal absorption, renal reabsorption and bone resorption, and the excessive

plasma Ca is stored in skeleton, and is excreted mainly *via* urine. These processes are mainly regulated by PTH, CT and $1,25(OH)_2D_3$, and these hormones are regulated by plasma Ca concentrations in turn. In this study, plasma Ca concentrations did not change with age in SAM mice. The similar results have also been found in rats (12, 13, 15). In humans, plasma Ca concentrations are unchanged (17, 30, 37) or decrease slightly (21, 23) with age. Although the Ca concentrations were not influenced by age in this study and in many previous reports for rats and humans, the levels of calcium-regulating hormones did significantly change with age (4-6, 12, 19, 21-23, 38, 40, 42). We found that resorption-stimulating hormones, PTH and $1,25(OH)_2D_3$, rose with age, and resorption-inhibiting hormone, CT, declined with age. Similar tendencies of the changes in PTH and CT were also found in men (4, 7, 23), although there was controversy for $1,25(OH)_2D_3$. In humans, the levels of $1,25(OH)_2D_3$ increased with age (6). However, other studies have reported that the levels of $1,25(OH)_2D_3$ decrease with age (23), and remain unchanged over a wide range of age (21, 30), or increase from age 35 up to age 65, followed by a decrease (5). The complexity and diversity of human foods may account for the disagreements among reports for the change in $1,25(OH)_2D_3$ levels. Ignoring the changes in $1,25(OH)_2D_3$, the plasma PTH level increases and CT decreases with age in mice as well as humans. This pattern of change is different from that in rats, in which both plasma PTH and CT increases with age (12, 19, 22, 38). Therefore, the mechanism underlying age-related bone loss in rats may be somewhat different from that for both mice and humans.

In this study, the pattern of changes in bone density and bone Ca content of SAM can be considered as an epitome of that found in men. Men's bone densities increase with age before middle age, then begin to decrease in the 40s and continues until old age (26). Because of the relatively constant levles of plasma Ca concentrations and the decline in bone densities and bone Ca contents in aged animals, we may speculate that the intestinal Ca absorption and/or renal Ca reabsorption may decrease with aging in SAM mice. In fact, the above speculation was confirmed by a recent study in our laboratory (unpublished data). In humans, it is also proven to be true (4-6, 21). There seems to be a tendency of reduced intestinal and renal responsiveness to $1,25(OH)_2D_3$ action, so that plasma PTH and $1,25(OH)_2D_3$ rise, and plasma CT and bone Ca content decrease with aging in SAM mice.

Skeletal ALP is an ectoenzyme of osteoblasts (8). The activity is proportional to the rate of collagen production and to the number of osteoblasts (20). Thus, the measurement of this enzymatic activity may be considered as a useful index for bone formation rate. In this study, the higher activities of the femoral ALP in young animals of both strains illustrated magnificent bone formation and turnover during this main period of skeletal building, and the subsequent decrease in the femoral ALP implied reduced bone formation and turnover rate during adulthood and senescence in our observations. A similar tendency was also found in rats (29).

Serum ALP activities were either high in young men (32), and low in old men (39), or were not correlated with age until the age of 90 (37). In women, serum ALP activities either increased with age (17, 37, 39), or were not correlated with age (41). In rats, serum ALP activities remained constant (13, 15). We found that the plasma ALP activities decreased during the early stage of the experiment, and then fluctuated in both strains. Therefore, there were no conclusive changes in plasma ALP in different species. It should be noted that serum ALP is derived not only from skeleton but also from other tissues, such as liver and kidney.

In ICR mice, bone formation declined and bone loss progressed after 15 M of age (16). Some studies reported that no bone loss was observed in the L4 vertebra of F344 rats until 18 M, which was past middle age in its life span (31 M) (2), and the symptoms were observed as late as 24 M of age (13, 14). In contrast, the femoral bone densities and Ca contents in SAM peaked from as early as 6 M and then decreased gradually, which show that SAM mice not only had a shortened life span but also had bone loss earlier than ICR mice and rats. In addition, the curves of age-related changes in the plasma CT levels, P concentrations, bone density and Ca contents were lower, while the curves in the plasma PTH levels were higher in SAMP8 as compared to those in SAMR1. These results imply that the male SAMP8 develops osteoporotic signs earlier than the male SAMR1.

In conclusion, the tendencies of age-related changes in the plasma P, PTH and CT levels found in SAM are similar to those found in men. Therefore, the male SAM is proved to be a satisfactory animal model for longitudinal studies related to osteoporosis for men. Besides, the comparisons of the age-related change curves in those parameters between SAMP8 and SAMR1, indicate that SAMP8 develops osteoporotic signs earlier as compare to SAMR1. In this respect, SAMP8 is more convenient to use and may be more useful in studying bone metabolism as compare to normal strain(s).

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