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Type 2 diabetic mice demonstrate slender long bones with increased fragility secondary to increased osteoclastogenesis

Yuki Kawashima [a,1], J. Christopher Fritton [b,1], Shoshana Yakar [a], Sol Epstein [a], Mitchell B. Schaffler [b], Karl J. Jepsen [b], Derek LeRoith [a,*]

[a] Division of Endocrinology, Diabetes and Bone Diseases, Mount Sinai School of Medicine, NY 10029, USA
[b] Leni and Peter W. May Department of Orthopedics, Mount Sinai School of Medicine, NY 10029, USA

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ABSTRACT

Type 2 diabetics often demonstrate normal or increased bone mineral density, yet are at increased risk for bone fracture. Furthermore, the anti-diabetic oral thiazolidinediones (PPARγ agonists) have recently been shown to increase bone fractures. To investigate the etiology of possible structural and/or material quality defects, we have utilized a well-described mouse model of Type 2 diabetes (MKR). MKR mice exhibit muscle hypoplasia from birth with reduced mass by the pre-diabetic age of 3 weeks. A compensatory hyperplasia ensues during early (5 weeks) development; by 6–8 weeks muscle is normal in structure and function. Adult whole-bone mechanical properties were determined by 4-point bending to test susceptibility to fracture. Micro-computed tomography and cortical bone histomorphometry were utilized to assess static and dynamic indices of structure, bone formation and resorption. Osteoclastogenesis assays were performed from bone marrow-derived non-adherent cells. The 8-week and 16-week, but not 3-week, male MKR had slender (i.e., narrow relative to length) femurs that were 20% weaker ($p<0.05$) relative to WT control femurs. Tissue-level mineral density was not affected. Impaired periosteal expansion during early diabetes resulted from 250% more, and 40% less of the cortical bone surface undergoing resorption and formation, respectively ($p<0.05$). Greater resorption persisted in adult MKR on both periosteal and endosteal surfaces. Differences were not limited to cortical bone as the distal femur metaphysis of 16 week MKR contained less trabecular bone and trabecular separation was greater than in WT by 60% ($p<0.05$). At all ages, MKR marrow-derived cultures demonstrated the ability for enhanced osteoclast differentiation in response to M-CSF and RANK-L. Taken together, the MKR mouse model suggests that skeletal fragility in Type 2 diabetes may arise from reduced transverse bone accrual and increased osteoclastogenesis during growth that is accelerated by the diabetic/hyperinsulinemic milieu. Further, these results emphasize the importance of evaluating diabetic bone based on morphology in addition to bone mass.

Introduction

Diabetes is an emerging epidemic that has consequences for musculoskeletal growth and development, and bone fracture risk [1–8]. Yet, Type 2 diabetics (non-insulin-dependent, T2D) often demonstrate increased bone mineral density (BMD) [9–13]. Furthermore, the use of anti-diabetic oral thiazolidinediones (PPARγ agonists) in T2D has recently been shown to increase fracture risk in the absence of a reduction in BMD [14]. These apparent paradoxes suggest that the increased bone fragility in T2D may not be discerned from bone mass, but may depend on aspects of bone structure or tissue quality that are not revealed from BMD. A number of factors have been postulated to be etiologically associated with poor bone

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine, an oxidized nucleoside of DNA; αMEM, α-modified minimum essential medium; AGE, advanced glycation end products; BMD, areal bone mineral density measured by DEXA; DEXA, dual energy X-ray absorptiometry; FVB/N, Friend virus B NIH inbreed strain of mouse, WT; IGF, insulin-like growth factor; IR, insulin receptor; J, cross-sectional polar moment of inertia; M-CSF, macrophage-colony-stimulating factor; Micro-CT, micro-computed tomography; MKR, Muscle creatine Kinase promoter/human IGF-I Receptor; MSC, marrow stromal cell; PPARγ, peroxisome proliferator-activated receptor γ; PYD, post-yield deflection; RAGE, Receptors specific for AGE proteins; RANKL, receptor activator of nuclear factor-κβ ligand; T1D, Type 1 diabetes mellitus; T2D, Type 2 diabetes mellitus; TRAP, tartrate resistant acid phosphatase; WT, wild-type, FVB/N; B.Pm, bone perimeter; BV/TV, bone volume fraction for cancellous bone; Ct.Ar, cross-sectional cortical area; Ct.Wi, cross-sectional cortical width; Er.Pm, cross-sectional eroded perimeter; Es.Pm, cross-sectional endosteal perimeter; dL.Pm, cross-sectional double labeled perimeter; sL.Pm, cross-sectional single labeled perimeter; L.Pm, cross-sectional labeled perimeter = dL.Pm + sL.Pm/2; MAR, mineral apposition rate; BFR, bone formation rate = MAR*L.Pm/B.Pm; Ps.Pm, cross-sectional periosteal perimeter; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Tt.Ar, cross-sectional total area; Ma.Ar, cross-sectional marrow area; TMDn, tissue mineral density measured by micro-CT.

* Corresponding author. Division of Endocrinology Diabetes and Bone Diseases, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA.
E-mail addresses: yuki.kawashima@mssm.edu (Y. Kawashima), james.fritton@mssm.edu (J.C. Fritton), shoshana.yakar@mssm.edu (S. Yakar), bonedocsol@aol.com (S. Epstein), mitchell.schaffler@mssm.edu (M.B. Schaffler), karl.jepsen@mssm.edu (K.J. Jepsen), Derek.leroith@mssm.edu (D. LeRoith).

[1] Both of these authors contributed equally to this work.

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accrual in diabetes, including an insulin-related direct reduction in bone formation and reduced force generating capacity of muscle.

Studies of bone turnover in animal models are most accurately assessed by histomorphometry. Several models have demonstrated diabetes-induced alterations in bone and could aid in elucidating the cellular and biomechanical mechanisms that may lead to increased risk of fragility fractures [15–20]. Models for Type 1 diabetes (T1D) are most prevalent and have demonstrated decreased breaking strength of femoral shafts when compared to controls [21,22]. However, many of these are drug-induced models confounded by side-effects that can result in significant health issues. For example, the pancreatic β-cell toxins streptozotocin or alloxan have been injected into both rats and mice to induce an uncontrolled diabetic state [23,24]. Growth trajectories are immediately altered such that diabetic animals fail to grow (i.e., the rates of long bone growth and body mass gains are significantly stunted) [25,26]. However, stunted longitudinal growth is not normally associated with diabetes. Most recently, using a model of streptozotocin-induced insulinopenia, Hamada et al. confirmed this affect on growth as well as a reduction in trabecular bone volume with reduction in both bone formation and bone resorption in young animals, changes that were reversed by insulin replacement therapy [25]. The main conclusion of that study was that in T1D, insulinopenia is the primary etiology for osteopenia, though secondary mechanisms, such as oxidative stress, may also play a role [25]. The cellular mechanisms behind these differences require further examination.

Models of T2D have thus far been less biologically insightful because many induce obesity (another state of insulin resistance) or are limited to rats [27–30]. There is limited evidence for increased fracture risk due to greater levels of obesity and obesity may even protect a subject from fracture, despite being associated with lower physical activity [3]. Obesity increases the load-bearing forces during locomotion and, absent a bone formation defect, elicits structural compensation, i.e., more weight requires a larger frame [31]. Obesity may also enhance endogenous production of estrogen, a hormone important for bone cell regulation in both sexes [32]. Non-obese T2D occurs spontaneously in rats such as the Torii, which was outbred from Sprague–Dawley in 1997, and also exhibits stunted longitudinal bone growth; however, the disease has unknown etiology and is associated with insulinopenia [28].

We have generated a non-obese transgenic mouse model of T2D (MKR mice) by blocking both the insulin and IGF-signaling pathways specifically in skeletal muscle. Blocking of both receptors in muscle abrogates downstream signaling, particularly the AKT pathway, which regulates glucose uptake into muscle. MKR mice are born with *naturally* occurring hyperinsulinemia and insulin resistance in muscle. Early in life (~2 weeks) the MKR mice develop dyslipidemia, and the mice eventually develop frank diabetes with hyperglycemia (~7–8 weeks) [33,34]. This scenario of disease development is similar to the human disease, where insulin resistance and hyperinsulinemia, with dyslipidemia eventually progress to diabetes. The MKR mouse model is useful since no exogenous agents are required to induce disease, thus removing potential confounding aspects. In addition, MKR mice are non-obese, excluding another confounding variable. In this study we employed the MKR mouse model to examine the effect of progression of T2D on skeletal integrity. Skeletal characterization was performed at the pre-diabetic (3 weeks of age), early diabetic (8 weeks) and established diabetic stages (16 weeks). Here we demonstrate that early onset of insulin resistance does not affect longitudinal growth but alters transverse expansion of skeletal structures, which worsens with the progression to the adult diabetic state.

Materials and methods

The generation of the MKR Type 2 diabetic mouse has been described elsewhere [33]. MKR male mice, on a FVB/N background, were bred to homozygosity, and were compared to wild-type FVB/N controls (WT). MKR mice demonstrate severe insulin resistance starting at birth, hyperinsulinemia and hyperlipidemia at 3–4 weeks of age but with normoglycemia [33]. At 6–8 weeks of age MKR mice develop diabetes with blood glucose levels of 250–400 mg/dl versus 130–160 mg/dl for WT. The mice were kept on a 12-h light/dark cycle and were fed with standard chow ad libitum. All studies were conducted in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine.

Bone histomorphometry

Cortical bone histomorphometry was carried out as described in detail elsewhere [35,36]. Briefly, control and MKR male mice at 3, 8 and 16 weeks of age were injected intraperitoneally with 10 mg/kg calcein (C-0875; Sigma) at 8 and 2 days prior to euthanasia to label bone forming surfaces. Left femurs were removed and fixed in 10% neutral buffered formalin, bulk-stained with Villanueva bone stain and embedded in poly-methylmethacrylate [35]. Embedded bones were sectioned transversely at the mid-diaphysis with a diamond-coated wafering saw, polished to a thickness of 30 μm with silicon carbide abrasive paper and mounted on glass slides. Cortical bone indices of osteoblast and osteoclast activity were measured using fluorescence and brightfield microscopy [35,37]. On both periosteal and endosteal surfaces, bone resorption was assessed from eroded surface (Er.Pm/B.Pm, %); bone formation was assessed by labeled surface (L.Pm/B.Pm, %), mineral apposition rate (MAR, μm/day), and bone formation rate (BFR/B.Pm, μm/day × 100). Sections were analyzed using an OsteoMeasure system (Osteometrics, Atlanta, GA, USA) connected to a Zeiss Axioskop microscope. A single observer blinded to the specimen identity made all measurements.

Micro-CT analysis

Three-dimensional micro-computed tomography (micro-CT) images of the contralateral (right) femurs for 3, 8 and 16 week old WT and MKR mice were obtained using an eXplore Locus SP Micro-CT system (GE Healthcare, London, Ontario, CA). Scans were performed at 15 μm isotropic voxel resolution as described elsewhere [38–40]. All scans were performed using a density calibration phantom containing air, water, and a hydroxyapatite standard (SB3; Gammex RMI, Middleton, WI, USA) to allow subsequent determinations of tissue mineral densities [39].

Mid-diaphyseal traits were quantified for a 2.5 mm diaphyseal region that was located immediately distal to the third trochanter. This site corresponded to the typical location of failure during the bending tests (see below). The mid-diaphyseal morphological traits measured were the tissue amounts (cortical area, Ct.Ar; marrow area, Ma.Ar; total area, Tt.Ar; cortical width, Ct.Wi), perimeters (endosteal, Es.Pm; periosteal, Ps.Pm) and the spatial distribution of mineralized tissue (polar moment of inertia, J). Moment of inertia is a measure of the proximity of the tissue to the geometric centroid of the cross-section. Total area was defined as the sum of the cortical and marrow areas. Traits were quantified for each cross-section and the values were averaged. Hind-limb long bone lengths were measured as the caliper distance between the proximal and distal most articulating surfaces (0.01 mm resolution). Micro-CT was also used to quantify tissue mineralization as described above [39]. Tissue mineral density (TMDn, mg/cc) was determined by converting grayscale values to mineral density values using a density calibration curve from the scanned phantom and then averaging mineral content values over all thresholded "bone" voxels.

Trabecular architecture was quantified for the proximal and distal femoral metaphyses. Cortical and trabecular bone were segmented manually and the trabecular volumes were filtered using a median-filtering algorithm and then thresholded. Trabecular traits measured include trabecular bone volume fraction (BV/TV), thickness (Tb.Th), and separation (Tb.Sp).