ORIGINAL ARTICLE

JBMR

Relationship of Testosterone and Osteocalcin Levels During Growth

Salman Kirmani, Elizabeth J Atkinson, L Joseph Melton III, B Lawrence Riggs, Shreyasee Amin, and Sundeep Khosla

College of Medicine, Mayo Clinic, Rochester, MN, USA

ABSTRACT

Recent studies in mice have demonstrated that osteocalcin (OCN) regulates testosterone (T) production in males but not in females. We hypothesized that this novel bone-testis axis may be most relevant during rapid skeletal growth to help maximize bone size. Thus we measured serum T, total and undercarboxylated (UC) OCN, and periosteal circumference at the radius in 56 boys (bone age 4 to 20 years). T was correlated with OCN (bone-age-adjusted $r = 0.30$, $p = .024$), with a similar trend for UC OCN. T began to increase in the boys at bone age 11 years, and OCN peaked at bone age 14 years. Thus we divided the boys into three groups: 4 to 10 years ($n = 16$), 11 to 14 years ($n = 18$), and 15 to 20 years ($n = 22$). In boys of bone age 11 to 14 years (but not the other two groups), OCN was correlated with T ($r = 0.57$, $p = .013$), with a similar trend for UC OCN; T, in turn, was correlated with periosteal circumference ($r = 0.75$, $p < .001$). Collectively, these findings support the recent observations in mice of a novel bone-testis axis. Moreover, our data suggest that in human males, this axis may be most relevant during rapid skeletal growth, when T levels are rising under the influence of the hypothalamic-pituitary axis and OCN is increasing due to skeletal growth. During this phase, OCN may further stimulate testicular T production, which, in turn, contributes to an increase in bone size. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: TESTOSTERONE; OSTEOCALCIN; ADOLESCENTS; BONE SIZE; MALES

Introduction

Recent provocative studies by Oury and colleagues[1] have demonstrated a novel connection between the skeleton and the testis. Using genetic studies in mice combined with in vitro analyses, these investigators showed that osteoblasts are able to induce testosterone (T) production by the testes but not T or estrogen production by the ovaries. Further, the influence of osteoblasts on the testis was shown to be mediated by osteocalcin (OCN), which, in its undercarboxylated (UC) form, was demonstrated to bind to a G protein–coupled receptor (GPCR6A) that was expressed in the testis but not the ovary. By increasing testicular T production, OCN also regulated fertility in males, principally via the effects of T in reducing germ cell apoptosis.[1] These studies are consistent with previous findings demonstrating that GPCR6A is a receptor for OCN[2] and that male *Gpcr6a* knockout mice are hypogonadal.[3] Thus work from two independent laboratories has demonstrated that the osteoblast secretory product OCN, via binding to GPCR6A on the testis, regulates T production and, indirectly, fertility in male but not female mice.

Since a major difference between the male and female skeleton is bone size, and since T has been shown to increase periosteal apposition and thus increase bone size in males,[4] the marked gender specificity of OCN effects on the gonads led us to hypothesize that this novel bone-testis axis may be most relevant during rapid skeletal growth in adolescent human males, helping to maximize bone size. In a previous study,[5] we used high-resolution peripheral quantitative computed tomographic (HRpQCT) imaging to define age-related changes in bone structure at the distal radius during adolescence in boys and found that, consistent with findings in rodents,[4] the serum T level was an independent predictor of periosteal circumference in boys but not in girls. Thus, in this study, we used our previously measured (by mass spectroscopy) T levels in the boys and related them to serum OCN and UC OCN levels. In addition, we used serum levels of another bone-formation marker, amino-terminal propeptide of type 1 collagen (P1NP), as an internal control in order to assess whether any associations observed between OCN and T levels were specific for OCN or related nonspecifically to changes in bone formation.

Received in original form March 25, 2011; revised form April 22, 2011; accepted May 4, 2011. Published online May 16, 2011.

Address correspondence to: Sundeep Khosla, MD, Endocrine Research Unit, Guggenheim 7-11, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA. E-mail: khosla.sundeep@mayo.edu

Journal of Bone and Mineral Research, Vol. 26, No. 9, September 2011, pp 2212–2216
DOI: 10.1002/jbmr.421

Methods

Study subjects

After approval by the Mayo Clinic Institutional Review Board, study subjects were recruited as described previously.[5] Written informed consent was obtained from all subjects older than 12 years of age and from a parent for all subjects younger than 18 years of age. For this analysis, we used data on 56 boys (chronologic age 6.9 to 20.8 years) in whom we had HRpQCT imaging without motion artifact as well as sufficient serum for the additional OCN and UC OCN measurements. None of the subjects had a chronic illness or dietary restrictions. No one was receiving supplements of calcium greater than 1000 mg/d or vitamin D greater than 200 IU/d. No subject had ever used sodium fluoride, calcitonin, bisphosphonates, or antiepileptic drugs or had a history of oral steroid use for more than 7 days. Skeletal maturity was assessed using plain hand and wrist X-rays, and subjects were divided into bone-age groups using the Tanner-Whitehouse III method,[6] although subjects who had completed skeletal maturation (bone age $>$ 16.5 years for boys) were classified according to chronologic age. Fasting (8 am) blood was drawn from all subjects and kept frozen at -80°C until analyzed. Blood samples were collected as the subjects were recruited, over the course of the year, and in random order in terms of age.

HRpQCT measurements

Details regarding the HRpQCT measurements in this cohort have been described previously.[5] For this analysis, we used the data on periosteal circumference at the radius from our earlier study. Briefly, measurements were obtained from the nondominant wrist on all subjects using the Xtreme CT (Scanco Medical, Brüttisellen, Switzerland). Using a scout view, a reference line was set at the proximal limit of the epiphyseal growth plate. For subjects whose epiphyseal plates had fused, the remnant of the plate was still visible to set the reference line. The scan was performed on a segment spanning 9.02 mm, starting at a distance 1 mm proximal to the reference line, thereby ensuring that despite differences in arm length, all subjects had the scans performed as close to the identical anatomic site as possible. Data were obtained using a 3D stack of 110 high-resolution CT slices with an isotropic voxel size and slice thickness of 82 μm, an effective energy of 40 keV, a field of view of 125.9 mm, and an image matrix of 1536 × 1536 pixels. The cortex was segmented from the grayscale image with a Gaussian filter and threshold. Cortical area was measured directly, and the periosteal circumference was calculated from the contour.[5]

Hormone and bone turnover measurements

Serum T was measured using tandem mass spectroscopy (API 5000, Applied Biosystems-MDS Sciex, Foster City, CA, USA)[7] [lower limit of detection, 1 ng/dL; interassay coefficient of variation (CV), 6%]. Serum OCN was measured using an enzyme immunoassay (Quidel, San Diego, CA, USA) (interassay CV $<$ 8%; CIS-US), and serum UC OCN was measured using an enzyme immunoassay with antibodies specific for the undercarboxylated form of osteocalcin (Takara Biotechnologies, Inc., Shiga, Japan) (interassay CV $<$ 10%).[8] Serum P1NP was measured using an enzyme-linked immunosorbent assay (Immunodiagnostic Systems, Ltd., Fountain Hills, AZ, USA) (interassay CV $<$ 10%).

Statistical analysis

Unadjusted and bone-age-adjusted associations between serum T levels and the various parameters were assessed using Spearman correlations. A p value of less than .05 was considered significant. The relationships between bone age with serum T, OCN, and UC OCN were explored using a Loess smoothing function. Analyses were performed using SAS Version 9.2 (SAS Institute, Inc., Cary, NC, USA) and R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Table 1 shows the unadjusted and bone-age-adjusted correlations of serum T levels with bone-formation markers and periosteal circumference at the radius in the study subjects. As is evident, following adjustment for bone age, serum OCN levels were significantly correlated with T levels, with a similar trend for the correlation between UC OCN and T levels. Of note, serum OCN and UC OCN levels also were significantly correlated ($r = .70$, $p < .001$). As an internal control, we used another bone-formation marker, serum P1NP, which was correlated with serum OCN ($r = 0.64$, $p < .001$). In unadjusted analyses, P1NP was inversely associated with T levels, but no significant correlation between P1NP and T levels was present following adjustment for bone age (Table 1). Thus the positive association of T with bone formation was specific for OCN and was not present for P1NP. We have previously published serum 25-hydroxyvitamin D levels in these subjects[5]; the mean (SD) 25-hydroxyvitamin D level was 28.9 (6.6) ng/mL, and serum 25-hydroxyvitamin D levels were not correlated with bone age ($r = -0.13$, $p = .337$).

Since the association between OCN and T levels was only present following adjustment for bone age, we performed additional analyses to further explore the role of age. As shown in Fig. 1, serum T levels began to increase in the boys at a bone age of 11 years. Serum OCN increased early in puberty and peaked at a bone age of approximately 14 years (Fig. 2*A*). Serum UC OCN (Fig. 2*B*) and P1NP (Fig. 2*C*) showed much more gradual increases in early puberty, but like OCN, both declined after a bone age of approximately 14 years. Thus, for the subsequent analyses, we divided the boys into three groups according to bone age: 4.00 to 10.99 years (group 1, prior to the onset of increases in T levels; $n = 16$), 11.00 to 14.99 years (group 2, the phase of rising T levels

Table 1. Spearman Correlation Coefficients ($r^{p\ \text{value}}$) Between Serum T Levels and Bone-Formation Markers and Periosteal Circumference at the Radius

	Unadjusted	Bone age adjusted
OCN	$-0.15^{0.285}$	$0.30^{0.024}$
UC OCN	$0.06^{0.675}$	$0.26^{0.056}$
P1NP	$-0.51^{<0.001}$	$0.14^{0.307}$
Periosteal circumference	$0.84^{<0.001}$	$0.32^{0.019}$

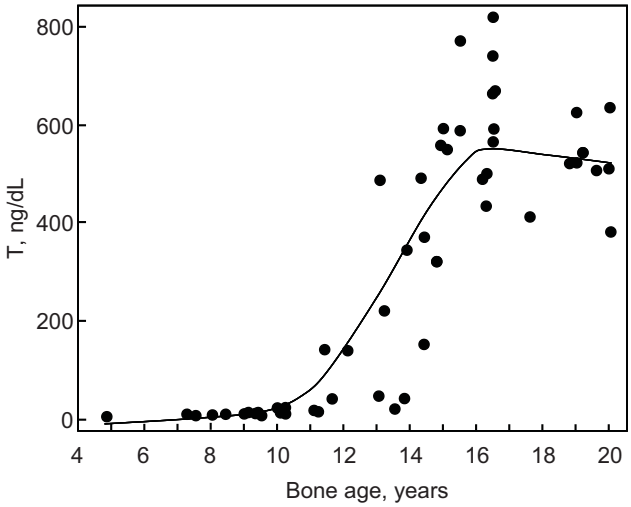

Fig. 1. Serum T levels as a function of bone age in the boys. Curve fitting was done using a Loess smoothing function (see "Methods").

and maximal bone formation; $n = 18$), and 15.00 to 20.99 years (group 3, the phase of declining bone formation; $n = 22$).

Table 2 shows the unadjusted and bone-age-adjusted correlations between serum T levels and bone-formation markers and periosteal circumference at the radius in the boys separated according to the three groups just described. In unadjusted analyses, the only significant associations were present in the boys in group 2 (11 to 14 years). Thus serum OCN was significantly associated with T levels in the boys in this group, with a similar trend for UC OCN. Importantly, there was no association between serum P1NP and T levels. Thus the overall, bone-age-adjusted association between OCN and T levels we observed initially (Table 1) was driven by the association between these variables in group 2. In addition, serum T levels were correlated with periosteal circumference in group 2. Of note, bone-age adjustment within group 2 reduced each of the associations by 50%, and these correlations were no longer significant following bone-age adjustment within the group in part owing to the smaller sample size (compared with the analogous analysis in all boys combined).

Table 2. Spearman Correlation Coefficients ($r^{p\ \text{value}}$) Between Serum T Levels and Bone-Formation Markers and Periosteal Circumference at the Radius in the Boys Separated by Bone-Age Groups

	Unadjusted	Bone age adjusted
Group 1 (4–10 years)		
OCN	$-0.05^{0.858}$	$-0.16^{0.574}$
UC OCN	$-0.38^{0.168}$	$-0.38^{0.183}$
P1NP	$0.27^{0.310}$	$0.04^{0.899}$
Periosteal circumference	$0.46^{0.070}$	$0.28^{0.308}$
Group 2 (11–14 years)		
OCN	$0.57^{0.013}$	$0.24^{0.361}$
UC OCN	$0.43^{0.071}$	$0.17^{0.524}$
P1NP	$0.08^{0.748}$	$0.02^{0.938}$
Periosteal circumference	$0.75^{<0.001}$	$0.36^{0.152}$
Group 3 (15–20 years)		
OCN	$0.21^{0.346}$	$0.04^{0.857}$
UC OCN	$0.24^{0.288}$	$0.13^{0.560}$
P1NP	$0.20^{0.372}$	$-0.01^{0.977}$
Periosteal circumference	$-0.17^{0.448}$	$-0.16^{0.496}$

Finally, we tested whether in group 2 serum OCN or UC OCN levels were correlated with periosteal circumference and whether this relationship was mediated by T levels. Serum OCN ($r = 0.49$, $p = .037$) but not UC OCN ($r = 0.26$, $p = .299$) levels were correlated with periosteal circumference; importantly, adjusting this correlation for T levels eliminated the association between OCN and periosteal circumference ($r = 0.12$, $p = .660$ following adjustment for T levels).

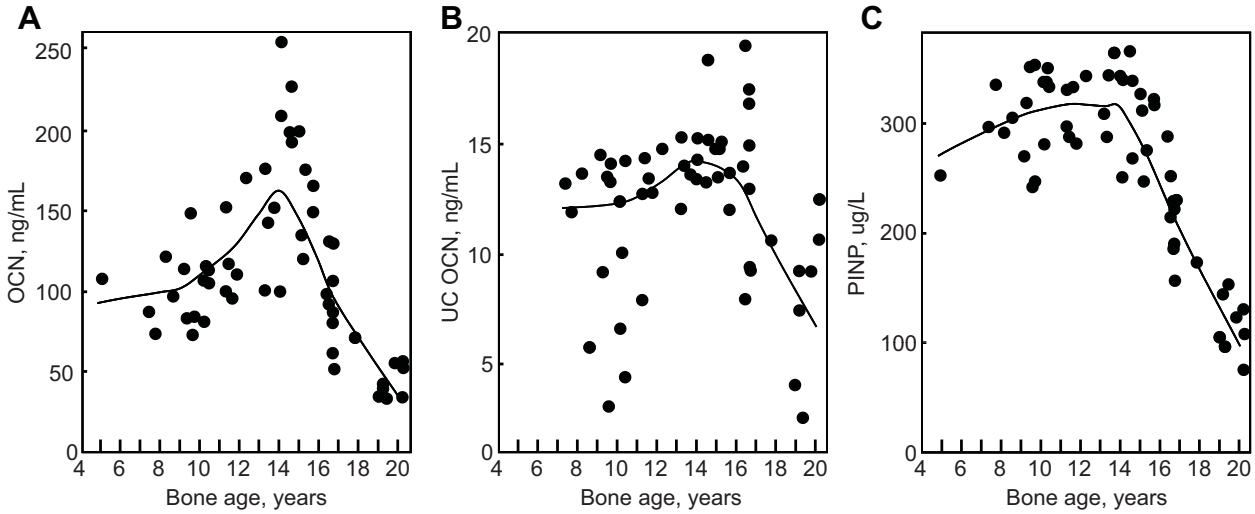

Fig. 2. (*A*) Serum OCN, (*B*) serum UC OCN, and (*C*) serum P1NP levels as a function of bone age in the boys. Curve fitting was done using a Loess smoothing function (see "Methods").

Discussion

We demonstrate in this study that in boys spanning the pubertal years of maximal skeletal growth, there is a significant association between serum OCN and T levels (with a similar trend for UC OCN) that is evident following adjustment for bone age. However, we observed no such association between T and levels of another bone-formation marker, P1NP, despite the fact that serum OCN and P1NP levels were significantly correlated in these subjects. These data thus indicate that the correlation between OCN and T we found was specific for OCN and not due to a nonspecific association with bone formation. In further analyses, separating the boys into three groups representing different physiologic stages [prior to the onset of increases in T levels (group 1), the phase of rising T levels and maximal bone formation (group 2), and the phase of declining bone formation (group 3)], we found that the overall association we observed was largely driven by the boys undergoing maximal increases in bone formation (assessed by serum OCN levels, group 2). Further, in all boys combined and in this group in particular, serum T levels were correlated with bone size (assessed by periosteal circumference, as measured by HRpQCT). Finally, serum OCN levels also were associated with periosteal circumference, but this relationship disappeared following adjustment for T levels, suggesting that T was the intermediary factor mediating the association between OCN and periosteal circumference.

Overall, our results are consistent with the very recent findings that demonstrated a role for OCN in regulating T production and, indirectly, fertility in male mice.[1] Our data extend these observations to humans and support our hypothesis that this novel bone-testis axis may be most relevant during rapid skeletal growth in adolescent males, helping to maximize bone size. Thus what the work of Karsenty and colleagues[1] may have uncovered is a novel feed-forward loop between bone and the testis that is designed to maximize bone size in males. Under this scenario, increased OCN production by osteoblasts during skeletal growth leads to increased T production specifically in males but not in females; T then acts on the skeleton to increase bone size in males.

An important, unresolved question is why we observed the association between serum OCN and T levels predominantly in group 2 (during midpuberty) but not in the younger or older boys. The explanation for this may lie in the possible interactions between bone (via OCN) and the hypothalamic-pituitary axis [via luteinizing hormone (LH)] in regulating testicular T production. Thus, while both LH and OCN act through GPCRs, each hormone functions through distinct receptors, and LH is likely the predominant driver of T production by the testis.[9] We did not measure LH levels in our study subjects because we only had a single 8 am blood draw, and LH levels show marked pulsatility.[10] In addition, particularly early in puberty, increases in LH secretion occur predominantly during sleep,[10] and there is an extensive existing literature on changes in LH production during puberty in males (summarized in ref. 10). Thus, based on previous studies,[10] the subjects in group 1 would be expected to have low LH levels; the subjects in group 2 would have rising, pulsatile LH levels (particularly at night); and the subjects in group 3 would have adult LH levels and secretory profiles. Our findings suggest that OCN may enhance T production predominantly during the phase of rising LH levels in puberty; once adult T and LH levels are achieved, the effect of OCN may wane, with T production controlled principally by LH. Since GPCR6A also appears to bind and mediate nongenomic effects of T,[11] it is possible that the rising T levels in late puberty feed back on the testis to attenuate the stimulation of T production by OCN. Clearly, however, further studies in rodents and humans are needed to test this hypothesis and to assess the possible interaction(s) between LH and OCN effects on the testis.

We also recognize several limitations to our study. First, even though the active form of OCN is UC OCN, the correlations we observed with T levels were consistently stronger for OCN than for UC OCN. This may have to do with the OCN immunoassay being more robust than the UC OCN immunoassay. Further studies, perhaps using better ways to measure UC OCN (eg, mass spectroscopy), are needed to evaluate this possibility. In addition, the antibodies used in the UC OCN assay do recognize undercarboxylated fragments of intact (1–49) OCN (eg, the 1–43 or 4–43 fragments),[12] thereby overestimating the amount of UC OCN present, and it is possible that this contributed to the weaker associations of T with UC OCN than with OCN. Alternatively, OCN may serve as a prohormone for local processing in the testis to UC OCN, which then would bind to the GPCR6A.[1] Second, association cannot prove causality. Thus, even though we observed a significant correlation between OCN and T levels (but not P1NP and T levels) following bone-age adjustment in all boys combined, and the unadjusted analyses showed that this association was being driven by the boys in the rapid phase of skeletal growth, we cannot exclude the possibility that OCN and T are correlated simply because they tend to increase similarly with age during this phase of growth (ie, bone age is the common factor relating increasing T and OCN levels in the age window of 11 to 14 years). However, as noted earlier, the correlation between T and OCN remained significant in all boys combined even following adjustment for bone age, indicating that this association was independent of bone age. Moreover, given the findings from two laboratories described earlier for a role of OCN in regulating T production by the testis,[1,3] there is certainly a plausible biologic basis for our observations in humans.

In summary, our data demonstrating an association between serum OCN, but not P1NP, and T levels, particularly during rapid skeletal growth in boys, are consistent with observations in mice[1,3] that osteoblasts may regulate the testis through the production of OCN. When combined with earlier studies demonstrating a role for OCN in regulating glucose homeostasis,[1,13] these data expand the role of the skeleton as an endocrine organ, with OCN being perhaps one of its most prominent hormones.

Disclosures

All the authors state that they have no conflicts of interest.

Acknowledgments

We wish to thank Ms Sara Achenbach for help with the statistical analyses, Ms Kelley Hoey for sample processing, Mr James Peterson for data management, Ms Margaret Holets for perform-

ing the HRpQCT scans, and the Mayo Immunochemical Core Laboratory for performance of the biochemical and hormonal assays. This work was supported in part by NIH Grants AR027065 and UL1-RR24150 (Center for Translational Science Activities), U.S. Public Health Service.

Authors' roles: All authors made substantial contributions to conception and design, analysis and interpretation of data; participated in drafting the manuscript or revising it critically for intellectual content; and approved the final version of the submitted manuscript. Salman Kirmani was responsible for data acquisition.

References

1. Oury F, Sumara G, Sumara O, et al. Endocrine regulation of male fertility by the skeleton. Cell. 2011;144:796–809.
2. Pi M, Faber P, Ekema G, et al. Identification of a novel extracellular cation sensing G-protein coupled receptor. J Biol Chem. 2005;280: 40201–40209.
3. Pi M, Chen L, Huang M-Z, et al. *Gprc6a* null mice exhibit osteopenia, feminization and metabolic syndrome. PLos ONE. 2008;3: e3858.
4. Turner RT, Wakley GK, Hannon KS. Differential effects of androgens on cortical bone histomorphometry in gonadectomized male and female rats. J Orthop Res. 1990;8:612–617.
5. Kirmani S, Christen D, van Lenthe GH, et al. Bone structure at the distal radius during adolescent growth. J Bone Miner Res. 2009;24: 1033–1042.
6. Tanner JM, Healy MJR, Goldstein H, Cameron N. *Assessment of Skeletal Maturity and Prediction of Adult Height: TW3 Method*. Philadelphia: Saunders, 2001.
7. Khosla S, Amin S, Singh RJ, Atkinson EJ, Melton LJ, Riggs BL. Comparison of sex steroid measurements in men by immunoassay versus mass spectroscopy and relationships with cortical and trabecular volumetric bone mineral density. Osteoporos Int. 2008;19:1465–1471.
8. Foresta C, Strapazzon G, De Toni L, et al. Evidence for osteocalcin production by adipose tissue and its role in human metabolism. J Clin Endocrinol Metab. 2010;95:3502–3506.
9. Smith LB, Saunders TK. The skeleton: the new controller of male fertility? Cell. 2011;144:642–643.
10. Hayes FJ, Crowley WF Jr. Gonadotropin pulsations across development. Horm Res. 1998;49:163–168.
11. Min P, Parrill AL, Quarles LD. GPRC6A mediates the non-genomic effects of steroids. J Biol Chem. 2010;285:39953–39964.
12. Gundberg CM, Nieman SD, Abrams S, Rosen H. Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin. J Clin Endocrinol Metab. 1998;83:3258–3266.
13. Fulzele K, Riddle RC, DiGirolamo DJ, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquistition and body composition. Cell. 2010;142:309–319.