CHANGES IN BONE MINERAL DENSITY DURING SEXUAL MATURATION IN MALE AND FEMALE RATS: CORRELATION WITH SERUM IGF-1, IGFBP-3, OSTEOCALCIN AND SEX STEROIDS

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Abstract

Bone (tibia, femur, and lumbar spine) and blood samples were obtained from 100 (50 males and 50 females) Wistar-Imamichi rats in groups aged 3, 5, 7, 9, 12, 15 and 20 weeks old to investigate the changes in bone mass during puberty in relation to insulin-like growth factor 1 (IGF-1), IGF binding protein (IGFBP) -3, osteocalcin (OC) and sex steroids in normal rats.

Sharp increases in BMD (bone mineral density) in the tibia, femur and lumbar appeared earlier in female than in male rats, and the BMD in females tended to be higher than in males between 5 and 9 weeks old. After 9 weeks old, BMD in males was higher than that in females, as BMD in males continued to increase whereas that in females tended to remain in a steady state after this stage. This sex-related difference in changes in BMD pattern is probably related to the serum concentrations of IGF-1, IGFBP-3, testosterone, and 17β -estradiol with maturation. In males, marked increases in serum IGF-1 and IGFBP-3 concentrations appeared earlier than that in serum testosterone level. IGF-1 and testosterone peaked at 9 weeks of age, and thereafter remarked in a steady state, whereas IGFBP-3 reached a peak at 7 weeks of age, and then declined gradually. In females, the changes in patterns of serum 17β -estradiol, IGF-1, and IGFBP-3 levels were very similar. The levels increased gradually from 3~5 weeks old, peaked at 9 weeks, and then decreased slowly thereafter. In contrast, serum OC concentrations remain relatively high from 3 to 9 and from 3 to 7 weeks old in males and females, respectively, although OC in both sexes declined gradually with aging.

These observations suggest that BMD development occurs earlier in female than in male rats. This sex-related difference in changes in the BMD pattern may result from the earlier onset of puberty in females, and from the sex-specific differences in concentrations of IGF-1, IGFBP-3 and sex steroids with maturation.

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key words : bone growth, IGF-1/IGFBP-3, sex steroid, rat

I. Introduction

During mammalian growth and development, marked increases in body length and body weight appear between birth and puberty. These changes depend mainly on the growth of the skeletal system, tissues and organs¹⁾, and require the con-

certed actions of sex steroids, growth hormones (GH), and insulin-like growth factor 1 (IGF-1)^{2, 3)}. Sex steroids induce increases in GH secretion during early puberty^{2, 3)} and serum concentrations of IGF-1 and predominant IGF binding protein (IGFBP) -3 reflect spontaneous GH secretion in puberty⁴⁾.

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Osteoporosis is a serious medical and socio-economic problem, and one of the means of minimizing the risk of future osteopenia and/or osteoporotic fracture is to maximize the accumulation of bone tissue during growth^{5,6)}. There is evidence that nutritional, mechanical and hormonal factors as well as genetic predisposition are important in the achievement of peak bone mass.

The domesticated growing rat has been used extensively as a model system in various studies to elucidate the adaptations of bone to exercise training and medical intervention. Marked changes in morphology and endocrine environments and the sexual divergence in growth rates begin at puberty in rats as well as in man⁷⁻⁹⁾. These physiological changes with maturation are evident in parameters such as body weight, head to tail length, bone length and the pattern of rat GH release⁹⁻¹¹⁾. However, little is known about the relation between bone mineral density (BMD) and endocrine changes with sexual maturation in rats. The aim of this study was to explore the changes in bone mass during puberty in relation to IGF-1, IGFBP-3, osteocalcin (OC; bone gla-protein) and sex steroids in normal male and female rats.

II. Materials and Methods

A. Animals

A total of 100 (50 males and 50 females), 3 ($n=8$), 5, 7, 9, 12, 15 and 20 weeks old ($n=7$ for each age group) male and female, Wistar-Imamichi strain rats were used in this study. The animals were fed in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) under a 12:12-h light-dark cycle and given free rat chow and water ad libitum until sacrifice. All rats were sacrificed by decapitation under mild ether anesthesia between 11:00~11:30 AM. In female rats ≥ 6 weeks old, the estrous cycle was confirmed using vaginal smears and sacrificed at estrus. Blood was immediately collected and stored at -80°C until biochemical determinations.

The tibia, femur and lumbar spine (L3 + L4) were then removed, cleaned of soft tissues, and kept for densitometric evaluation.

B. Bone length

The tibial and femoral lengths were measured from the tibial plateau to the end of the lateral malleolar process, and the top of femoral head to the end of the medial condyle, respectively, a using precision calipers.

C. Densitometric Study

BMD, the bone mineral content (BMC) per area, was measured in the tibia, femur and lumbar spine. Bone samples were positioned exactly in water at a depth of 2.5 cm according to Ammann et al.¹²⁾. A dual-energy X-ray absorptiometer (DXA) (DCS-3000, Aloka, Tokyo, Japan) was used with the ultra-slow mode adapted for measurement of small animals. The whole tibia, femur and lumbar spine were scanned for investigation to take into account the changes in bone densities at levels different skeletal sites.

D. Measurement of hormones

Serum 17β -estradiol and testosterone levels were determined in females and males, respectively. IGF-1, IGFBP-3 and OC were determined in both sexes.

Serum 17β -estradiol and testosterone were determined using an enzyme immunoassay kit (EIA) (Cayman Chemical Co., MI, U.S.A.) in duplicate samples. The assay sensitivity of 17β -estradiol was 7.8 pg/ml, and the intra- and interassay coefficients of variation were 3.4% and 6.8%, respectively. The assay sensitivity of testosterone was 3.9 pg/ml, and the intra- and interassay coefficients of variation were 4.8% and 7.8%, respectively. The minimum detectable amount was 4.2 pg/ml.

Serum IGF-1 was determined using a radioimmunoassay kit (RIA) (Eiken Co., Tokyo, Japan) in duplicate samples after extraction with hydrochloric acid. The assay sensitivity was 0.1 ng/ml,

and the intra- and interassay coefficients of variation were 5.3 and 11.1%, respectively.

Serum IGFBP-3 was determined, using an immunoradiometric assay kit (IRMA) (Eiken Co.) in duplicate samples. The assay sensitivity was 2 ng/ml, and the intra- and interassay coefficients of variation were 6.1% and less than 10.0%, respectively.

Serum osteocalcin was determined using a RIA kit (Biomedical Technologies Inc., MA, U. S. A.) in duplicate samples. The assay sensitivity was 0.5 ng/ml, and the intra- and interassay coefficients of variation were 4.8% and less than 11.0%, respectively.

E. Statistical Analysis

All data are expressed as means and standard deviation (SD). Unpaired Student's *t*-test was used for comparisons between males and females. One-way analysis of variance (ANOVA) followed by Bonferroni or Scheffe's test when necessary were used to test the significance of the difference between results at various age groups. $P < 0.05$ was regarded as significant for all statistical tests.

III. Results

A. Body weight

The body weight increased with age in both male and female rats, but the increase in females was slower after about 9 weeks old (Fig. 1A). Males weighed more than females, and a sex difference in body weight, was observed after 5 weeks old ($P < 0.001$). As shown in Figure 1B, body weight gain in males showed a peak at 8~9 weeks old, declining thereafter. In contrast, the body weight gain in females showed a peak at 4~5 weeks old, and then declined.

B. Bone length

In both males and females, the tibial length increased gradually, this increase was slower from 9~12 weeks old (Fig. 2). Similar results

Fig. 1. Changes in body weight (A) and gained body weight (B) in male and female rats. Overall significance of differences between age groups by analysis of variance are as follows: body weight (male), $P < 0.001$; body weight (female), $P < 0.001$; gained BW (male) $P < 0.001$; gained BW (female), $P < 0.001$. *: $P < 0.01$; **: $P < 0.001$; compared with age-matched female rats (Unpaired *t*-test).

Fig. 2. Changes in tibial length in male and female rats. Overall significance of differences between age groups by analysis of variance are as follows: male, $P < 0.001$; female $P < 0.001$. *: $P < 0.01$; **: $P < 0.001$; compared with age-matched female rats (Unpaired *t*-test).

were obtained for the femur (data not shown).

C. Bone mineral density (BMD)

The mean BMD values of the tibia (Fig. 3 A), femur (Fig. 3 B) and lumbar spine (L3 · L4) (Fig. 3 C), were not significantly different between 3-week-old males and females. Between 5 and 9 weeks old, females had a slightly but not significantly higher mean value than males. After 9 weeks

Fig. 3. Changes in BMD in the tibia (A), Femur (B) and lumbar spine (L3·L4) (C) in male and female rats. Overall significance of differences between age groups by analysis of variance are as follows: tibia (male), $P<0.001$; tibia (female), $P<0.001$; femur (male), $P<0.001$; femur (female), spine (male), $P<0.001$; spine (female) $P<0.001$. *: $P<0.05$ **: $P<0.01$, ***: $P<0.001$ compared with age-matched female rats (Unpaired t -test).

old, a tendency toward a greater BMD in males than in females was observed at all three sites.

D. Blood analyses

As shown in Figure 4 A, serum testosterone level in males increased markedly and rapidly at 5~7 weeks of age, and peaked at 9 weeks, thereafter reaching a plateau.

Serum 17β -estradiol level in females increased at 5 weeks old, with a peak at 9 weeks (Fig. 4 B).

Fig. 4. Changes in serum levels of testosterone in male rats (A) and 17β -estradiol on female rats (B). Overall significance of differences between age groups by analysis of variance are as follows: testosterone, $P<0.001$; 17β -estradiol, $P<0.01$ (Unpaired t -test).

As shown in Figure 5 A, serum IGF-1 levels in male were elevated markedly at 5~7 weeks old and then followed the same pattern as serum testosterone ($r=0.363$, $n=35$, $P<0.05$). In female, IGF-1 increased markedly as well as in males

Fig. 5. Changes in serum IGF-1 (A) and IGFBP-3 (B) levels in male and female rats. Overall significance of differences between age groups by analysis of variance are as follows: IGF-1 (male) $P < 0.001$; IGF-1 (female), $P < 0.001$; IGFBP-3 (male), $P < 0.001$; IGFBP-3 (female), $P < 0.001$. *: $P < 0.05$, **: $P < 0.001$ (Unpaired t -test).

from 5 weeks old, peaking at 9 weeks, and then showed a rapid significant decrease ($P < 0.001$, 9 weeks old vs. 12 weeks old). A sex-specific difference in IGF-1 level was observed from 12 weeks old as that in males was significantly higher than that in females ($P < 0.001$, $P < 0.05$ and $P < 0.05$ for 12, 15 and 20 weeks old age, respectively).

Serum IGFBP-3 levels in males increased markedly at 5 weeks, and peaked at 7 weeks old at a higher level than that in females ($P < 0.001$), and then declined gradually (Fig. 5B). In females, IGFBP-3 was first elevated at 5 weeks of age, peaked at 9 weeks, later than in males, and then decreased (Fig. 5B).

Fig. 6. Changes in serum osteocalcin levels in male and female rats. Overall significance of differences between age groups by analysis of variance are as follows: male, $P < 0.001$; female, $P < 0.001$.

As shown in Figure 6, serum OC maintained relatively high levels from 3 to 9 weeks old and from 3 to 7 weeks old in males and females, respectively, although serum OC level declined gradually with aging in both sexes. After 15 weeks old, serum OC showed a steady level in both sexes. Although females tended to have a higher mean value than males between 5 and 7 weeks old, there were no significant sex-related differences in serum OC values.

IV. Discussion

Mammals undergo marked growth and development remarkably between birth and puberty¹⁾. In human, bone mass also increases markedly during the process of sexual maturation. The concerted actions of sex steroids, GH, and IGF-1 are required for a normal pubertal growth spurt, and are also important for skeletal growth^{2,3)}. Recently, the development of noninvasive, precise, and sensitive techniques such as a DXA has enabled assessment of changes in bone mass with puberty in man. However, there is little information available pertaining to rat pubertal changes in bone mass.

In the present study, we evaluated the changes in BMD during puberty in relation to serum

IGF-1, IGFBP-3, testosterone, 17β -estradiol, and osteocalcin levels in rats.

Our results demonstrated that female rats tended to have a higher mean BMD value than male rats between 5 to 9 weeks old. Between 15~20 weeks, BMD continued to increase substantially in male rats, whereas a tendency to reach a steady state was observed in male rats. The only point left to discuss now is the sex-specific difference of the size of the bone. This is because the measurement of BMD with DXA is greatly influenced by the size of the bone. However, although the size of female body is smaller than that of male body, BMD in females is transiently higher than that in males between 5 to 9 weeks, being probably related to their earlier onset of puberty in female^{13~15}).

Changes in the BMD pattern may be related to the serum concentrations of IGF-1 and sex steroids with sexual maturation, because IGF-1 and sex steroids are closely related to serum GH which is an important hormone for growth^{2,3}). IGF-1 is thought to mediate the growth-promoting effect of GH, and rising levels of circulating IGF-1 is a common event among animals during sexual maturation^{16~19}). IGF-1 is mainly produced in the liver, but is also synthesized locally in bone²⁰). It has been shown that osteoblasts express IGF-1 receptors and that IGF-1 can stimulate these cells in vitro, it enhancing osteoblastic collagen synthesis and matrix appositional rates^{21,22}). Systemic IGF-1 has been shown to stimulate longitudinal growth in vivo in GH-deficient rodents²³). However, the biological significance of circulating IGF-1 has not been clarified, and the relative importance of endocrine and paracrine actions of IGF-1 on bone during puberty is unknown at present. It is known that most of the circulating IGF-1 is bound to specific binding proteins (IGFBP-1 to -6)²⁴), the predominant form in blood being IGFBP-3. IGFBP-3 levels reflect the total concentration of circulating

IGFs (IGF-1 + IGF-2) and do not seem to be dependent on IGF-1, although IGF-1 and IGFBP-3 are closely related to GH secretion status^{2~4}). The present study showed that the changes in IGF-1 and IGFBP-3 levels are different in males, whereas those in females are very similar. This sex-related difference may be due to the differential effects of the predominant sex steroids, testosterone and 17β -estradiol in males and females, respectively, on IGF-1 synthesis. The anabolic actions of testosterone and its positive effect on IGF-1 production may contribute to enhanced growth in males²⁵). Conversely, at low levels, estradiol can stimulate GH and IGF-1 production and stimulate growth, but at higher levels, estradiol may negatively affect IGF-1 production and thus inhibit growth^{26,27}). Furthermore, sex steroids are considered to be important in the skeletal maturation during growth and in the prevention of bone loss associated with aging. Recently, estrogen receptors were located primarily on osteocytes²⁸). In male rats, except that there was a time-lag of marked rises in serum levels of testosterone and IGF-1, the changes in both levels were very similar. Previous studies in man⁸) and chimpanzees¹⁸) showed that serum testosterone level is correlated with that of IGF-1. The rapid increase in BMD value from 3 weeks old observed in this study, may be related to serum testosterone and IGF-1 concentrations, since IGF-1 and testosterone directly affect bone formation^{21,22}). However, after 12 weeks old with relatively high serum IGF-1 and testosterone levels, the rate of increase in BMD value declined. Although the results appear to be contradictory, serum IGFBP-3 concentrations may offer the key to understanding this discrepancy. In this study, IGFBP-3 levels increased rapidly between 5 and 7 weeks old, concurrent in with those in IGF-1, and then IGFBP-3 levels decreased markedly, whereas IGF-1 levels remained high. A previous study suggested that a complex

between IGFBP-3 and IGF-1 may be more active than free IGF-1 in the regulation of bone metabolism, and that IGFBP-3 may influence the activity of IGF-1 at the receptor level²⁹⁾. Therefore, it is possible that the decline of IGFBP-3 after 7 weeks induces a decrease in the activity of IGF-1 at the receptor level, and decreased IGFBP-3 levels may cause reduction of the rise in BMD. In female rats, serum IGF-1, IGFBP-3 and 17β -estradiol levels increased rapidly at 5 weeks with a peak at 9 weeks, concurrently with the rise in BMD. Thereafter, serum IGF-1 and IGFBP-3 levels were significantly decreased compared with the peak levels ($P < 0.001$), whereas serum 17β -estradiol remained relatively high. These results indicate that at 9 weeks old 17β -estradiol levels may be above the upper threshold that stimulates IGF-1 production. As a result, the levels of IGF-1 were inhibited after 9 weeks old, preventing and further increase in BMD.

We also measured serum OC as a marker of bone formation³⁰⁾ OC, i. e. bone gla-protein, is synthesized in osteoblasts and is a major noncollagen protein of bone matrix³¹⁾. The serum levels of OC may therefore reflect the level of osteoblastic activity in the bone and be related to the rate of bone turnover³²⁾. Several human studies have shown that increases in serum OC occur at the expected age of the growth spurt in both sexes, and the peak values occurs at the same age as the maximal height velocity⁸⁾. Furthermore, it has been reported that the rise of serum OC is closely related to the increase in forearm BMD³³⁾. It has also been reported that serum OC is highly correlated with serum IGF-1 levels in children⁸⁾. In this study, OC decreased with aging from 3 weeks old and did not increase during puberty in both male and female rats. One of the reasons for these discrepancies between human and rats may be that the pattern of longitudinal growth in rats is different from that in man. In man, peak height

velocity occurs very close to the corresponding period of sexual maturation³⁴⁾. In contrast, although we did not measure the head to tail length, the rates of longitudinal growth of tibia and femur declined from 5 weeks old and no peak velocity was found during puberty in rats. Another reason may be the differences in bone remodeling systems, because rats do not have haversian canals. Our findings are in agreement with the previous human study⁸⁾ that OC concentrations during early puberty were shown to be higher than those at later stages of puberty. The details of the pattern of changes in OC with aging in rats remain to be clarified. Further histomorphometric analyses will facilitate understanding of the mechanisms in more detail.

We conclude that skeletal growth occurs earlier in female than in male rats. This sex-specific difference of changes in the BMD pattern may result from the earlier onset of puberty in females, and from the sex-related differences in concentrations of IGF-1, IGFBP-3 and sex steroids with maturation.

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