The influence of leptin on trabecular architecture and marrow adiposity in GH-deficient rats

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Abstract

The relationship between the degree of GH deficiency and impaired bone integrity is not simple and may be influenced by related endocrine variables. To test the hypothesis that elevated adiposity and hyperleptinaemia are contributory factors, we quantified femoral trabecular organisation in two models of GH deficiency with divergent degrees of adiposity – the moderately GH-deficient/hyperleptinaemic transgenic growth retarded (Tgr) rat and the profoundly GH-deficient/hypoleptinaemic *dw/dw* rat. Trabecular density (bone volume/total volume) and surface were reduced by 16% in *dw/dw* males, with a more fragmented trabecular lattice. This impairment was more pronounced in Tgr rats, with trabecular number and density further reduced (by an additional 21%) and relative surface (bone surface/bone volume), trabecular convexity (structural modal index) and fragmentation (pattern factor) increased. To establish whether the presence of obesity/hyperleptinaemia exacerbates bone

impairment in GH deficiency, trabecular structure was assessed in *dw/dw* rats following diet-induced obesity (DIO). DIO had minimal effect on trabecular architecture, the increased concavity of trabecular surfaces being the only observable effect. Similarly, infusion of leptin into the tibial bone marrow cavity had no effect on trabecular organisation or tibial growth in wild-type rats. However, while this procedure also failed to affect trabecular architecture or osteoclast number in *dw/dw* rats, distal osteoblast surface was increased by 23%, marrow adipocyte number and epiphyseal plate width being reduced (by 40 and 5% respectively), without increasing caspase-3 immunoreactivity. These findings suggest that while leptin may directly inhibit adipocyte differentiation and favour osteoblast production, hyperleptinaemia makes only a minimal contribution to the impairment of bone structure in GH deficiency.

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Introduction

GH is not only the principle endocrine determinant of post-natal skeletal growth, but exerts a significant influence on bone remodelling and mineralisation (Bouillon 1991, Ohlsson *et al.* 1998). Thus, the profound impairment of linear growth seen in GH-deficient humans is accompanied by a significant reduction in bone mineral density (Holmes *et al.* 1994) and an increase in fracture risk (Rosén *et al.* 1997). However, it is becoming clear that the relationship between the degree of GH deficiency and the impairment of bone strength is not simple. For example, we have shown that the compromised strength of the femoral cortex in the moderately GH-deficient transgenic growth retarded (Tgr) rat is similar to that in the profoundly GH-deficient dwarf (*dw/dw*) rat (Stevenson *et al.* 2009). This suggests that the impairment of bone strength in GH deficiency may be partially determined by other related variables.

GH deficiency in humans is normally accompanied by increased fat accumulation and elevated circulating leptin (Jørgenson *et al.* 1996, Fisker *et al.* 1997). This obese phenotype, which is observed in childhood onset GH deficiency (Growth Hormone Research Society 2000, Raine *et al.* 2006), is also present in the Tgr rat (Evans *et al.* 2003, Davies *et al.* 2007), but we have shown that the more profoundly GH-deficient *dw/dw* rat is surprisingly lean and hypoleptinaemic (Davies *et al.* 2007). This difference is potentially significant, since low levels of circulating leptin are associated with prevention of bone loss (Martin *et al.* 2007), and high leptin and extreme obesity are accompanied by decreased bone formation (Martin *et al.* 2007, Núñez *et al.* 2007). However, developmental manipulations to elevate adiposity and circulating leptin in the *dw/dw* rat failed to affect the biomechanical properties of the femoral cortex (Stevenson *et al.* 2009), suggesting that, in the cortex at least, adiposity is not a determinant of strength. Evidence is now

emerging that the actions of leptin in bone are site specific, enhancing the formation of cortical bone (Hamrick *et al.* 2005, Guidobono *et al.* 2006) but inducing bone loss in trabecular structures (Ducy *et al.* 2000, Guidobono *et al.* 2006). Since trabecular architecture is a more dynamically regulated structure than cortical bone, we have now used micro-computed tomography (μ -CT) to test the hypothesis that the presence of increased adiposity and hyperleptinaemia contribute to the impairment of trabecular architecture in GH deficiency.

Given that the processes of skeletal growth and bone remodelling in rats and humans are surprisingly similar (Frost & Jee 1992), we have measured the population, geometry and organisation of femoral trabeculae in peripubertal male wild-type (wt), Tgr and *dw/dw* rats. In the Tgr rat, expression of human GH in the arcuate GH releasing hormone neurones produces moderate GH deficiency (Flavell *et al.* 1996, Wells *et al.* 1997), accompanied by obesity and hyperleptinaemia (Fig. 1; Evans *et al.* 2003, Davies *et al.* 2007). In contrast, an unknown spontaneous mutation in the *dw/dw* rat leads to profound GH deficiency (Charlton *et al.* 1988, Legraverend *et al.* 1992) and a similar degree of growth retardation, accompanied by leanness and hypoleptinaemia (Fig. 1; Davies *et al.* 2007). In order to determine whether elevation of fat mass and circulating leptin exacerbates the impairment of the bone phenotype in profound GH deficiency, we have quantified trabecular architecture in *dw/dw* rats following diet-induced obesity (DIO; Davies *et al.* 2007). In addition, we have examined the potential direct effects of leptin on trabecular architecture and marrow adiposity following chronic infusion of leptin into the tibial marrow cavity of Sprague–Dawley (SD) and *dw/dw* rats. Our data suggest that while leptin may favour the differentiation of osteoblasts, hyperleptinaemia exerts only a minimal influence on the impaired trabecular architecture and reduced skeletal growth in GH deficiency.

Figure 1 Diagrammatic representation of the GH and adiposity status of the rat models of GH deficiency used in the present study. Scaled silhouettes (with adiposity in grey) show relative body length and adiposity (based upon % body weight of retroperitoneal fat) with circulating GH, IGF1 and leptin as normalised to their wt (AS) controls (Charlton *et al.* 1988, Legraverend *et al.* 1992, Flavell *et al.* 1996, Wells *et al.* 1997, Evans *et al.* 2003, Davies *et al.* 2007).

Materials and Methods

GH-deficient rats

The animal procedures described below, including those involving genetically modified animals, conformed to the institutional and national ethical guidelines for animal experimentation at the respective institutions and were specifically approved by local ethical review. Homozygous *dw/dw* rats in study 2 were housed in the Division of Biological Services, National Institute of Medical Research (NIMR, London, UK) under conditions of 12 h light:12 h darkness (lights on at 0600 h), and those in study 4 were supplied from NIMR to the School of Biosciences, Cardiff University. The hemizygous Tgr rats, wt (Albino Swiss, AS) littermates and homozygous *dw/dw* (maintained on the same wt background) rats used in study 1 were derived from the original colonies at NIMR and were bred in the Transgenic Unit (School of Biosciences, Cardiff University) under conditions of 14 h light:10 h darkness (lights on at 0500 h), Tgr rats being identified by PCR analysis of a tail biopsy. SD rats used in study 3 were purchased from Charles River (Margate, UK) and maintained under the same conditions in the School of Biosciences, Cardiff University. Rats were permitted *ad libitum* access to food and water, standard chow diet (Cardiff) consisting of 4.0% fat, 14.2% protein, 4.5% fibre, 63.9% carbohydrate and 4.7% ash (metabolisable energy: 12.99 MJ/kg; Rodent Maintenance Diet 2014; Harlan Teklad, Harlan, UK) and standard chow diet (NIMR) consisting of 3.4% fat, 18.8% protein, 3.7% fibre, 60.3% carbohydrate and 3.8% ash (metabolisable energy: 15.6 MJ/kg). The high-fat diet used in study 2 was made by mixing normal rat chow (NIMR) with 60% fat containing chow (Special Diet Services, Witham, UK) to give a diet consisting of 41.1% fat, 19.6% protein, 29% carbohydrate and 2.3% ash (metabolisable energy: 24.0 MJ/kg). The number of animals used in these studies was calculated from our previously published endocrine and metabolic studies (Evans *et al.* 2003, Thompson *et al.* 2004, Davies *et al.* 2007) using the Lenth's Power and Sample Size software (Lenth RV (2006–2009) Java Applets for Power and Sample Size (Computer software), retrieved 20/02/2003 from <http://www.stat.uiowa.edu/~rlenth/Power>), with the power set at >75% and assuming S.D.s of 32 and 36% for two-group *t*-test and one-way ANOVA four-group comparisons respectively.

Study 1: transpubertal development of trabecular organisation in Tgr and *dw/dw* rats

Groups of non-fasting 3-, 6-, 9- and 52-week-old male wt, Tgr and *dw/dw* rats (*n*=3–6) from the Cardiff colonies were weighed, anaesthetised with halothane and killed by decapitation. Left femurs were dissected, wrapped in isotonic saline-soaked gauze and stored at –20 °C prior to analysis of trabecular structure by μ -CT (see below).

Study 2: the effect of a high-fat diet on trabecular organisation in dw/dw rats

To determine the contribution of elevated adiposity on impaired bone microarchitecture, trabecular organisation was measured in *dw/dw* rats after the induction of obesity by pubertal exposure to elevated dietary fat. Five- to seven-week-old female *dw/dw* and wt rats ($n=5$ per group for *dw/dw* rats and $n=6$ per group for wt rats) were fed normal chow or a high-fat diet for 4 weeks and weighed weekly. This procedure, which fails to elevate fat deposition in wt rats, doubles abdominal fat in *dw/dw* rats, without suppressing the low level GH secretion (Davies *et al.* 2007). At the end of the treatment period, the rats were concussed and decapitated, and left femori were excised and stored as above.

Study 3: the effect of an intra-bone marrow infusion of leptin on trabecular organisation in wt rats

To establish whether elevated leptin has any direct effect on bone microarchitecture, trabecular organisation was analysed in male SD rats (weighing 330–356 g) after an intra-bone marrow infusion of leptin. This procedure permits chronic infusion into the tibial marrow cavity without significant systemic overflow (Thompson *et al.* 2004). In brief, a stainless steel cannula, attached via a polythene cannula to an osmotic minipump (ALZET model 2002; Alza Corp., Palo Alto, CA, USA) primed to deliver vehicle (sterile saline containing BSA (1 mg/ml) and heparin 5 units/ml; at 0.55 µl/h) or vehicle containing recombinant rat leptin (5 µg/day; Sigma) for 14 days, was introduced into the marrow cavity of the right tibiae under halothane anaesthesia and secured with cyanoacrylate cement. This dose of leptin was based upon our previous descriptions of the relative doses of i.v. and intra-bone marrow-infused ghrelin required to influence marrow adiposity (Thompson *et al.* 2004). Following surgery, the rats were housed in groups of six (mixed treatments) and weighed daily. At the end of the infusion period, the rats were concussed and decapitated, trunk blood being collected, centrifuged and plasma was stored at -20°C for subsequent measurement of plasma leptin concentration by RIA. Right tibiae were excised; the lengths were measured with a hand-held micrometer, fixed in 10% buffered formal saline for 2 days and stored in 70% ethanol. After μ -CT analysis, tibiae were decalcified as above for subsequent quantification of marrow adiposity and epiphyseal plate width.

Study 4: the effect of an intra-bone marrow infusion of leptin on trabecular organisation in dw/dw rats

To establish whether a direct effect of leptin on bone microarchitecture can only be seen under conditions of GH deficiency, male *dw/dw* rats (weighing 97.3–113.3 g) received a 14-day intra-bone marrow infusion of vehicle (0.55 µl/h) or leptin (5 µg/day) as in study 3. At the end of the infusion

period, infused *dw/dw* rats were killed, and tissue was collected and processed as in study 3 above.

Trabecular analysis

Bones were scanned with a commercially available high-resolution *ex vivo* μ -CT system (Skyscan 1072, Kontich, Belgium) at 50–100 kV and 98–197 µA. During data acquisition, a 1 mm aluminium filter was employed in conjunction with a resolution of 12–17 µm, a rotation step of 0.05 or 0.9° and a rotation angle of 180°, these parameters were kept constant within each of the studies. The complete secondary spongiosa of each bone was analysed using the CT analyser (CT-An; <http://www.skyscan.be/products/nrecon.htm>), the area of interest being defined as a set number of slices at a fixed distance from the epiphyseal plate. Trabecular bone was separated from cortical bone within the area of interest by using the freehand drawing tool in CT-An. Representative three-dimensional reconstructions of trabecular bone in the distal femori from 6-week-old male wt, Tgr and *dw/dw* rats are shown in Fig. 2.

Additional tissue analyses

Following μ -CT, the tibia from studies 3 and 4 was decalcified (in 10% EDTA in 0.3 M NaOH for 3 weeks) and embedded in paraffin wax. Longitudinal anterior–posterior sections (7 µm) were taken, and alternate sections were stained with Masson's trichrome or toluidine blue. Epiphyseal plate width was measured on Masson's trichrome-stained sections under light microscopy using the Leica Q-win software (mean of three measurements/section; three sections/bone for each animal). Bone marrow adiposity was quantified on toluidine blue-stained sections as previously described (Gevers *et al.* 2002, Thompson *et al.* 2004). Briefly, digital images of mid-diaphyseal marrow ($1 \times 343\,313\,\mu\text{m}^2$ field/section, three sections/tibia; taken with a Leica DFC300FX digital camera mounted on a Leica DMLB microscope) were analysed with National Institutes of Health (NIH) Image (version 1.62 for Macintosh, available at <http://rsb.info.nih.gov/nih-image/>) to quantify adipocyte density (cells/field), adipocyte size and degree of adiposity (total adipocyte area as a percentage of the field area).

Three additional 7 µm sections were collected from the decalcified tibia in study 4 for quantification of osteoblast surface, osteoclast number and apoptosis. Osteoblast surface was determined using a histomorphometric approach modified from that originally developed for undecalcified sections (Samuels *et al.* 1999), while osteoclast number was quantified on tartrate-resistant acid phosphatase (TRAP)-stained sections using a previously described protocol (Still *et al.* 2008). Apoptotic nuclei were identified by light microscopy after immunocytochemistry for cleaved (activated) caspase-3 (using NEB/CST antibody 9661, New England Biolabs, Hitchin, Herts, UK).

Figure 2 Representative three-dimensional reconstructions of trabecular bone in distal femori of 6-week-old male wild-type (wt), Tgr and *dw/dw* rats.

Plasma leptin concentration

Plasma leptin concentration was determined in samples from studies 3 and 4 by RIA (Linco Research, Inc., St Charles, MO, USA). Mean inter- and intra-assay coefficients of variation were 3.0–5.7 and 2.0–4.6% respectively.

Statistical analysis

All data are expressed as mean $\pm$ S.E.M.; statistical comparisons being performed by Student's *t*-test or ANOVA plus Bonferroni/Dunnnett's *post-hoc* test as indicated in the table and figure legends.

Results

*Study 1: transpubertal development of trabecular organisation in Tgr and *dw/dw* rats*

An age-related decline in trabecular number in the distal femori of wt males (Fig. 3A; $P < 0.05$) was accompanied by a progressive increase in trabecular surface (Fig. 3D; $P < 0.001$). However, when corrected for bone volume, relative surface (bone surface (BS) / bone volume (BV)) declined dramatically between 9 and 52 weeks ($P < 0.01$; data not shown), with a reciprocal increase in trabecular thickness (Fig. 3C). This was accompanied by a dramatic shift in trabecular cross section towards a more plate-like shape (a decline in structural modal index (SMI); Fig. 3E; $P < 0.001$), and an increase in trabecular connectivity (a decline in pattern factor; Fig. 3F; $P < 0.001$). Trabecular density (Fig. 3B) and alignment (degree of anisotropy (data not shown)) remained relatively constant with age.

While the overall pattern of age-related changes in trabecular organisation was largely replicated in both models of GH deficiency, there was a significant impairment of trabecular architecture, particularly in the Tgr model of moderate GH deficiency. Tgr males showed a more pronounced decline in trabecular number (Fig. 3A; $P < 0.01$ at 52 weeks), with a 37% reduction in trabecular density (Fig. 3B; $P < 0.05$ at 9 and 52 weeks). Trabecular surface failed to increase after 6 weeks, being 58% lower at 52 weeks (Fig. 3D; $P < 0.01$), but this was largely due to a lack of expansion in bone volume, as relative surface (BS/BV) was only different (10% higher) at 52 weeks ($P < 0.05$; data not shown). The trabeculae in Tgr males showed a significantly more cylindrical shape at 52 weeks (SMI 41% higher; Fig. 3E; $P < 0.05$) with a significantly more fragmented lattice at 9 and 52 weeks (pattern factor doubled at 52 weeks; Fig. 3F; $P < 0.01$).

Surprisingly, trabecular architecture in the *dw/dw* model of profound GH deficiency showed an intermediate bone phenotype between that in Tgr rats and in fully GH-replete wt rats. The only significant differences were in trabecular density at 52 weeks (16% lower; Fig. 3B; $P < 0.05$), trabecular surface, but not in relative surface, at 9 and 52 weeks (40% lower; Fig. 3D; $P < 0.01$) and lattice fragmentation at 52 weeks (43% increase in pattern factor; Fig. 3F; $P < 0.01$). At all of these points, trabecular organisation was significantly less impaired than in age-matched Tgr males.

*Study 2: the effect of a high-fat diet on trabecular organisation in *dw/dw* rats*

In order to examine whether the bone phenotype of the *dw/dw* model could be exacerbated by increasing fat mass, we

Figure 3 Analysis of trabecular architecture in the distal femur of 3-, 6-, 9- and 52-week-old male wt, Tgr and *dw/dw* rats as assessed by μ -CT. Parameters of trabecular morphology and organisation shown are trabecular number (A), density (BV/TV; (B)), thickness (C), surface area (D), structural modal index (indicating the prevalence of rod- or plate-like cross-sectional shape, where an ideal plate = 0; cylinder = 3; sphere = 3; (E)) and pattern factor (an index of connectivity where higher values represent a more fragmented lattice; (F)). Values shown are mean $\pm$ S.E.M. ($n=3$ (Tgr, 3 weeks); 4 (wt, 6 weeks); 5 (wt, 9 and 52 weeks; Tgr, 6, 9 and 52 weeks; *dw/dw*, all ages); with statistical comparisons performed by one-way ANOVA and Bonferroni's *post-hoc* test; ^a $P<0.05$, ^{aa} $P<0.01$ versus wt (same age); ^b $P<0.05$, ^{bb} $P<0.01$ versus Tgr (same age).

quantified trabecular architecture in *dw/dw* rats following pubertal exposure to elevated dietary fat. This manipulation, which normalises abdominal adiposity and circulating leptin (Davies *et al.* 2007), had little effect on trabecular structure. Chow-fed *dw/dw* rats had a slightly higher trabecular density (6% increase in bone volume/total volume (BV/TV); Fig. 4B; $P<0.01$), with a decrease in trabecular surface (25% lower; Fig. 4D; $P<0.01$), but not in relative surface. Interestingly, SMI in female rats was in the negative range (Fig. 4E), indicating the preponderance of concave surfaces. This was the only variable to be significantly influenced by elevated dietary fat, becoming even more concave in fat-fed *dw/dw* rats ($P<0.05$). There were no differences in trabecular pattern factor (Fig. 4F) or orientation (degree of anisotropy; data not shown).

Study 3: the effect of an intra-bone marrow infusion of leptin on trabecular organisation in SD rats

Since it is possible that elevated circulating leptin may have confounding direct and indirect effects on bone, we investigated the possible direct effects of leptin on trabecular organisation following an intra-bone marrow infusion of

leptin in male SD rats. Our measurements of circulating leptin, body weight gain and intra-abdominal adiposity confirmed that this procedure did not result in appreciable systemic leakage of infusate (Table 1). In SD rats, intra-bone marrow infusion of leptin had no significant effect on either tibial growth (tibial length or epiphyseal plate width) or marrow adiposity (Table 1). Similarly, leptin infusion had little impact on the parameters of trabecular architecture in the treated tibiae (Table 1), trabecular thickness increasing by 29% ($P<0.05$), with mean trabecular connectivity (pattern factor) in leptin-treated tibiae only 30% of that in vehicle-treated rats ($P=0.282$; Table 1).

Study 4: the effect of an intra-bone marrow infusion of leptin on trabecular organisation in *dw/dw* rats

To establish whether a direct effect of leptin on bone microarchitecture can only be seen under conditions of GH deficiency, trabecular organisation was analysed in male *dw/dw* rats after a similar intra-bone marrow infusion of leptin. As in SD rats, this procedure had no effect on circulating leptin, body weight gain or intra-abdominal adiposity (Table 1). However, although tibial length was

Figure 4 The effect of elevated dietary fat on trabecular architecture in the distal femur of 9- to 13-week-old female wt and *dw/dw* rats as assessed by μ -CT. Parameters of trabecular morphology and organisation shown are trabecular number (A), density (BV/TV; B), thickness (C), surface area (D), structural modal index (E) and pattern factor (F). Values shown are mean $\pm$ S.E.M. ($n = 5$ (*dw/dw* rats) and 6 (wt rats); and statistical comparisons described in the text are made using one-way ANOVA and Bonferroni comparison); ^{aa} $P < 0.01$ versus chow-fed wt; ^b $P < 0.05$ versus chow-fed *dw/dw*; ^{cc} $P < 0.01$ versus fat-fed wt.

unaffected, epiphyseal plate width was reduced by 5% following leptin infusion (Table 2; $P < 0.05$). In addition, proportionate marrow adiposity in leptin-treated tibiae was reduced by 40% ($P < 0.05$). This reduction in adiposity appeared to be due to a decrease in the number of the marrow adipocytes (58% of that in vehicle-treated bones; $P < 0.05$; Table 2), mean adipocyte size being unaffected ($P = 0.551$; Table 2). Immunocytochemistry for activated caspase-3 revealed that the small number of apoptosing cell nuclei in the hypertrophic zone of the growth plate of *dw/dw* rats was unaffected by leptin infusion (Supplementary Figure 1, see section on supplementary data given at the end of this article). Similarly, the low frequency of apoptosing adipocytes in tibial marrow was unaffected by leptin treatment (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Despite these direct effects of leptin, none of the parameters of trabecular morphology or organisation were significantly altered in *dw/dw* rats by leptin treatment (Fig. 5A–E). This lack of effect was confirmed by histomorphometric analysis of both the proximal and distal tibiae (Table 2). However, while osteoblast surface in the proximal tibia did not decline significantly in leptin-infused rats (Fig. 5F; $P = 0.10$),

osteoblast surface in the distal tibia was increased by 23% (Fig. 5G; $P < 0.01$). Quantification of osteoclast number in TRAP-stained sections revealed that intra-bone marrow infusion of leptin had no effect on the number of osteoclasts in *dw/dw* tibiae (data not shown).

Discussion

The contribution of leptin to the complex array of signals regulating the integrity of bone structure remains controversial. However, given the evidence that exposure to elevated leptin induces bone loss in trabecular structures (Ducy *et al.* 2000, Guidobono *et al.* 2006) and that rat bone has proved a useful model for the growing human skeleton (Frost & Jee 1992), we examined the potential contribution of elevated leptin to the disturbance of trabecular architecture in rat models of GH deficiency. Our data indicate that while elevated truncal adiposity and circulating leptin normally seen in GH deficiency make a negligible contribution to the impairment of trabecular structure in long bones, elevating leptin in the marrow compartment may reduce the number of marrow adipocytes and increase osteoblast surface.

Table 1 The effect of a 14-day intra-bone marrow infusion of recombinant rat leptin (5 µg/day) on growth, adiposity and trabecular microarchitecture (as assessed by micro-computed tomography) in male Sprague–Dawley rats (study 3). Values shown are mean $\pm$ S.E.M. ($n=5$ for both groups)

	Vehicle ($n=5$)	Leptin ($n=5$)
Body weight gain (g)	61.6 $\pm$ 7.6	68.6 $\pm$ 4.2
R-P fat pad weight (g)	2.47 $\pm$ 0.27	2.68 $\pm$ 0.46
Plasma [leptin] (ng/ml)	6.90 $\pm$ 0.96	7.34 $\pm$ 1.15
Treated tibia		
Length (mm)	42.3 $\pm$ 0.1	41.9 $\pm$ 0.5
Epiphyseal plate width (µm)	339 $\pm$ 19	333 $\pm$ 8
Marrow adiposity (%)	4.64 $\pm$ 0.75	4.51 $\pm$ 0.93
Adipocyte number (cells/field)	40 $\pm$ 7	47 $\pm$ 10
Adipocyte size (µm ²)	467 $\pm$ 71	412 $\pm$ 74
Trabecular number (1/mm)	2.78 $\pm$ 0.24	3.20 $\pm$ 0.24
Trabecular density (BV/TV)	0.303 $\pm$ 0.039	0.367 $\pm$ 0.037
Trabecular thickness (µm)	107.7 $\pm$ 5.1	138.8 $\pm$ 3.5*
Trabecular surface (mm ²)	342.6 $\pm$ 26.9	354.5 $\pm$ 20.5
Trabecular SMI	1.88 $\pm$ 0.18	1.51 $\pm$ 0.23
Pattern factor (1/mm)	4.67 $\pm$ 2.07	1.39 $\pm$ 1.95

R-P, retroperitoneal. Statistical comparisons performed by unpaired Student's *t*-test (* $P<0.05$ versus vehicle-treated).

We have previously reported that cortical strength is significantly compromised in both juvenile and adult GH-deficient rats (Evans *et al.* 2003, Stevenson *et al.* 2009). However, it is clear from the present data that, during the early pubertal period (before 6 weeks of age), this impairment of cortical strength is not matched by impaired trabecular architecture, even in the model of profound GH deficiency. This divergent influence of GH on the regulation of cortical and trabecular bone has also been reported in GH receptor/binding protein-null mice, which show normal trabecular bone volume (Venken *et al.* 2007) despite a marked reduction in cortical diameter and strength (Stevenson *et al.* 2009). Following the rapid growth phase (from 9 weeks of age), however, the number, shape and connectivity of the femoral trabeculae become progressively impaired in GH-deficient rats.

The most striking feature of this impairment is that it was always most pronounced in the model of moderate GH deficiency, with Tgr rats having fewer, more convex trabeculae in a more fragmented lattice than that observed in the profoundly GH-deficient *dw/dw* model. This difference in trabecular structure between the two models was more pronounced than our measurements of cortical strength (Stevenson *et al.* 2009). When seen in the light of the similar degree of insulin-like growth factor 1 (IGF1) deficiency reported in these models (Davies *et al.* 2007; Fig. 1), our current data suggested to us that another factor either exacerbated lattice fragmentation in Tgr rats, or partially alleviated the impairment in *dw/dw* rats.

We have recently shown that, in contrast to the obese/hyperleptinaemic phenotype of the Tgr model, *dw/dw* rats are lean and hypoleptinaemic (Davies *et al.* 2007). We therefore investigated whether elevating truncal

adiposity and circulating leptin in *dw/dw* rats would further impair trabecular structure in this model. This was achieved by maintaining *dw/dw* rats on a high-fat diet, which doubled abdominal adiposity and trebled circulating leptin, without influencing circulating IGF1 or skeletal growth (Davies *et al.* 2007). However, this treatment had negligible effect on trabecular architecture, the only significant change being an increased preponderance of concave surfaces (i.e. a less fragmented lattice, less like the Tgr model), implying that truncal adiposity and circulating leptin do not impair trabecular structure in GH deficiency. While the absence of a significant effect may be due to the relatively short period of exposure to elevated dietary fat, our conclusion is supported by our observation that prolonged elevation of abdominal adiposity and plasma leptin in *dw/dw* rats following neonatal monosodium glutamate treatment also has no effect on trabecular architecture (unpublished data), despite impairing cortical strength (Stevenson *et al.* 2009).

However, the published evidence suggests that leptin influences bone by several mechanisms. Supra-physiological levels of leptin are thought to suppress bone formation by a centrally mediated pathway involving the hypothalamus and the sympathetic nervous system (Ducy *et al.* 2000, Hamrick & Ferrari 2007), while leptin within the normal range increases osteoblast number and enhances osteoblast activity via a direct mechanism (Thomas *et al.* 1999, Lee *et al.* 2002). Indeed, it has been reported that the leptin receptor (Hamrick *et al.* 2005) and even leptin itself (Laharrague *et al.* 1998) are both expressed in bone. In order to determine the possible contribution of a direct action of leptin to the bone

Table 2 The effect of a 14-day intra-bone marrow infusion of recombinant rat leptin (5 µg/day) on growth, adiposity and trabecular architecture in the proximal and distal tibiae (as assessed by histomorphometric analysis) of male *dw/dw* rats (study 4). Values shown are mean $\pm$ S.E.M. ($n=6$ for both groups)

	Vehicle ($n=6$)	Leptin ($n=6$)
Body weight gain (g)	28.0 $\pm$ 0.6	27.7 $\pm$ 2.0
R-P fat pad weight (g)	0.40 $\pm$ 0.03	0.37 $\pm$ 0.05
Plasma [leptin] (ng/ml)	2.99 $\pm$ 0.60	3.30 $\pm$ 0.84
Treated tibia		
Length (mm)	31.9 $\pm$ 0.2	32.1 $\pm$ 0.2
Epiphyseal plate width (µm)	313 $\pm$ 6	297 $\pm$ 4*
Marrow adiposity (%)	6.38 $\pm$ 0.75	3.78 $\pm$ 0.36*
Adipocyte number (cells/field)	87 $\pm$ 15	50 $\pm$ 6*
Adipocyte size (µm ²)	304 $\pm$ 34	276 $\pm$ 30
Proximal tibia		
Trabecular number	4.2 $\pm$ 1.2	4.0 $\pm$ 0.6
Trabecular thickness (µm)	29.0 $\pm$ 7.2	30.6 $\pm$ 6.3
Trabecular density (BV/TV; %)	12.8 $\pm$ 4.8	11.7 $\pm$ 4.4
Distal tibia		
Trabecular number	2.0 $\pm$ 0.9	2.2 $\pm$ 0.9
Trabecular thickness (µm)	19.3 $\pm$ 11.7	25.2 $\pm$ 7.7
Trabecular density (BV/TV; %)	6.4 $\pm$ 5.4	6.4 $\pm$ 4.0

R-P, retroperitoneal. Statistical comparisons performed by unpaired Student's *t*-test (* $P<0.05$ versus vehicle-treated).

Figure 5 The effect of a 14-day intra-bone marrow infusion of recombinant rat leptin on trabecular architecture in proximal and distal tibia of male *dw/dw* rats. Parameters of trabecular morphology (trabecular number (A), density (BV/TV; (B)), thickness (C) and structural modal index (D)) and organisation (pattern factor (E)) were assessed by μ -CT and are shown together with osteoblast surface (assessed by histomorphometry) in the proximal (F) and distal (G) tibiae. Values shown are mean $\pm$ S.E.M. ($n=6$ for both groups); with statistical comparisons performed by unpaired Student's *t*-test (^{aa} $P<0.01$ versus vehicle-treated *dw/dw*).

phenotype in GH deficiency, we performed intra-bone marrow infusions of leptin into the tibiae of normal and *dw/dw* rats. This technique, initially designed in our laboratory to show the adipogenic action of ghrelin in marrow (Thompson *et al.* 2004), included the use of heparin

to maintain cannula patency. While there is evidence that heparin induces adipogenesis and inhibits osteoblastogenesis (Hurley *et al.* 1990, Osip *et al.* 2004), this was unlikely to influence the results of the current study unduly as heparin was also included in the control infusate. These experiments revealed that leptin had no direct effect on tibial trabecular architecture in either GH-replete or GH-deficient rats; a result demonstrated by both μ -CT and histomorphometry.

It is possible, of course, that the tibial trabeculae targeted by this infusion strategy respond differently to those in the distal femori, but reports that tibial and femoral trabeculae respond similarly to peripheral leptin exposure (Hamrick *et al.* 2004, 2007) suggest that this is not the case. It is also possible that the dose/duration of our intra-bone marrow leptin infusion was insufficient to elicit an observable effect on trabecular architecture, but the available evidence here is inconclusive. While the significant elevation in osteoblast surface (a more dynamic variable) in *dw/dw* rats suggests that this may be the case, the accompanying reduction in processes of cell differentiation/proliferation (epiphyseal plate width and marrow adiposity) suggests the reverse.

These considerations aside, we believe our data represent the first demonstration of these direct actions of leptin *in vivo*. Therefore, although evidence for a role of the central and sympathetic nervous systems in mediating the suppressive actions of leptin on bone is compelling (Ducy *et al.* 2000, Hamrick & Ferrari 2007), our data combined with that demonstrating that leptin enhances osteoblast number and activity *in vitro* (Thomas *et al.* 1999, Lee *et al.* 2002) indicate that a positive direct action of leptin in bone cannot be excluded.

The effects of the intra-bone marrow infusion of leptin on epiphyseal plate width and marrow adiposity are worthy of comment in their own right. It has been reported that i.p. leptin treatment promotes longitudinal skeletal growth (Steppan *et al.* 2000). This action appears to involve a central component (Iwaniec *et al.* 2007), possibly via increased activity of the GH-IGF1 axis (Tannenbaum *et al.* 1998), and a direct action, inducing differentiation of endochondrial chondrocytes (Nakajima *et al.* 2003, Kishida *et al.* 2005) and elevating IGF1 (Dumond *et al.* 2003) and parathyroid hormone-related peptide expression (Gat-Yablonski *et al.* 2007). In contrast, our intra-bone marrow infusion of leptin, which was unlikely to influence the low levels of circulating GH, reduced epiphyseal plate width in *dw/dw* rats, supporting the recent observation that high doses of peripherally administered leptin reduced femoral growth (Martin *et al.* 2007). Although this action was initially thought to be centrally mediated (Martin *et al.* 2007), our data indicate that a direct peripheral component cannot be excluded and that this action of leptin is masked in the context of a fully replete GH-IGF1 axis.

Similarly, the intra-bone marrow infusion of leptin halved marrow adiposity in *dw/dw* rats, an effect that appears to be due primarily to a reduction in adipocyte

number. It has previously been demonstrated that central leptin treatment not only inhibits pre-adipocyte differentiation (Venken *et al.* 2007), but also induces adipocyte apoptosis in both extramedullary white adipose tissue and bone marrow (Qian *et al.* 1998, Hamrick *et al.* 2007). However, our measurement of activated caspase-3 immunoreactivity indicates that intra-bone marrow infusion of leptin did not induce adipocyte apoptosis in bone marrow, implying that direct application of leptin inhibits pre-adipocyte differentiation. Given that adipogenesis and osteoblastogenesis are thought to be reciprocally regulated (Muruganandan *et al.* 2009), our observation that this treatment also elevates osteoblast surface at least in the distal tibia supports this proposition. When combined with our previous studies, it is becoming clear that marrow adipocytes in the *dw/dw* model are not only exquisitely sensitive to GH exposure (Gevers *et al.* 2002), but are also regulated by endocrine signals of metabolic insufficiency (ghrelin (Thompson *et al.* 2004, Wells 2009)) and metabolic excess (leptin). The significance of this phenomenon for the physiology of marrow and bone is unclear, but the combination of reduced adiposity and increased osteoblast surface suggests that, under conditions of metabolic excess, leptin may shift the balance of mesenchymal stem cell descendents away from fat storage and towards bone formation.

These findings fail to answer the question as to why the trabecular phenotype of the Tgr rat is more significantly impaired than that of the more profoundly GH-deficient *dw/dw* rat. Although a number of other obesity-related variables (e.g. reduced adiponectin decreased insulin sensitivity and elevated inflammatory cytokines) are known to influence osteoblastogenesis, we have no direct evidence of changes in these determinants in this model. However, Tgr males are known to display both reduced pituitary prolactin (PRL; Flavell *et al.* 1996) and hypergonadotrophism (without any alteration in either circulating testosterone or testicular function; Davies *et al.* 2006). However, neither of these endocrine disturbances are likely to exert a significant influence on the bone phenotype in this model since bone loss accompanies elevated PRL (Shibli-Rahhal & Schlechte 2009) and reduced LH signalling (Yarram *et al.* 2003). Similarly, although genetic background is known to exert a significant influence upon trabecular architecture (Sabovich *et al.* 2008), this is not a contributory factor in the current study as both the *dw/dw* and Tgr strains were maintained on the same inbred strain.

In summary, we have shown that features of femoral trabecular architecture are more profoundly impaired in the Tgr model of moderate GH deficiency than in the *dw/dw* model of profound GH deficiency. Although direct leptin exposure reduced marrow adiposity and increased osteoblast surface, the divergent abdominal fat mass and circulating leptin levels in these two models do not appear to contribute to the impairment of trabecular structure. Indeed, when combined with our previous report (Stevenson *et al.* 2009), the present data indicate that, in the long bones at least,

adiposity status has a more profound influence on the structural properties of cortical bone than on the micro-architecture of trabecular bone.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-10-0178>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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