

A novel rat model for the study of deficits in bone formation in type-2 diabetes

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Background There is evidence to suggest that impairment in bone formation and/or turnover is associated with the metabolic abnormalities characteristic of type-2 diabetes mellitus. However, bone regeneration/repair in type-2 diabetes has not been modeled. Using Zucker Diabetic Fatty (ZDF) rats (a model of type-2 diabetes) for tibial distraction osteogenesis (DO), we hypothesized that bone formation within the distraction gap would be impaired.

Animals and methods Rats were examined for body weight, glycosuria, and glyosemia to confirm the diabetic condition during the study. The rats received placement of the external fixators and osteotomies on the left tibia. Distraction was initiated the following day at 0.2 mm twice a day and continued for 14 days. The lengthened tibiae were harvested and distraction gaps were examined radiographically and histologically.

Results We found significant reduction in new bone formation in the distraction gaps of the ZDF rats, both radiographically and histologically, compared to lean rats. We found a decrease in a marker of cellular proliferation in the distraction gaps and increased adipose volume in adjacent bone marrow of the ZDF rats.

Interpretation Our findings suggest that this model might be used to study the contributions of leptin resistance, insulin resistance and/or hyperglycemia to impaired osteoblastogenesis *in vivo*.

Type-2 diabetes mellitus is a heterogeneous disease with chronic hyperglycemia as a common denominator. Recent studies have revealed that impaired insulin secretion and reduced insulin sensitivity are the most important pathophysiological factors that contribute to development of type-2 diabetes (Gerich 2003). In type-2 diabetes, as in type 1 diabetes, there is often development of later complications in many tissues and organs due to metabolic and/or hormonal disturbances and microangiopathy. To date, at least 8 major complications have been found in both type-1 and type-2 diabetes, including retinopathy, nephropathy, cardiovascular disease, neuropathy, and wound healing problems (Bailes 2002).

Diabetic bone complications, such as diabetic osteoporosis and impaired fracture repair, have been noted. In type 1 diabetes, diabetic osteoporosis has been well documented in patients (Chau et al. 2003) and animal models (Verhaeghe et al. 1989, Bouillon 1992). In contrast, studies of bone mineral density in patients with type-2 diabetes are less clear and often contradictory. Increased (Barret-Connor 1992), decreased (Takizawa et al. 2003), and unchanged (Weinstock et al. 1989) bone volumes have been observed. In addition, several studies have demonstrated that bone fracture healing is significantly impaired in type 1 diabetic animal models (Lu et al. 2003, Tyndall et al. 2003). However, there have been no studies addressing

bone regeneration during distraction osteogenesis (DO) in type-2 diabetes reported in the literature.

The Zucker Diabetic Fatty (ZDF) rat is an inbred rat model with a known homozygous functional defect in the leptin receptor that spontaneously develops type-2 diabetes. This is characterized by hyperglycemia, hyperinsulinemia, insulin resistance, and obesity in all males (Shibata et al. 2000). Recent studies have shown that many diabetic complications are found in the ZDF rats, including periodontal disease (Ryan et al. 1999) and diabetic osteopenia (Shibata et al. 2000, Picherit et al. 2003). Decreased serum osteocalcin has been found in ZDF rats with diabetic osteopenia, which suggests that reduced bone density in ZDF rats may be associated with depressed osteoblast activities that would result in a decline in bone formation. We postulate that the ZDF rat model is potentially a good model to study skeletal complications of diabetes for two reasons: (1) it shares a number of common metabolic disturbances (leptin signaling deficiency, insulin resistance, hyperglycemia, hyperinsulinemia) with patient populations, and (2) the evolution of type-2 diabetes in this model enables separate study of these variables in the same model by judicious choice of the age of the rats at the start of the protocol (Shibata et al. 2000). For example, at weeks 4–5 the ZDF rats have not developed the diabetic symptoms while they are of course leptin resistant compared to leptin-sensitive controls. At the age of 6–7 weeks they are hyperinsulinemic but still normoglycemic, and at age 9–15 weeks they are insulin-resistant and hyperglycemic. The natural development of the pathology in this model thus lends itself to testing for the effects of systemic mediators.

A rat model for DO has been used to isolate and study osteoblastogenesis *in vivo*, and to study bone repair processes associated with osteoporotic fractures (Aronson et al. 1997a, 2001, Brown et al. 2002, Perrien et al. 2002). Using the rat tibia, DO has been shown to induce rapid bone formation by stretching a fracture callus at a prescribed rate and rhythm. After 14 days of distraction, the distraction gap can be histologically divided into 5 zones. The fibrous interzone (FIZ), a central part of the callus, is composed of fibroblasts and mesenchymal cells surrounded by collagen bundles parallel to the stretching direction. The FIZ is bordered on both

sides by the zones of primary matrix front (PMF) where proliferating and differentiating osteogenic cells accumulate and where bone matrix formation first occurs. The PMFs are then bordered by the zones of microcolumn formation (MCF) where osteoblasts and osteocytes are embedded in mineralized matrix and new bone expands parallel to the distraction force with invading vascular sinusoids. Since DO stretches cellular bone formation into temporal and spatial zones (from proliferation to differentiation), allowing detailed *in vivo* examination of osteoblastogenesis separate from osteoclastic activity, this model has become a useful method to study the effects of numerous experimental variables on osteoblastogenesis *in vivo* (Aronson et al. 2001, Brown et al. 2002, Perrien et al. 2002).

We used DO created at the mid-diaphysis of the tibia of 9–11 week-old male ZDF rats, to test the hypothesis that osteoblastogenesis within the distraction gap will be significantly impaired in association with a concurrence of leptin signaling deficiency, insulin resistance, and hyperglycemia.

Animals and methods

Animals

10 male ZDF rats (ZDF Gmi / -fa/fa), 360 g, and 10 male Zucker lean rats (ZDF/Gmi-lean +/-), 280 g, were purchased from Charles River Lab (Wilmington, MA) when they were 8 weeks old. Rats were caged individually and given a rodent chow diet (Purina Cat. no: 5008; Charles River Lab) and tap water. All animals were examined for body weight, glycosuria, and glycosemia to confirm the diabetic condition during the study. Keto-Diastix Reagent Strips (Bayer, Elkhart, IN) and OneTouch Ultra test strips (Lifescan, Milpitas, CA) were used to test glycosuria and glycosemia, respectively. All research protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

Distraction osteogenesis

1 week after acclimation, all rats underwent an intraperitoneal injection of sodium pentobarbital anesthesia (50 mg/kg) for surgical application of a two-ring external fixator to the left tibia using four transosseous pins and a mid-diaphyseal, low-energy

osteotomy as previously described (Aronson et al. 1997b). Daily manual distraction was initiated the following morning (one day latency) at 0.2 mm b.i.d. (0.4 mm/day) and continued for 14 days. At sacrifice under anesthesia, the rats were decapitated for collection of trunk blood and then the lengthened tibiae were surgically removed for further examination.

Radiographic analysis

As previously described, after removal of soft tissues except periosteum, the lengthened tibiae were fixed in 10% neutral buffered formalin with the external fixator intact (Aronson et al. 1997a, b, 2001, Brown et al. 2002, Perrien et al. 2002). After 48 h of fixation, the lengthened tibiae were removed from the fixators and positioned for high-resolution single-beam radiography. The radiographs were video-captured on a light microscope under low-power magnification. For radiographic analysis, the area and relative density of partially to completely “mineralized” new bone in the distraction gaps were measured with NIH Image Analysis software version 1.62. The “mineralized” new bone area in the distraction gap was measured by selecting and outlining regions with a radiodensity defined as being equivalent to or greater than the average background density (1–20, gray-scale). The total area of the gap was measured by outlining the distraction gap from proximal to distal cortices. The percentage of new bone area in the distraction gap was calculated by dividing the new bone area by the total gap area. The relative density of “mineralized” new bone was calculated as a percentage of the adjacent host bone density as follows (Aronson et al. 1997a, 2001, Brown et al. 2002, Perrien et al. 2002). First, selected regions of interest (3 mm²), one in both the adjacent proximal and distal original bone, were scanned by density slice. These areas were covered by using the red color threshold in the toolbox and the density of the areas was recorded. The mean density of proximal and distal areas was used as the reference density. Next, the distraction gap was selected, the density slice was chosen, the red threshold was adjusted to just cover the gap, and the density was recorded and divided by the reference value.

Histological analysis

As previously described, after taking radiographs, the tibiae were fixed in 10% neutral buffered formalin for an additional 24 h (Aronson et al. 1997a, b, 2001, Brown et al. 2002, Perrien et al. 2002). The specimens were then decalcified in 5% formic acid and finally embedded in paraffin. 5- μ m sagittal sections were cut and stained with hematoxylin and eosin (H&E). The samples chosen for histological analysis were sectioned from the mid-sagittal plane, containing the distraction gap and all four cortices with intact marrow spaces at both the proximal and distal ends. For quantitative analysis of new bone formation, the area of endosteal new bone and periosteal new bone was measured and calculated as a percentage of the total gap area using Image J software version 1.30 (rsb.info.nih.gov/ij/). We defined endosteal new bone as that arising within cortices (marrow space) and periosteal new bone as that arising from the periosteum.

To investigate the potential relationship between bone formation and bone marrow function during DO, the adipose tissues in the proximal and distal bone marrow were measured using NIH Image Analysis software. Images from histological H&E slides were video-recorded under 20 \times magnification. For each specimen, six 0.18-mm² regions of interest (ROIs, calibrated for the objective with a micrometer) were selected, three proximal and three distal, within the bone marrow adjacent to the distraction gap (Figure 1). The images were imported into the NIH Image Analysis program, and using the gray density threshold icon, the fat cells and nucleated marrow cells were distinguished and counted by visually matching the variable red areas with the fat cells and nucleated cells, respectively. Finally, the numbers of fat cells and nucleated cells of each ROI were calculated, and the percentage of fat cells was calculated by dividing by the total number of cells in the ROIs.

Immunohistochemistry

Immunohistochemistry was performed using a streptavidin-biotin immunoperoxidase method. Briefly, the decalcified sections were deparaffinized in xylene, rehydrated in ethanol, and washed in PBS containing 0.02% Triton-100. The sections were incubated in 1 \times Antigen Retrieval Citra solution (Biogenex, San Ramon, CA), which

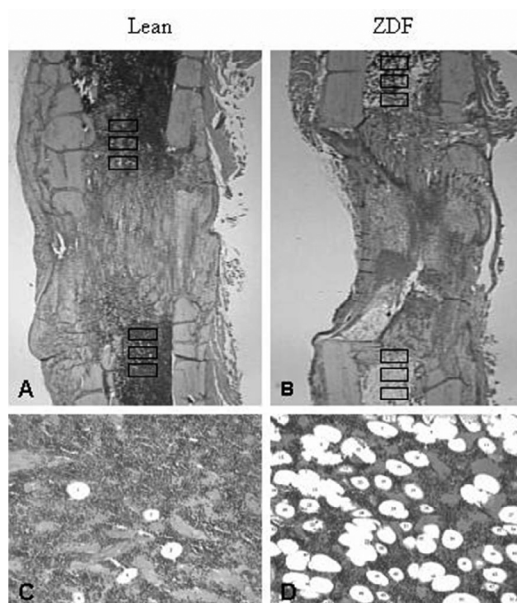


Figure 1. A and B: Histological examination of adipocytes in the bone marrow adjacent to distraction gaps of lean and ZDF rats. Regions of interest (ROIs) at each end of the bone marrow are indicated by boxes (original magnification 1.25 \times). C and D: Higher-power magnification (20 \times) of one box each from the lean and ZDF rats represents a sample in which the adipocytes (white areas) were counted.

had been previously heated to 95 $^{\circ}$ C for 15 min to retrieve the epitope. The slides were then incubated with Peroxo-Block solution (Zymed, San Francisco, CA) for 15 min to block endogenous peroxidase activity. PC-10, a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Biogenex) was used to examine cell proliferation in distraction gaps. A normal mouse IgG (Dako) was used as a negative control. The prepared sections were incubated at 4 $^{\circ}$ C overnight with one of these two antibodies. Following two washes in PBS containing 0.02% Triton-100, all sections were exposed to a 1/250 dilution of biotinylated horse anti-mouse antibody (Vector Labs, Burlingame, CA) at room temperature for 20 min. The sections were then incubated with peroxidase-conjugated streptavidin complex at room temperature for 20 min and color was developed by exposure to peroxidase substrate (Vector Labs). Finally, the sections were counterstained with hematoxylin, then dehydrated and mounted using permanent mounting medium.

Semiquantitation of cell proliferation

The immunostained slides were examined under bright-field microscopy. The digital pictures were captured at 40 \times magnification using a Polaroid DMC 1e digital camera (Polaroid Corp., Cambridge, MA) and the proprietary software package. The expression of PCNA in the respective pictures was quantitatively analyzed at 6.58 pixels/ μ m using Image J software. A blinded observer then counted the number of positive cells and negative cells within each previously defined zone (FIZ, PMF, and MCF). In each zone, a fixed-area ROI measuring 1 mm² was examined. The data were used to calculate the total cellular density (total cells/mm²), density of positive cells (positive cells/mm²), and percentage of positive cells (positive cells/total cells \times 100) for each specimen.

ELISA

Serum concentrations of osteocalcin were measured using an ELISA (Rat Osteocalcin EIA Kit; Biomedical Technologies Inc. Stoughton, MA) according to the manufacturer's instructions. Values were assayed in duplicate and calibrated against a rat osteocalcin standard (0.78–50 ng/mL). The sensitivity was 0.5 ng/mL and the variations in intra- and interassay values were lower than 4% and 7%, respectively.

Blood insulin levels were analyzed using a Rat Insulin ELISA Kit (Crystal Chem Inc., Chicago, IL) according to a protocol provided by the manufacturer.

Statistics

For statistical analysis, differences between group means were determined by Student's t test. All data are reported as mean (SD). Differences were considered significant when $p < 0.05$.

Results

Diabetic rats

All rats survived the surgery and distraction period without complications such as infection or ankle fracture. The ZDF rats gained approximately 10 more grams, while the Zucker lean rats remained at a similar level of body weight during the study. Blood glucose levels of the Zucker lean

rats remained within the physiological range at an average value of 109 (5.1) mg/dL. Glycosuria was not detected in these lean rats. The ZDF rats exhibited glycosuria (1,000–2,000 mg/dL) during the study but did not develop ketonuria (< 5 mg/dL). Blood glucose levels for the ZDF rats were above the physiological range, at a value of 347 (21) mg/dL. The ZDF rats also showed a higher serum level of insulin (5.19 (0.91) ng/mL vs. 1.96 (0.47) ng/mL, $p = 0.02$) and a lower serum concentration of osteocalcin (94 (6.8) ng/mL vs. 278 (17) ng/mL, $p < 0.001$) as compared to the lean rats.

Radiographic data

The radiographs revealed that new bone formation in the distraction gaps was less in the ZDF rats than in the lean rats (Figure 2a). The quantitative analysis showed a significant decrease in the area of “mineralization” (area above threshold) of distraction gaps in the ZDF rats when compared to the lean rats ($p < 0.001$). The relative density of mineralization of the distraction gaps was also significantly lower in the ZDF rats than in the lean rats ($p = 0.03$) (Figure 2b).

Histological data

The histological examination revealed that, following 14 days of distraction, the distraction gaps of the Zucker lean rats were almost completely bridged by endosteal new bone alternating with a high density of collagen bundles parallel to the distraction force. On the other hand, in the ZDF rats the distraction gaps were mainly filled with loose, slim collagen bundles intermixed with fibroblast-like cells and other types of cells. A greatly reduced degree of bone formation—and in some cases a complete absence of new bone formation—was observed in the distraction gaps of the ZDF rats (Figure 3a). The quantitative analysis demonstrated that there was a significant deficiency of bone formation not only in endosteal new bone formation ($p = 0.001$), but also in periosteal new bone formation (5.3% (1.8%) vs. 16.1% (2.9%), $p = 0.008$), in the ZDF rats as compared to the Zucker lean rats (Figure 3b).

We found an increase in the number and percentage of adipocytes in the proximal bone marrow in the ZDF rats compared to the lean rats ($p < 0.001$ and $p < 0.001$). Similarly, both the number and

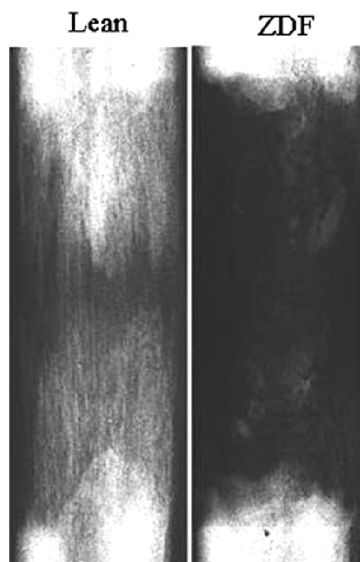


Figure 2a. Representative radiographs showing the distraction gaps of a lean rat and a ZDF rat. Notice the reduced amount of mineralization in the DO gap of the ZDF rat (original magnification 1.25 \times).

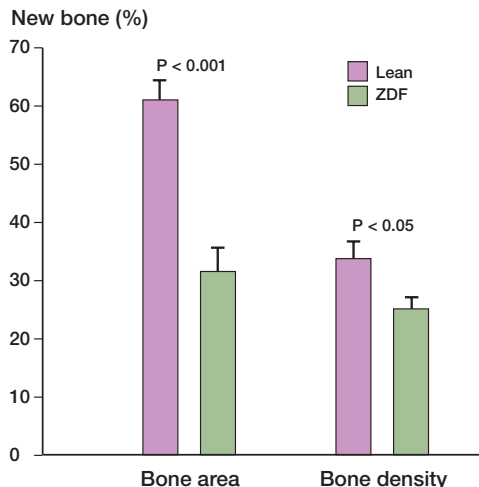


Figure 2b. Quantitative analysis revealed that both the area and relative mineral density of new bone in the distraction gaps of the ZDF rats were significantly reduced compared to those of the lean rats (31% (4.1%) vs. 61% (3.5%), $p < 0.001$) and (25% (1.8%) vs. 34% (2.8%), $p = 0.03$).

percentage of fat cells were increased in the distal bone marrow of the ZDF rats relative to the figures for the lean rats, but only the increase in the percentage of fat cells reached statistical significance

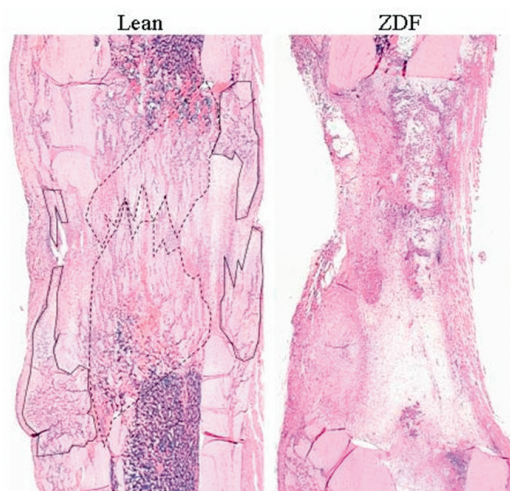


Figure 3a. Representative histological sections of the distraction gaps of a ZDF rat and a lean rat after 14 days of distraction (original magnification 1.25×). The dashed lines delineate the area of endosteal new bone (ENB) and the solid lines delineate the area of periosteal new bone (PNB).

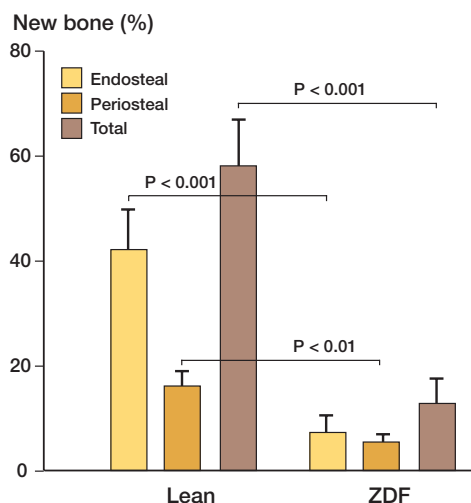


Figure 3b. Quantitative analysis of all histological sections revealed reduction in ENB formation (7.3% (3.3%) vs. 42.2% (7.8%), $p = 0.001$), in PNB formation (5.3% (1.8%) vs. 16.1% (2.9%), $p = 0.008$), and in both ENB and PNB formation ($p < 0.001$) in ZDF rats as compared to lean rats.

Table 1. Number (SD) of adipocytes and total cells in bone marrow

	Proximal marrow			Distal marrow		
	Total cells/mm ²	Fat cells/mm ²	% fat cells	Total cells/mm ²	Fat cells/mm ²	% fat cells
ZDF	15448 (2023)	919 (53)	4.9	14956 (2444)	1203 (267)	9.2
Lean	41605 (1991)	447 (62)	0.9	38218 (1673)	619 (131)	1.7
P-value	<0.001	<0.001	<0.001	<0.001	0.07	0.008

($p = 0.008$). In contrast, the total number of marrow cells was significantly reduced in the proximal ($p < 0.001$) and distal ($p < 0.001$) bone marrow (Figure 1 and Table 1) of the ZDF rats compared to the Zucker lean rats.

Immunohistochemistry

The immunohistochemical stain for PCNA showed different expression patterns of PCNA+ cells in the DO gaps of ZDF rats than in Zucker lean rats. In the FIZ of both groups, PCNA+ cells were scattered and intermixed with PCNA- cells and collagen bundles (Figure 4 A and B). In the PMF of the lean rats, PCNA+ cells were highly organized to form an intensive cellular level in front of the newly formed bone (Figure 4 E). In the PMF of

the ZDF rats, the PCNA+ cells were found to be fewer in number, but remained associated with new osteoid formation (Figure 4 F).

The semiquantitative analyses of cellular proliferation in the distraction gaps of the two groups are summarized in Table 2. The numbers of PCNA+ cells in the FIZ and the PMF were lower in the ZDF rats than in the Zucker lean rats ($p = 0.002$ and $p = 0.02$, respectively). Similarly, the total cellular densities of the FIZ and PMF in the ZDF rats were also lower than those in the Zucker lean rats. However, the difference was significant only in the fibrous interzone ($p = 0.04$). Finally, the percentages of PCNA+ cells in the FIZ and PMF of the ZDF rats were lower than in those of the Zucker lean rats ($p = 0.02$, $p = 0.01$).

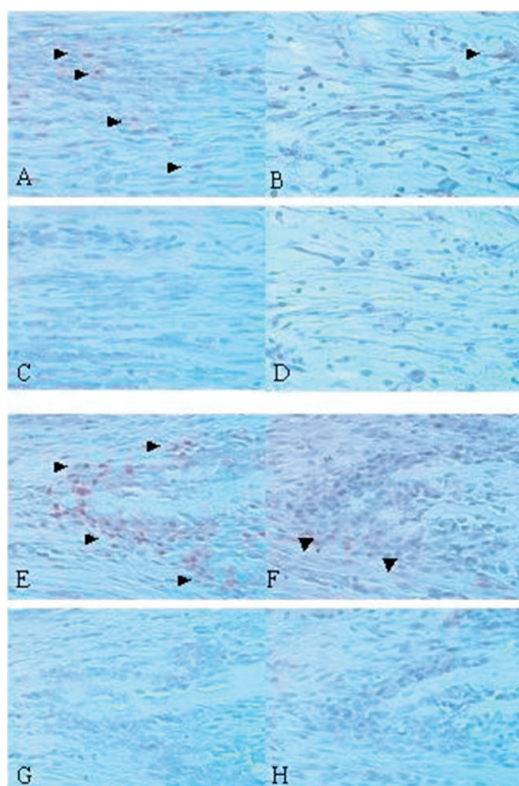


Figure 4. Immunohistochemical detection of PCNA-labeled cells in the distraction gaps of ZDF and lean rats following 14 days of distraction. Higher proportions of PCNA+ cells were found in the fibrous interzone (FIZ) (panel A: lean; panel B: ZDF) and the primary matrix front (PMF) (panel E: lean; panel F: ZDF) of distraction gaps of Zucker lean rats than of ZDF rats. Panels C, D, G, and H show the same areas of the sections incubated with normal mouse IgG instead of the mouse monoclonal antibody, PC-10.

Discussion

The ZDF is an inbred rat model that spontaneously develops type-2 diabetes. This occurs due to an

amino acid substitution at residue 269 in the extracellular domain of the leptin receptor (proline instead of glutamine) which renders it nonfunctional (Chua et al. 1996). All male ZDF rats develop obesity, hyperleptinemia, hyperglycemia, hyperinsulinemia, and insulin resistance when fed a rodent diet of Purina 5008. Recent studies have shown that many diabetic complications are found in ZDF rats, including periodontal disease (Ryan et al. 1999) and diabetic osteopenia (Shibata et al. 2000, Picherit et al. 2003).

Our findings have confirmed that ZDF rats exhibit hyperleptinemia, hyperglycemia, hyperinsulinemia, glycosuria, and obesity between 9–11 weeks of age. We have demonstrated significant decreases in endosteal new bone formation and periosteal new bone formation in and around distraction gaps of the ZDF rats when compared with lean (leptin-sensitive) rats. This correlates at weeks 9–11 with leptin signaling deficiency, insulin resistance, and hyperglycemia—all three of which are candidates for mediating inhibition of bone formation.

In addition, we recorded several observations that correlated with the decrease in new bone formation. (1) Histologically, we noted a reduction in the amount and density of collagen fibers in the FIZ zones in the distraction gaps of ZDF mice relative to the results in control mice. This might reflect either a reduced number of fibroblast-like cells and/or a decrease in matrix formation. A semiquantitative decrease in cell density was noted in the FIZ and PMF zones in the ZDF mice relative to control mice. (2) A semiquantitative decrease in PCNA+ stained cells in the FIZ and PMF zones of the ZDF rats was noted. The PCNA+ cells in the PMF zones of normal rats have been hypothesized to be pre-osteoblasts, based on their molecular

Table 2. PCNA staining in DO gaps. Number (SD)

Group		PCNA+ cells/mm ²	Total cells/mm ²	% of PCNA+ cells
FIZ	Lean	588 (12)	4155 (170)	14.2 (0.32)
	ZDF	67 (66)	2851 (408)	2.9 (2.9)
	T-test	P<0.01	P<0.05	P<0.05
PMF	Lean	1603 (325)	4314 (547)	35.8 (4.4)
	ZDF	654 (157)	3650 (264)	18.3 (4.2)
	T-test	P<0.05	P=0.3	P<0.05

signatures (Sato et al. 1998). In addition, we have unpublished data demonstrating that BrdU-labeled cells co-localize with PCNA+ cells in the PMF and that 24–48 h later BrdU+ cells appear in the tips of the new bone columns in the MCF surrounded by osteoid. This suggests that at least some of the PCNA+ cells in the PMF of the control gaps are pre-osteoblasts. Thus, we hypothesize that the smaller numbers of PCNA+ cells associated with the smaller amount of new bone formation in the ZDF rats are also pre-osteoblasts, but the identity of these cells and of the other fibroblastic PCNA+ cells in the FIZ remains in doubt. (3) Serum osteocalcin levels of the ZDF rats were significantly lower than the control levels. Whether this was due to lower production in the distraction gaps or a general homeostatic reduction in bone formation is not known. (4) The data demonstrated a semiquantitative increase in adipocytes in bone marrow adjacent to the distraction site at day 14 of distraction. The relevance of this observation is unknown, since the adjacent marrow may not communicate with the distraction gap processes during the later days of DO. However, it does allow us to speculate on the question of the possible contribution of marrow adipocytes during the early days (1–7) of DO, when the marrow is contiguous with the newly forming DO gap. Taken together, these results are consistent with the hypothesis that impaired bone formation in the ZDF rats is associated with reduced proliferation and diminished function of osteogenic cells at different stages during DO.

Several studies have indicated that the diabetic osteoporosis in ZDF rats appears to be a “low-turnover” osteoporosis associated with depression of osteoblastic activities. Shibata et al. (2000) have shown that serum osteocalcin levels, a marker of bone formation, and urinary deoxy-pyridinoline (DPD) levels, a marker of bone resorption, in ZDF rats are lower than those in lean rats. In addition, the ZDF rats excreted urinary calcium at high levels compared to lean rats. More recently, Picherit et al. (2003) reported that, at 24 weeks of age, the total, diaphyseal, and metaphyseal femoral bone densities of ZDF rats were significantly lower than those of littermate lean rats while in 12-week old animals, these parameters were no different in the two groups. Similarly, serum osteocalcin levels were

lower in the ZDF rats than in lean controls at both 12 and 24 weeks of age, but the difference was statistically significant only in 24-week-old animals. A recent study has suggested that deficiency in leptin signaling contributes a small negative effect to bone homeostasis, which was detected only at 15 weeks of age while the effects reported here were significant at week 9 (Tamasi et al. 2003). It is possible that the DO protocol challenges the bone formation processes more severely than normal remodeling and would therefore uncover underlying osteoblastogenic deficits earlier.

The mechanisms by which type-2 diabetes inhibits osteoblastogenesis, resulting in impaired bone formation, are unclear. However, several metabolic disturbances postulated to affect bone formation negatively are characteristic of 9-week-old male ZDF rats—including deficiency in leptin signaling, insulin resistance, and hyperglycemia (McCarthy et al. 1999, Zayzafoon et al. 2002, Tamasi et al. 2003). Evidence has accumulated to suggest that in type-2 diabetes, bone formation and/or turnover are impaired due to glucose metabolic and hormonal disturbances that might modulate osteoblastic activities. Several studies have demonstrated that type-2 diabetic osteoporosis is associated with depression of osteoblastic activities, resulting in a decrease in bone formation (Inaba et al. 1999, Takizawa et al. 2003). In *in vitro* studies, elevated extracellular glucose has been shown to change osteoblast phenotype by inhibiting osteocalcin expression (Inaba et al. 1995, Terada et al. 1998, Zayzafoon et al. 2002). Irreversible advanced glycosylation end-products (AGEs) are generated by even sporadic elevations in blood glucose and appear to act through specific receptors (RAGEs) to increase the levels of inflammatory cytokines (tumor necrosis factor- α and interleukin-6), which have been shown to negatively affect the balance of bone formation/resorption. Moreover, recent studies have shown that AGE-specific binding sites are present in osteoblastic-like cells and that AGEs directly inhibit osteoblast proliferation and differentiation in cultured osteoblastic cells from mice and rats (McCarthy et al. 1999). In addition, type-2 diabetic osteoporosis is associated with altered calcium and vitamin D metabolism. Gregorio et al. (1994) have demonstrated that bone mineral content is

reduced in type-2 diabetic patients. Takizawa et al. (2003) have also found diabetic osteopenia in patients with type-2 diabetes. Both groups have found a negative calcium balance caused by renal calcium leak in such diabetic patients. Also, since leptin resistance is a feature of type-2 diabetes and several labs have indicated that leptin may be involved in the process of bone formation (Maor et al. 2002, Tamasi et al. 2003), a skeletal role for leptin in type-2 diabetes is a possibility. Two opposing mechanisms have been suggested: (1) leptin may work locally to promote osteoblastogenesis, and (2) leptin may work through a central hypothalamic relay to produce an osteoblastic inhibitory factor (Ducy et al. 2000). Leptin has also been demonstrated to enhance wound healing of the skin in leptin-deficient diabetic mice (Ring et al. 2000). To date, there have been no studies investigating the role of leptin in bone formation during DO.

As noted previously, we speculate that in comparison to other models, this model may be useful in determining the effects of the systemic candidates and subsequently the changes in local mediators due to the evolution of type-2 diabetes in this model. Several approaches can be suggested: (1) to test for the effects of leptin signaling deficiency alone in 5–7-week-old male ZDF rats and age-matched controls, (2) to test the effects of insulin replacement and/or insulin sensitizers on 9–11-week-old ZDF rats, and (3) to test the effects of exogenous growth factors and/or cytokine blockers. We caution that comparisons between results at different ages would need to take the known age differences into account (Aronson et al. 2001).

In conclusion, our results demonstrate that new bone formation is impaired in 9–11-week-old male ZDF rats and is associated with reduced serum osteocalcin levels, leptin signaling deficiency, insulin resistance, and hyperglycemia. These results are consistent with the results of a recent paper that demonstrated both reduced bone formation and decreased serum osteocalcin levels in the db/db diabetic mouse model, which is the homolog of the ZDF rat model studied here (He et al. 2004). We propose that this model of bone formation in type-2 diabetes will be a useful addition to other models for future mechanistic studies of the underlying deficits in osteoblastogenesis.

Contributions of authors

All authors contributed both intellectually and physically to this report. JA, PAK, JLF, KMT, RCB, CKL: co-wrote the grant to fund the study. ZL, ECW, LL: animal surgery. DSP, GEC, RAS: performed assays and analysis of the study samples.

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