Delayed bone regeneration and low bone mass in a rat model of insulin-resistant type 2 diabetes mellitus is due to impaired osteoblast function

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First published September 6, 2011; doi:10.1152/ajpendo.00378.2011.—Patients with diabetes mellitus have an impaired bone metabolism; however, the underlying mechanisms are poorly understood. Here, we analyzed the impact of type 2 diabetes mellitus on bone physiology and regeneration using Zucker diabetic fatty (ZDF) rats, an established rat model of insulin-resistant type 2 diabetes mellitus. ZDF rats develop diabetes with vascular complications when fed a Western diet. In 21-wk-old diabetic rats, bone mineral density (BMD) was 22.5% (total) and 54.6% (trabecular) lower at the distal femur and 17.2% (total) and 20.4% (trabecular) lower at the lumbar spine, respectively, compared with nondiabetic animals. BMD distribution measured by backscattered electron imaging postmortem was not different between diabetic and nondiabetic rats, but evaluation of histomorphometric indexes revealed lower mineralized bone volume/tissue volume, trabecular thickness, and trabecular number. Osteoblast differentiation of diabetic rats was impaired based on lower alkaline phosphatase activity (~20%) and mineralized matrix formation (~50%). In addition, the expression of the osteoblast-specific genes bone morphogenetic protein-2, RUNX2, osteocalcin, and osteopontin was reduced by 40–80%. Osteoblast biology was not affected based on tartrate-resistant acidic phosphatase staining, pit formation assay, and gene profiling. To validate the implications of these molecular and cellular findings in a clinically relevant model, a subcritical bone defect of 3 mm was created at the left femur after stabilization with a four-hole plate, and bone regeneration was monitored by X-ray and microcomputed tomography analyses over 12 wk. While nondiabetic rats filled the defects by 75%, diabetic rats showed delayed bone regeneration with only 26% defect filling. In conclusion, we identified suppressed osteoblastogenesis as a cause and mechanism for low bone mass and impaired bone regeneration in a rat model of type 2 diabetes mellitus.

bone defect; bone matrix mineralization; bone regeneration; type 2 diabetes mellitus; osteoblast; osteoclast

DIABETES MELLITUS TYPE 2 AND the associated metabolic syndrome have become epidemic clinical and economic health problems (1). Morbidity and mortality of diabetes mellitus are determined by vascular complications, including cardiovascular disease, retinopathy, nephropathy, and polyneuropathy (11). Skeletal sequelae of long-standing diabetes mellitus include Charcot neuroarthropathy and the diabetic foot syndrome, which may require amputation. More recently, osteoporosis with an increased risk of fragility fractures has emerged as a complication in patients with long-standing or poorly controlled type 1 and type 2 diabetes mellitus (8). Several factors may contribute to poor bone health in patients with diabetes mellitus, including hypercalcemia in periods of glucosuria in type 1 and 2 diabetes mellitus (20), delayed bone mass accrual and a lower peak bone mass in type 1 diabetes mellitus (8), poor bone quality despite higher bone mineral density (BMD) in type 2 diabetes mellitus (26), and a higher propensity to falls and osteoporotic fractures in long-standing type 1 and 2 diabetes, in particular when neuropathy, retinopathy, or nephropathy are present (3). After sustaining fractures, patients with diabetes type 2 have a higher risk of wound infection, delayed fracture healing, a higher incidence of nonunion, and impaired osseointegration (22), which translate in prolonged hospitalization and decreased mobility.

Despite a large body of clinical and epidemiological evidence linking diabetes mellitus to poor bone health, there are limited data on the mechanisms whereby diabetes mellitus affects bone mass and bone quality. This may be, at least in part, due to the lack of appropriate rodent models of type 2 diabetes mellitus, which accounts for over 90% of diabetes mellitus in humans. Recently, two groups (17, 21) have reported on the skeletal phenotype of Zucker diabetic fatty (ZDF) rats. Male ZDF rats start to develop obesity and type 2 diabetes mellitus at 9 wk of age after exposure to a high-fat and high-carbohydrate diet. The phenotype is characterized by progressive insulin resistance with hyperglycemia and initial hyperinsulinemia and results in the development of the full spectrum of the metabolic syndrome, including dyslipidemia and diabetic vascular complications, which is reminiscent of the metabolic syndrome in humans. Skeletal abnormalities of these ZDF rats include a low bone mass and low bone formation (17). However, the biological basis for these observations and the functional consequences of these alterations has remained poorly defined.
To analyze the biological basis for abnormal bone mass in insulin-resistant type 2 diabetes mellitus, we elected the ZDF rat model, since it recapitulates most of the metabolic and microvascular features of type 2 diabetes mellitus in humans. We hypothesized that osteoblast function is impaired in this model that should result in an altered bone matrix mineralization and abnormal bone regeneration in vivo using a subcritical femoral defect model. Our study analyzes BMD, bone matrix mineralization, bone cell function, and bone defect regeneration in a rat model of type 2 diabetes mellitus.

MATERIALS AND METHODS

Animal maintenance. All research protocols were approved by the Institutional Animal Care Committee (registration no. 24D-9168.11-1/2008–30). Male ZDF rats (ZDF Gmi/afa) and male Zucker lean rats (ZDF Gmi(+/+)) were purchased from Charles River Laboratories (Sulzfeld, Germany) at the age of 9 wk. Male ZDF afa/afa rats spontaneously develop type 2 diabetes mellitus between the age of weeks 9 and 11. The Zucker lean rats (ZDF−/−) are nondiabetic and served as the control. The animals were housed under standard laboratory conditions with a 12:12-h light-dark cycle and a controlled room temperature of 20–21°C. The animals had free access to water, and both groups were fed a high-fat, high-carbohydrate chow (Purina 5008).

Critical size defect model. At the age of 9 wk, the left femur was shaved and disinfected. Under general anesthesia with intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine, a four-hole plate (Stryker, Hamburg, Germany) was fixed with four screws (1.5 × 5 mm) on the left femur. A 3-mm cross-sectional subcritical defect was created at the midshaft using an oscillating saw between the second and the third screw. The wound was closed, and a postoperative X-ray was performed. The animals were returned to free cage activity. During surgery and 24 h later, all animals received subcutaneous injections of antibiotics (sulfadoxin and trimethoprim, 5.1, 15 mg/kg) to minimize the risk of infections. After surgery, both groups were maintained on a high-fat, high-carbohydrate chow (Purina 5008). Twelve weeks after surgery, the animals were killed under general anesthesia, blood samples were collected, and the bones were removed for further analysis. To verify the diabetic state of the animals at 3, 8, 10, and 12 wk after surgery, plasma glucose levels of nondiabetic (n = 6) and diabetic (n = 6) animals were measured.

Serum analysis. Serum measurements of glucose, calcium, phosphate, creatinine, alkaline phosphatase (AP), and bone-specific AP were performed using standard methods. Serum parathyroid hormone (PTH) [intra-assay coefficient of variation (CV): 3.1%; interassay CV: 5.1%] and insulin (intra-assay CV: 3.9%; interassay CV: 9.3%) levels were determined using immunoassays from DGR Diagnostics (Marburg, Germany). Serum procollagen I NH2-terminal peptide (PINP) (intra-assay CV: 2.1%; interassay CV: 7.4%), osteocalcin (intra-assay CV: 2.2%; interassay CV: 5.5%), and COOH-terminal collagen cross links (CTX) (intra-assay CV: 2.9%; interassay CV: 5.6%) levels were measured with ELISAs from Immunodiagnostic Systems (Frankfurt, Germany).

X-ray, peripheral quantitative computed, and microcomputed tomography analysis. X-rays were performed after surgery to control the defect size and the position of the plate and screws as well as 3, 8, 10, and 12 wk after surgery to document the progress of defect healing. Defect areas were quantified from the X-ray images using ImageJ software (National Institutes of Health). Peripheral quantitative computed tomography (pQCT) of the right femur and the lumbar spine was performed before surgery in vivo and 12 wk later ex vivo using a Stratec XCT Research M + pQCT (Stratec Medical Systems). pQCT scans were performed at the nonoperated right femur at the metaphyseal and diaphyseal area and the fourth lumbar vertebra. The bones were measured in plastic tubes filled with 70% ethanol. The regions of interests were predefined, and contour mode I and peel mode 20 were used. The percentage of trabecular bone was set to 50% of the cross-sectional area in the proximal tibia. A threshold of 710 mg/cm3 was used for calculation of cortical BMD. For nondestructive analysis of newly formed bone inside the defect zone, a Scanco vivaCT 75 system (Scanco Medical, Bruttisellen, Switzerland) and a Synchrotron microcomputed tomography (SRμCT) device at BESSY II (Berlin, Germany) were used. Femur explants with metallic plates were measured with a voxel resolution of 20 μm (SRμCT 9 μm) and X-ray energy of 70 keV (SRμCT 55 keV, monochromatic). A fixed constant threshold was applied to all samples. The digital volumes were aligned to the long bone axis, and a reference area for stationary bone was measured at the top and bottom slices of the callus volume. The relative amount of newly formed bone within the callus was calculated based on the medium bone amount for all slices along the axis divided by the reference values using ImageJ. Standard deviation of the average bone amount for each sample was calculated using individual slices along callus in the μCT volumes.

Quantitative backscattered electron imaging. To assess the mineral content of bone at the material level, i.e., bone mineralization density distribution (BMDD), quantitative backscattered electron imaging (qBEI) was performed at the distal femur. The right (nonoperated) distal femurs of diabetic (n = 6) and nondiabetic (n = 6) animals were fixed and stored in 70% ethanol before embedding in polymethylmethacrylate. Longitudinal bone sections containing cancellous and cortical bone were processed. Trabecular and cortical BMDD was determined by qBEI using a digital scanning electron microscope (DSM 962; Zeiss, Oberkochen, Germany) equipped with a four-quadrant semiconductor backscattered electron detector as described previously (23). The accelerating voltage of the electron beam was adjusted to 20 kV, the probe current to 110 pA, and the working distance to 15 mm. The cancellous and cortical bone areas were imaged at 100-fold nominal magnification (corresponding to a pixel resolution of 2 μm/pixel). From these digital images, gray level histograms were deduced, displaying the percentage of bone area occupied by pixels of a certain gray level. The transformation of these into calcium weight percent (wt/100 wt) histograms led to a bin width of 0.17 wt/100 wt calcium. A technical precision of 0.3% was achieved. BMDD parameters like the mean (weighted mean, CaMean) and the standard deviation (CaSD), peak position of the BMDD calcium concentration (CaPeak), and the width of the distribution (CaWidth, full width at half-maximum) reflecting the heterogeneity in matrix mineralization, the fraction of low mineralized bone (CaLow), which is the percentage of the area below 17.7 wt/100 wt Ca (corresponding to the 5th percentile of human reference BMDD), and the fraction of high mineralized bone (CaHigh), which is the percentage of the area above 25.3 wt/100 wt Ca (corresponding to the 75th percentile of human reference BMDD), were derived from the histogram. CaHigh was only determined for metaphyseal bone.

Structural histomorphometric parameters [mineralized bone volume/tissue volume (mBV/TV), trabecular thickness (TbTh) and trabecular number (TbN)] were determined by analyzing a tissue area between 0.5 and 1.5 mm below the growth plate of metaphyseal bone at the distal femur from nondiabetic vs. diabetic rats (n = 6) using qBEI.

Assessment of osteoblast and osteoclast functions. Bone marrow cells were rinsed with DMEM containing 10% FCS. Colony-forming units (CFU)-fibroblast were determined by seeding cells at a density of 200,000/cm2 and culturing them in DMEM with 10% FCS and 1% penicillin/streptomycin for 14 days. Thereafter, cells were stained with 1% crystal violet (Sigma), and formed colonies were counted. In addition, the specifically bound dye was eluted with 0.2% Triton X-100 and measured using a spectrophotometer at 590 nm. Cell vitality was determined using Cell Titer Blue (Promega). Bone marrow stromal cells were differentiated toward osteoblasts using DMEM containing 25 mM of glucose supplemented with 10% FCS, 1% penicillin/streptomycin, 100 μM ascorbate phosphate, 5 mM β-glyc-
erol phosphate, and 10 mM dexamethasone. CFU-osteoblasts were assayed by seeding 200,000 cells/cm² and culturing them for 21 days. Osteoblastic colonies were quantified by counting alizarin red-stained colonies and subsequently eluting the dye with 100 mM cetylpyridinium chloride (Sigma). For all other experiments, cells were seeded at a density of 1 x 10⁶ cells/cm². AP activity was assessed after 14 days. Therefore, cells were lysed and centrifuged for 30 min at 25,000 g at 4°C. Aliquots of each sample were incubated with 100 μL AP substrate buffer for 30 min at 37°C. The reaction was stopped with 100 mM NaOH. Optical density was measured at a wavelength of 405 nm and normalized to the total protein content determined by the BCA method. After 21 days of culture, matrix formation was analyzed by alizarin red S staining. Osteoblasts were fixed in 10% paraformaldehyde for 30 min and stained with 1% alizarin red S (pH 5.5; Sigma) for 10 min. Excess dye was removed with distilled water, and the amount of incorporated dye was eluted with 100 mM cetylpyridinium chloride (Sigma) for 30 min at room temperature. Aliquots were taken and measured spectrophotometrically at 450 nm in triplicates.

For osteoclast assessment, bone marrow cells were collected and plated at a density of 2 x 10⁶ cells/cm² in α-minimal essential medium containing 5.5 mM of glucose, 10% FCS, and 1% penicillin/streptomycin. Following attachment, medium was replaced with basal medium supplemented with macrophage colony-stimulating factor (M-CSF, 25 ng/ml; R & D Systems) for 2 days. Thereafter, cells were cultured for 6 days in differentiation medium containing M-CSF (25 ng/ml) and receptor activator of NF-κB ligand (50 ng/ml; R&D Systems). Tartrate-resistant acidic phosphatase (TRAP) staining was performed using the leukocyte acid phosphatase kit (Sigma). To assess resorption activity, osteoclast precursor cells were plated in 24-well plates coated with osteoblast-derived matrix as reported elsewhere (19). After 8 days, bone matrix was stained with von Kossa, and the resulting resorption pits were quantified by densitometry.

**RNA isolation and real-time PCR analysis.** Total RNA was isolated using the High Pure RNA Isolation Kit (Roche). In reverse transcriptase reactions, equal amounts of total RNA (500 ng) were incubated for 3 min at 70°C and subsequently reverse-transcribed into cDNA using random hexamer primers for 1 hr at 42°C. For real-time PCR reactions, the following primer pairs for amplification were used: RUNX2 (sense: 5'-CCACGGCCCTCCCTGAACT-3'; antisense: 5'-GTTGGAAGACAGCCGGT-3'), osteopontin (sense: 5'-ggagaaaggagccagagttggcc-3'; antisense: 5'-atggctttcattggagttgc-3'), osteocalcin (5'-GAGGGCCAGTAAAAAGGTTGGA-3'), bone morphogenetic protein (BMP)-2 (sense: 5'-ACATCCACTCCACAAACAGGACG-3'; antisense: 5'-GTCATCCACCCCACTCAC-3'), and TRAP5b (sense: 5'-gagaagacgggagtcgtagttg-3'; antisense: 5'-ggaagacgggagtcgtagttg-3'). The mRNA expression was normalized to β-actin.

**Statistical analysis.** Results are presented as means ± SE. All experiments were repeated at least three times. Single-group comparisons were performed using a Student’s t-test. P values of <0.05 were considered statistically significant.

**RESULTS**

**Characteristics and laboratory findings.** At baseline at an age of 9 wk, i.e., immediately before surgery, mean serum glucose levels were 9.0 ± 0.62 mM/l for nondiabetic rats and 30.8 ± 0.60, mM/l for diabetic rats (P < 0.01). Moreover, the body weight of ZDF rats was higher (367 ± 4.6 g) for diabetic rats than for nondiabetic rats (296 ± 5.6 g) (P < 0.01). Total, cortical/subcortical, and trabecular BMD at the distal femur, total BMD, and cortical thickness of the femoral shaft were not statistically different at 9 wk between diabetic and non-diabetic ZDF rats as measured in vivo by pQCT.

When exposed to a diet with a high degree of saturated fat and carbohydrate (Purina 5008) for 12 wk, serum glucose levels of diabetic ZDF rats (21 wk old) increased to values of 40 mM/l (Table 1) while serum glucose levels of non-diabetic ZDF rats fed Purina 5008 rose to 19 mM/l. In 21-wk-old rats, serum insulin levels were similar in nondiabetic and diabetic ZDF rats, a finding that has been reported previously (27). Of note, the body weight was similar between the two groups, presumably because of the persistent hyperglycemia and glucosuria of diabetic rats (27). The clinical and laboratory findings of 21-wk-old nondiabetic and diabetic ZDF rats are summarized in Table 1. Diabetic ZDF rats showed significant higher blood glucose levels (P < 0.01). Serum calcium levels were increased markedly in diabetic rats compared with non-diabetic rats (P < 0.01), and there was a trend toward higher serum PTH levels, whereas serum phosphate levels were not different. Serum urea levels, but not creatinine levels, were elevated in the diabetic animals, indicating renal impairment (Table 1). Serum levels of P1NP (P = 0.016) and osteocalcin (P = 0.001), two markers of bone formation, were lower in diabetic rats compared with nondiabetic rats, whereas serum CTX levels, a bone resorption marker, were threefold higher in diabetic compared with nondiabetic ZDF rats (P = 0.013) (Table 1).

**Lower BMD at all skeletal sites in diabetic rats.** At 21 wk of age, detailed pQCT analysis revealed significantly lower total BMD at the distal femur, the femoral shaft, and the lumbar spine in diabetic rats compared with nondiabetic rats (Fig. 1). At the distal femur, total BMD (Fig. 1A), trabecular BMD (Fig. 1B), and cortical/subcortical BMD (Fig. 1C) were 22.5% (P < 0.01), 54.6% (P < 0.01), and 15.9% (P < 0.05) lower, respectively, in diabetic rats compared with nondiabetic rats. At the femoral shaft, cortical BMD and cortical thickness were 2.3% (P < 0.05) and 17.7% (P < 0.01) lower in diabetic rats, respectively (Fig. 1, D and E). At the fourth lumbar vertebrae, total BMD (−17.2%, P < 0.01), trabecular BMD (−20.4%, P < 0.01), and cortical/subcortical BMD (−15.8%, P < 0.01) were significantly lower in diabetic rats compared with nondiabetic rats (Fig. 1, F–H).

**Lack of alteration in bone mineralization of diabetic rats.** BMDD of trabecular and cortical bone was very similar between collagen links.

**Table 1. Body weight and laboratory characteristics of 21-wk-old nondiabetic (+/+ and diabetic (fa/fa) ZDF rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nondiabetic ZDF (+/+ Rats)</th>
<th>Diabetic ZDF (fa/fa) Rats</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Body wt. g</td>
<td>409 ± 7.80</td>
<td>423 ± 8.40</td>
<td>0.265</td>
</tr>
<tr>
<td>Blood glucose levels, mM/l</td>
<td>18.7 ± 1.18</td>
<td>39.5 ± 0.72</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum insulin levels, ng/l</td>
<td>0.35 ± 0.07</td>
<td>0.43 ± 0.07</td>
<td>0.249</td>
</tr>
<tr>
<td>Serum calcium levels, mM/l</td>
<td>2.50 ± 0.06</td>
<td>2.70 ± 0.03</td>
<td>0.006</td>
</tr>
<tr>
<td>Serum phosphate levels, mM/l</td>
<td>2.39 ± 0.12</td>
<td>2.43 ± 0.12</td>
<td>0.784</td>
</tr>
<tr>
<td>Serum creatinine levels, μM/l</td>
<td>55.3 ± 2.37</td>
<td>38.3 ± 1.50</td>
<td>0.724</td>
</tr>
<tr>
<td>Serum uric acid levels, mM/l</td>
<td>7.14 ± 0.21</td>
<td>8.49 ± 0.40</td>
<td>0.006</td>
</tr>
<tr>
<td>Serum PTH levels, pg/ml</td>
<td>783 ± 110</td>
<td>962 ± 120</td>
<td>0.283</td>
</tr>
<tr>
<td>Serum PINP levels, ng/ml</td>
<td>15.5 ± 1.46</td>
<td>11.0 ± 0.93</td>
<td>0.016</td>
</tr>
<tr>
<td>Serum osteocalcin levels, pg/ml</td>
<td>458 ± 49.4</td>
<td>175 ± 21.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum CTX levels, ng/ml</td>
<td>35.9 ± 2.6</td>
<td>96.3 ± 17.0</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Values are means ± SE. ZDF, Zucker diabetic fatty; PTH, parathyroid hormone; PINP, procollagen 1 N-terminal peptide; CTX, COOH-terminal collagen cross-links.
displayed characteristic modifications in BMDD dependent on bone tissue site: a shift of the BMDD curves toward higher matrix mineralization (to the right) was observed from metaphyseal to epiphyseal and to cortical bone as reflected by an increase in the mean calcium content (CaMean) and the peak position of the BMDD (CaPeak). At the same time, the heterogeneity in matrix mineralization (CaWidth) became narrower, and the fraction of low mineralized bone matrix (CaLow)

Table 2. BMDD parameters of metaphyseal, epiphyseal, and cortical midshaft bone of distal femurs from 21-wk-old nondiabetic vs. diabetic rats

<table>
<thead>
<tr>
<th>BMDD Parameters</th>
<th>Metaphyseal</th>
<th></th>
<th></th>
<th>Epiphyseal</th>
<th></th>
<th></th>
<th>Cortical</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nondiabetic</td>
<td>Diabetic</td>
<td></td>
<td>Nondiabetic</td>
<td>Diabetic</td>
<td></td>
<td>Nondiabetic</td>
<td>Diabetic</td>
<td></td>
</tr>
<tr>
<td>CaMean, wt/100 wt Ca</td>
<td>23.7 ± 0.24</td>
<td>24.24 ± 0.17</td>
<td></td>
<td>24.12 ± 0.32</td>
<td>24.6 ± 0.15</td>
<td></td>
<td>25.2 ± 0.20</td>
<td>25.5 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>CaPeak, wt/100 wt Ca</td>
<td>24.0 ± 0.23</td>
<td>24.4 [24.26; 24.87]</td>
<td></td>
<td>24.70 ± 0.27</td>
<td>25.1 ± 0.14</td>
<td></td>
<td>25.6 ± 0.21</td>
<td>25.7 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>CaWidth, Dwt/100 wt Ca</td>
<td>3.32 ± 0.08</td>
<td>4.07* [3.38; 4.33]</td>
<td></td>
<td>2.95 [2.77; 2.95]</td>
<td>2.95 ± 0.06</td>
<td></td>
<td>3.12 [3.03; 3.21]</td>
<td>3.06 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>CaLow, % BAr</td>
<td>4.00 ± 0.24</td>
<td>4.56 ± 0.22</td>
<td></td>
<td>2.83 ± 0.31</td>
<td>2.93 ± 0.19</td>
<td></td>
<td>1.59 ± 0.06</td>
<td>1.73 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>CaHigh, % BAr</td>
<td>25.0 ± 3.24</td>
<td>38.3 ± 3.02*</td>
<td></td>
<td>NM</td>
<td>NM</td>
<td></td>
<td>NM</td>
<td>NM</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± SE or the median with the 25th and 75th percentiles in square brackets if data did not pass the normality test (n = 6). BMDD, bone mineralization density distribution; BAr, bone ares; NM, not measured. *P < 0.05 between controls and diabetic animals using unpaired t-tests or Mann-Whitney rank sum tests, if data were not normally distributed. For abbreviations of the BMDD parameters, please see MATERIALS AND METHODS.
Diabetes mellitus has been increasingly recognized as a risk factor for osteoporosis and fragility fractures in humans. Poor glycemic control over a long time and the presence of vascular complications have been identified as potential determinants of low bone mass and increased risk of fractures (8). Clinically, patients with diabetes mellitus carry a higher risk of delayed bone regeneration.

Using a well-established rat model of type 2 diabetes mellitus, we show that bone formation is impaired by type 2 diabetes due to a specific osteoblast defect that leads to decreased bone mass and delayed bone regeneration.

Table 3. Structural histomorphometric parameters of metaphyseal bone of distal femurs from nondiabetic vs. diabetic rats

<table>
<thead>
<tr>
<th>Structural Parameters</th>
<th>Nondiabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV, %</td>
<td>26.5 ± 2.73</td>
<td>13.8 ± 1.40*</td>
</tr>
<tr>
<td>TbTh, μm</td>
<td>86.3 ± 5.71</td>
<td>60.7 ± 3.03*</td>
</tr>
<tr>
<td>TbN, 1/mm</td>
<td>3.04 ± 0.18</td>
<td>2.25 ± 0.15*</td>
</tr>
</tbody>
</table>

Values were derived from quantitative backscattered electron imaging of a tissue area between 0.5 to 1.5 mm below the growth plate and represent the means ± SE; n = 6 rats in each group. BV/TV, bone volume/total volume; TbTh, trabecular thickness; TbN, trabecular number. *P < 0.01 between controls and diabetic animals using unpaired t-tests.

DISCUSSION

Using a well-established rat model of type 2 diabetes mellitus, we show that bone formation is impaired by type 2 diabetes due to a specific osteoblast defect that leads to decreased bone mass and delayed bone regeneration.

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Delayed defect regeneration in diabetic vs. nondiabetic rats.

To validate the functional implications of these observations in a translational rodent model, a bone defect of 3 mm was created by microsurgical techniques at the left femur after stabilizing the femur with a four-hole plate (Fig. 5A). Defect regeneration was monitored in vivo by X-ray over the course of 12 wk and with μCT analysis postmortem. While nondiabetic rats partially filled the defects by a mean of 57% after 12 wk, diabetic rats showed delayed new bone defect filling, resulting in only 21% filling of the defect (P < 0.01, Fig. 5, B and C). Longitudinal X-ray analysis was consistent with these data and indicated that decreased defect healing in diabetic rats was mainly due to delayed bone regeneration in the first 8 wk postoperatively, after which the bone defects of diabetic and nondiabetic rats healed at a similar rate (Fig. 5D).

Impaired osteoblastic functions in diabetic rats.

To assess the underlying cellular mechanism for the observed decrease in BMD in type 2 diabetes mellitus, osteoblast and osteoclast biology was analyzed. Bone marrow cells from diabetic and nondiabetic rats displayed a similar vitality and contained an equal number of precursor cells as assessed by colony-forming assays (data not shown), indicating that there was a similar supply of osteoblastic precursor cells. Osteoblast differentiation was impaired by type 2 diabetes mellitus based on a 55% lower mineralized matrix formation in cells from diabetic compared with nondiabetic rats after 21 days of differentiation (Fig. 3A). In addition, AP activity of osteoblasts was reduced by 20% (Fig. 3B). Furthermore, osteoblast-specific transcription factors, growth factors, and extracellular matrix proteins such as RUNX2, osteopontin, BMP-2, and osteocalcin were reduced by 40–80% after osteogenic differentiation for 7 and 21 days, respectively (Fig. 3, C–F).

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DISCUSSION

Using a well-established rat model of type 2 diabetes mellitus, we show that bone formation is impaired by type 2 diabetes due to a specific osteoblast defect that leads to decreased bone mass and delayed bone regeneration.

Diabetes mellitus has been increasingly recognized as a risk factor for osteoporosis and fragility fractures in humans. Poor glycemic control over a long time and the presence of vascular complications have been identified as potential determinants of low bone mass and increased risk of fractures (8). Clinically, patients with diabetes mellitus carry a higher risk of delayed bone regeneration.

Delayed defect regeneration in diabetic vs. nondiabetic rats.

To validate the functional implications of these observations in a translational rodent model, a bone defect of 3 mm was created by microsurgical techniques at the left femur after stabilizing the femur with a four-hole plate (Fig. 5A). Defect regeneration was monitored in vivo by X-ray over the course of 12 wk and with μCT analysis postmortem. While nondiabetic rats partially filled the defects by a mean of 57% after 12 wk, diabetic rats showed delayed new bone defect filling, resulting in only 21% filling of the defect (P < 0.01, Fig. 5, B and C). Longitudinal X-ray analysis was consistent with these data and indicated that decreased defect healing in diabetic rats was mainly due to delayed bone regeneration in the first 8 wk postoperatively, after which the bone defects of diabetic and nondiabetic rats healed at a similar rate (Fig. 5D).

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fracture healing as well as developing nonunion and impaired osseointegration (3, 22). Various individual skeletal and extraskeletal factors have been implicated to contribute to poor bone health (8, 20, 26) in diabetes, however, they remain poorly defined at the cellular and molecular level. Studies of the complex relationship between bone metabolism in type 2 diabetes mellitus were limited by the lack of appropriate rodent models. The ZDF fa/fa rats represent a suitable, diet-induced model.

Fig. 3. Impaired osteoblast functions in diabetic rats. Osteoblasts from nondiabetic (+/+ ) and diabetic (fa/fa) ZDF rats were obtained by differentiating bone marrow stromal cells using dexamethasone, β-glycerol phosphate, and ascorbic acid as indicated. A: alizarin red S staining of the mineralized matrix was performed after 7 and 21 days, n = 3–5. B: alkaline phosphatase (ALP) activity was measured after 14 days and normalized to the total protein content, n = 3–5. C–E: RNA was extracted at day 7, reverse transcribed, and subjected to real-time PCR analysis. Gene expression of RUNX2 (C), osteopontin (D), and bone morphogenetic protein (BMP)-2 (E) was normalized to β-actin, n = 4–6. F: RNA was extracted at day 21, reverse transcribed, and subjected to real-time PCR analysis. Gene expression of osteocalcin was normalized to β-actin, n = 4–6. Data are presented as means ± SE. *P < 0.05, +/+ vs. fa/fa.

Fig. 4. Osteoclast function is not altered in diabetic rats. Osteoclasts from nondiabetic (+/+ ) and diabetic (fa/fa) ZDF rats were generated by differentiating bone marrow cells using macrophage colony-stimulating factor and receptor activator of NF-κB ligand for 7 days. Osteoclast differentiation was assessed by counting tartrate-resistant acidic phosphatase (TRAP)-positive multinucleated cells (A, n = 6), by performing resorption pit assays (B, n = 3; left, representative pit image; right, quantitative analysis of pit size), and by analyzing TRAP mRNA levels (C, n = 6). Data are represented as means ± SE.
rodent model of type 2 diabetes mellitus, since they recapitulate most features of poorly controlled type 2 diabetes mellitus in humans (17, 21). Although hyperglycemia was consistently documented in diabetic rats, serum insulin levels after 21 wk were comparable as reported (27). Thus, this reflects a late stage of long-lasting type 2 diabetes mellitus at which there is a relative \( \beta \)-cell failure secondary to prolonged hyperinsulinemia and insulin resistance. In addition, mild renal impairment was already present (27). Unexpectedly, diabetic rats displayed mild hypercalcemia, which was not fully explained by PTH variation and was not associated with phosphate abnormalities. These findings have no correlate in humans with type 2 diabetes mellitus where hypercalcemia in diabetic individuals is mainly due to comorbidity (e.g., primary hyperparathyroidism) or comedication (e.g., thiazide diuretics).

Diabetic ZDF rats display reduced BMD at different skeletal sites, lower histomorphometric indexes of BV/TV, and lower bone formation markers osteocalcin and P1NP in vivo that are paralleled by impaired osteoblastic transcription factors and genes, less efficient differentiation, and markedly reduced formation of mineralized matrix. Of note, our study showed that trabecular BMD was particularly decreased in ZDF rats. Interestingly, the suppressive effect of the diabetic microenvironment on osteogenic differentiation persists even after culture for up to 3 wk ex vivo. Our data are in accordance with earlier findings obtained in ZDF rats that demonstrated impaired bone mass and bone strength in diabetic ZDF rats (17, 21) and provide a cellular explanation for these observations, which identify a predominant defect of osteoblastogenesis. This is in line with previous in vitro (9, 7) and in vivo studies involving rodent models of type 1 diabetes mellitus (2, 18). Our findings are also consistent with the observations made in spontaneously diabetic Torii rats, a model of nonobese type 2 diabetes mellitus that displayed a lower bone formation rate and decreased gene expression of AP and osteocalcin (6). These changes were reversed by insulin treatment.

We did not observe altered osteoclast functions ex vivo, in contrast to other reports, which suggested that a diabetic microenvironment tends to inhibit osteoclast differentiation and function (30, 16, 29, 4, 12). However, none of these studies used an in vivo model of rats with type 2 diabetes mellitus. A potential limitation of our bone cell differentiation assays is that heterogeneous cell populations are obtained from the bone marrow harboring self-renewing stem cells and committed progenitor cells. This may also explain the higher susceptibility of osteoblasts to the inhibitory effects of diabetes mellitus if one considers that a significant proportion of cells, ranging from stem cells to bipotent progenitor cells, may be primed to enter the adipogenic differentiation pathway instead of the osteogenic pathway and that this lack of osteogenic precursor cells cannot be compensated during the culture period. In fact, recent studies indicate that several established oral antidiabetic drugs may specifically modulate osteoblast differentiation. While metformin stimulates osteoblast differentiation through the transactivation of RUNX2 via the AMPK/USF-1/SHP regulatory cascade (10), glitazones activate the peroxisome proliferator-activating receptor-\( \gamma \) and may direct bone marrow...
stromal cells toward the adipocytic lineage at the cost of osteoblasts (13).

The lack of significant differences in BMDD between diabetic and nondiabetic rats with the exception of metaphyseal bone suggests that the bone mineralization at the material level is not affected in diabetic animals. Thus, lower BMD in diabetic rats is most likely caused by a decrease in bone volume/mass alone but not by impaired matrix mineralization as evidenced by qBEI. The variations in BMDD found between metaphyseal, epiphyseal, and cortical bone are due to the differences in average tissue age at these specific skeletal sites. Younger bone tissue is less mineralized than older bone tissue (24). Thus, in the investigated femurs of the diabetic and nondiabetic rats, the metaphyseal bone tissue is the least mineralized and therefore the youngest because of ongoing (re)modeling and the cortical bone the highest mineralized and therefore the oldest one because cortical bone is less (re)modeled. These differences in mineralization are consistent with previous observations in mouse femurs (5). Of note, the increased CaWidth and CaHigh found in metaphyseal bone for diabetic rats suggests that the diabetic rats have a disturbed endochondral ossification in the subchondral region of the growth plate, probably related to the diminished bone formation, leading to a higher portion of residual mineralized cartilage in the metaphyseal bone. Thus, bone mineralization in diabetic rats appears to be intact and does not account for their low BMD.

While nondiabetic rats partially filled the bone defects, diabetic ZDF rats showed delayed bone regeneration of the subcritical bone defect. These findings are consistent with the ex vivo studies demonstrating markedly reduced osteoblastic function in diabetic animals. Bone regeneration that occurs in areas of bone defects is mainly driven by osteoblastic bone formation. Our data indicate that this process is particularly impaired in diabetic rats within the first 6 wk of the bone defect, which may indicate altered recruitment of osteoblastic precursor cells under diabetic conditions. In fact, Lu et al. (18) reported in a type I mouse model that delayed bone formation was due to inadequately low expression of genes that control osteoblast differentiation such as RUNX-2 and Dlx5, whereas immature mesenchymal tissue with putative osteoblastic precursors was sufficient. Impaired bone healing with reduced bone formation parameters was also observed in a bone histomorphometric study of GK rats with type 2 diabetes mellitus with titanium implants placed in the femora (28). Thus, based on these findings, osteoinductive biomaterials or bone-anabolic therapies that stimulate osteoblastic differentiation and function locally or systemically may be promising strategies to target impaired bone formation. This should be formally assessed in patients with type 2 diabetes mellitus.

Our study has several limitations. First, we used relatively young rats that underwent surgery at 9 wk and were studied after 21 wk of age. Therefore, our observations cannot be extrapolated to older rats. However, in light of the severity of diabetes mellitus, we minimized the risk of increased perioperative infections and lethality that was observed in older animals with advanced diabetic complications (data not shown). Second, we left the diabetes mellitus untreated for 12 wk in the ZDF rats and did not attempt to alter the course of disease using dietary intervention, oral antidiabetic drugs, or insulin. Certainly, the rapid, rather than insidious, onset of type 2 diabetes mellitus with persistently elevated serum glucose levels of 40 mmol/l for 8 wk represents an extreme metabolic situation that cannot fully translate into clinical medicine. However, despite these inherent differences, this model may recapitulate some of the endocrine and vascular consequences of poorly controlled type 2 diabetes mellitus that may be diagnosed 5 to 10 years after its onset and is frequently only recognized through cardiovascular complications. Third, we did not assess chondrocyte functions and involved pathways (14, 15) or the effect of altered enzymatic and nonenzymatic collagen cross-linking on bone strength (25), two aspects that may contribute to poor bone quality in type 2 diabetes. Fourth, we employed whole bone marrow rather than more highly enriched precursors for the in vitro differentiation assays. Despite these limitations, most findings of our study may be relevant also for skeletal health in patients with diabetes mellitus.

In conclusion, our study identified suppressed osteoblastogenesis as a key mechanism for low bone mass and impaired bone regeneration in a rat model of type 2 diabetes mellitus while bone matrix mineralization is intact. Local or systemic strategies that specifically target this mechanism may improve bone health and bone regeneration in diabetes.

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DISCLOSURES

The authors have no conflict of interest.

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