Adiponectin Is a Negative Regulator of Bone Mineral and Bone Strength in Growing Mice

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Obesity is associated with increased bone mineral density (BMD) but the mechanism for this is unclear. Serum levels of the adipokine adiponectin are inversely correlated with obesity, but results from studies on its relationship to bone mass are conflicting. The objective of this study was to compare bone mineral content (BMC), BMD and biomechanical strength properties of femur and lumbar vertebrae in 8- and 16-week old adiponectin transgenic mice (AdTg). These mice exhibit significantly elevated circulating adiponectin but have similar body weights compared to wild-type (WT) littermates that were used as controls. Female AdTg mice displayed significantly lower femur BMC at 8 and 16 weeks of age and femur neck peak load was significantly lower in 8-week old AdTg mice of both genders compared to controls. The peak load from compression testing of an individual lumbar vertebra was significantly lower in female AdTg mice compared to WT at 8 weeks, and this difference persisted at 16 weeks of age. In addition, lumbar vertebrae BMC was significantly lower in 16-week old male AdTg mice compared to WT although vertebra peak load was not different. Serum adiponectin levels were inversely correlated with femur BMC. In summary, elevated circulating adiponectin inhibits the acquisition of bone mass in growing mice and results in decreased biomechanical measures of functional strength that are surrogate measures of susceptibility to fractures. These results support a role for circulating adiponectin as a metabolic link that can explain, at least in part, the positive relationship between obesity and both bone mass and reduced susceptibility to fractures. Exp Biol Med 233:1546–1553, 2008

Key words: adiponectin; bone mineral content; biomechanical bone strength; development; mice

Introduction

Excess body weight and elevated body mass index (BMI) are strongly correlated with high bone mineral density (BMD) (1). Conversely, a decrease in body weight is associated with bone loss (2) and a low BMI is associated with an increased risk of fractures (3). The mechanism for the positive correlation between obesity and BMD is unclear. Although increased mechanical load on the skeleton resulting from an increase in fat and lean mass are thought to partially explain this observation (4), it is generally accepted that there are other factors involved. Increased adipose tissue may influence bone metabolism by altering hormonal factors in the circulation and through the secretion of adipokines (5, 6).

Adiponectin is the most abundant protein secreted from adipose tissue (7), and its concentration in the circulation is much higher than that of other hormones and cytokines (8). This 30 kDa protein circulates as various complexes and mediates its effects through binding to its specific receptors AdipoR1 and AdipoR2 that have differential tissue distribution (9). Adiponectin synthesis and secretion are dependent on many factors and its levels in serum are higher in women than in men (10). Unlike other adipokines, serum adiponectin levels are decreased in conditions associated with obesity and the metabolic syndrome including insulin resistance (11). Serum adiponectin levels are negatively associated with obesity, particularly visceral adiposity (12). Moreover, adiponectin levels increase in response to weight loss (13).

Human osteoblasts have been reported to express adiponectin and its receptors AdipoR1 and AdipoR2 suggesting that this protein may be a metabolic factor linking obesity to bone metabolism (14). In one study, treatment of human osteoblasts with adiponectin resulted in increased osteoblast proliferation and differentiation, increased alkaline phosphatase activity (ALP), a marker of bone formation, and increased mineralization suggesting a positive effect of adiponectin on bone (15). In another study, adiponectin was shown to increase osteoclast formation by

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stimulating the production of receptor activator of nuclear factor-κB ligand (RANKL) that stimulates osteoclast differentiation and activity and by inhibiting the production of osteoprotegerin (OPG), an inhibitor of osteoclastogenesis, in osteoblasts (16). The *in vivo* data are conflicting. Serum adiponectin has been reported to be negatively associated with BMD at a number of skeletal sites, in both men and women, even after adjusting for fat mass (17, 18). In addition, adiponectin was shown to be positively correlated with serum concentrations of bone-specific ALP, and with cross-linked N-telopeptides of type collagen (NTX), a bone resorption marker, suggesting that adiponectin is associated with increased bone turnover (18). Jurimae *et al*. (19) similarly reported a significant negative association between adiponectin and whole body BMC and BMD as well as lumbar spine BMD in both pre-and postmenopausal women, whereas some studies suggest that serum adiponectin is a predictor of BMD in post- but not premenopausal women (20–22). Tamura *et al*. (23) reported a significant positive correlation between serum adiponectin and BMD at the distal radius, but no effect at the femur neck or lumbar spine in patients with type 2 diabetes.

Few animal studies have assessed a role for adiponectin in bone development and those results are also conflicting. Oshima *et al*. (24) treated 8-week old male C57BL/6J mice with an adenovirus producing adiponectin or the lacZ reporter gene (controls) for 2 weeks to determine the effects of adiponectin on bone metabolism. Treatment with adiponectin-adenovirus resulted in increased trabecular, but not cortical bone mass, a decreased number of osteoclasts and reduced circulating NTX compared to LacZ-adenovirus-treated mice suggesting that adiponectin increased bone mass by suppressing osteoclastogenesis (24). In another study, there were no differences in bone mass or turnover in 8-week old male adiponectin transgenic mice that overexpress adiponectin specifically in the liver compared to their wild-type (WT) littermates (25). Furthermore, they reported no differences in BMD of the femur, tibia and vertebrae and no differences in bone formation or resorption parameters in 8-week old male adiponectin-deficient mice and their WT controls (25). *In vitro* cultures of bone marrow cells isolated from the adiponectin-deficient mice, however, displayed reduced osteogenesis compared to WT mice (25). The discrepancies in the *in vitro* and *in vivo* data suggest that adiponectin may have both direct and indirect effects on bone that may differ depending on the presence of adiponectin receptors or other metabolic markers in serum.

The objective of the present study was to compare BMC, BMD and biomechanical strength properties of femur and lumbar vertebrae in adiponectin transgenic mice (AdTg) (26). We chose this mouse model because the AdTg mice have significantly elevated circulating adiponectin levels compared to their WT littermates used as controls (26), but exhibit similar body weights therefore allowing us to determine a specific effect of elevated circulating adiponectin in the absence of obesity. There is a sexual dimorphism in these mice whereby females have significantly higher circulating adiponectin levels than males (26). For this reason, we assessed the bone phenotype of both male and female AdTg and WT mice at 8 and 16 weeks of age to determine the role of adiponectin on accumulation of bone mineral and functional measures of bone strength that are indicators of fracture risk. Mice were studied at two distinct stages of bone metabolism representing a stage when bone development is occurring (8 weeks) and young adulthood when peak bone mass has been attained (16 weeks).

Materials and Methods

Animals. The generation of AdTg mice has been previously described (26). We received a group of male AdTg mice that were on an FVB/N background as a generous gift from Dr. Philipp Scherer (University of Texas Southwestern Medical Center, Dallas, TX). These mice were mated with female FVB/N mice in order to propagate the transgene in a pure FVB background. All genotyping to distinguish AdTg and WT mice was performed by PCR using genomic DNA isolated from the tails of weaned mice and a probe that recognizes the adiponectin transgene (personal communication). The animals were housed in the Department of Comparative Medicine at the University of Toronto. Mice were housed at 22°C and 50% humidity on a 12:12 h light-dark cycle and fed a standard rodent chow diet and water *ad libitum*. Groups of male and female AdTg and WT littermates used as controls, were killed at 8 and 16 weeks of age (n = 12–14/group). Femurs and lumbar vertebrae (LV1–LV4) were excised. All protocols for animal use and treatment were reviewed and approved by the University of Toronto Animal Care Committee and were in compliance with the guidelines of the Canadian Council on Animal Care (27).

Serum Adiponectin and Insulin. At the time of necropsy, blood was obtained from 16-week old mice in the fed state for the determination of serum levels of adiponectin and insulin. Adiponectin and insulin were measured by radioimmunoassay (Linco Research Inc., St. Charles, MO).

Femur and Lumbar Vertebrae (LV1–LV4), BMC and BMD. Whole left femurs and intact spines (LV1–LV4) were placed on a plastic tray and scanned in air for determination of BMD using PIXImus dual energy X-ray absorptiometry (DEXA) (LUNAR Corporation, GE Medical Systems, Mississauga, Ontario, Canada) and a specialized software program (Lunar Software Version 1.46) (28).

Biomechanical Strength Testing. Biomechanical strength testing was performed at femur midpoint, femur neck and lumbar vertebra 3 as previously described (28). Femurs and LV3 were soaked in physiological saline (9 g NaCl/L) for 3 hours at room temperature prior to testing. Three point bending at the femur midpoint and femur neck,

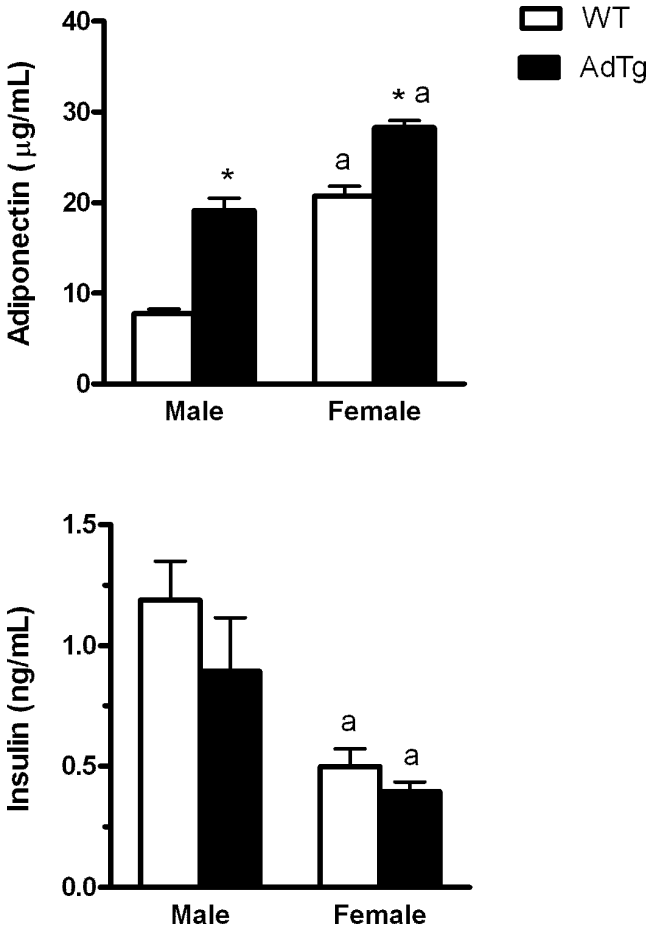

Figure 1. Serum adiponectin and insulin levels in fed 16-week old AdTg and WT mice. Values are expressed as mean ± SEM, $n = 9$–12/group. * Significantly different from WT mice of the same gender; [a] significantly different from males of the same genotype. Statistical significance was accepted at $P < 0.05$.

and compression testing of LV3 were performed using a material testing system (Model 4442 Universal Testing System, Instron, Canton, MA) and a specialized software program (Instron Series IX Automated Materials Tester-Version 8.15.00, Instron).

Three Point Bending at the Femur Midpoint. Femur weight was measured by electronic scale, and femur length was measured using an electronic precision caliper (Cedarlane Laboratories Ltd., Hornby, ON) to determine the location of the femur midpoint. Femurs were positioned such that the posterior side was placed on two base supports of the bending jig separated by 6 mm with the midpoint directly under the crosshead. The crosshead was then lowered at a constant speed of 2 mm/min, applying force to the femur midpoint until fracture occurred. The tips of the bending jig are rounded to reduce shear forces during the test. Yield load, stiffness and peak load were determined from the load-displacement curve (28).

Femur Neck Fracture. After three point bending was performed, individual femurs were placed in a customized holder and the crosshead was lowered at a constant speed of 2 mm/min such that direct force was applied directly onto the proximal femur head until fracture occurred (28).

Compression Testing of Lumbar Vertebra 3 (LV3). LV3 was isolated from the lumbar vertebrae (LV1–LV4) and positioned at the center of a smooth stainless steel plate. A second stainless steel plate was lowered onto LV3 at a constant speed of 2 mm/min until compression of LV3 was achieved. Peak load was identified as the first peak of the load-displacement curve (28).

Statistical Analyses. All data are expressed as mean ± SEM. Two-way ANOVA was performed with genotype and gender as the main effects. Interaction effects (genotype x gender) were also assessed. A Student-Newman Keul's test was used when a significant interaction effect was observed. To determine the relationship between BMC and biomechanical strength properties, or BMC and adiponectin, simple linear regression analysis was performed. All statistical analyses were performed using Sigma Stat (Jandel Corp., San Rafael, CA, USA) and differences were considered significant at $P < 0.05$.

Results

Body Weights, Serum Adiponectin and Insulin. At 8 weeks of age, there were no significant differences in body weight due to genotype (29.1 ± 0.7 g *vs* 28.6 ± 0.6 g for male WT *vs* AdTg and 22.8 ± 0.5 g *vs* 23.2 ± 0.5 g for female WT *vs* AdTg) although males were significantly heavier than females within both genotypes. Similarly, body weight did not differ between 16-week old WT and AdTg mice (31.8 ± 1.1 g *vs* 29.4 ± 0.8 g for male WT *vs* AdTg and 24.4 ± 0.6 g *vs* 24.1 ± 1.0 g for female WT *vs* AdTg) and males were significantly heavier than females within both genotypes. As shown in Figure 1, both genotype and gender had significant effects on serum adiponectin and there was a significant interaction between these two parameters. Circulating adiponectin was significantly higher in both male and female AdTg mice compared to WT controls and was significantly elevated in female mice of both genotypes compared to males (Fig. 1). Serum insulin levels were not significantly affected by genotype, and within each genotype, females had significantly lower circulating insulin than males (Fig. 1).

Femur Outcomes: BMC, BMD, and Biomechanical Strength Properties. In 8-week old mice, there was a significant effect of genotype on whole femur BMC with female AdTg mice having lower femur BMC compared to WT mice (Table 1). There was no effect of gender on whole femur BMC. Whole femur BMD was not affected by genotype, but female WT mice had significantly higher femur BMD than their male counterparts. Yield load at the femur midpoint was significantly lower in 8-week old male AdTg mice than in WT controls, but there was no effect of genotype on femur midpoint stiffness or peak load (Table 1). For gender effects, female AdTg mice had significantly higher femur yield load than male AdTg mice, and femur

Table 1. Femur BMC, BMD, and Biomechanical Strength Properties in AdTg and WT Mice[a]

	Males		Females		2-way ANOVA (*P* value)		
	WT	AdTg	WT	AdTg	Genotype	Gender	Genotype x gender
8 weeks							
Whole femurs							
BMC (mg)	29.49 ± 0.67	27.47 ± 0.83	28.8 ± 0.90	25.17 ± 0.6*	< 0.001	NS	NS
BMD (mg/cm^2)	65.45 ± 1.10	65.36 ± 1.3	69.67 ± 0.95[a]	66.29 ± 0.98	NS	0.026	NS
Femur midpoint							
Yield load (N)	10.24 ± 0.34	9.09 ± 2.8*	11.24 ± 0.6	10.5 ± 0.4[a]	.025	.005	NS
Stiffness (N/mm)	152.28 ± 6.5	152.7 ± 5.1	170.1 ± 6.9	165.8 ± 6.4	NS	.017	NS
Peak load (N)	21.08 ± 0.77	20.20 ± 0.6	21.15 ± 0.8	19.55 ± 0.7	NS	NS	NS
Femur neck							
Peak load (N)	15.34 ± 0.78	13.62 ± 0.47*	13.11 ± 0.52[a]	11.21 ± 0.48*,[a]	0.003	< 0.001	NS
16 weeks							
Whole femurs							
BMC (mg)	32.95 ± 0.93	29.81 ± 1.75	31.20 ± 0.73	27.16 ± 1.20*	.004	NS	NS
BMD (mg/cm^2)	71.71 ± 1.42	66.53 ± 1.86*	71.31 ± 0.78	67.95 ± 1.42	.005	NS	NS
Femur midpoint							
Yield load (N)	11.96 ± 0.55	11.40 ± 0.73	12.13 ± 0.63	11.44 ± 0.55	NS	NS	NS
Stiffness (N/mm)	178.48 ± 10.45	179.5 ± 6.30	182.70 ± 4.86	184.4 ± 4.77	NS	NS	NS
Peak load (N)	23.88 ± 0.81	22.43 ± 0.86	22.44 ± 0.70	21.27 ± 0.80	NS	NS	NS
Femur neck							
Peak load (N)	15.07 ± 1.53	13.25 ± 1.95	14.45 ± 1.13	11.31 ± 1.60	NS	NS	NS

[a] Values are expressed as mean ± SEM, $n = 10$–14/group. NS, not statistically significant, $P > 0.05$.
* Significantly different from WT mice of the same gender, and [a] significantly different from males of the same genotype ($P < 0.05$).

stiffness was higher overall in female mice compared to males (Table 1). There was no significant effect of gender on femur midpoint peak load in 8-week old mice. At the femur neck, both genotype and gender had a significant effect on peak load. Eight-week old AdTg mice of both genders had significantly lower femur neck peak load compared to their respective WT controls, and female mice of both genotypes had significantly lower femur neck peak load than males (Table 1). There were no significant interactions between genotype and gender for any of the measures of biomechanical strength properties of the femur midpoint and femur neck.

In 16-week old mice, there was a significant effect of genotype but not gender on whole femur BMC and BMD (Table 1). AdTg mice had lower whole femur BMD and BMC than WT mice (Table 1). There were no significant effects of either genotype or gender on femur midpoint yield load, stiffness, peak load and femur neck peak load and no genotype x gender interactions in 16-week old mice (Table 1).

BMC and BMD of LV1–LV4 and Peak Load of LV3. In 8-week old mice, there were no effects of gender or genotype on BMC or BMD of LV1–LV4. For LV3 peak load, there was a significant interaction between genotype and gender. Female 8-week old AdTg mice had significantly lower LV3 peak load compared to female WT controls and to male AdTg mice (Table 2).

At 16 weeks of age, male AdTg mice had significantly

Table 2. BMC and BMD of Lumbar Vertebrae (LV1-LV4) and Peak Load of LV3 in AdTg and WT Mice[a]

	Males		Females		2-way ANOVA (*P* value)		
	WT	AdTg	WT	AdTg	Genotype	Gender	Genotype x gender
8 weeks							
LV1–LV4							
BMC (mg)	37.88 ± 0.8	36.38 ± 1.5	38.21 ± 1.4	40.0 ± 1.8	NS	NS	NS
BMD (mg/cm^2)	58.20 ± 0.9	58.43 ± 1.2	60.16 ±1.3	61.91 ± 2.0	NS	NS	NS
Peak load LV3 (N)	48.58 ± 2.5	53.05 ± 2.2	53.4 ± 3.4	45.1 ± 2.8*,[a]	NS	NS	.025
16 weeks							
LV1–LV4							
BMC (mg)	47.16 ± 1.93	39.39 ± 2.50*	50.15 ± 2.09	48.30 ± 1.96[a]	.029	.008	NS
BMD (mg/cm^2)	66.09 ± 1.88	63.16 ± 2.20	71.46 ± 1.84[a]	69.04 ± 1.78[a]	NS	.006	NS
Peak Load LV3 (N)	50.23 ± 1.87	46.12 ± 2.26	57.56 ± 2.55[a]	50.31 ± 2.98*	.026	.024	NS

[a] Values are expressed as mean ± SEM, $n = 10$–14/group. NS, not statistically significant, $P > 0.05$.
* Significantly different from WT mice of the same gender, and [a] significantly different from males of the same genotype ($P < 0.05$).

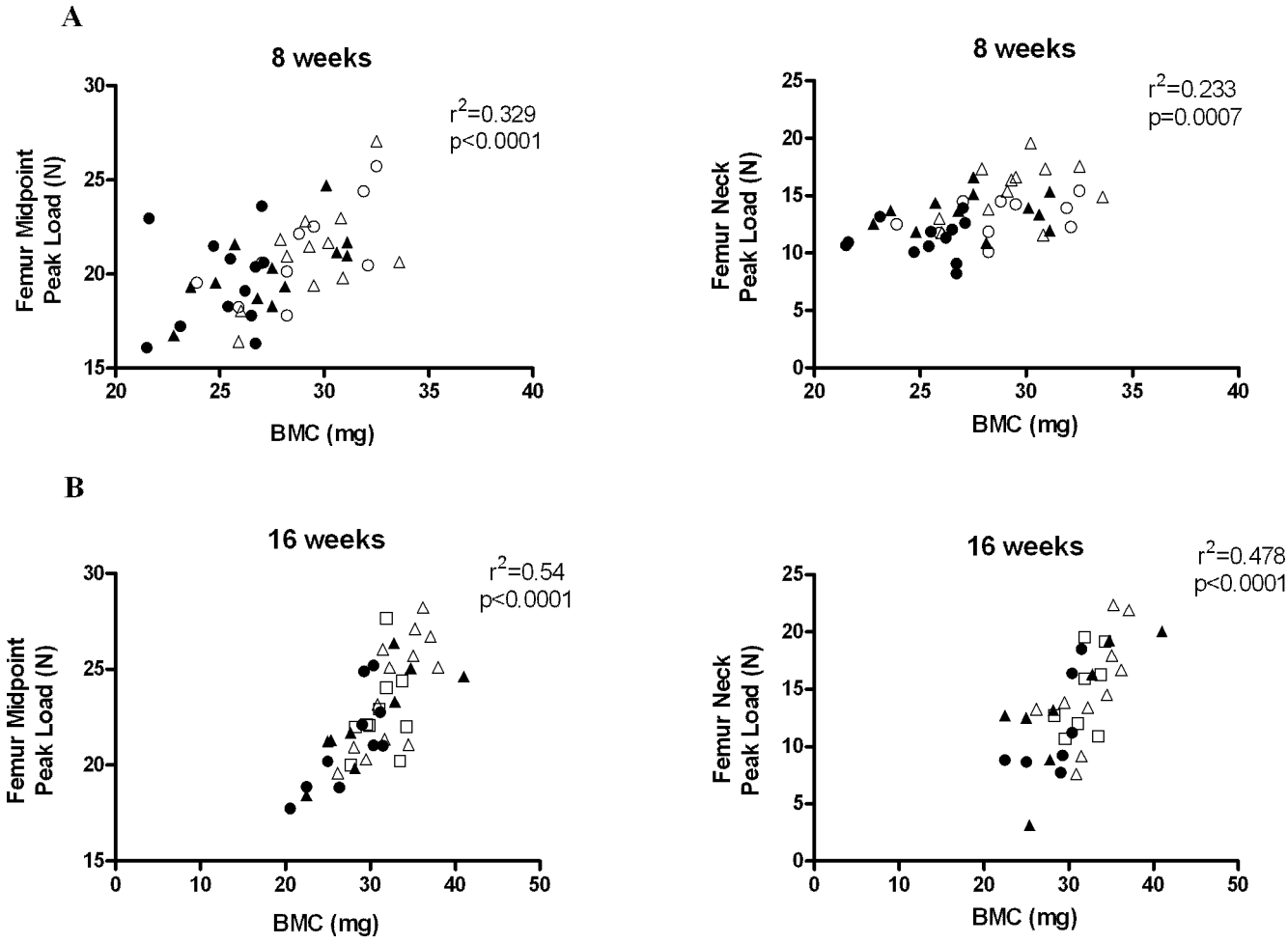

Figure 2. Relationship between whole femur BMC and selected measures of femur biomechanical strength in 8-week old (A) and 16-week old (B) mice. Symbols used: △, WT male; ▲, AdTg male; ○, WT female; and ●, AdTg female.

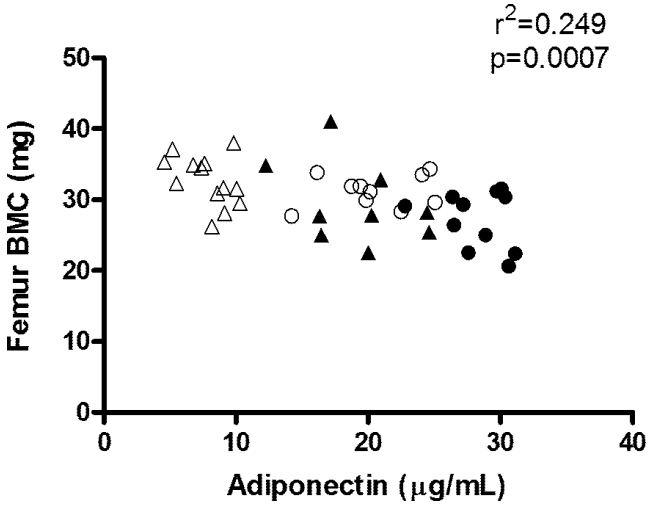

Figure 3. Relationship between serum adiponectin and whole femur BMC in 16-week old mice. Symbols used: △, WT male; ▲, AdTg male; ○, WT female; and ●, AdTg female.

lower LV1–LV4 BMC than WT mice. Female AdTg mice had significantly higher LV1–LV4 BMC than males, and BMD was significantly higher in females within both genotypes compared to males (Table 2). LV3 peak load was significantly lower in female AdTg mice compared to female WT controls, and amongst WT mice, it was higher in females than in males (Table 2).

Relationship of Whole Femur BMC and Femur Biomechanical Strength Properties. Using linear regression analyses, we found a significant positive relationship between whole femur BMC and various measures of femur biomechanical strength properties in both 8- and 16-week old mice. Whole femur BMC was significantly correlated with femur midpoint peak load and femur neck peak load in both 8- and 16-week old mice (Fig. 2). In addition, whole femur BMC was significantly and positively correlated with femur midpoint yield load ($r^2 = 0.26$, $P = 0.0006$) at 16 weeks of age. There was no correlation between whole femur BMC and stiffness in either 8- or 16-week old mice.

Relationship of Serum Adiponectin and Whole Femur BMC. There was a significant inverse association

between circulating adiponectin levels and whole femur BMC in 16-week old mice (Fig. 3). Adiponectin was also significantly and inversely correlated with circulating insulin (data not shown). Since adiponectin has been shown to be a potent insulin sensitizer, we determined the ratio of circulating adiponectin to insulin (Ad/I) and whether there were any significant correlations with this measurement and whole femur BMC. There was a significant inverse correlation between Ad/I and whole femur BMC ($r^2 = 0.303$, $P = 0.0002$).

Discussion

The objective of this study was to compare BMC, BMD and biomechanical strength properties of femur and lumbar vertebrae in AdTg mice and their WT littermates. The main findings of this work are that AdTg mice that have elevated circulating adiponectin levels exhibit reduced BMC and bone strength compared to WT mice at skeletal sites rich in cortical or trabecular bone. Since the findings were similar at 8 and 16 weeks of age, our results suggest that circulating adiponectin plays a negative role in the acquisition of bone during growth, as well as the attainment of peak bone mass by young adulthood.

There is ample evidence showing that obesity is strongly correlated with increased bone mass (3, 29–33) and that reductions in body weight are related to bone loss (2, 34–36). It would, therefore, be expected that circulating adiponectin, an adipokine that is negatively associated with obesity, would have negative effects on bone. Indeed, our data supports a role for circulating adiponectin as a potential metabolic link between obesity and bone mass. Furthermore, our results confirm those from a number of human studies that report significant negative correlations between circulating adiponectin and bone mass (17–20).

Our data are in contrast to that of two other mouse models of adiponectin overexpression that reported different effects. Oshima *et al*. (24) reported an increase in trabecular but not cortical bone in mice that were treated with an adenovirus overexpresssing adiponectin for 2 weeks. In contrast to that study, Shinoda *et al*. (25), reported no differences in bone mass or bone turnover in an adiponectin transgenic mouse model that overexpresses mouse globular adiponectin in the liver when compared to WT littermates. In this transgenic mouse model, globular adiponectin levels have been reported to be elevated compared to WT mice, but expressed at lower circulating levels than full-length adiponectin that does not differ between WT and transgenic mice (37). It is possible that the full effects of adiponectin on bone may be mediated by full-length adiponectin, rather than the globular form. In the present study, we used adiponectin transgenic mice that express a dominant mutation in the collagenous domain of adiponectin that results in increased levels of innate adiponectin complexes, including full-length adiponectin (26). In both of the aforementioned studies of adiponectin overexpression in mice (24, 25), biomechanical measures of the functional strength of bone, a good indicator of fracture risk, were not assessed, whereas in our study we observed differences not only in bone mass but also in bone strength between AdTg and WT mice.

The mechanisms by which adiponectin mediates effects on bone mass are unclear. It is possible that circulating adiponectin indirectly inhibits bone mass by increasing insulin sensitivity and inhibiting the action of insulin in tissues (5). BMD has been shown to be positively associated with elevated circulating insulin and inversely correlated with insulin sensitivity (38, 39). Shinoda *et al*. (25) reported that adiponectin treatment inhibited osteogenesis in cultures of bone marrow cells derived from WT mice but that this inhibitory effect was not observed in the presence of insulin. In the present study, although serum insulin levels were not significantly lower in AdTg mice compared to WT mice, circulating adiponectin was significantly and inversely correlated with circulating insulin, and the ratio of adiponectin to insulin was significantly and inversely correlated with whole femur BMC. These data suggest that chronic elevations in circulating adiponectin may lead to reductions in bone mass by modulating insulin action on bone.

It has also been suggested that the relationship between adiponectin and bone mass may be influenced by sex hormones since adiponectin has divergent effects in pre- and postmenopausal women (20). Previous studies in mice investigated only one time point, and this time point was 8 weeks of age, when accumulation of mineral and matrix is rapidly occurring. Moreover, previous studies have not examined gender differences. In general, we observed a stronger inhibitory effect of adiponectin overexpression on bone mass and strength in female mice compared to males. In contrast to males, female AdTg mice exhibited a lower femur BMC at 8 weeks compared to their controls, and this difference persisted at 16 weeks of age. In addition, LV3 peak load at 8 weeks of age was lower specifically in female AdTg mice compared to controls, and this effect also persisted at 16 weeks of age. Thus, it appears that the higher adiponectin levels in female AdTg compared to WT mice, coupled potentially with interactions with sex steroids, impact femur bone mineral and peak load of LV3, a surrogate measure of susceptibility to vertebral compression fracture, early in development and that this insult persists into young adulthood, a time at which peak bone mass is attained in mice.

In conclusion, the present findings show that elevated circulating adiponectin is associated with lower bone mass and weaker bones in growing mice, in the absence of excess body weight. The marked inverse relationship between serum adiponectin and femur midpoint peak load is particularly important as peak load is a surrogate measure of fracture risk. We suggest that circulating adiponectin, the most abundant protein secreted from adipoctyes, is a metabolic link that can explain, at least in part, the known

positive relationship between obesity and both bone mass and reduced susceptibility to fractures (3). The differences in femur BMC and LV3 peak load between female and male AdTg mice and their respective controls should be further assessed in order to determine whether these differences put the female transgenic mice, like humans, at a greater risk of deterioration of bone tissue during later life.

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