Insulin-dependent diabetes mellitus decreases osteoblastogenesis associated with the inhibition of Wnt signaling through increased expression of Sost and Dkk1 and inhibition of Akt activation

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Abstract. Insulin-dependent diabetes mellitus (IDDM) is known to be associated with an increased risk of osteopenia. However, the cellular and molecular mechanisms for IDDM-induced alterations of the bone are not well understood. The effects of IDDM on bone metabolism were investigated using rats rendered diabetic by an injection of streptozotocin (STZ). After 4 weeks, the diabetic rats exhibited bone loss, low levels of osteocalcin, insulin-like growth factor-I (IGF-I) and bone alkaline phosphatase (ALP) activity with normal levels of bone tartrate-resistant acid phosphatase (TRAP) and cathepsin K activity, and urinary excretion of deoxypyridinoline (Dpd). Histological analysis showed a decrease in the number of osteoblasts with a normal number of osteoclasts in the metaphysis of the proximal tibia. The decreased expression of ALP, osteoclacin and collagen mRNA was associated with a decrease in the expression of runt-related transcription factor 2 (Runx2), Osterix and distal-less homeobox 5 (Dlx5) and an unaltered expression of bone morphogenic protein-2 (BMP2). The protein levels of Runx2, phosphorylated glycogen synthase kinase 3β (GSK3β), active β-catenin and β-catenin decreased. The activation of Akt was inhibited. The mRNA and protein levels of sclerosteosis (Sost) and Dickkopf 1 (Dkk1), inhibitors of Wnt signaling, increased. The mRNA expression of IGF-I and the IGF-I receptor (IGF-IR) was suppressed. These changes observed in the bone of diabetic rats were reversed by treatment with insulin, but not by normalization of the circulating IGF-I levels by treatment with IGF-I. These results suggest that insulin-deficiency in IDDM decreases osteoblastogenesis associated with inhibition of Wnt signaling through the increased expression of Sost and Dkk1 and the inhibition of Akt activation.

Introduction

Insulin-dependent diabetes mellitus (IDDM, type I diabetes) is known to be associated with decreased bone mass, osteoporosis, and increased fracture rates (1-3). Streptozotocin (STZ)-induced diabetes in rats is a well-recognized model for IDDM (4,5). In these rats, bone histology and biochemical markers of bone formation and resorption indicate decreased osteoblastic activity with normal (5), decreased (6,7), or increased (8-10) osteoclastic activity. Although these observational studies have provided consistent findings regarding deficits in bone integrity attributable to the diabetic state, little is known about the specific cellular and molecular mechanisms underlying the changes in bone metabolism in IDDM.

Bone formation is a tightly regulated process characterized by a sequence of events starting with the commitment of osteoprogenitor cells, and their differentiation into pre-osteoblasts and then into mature osteoblasts, which express high levels of alkaline phosphatase (ALP), osteocalcin and collagen. Osteoblast commitment, differentiation, and function are all governed by several transcription factors, resulting in the expression of phenotypic genes and in the acquisition of the osteoblast phenotype (11). The factors capable of initiating osteoblastogenesis from uncommitted progenitors are the bone morphogenetic proteins (BMPs) (12). BMPs stimulate the transcription of runt-related transcription factor 2 (Runx2), a gene encoding an osteoblast-specific transcription factor, (13). Runx2 is essential for the differentiation of mesenchymal cells into osteoblasts and inhibits differentiation into adipocytes and chondrocytes (14). Osterix (Osx) acts downstream of Runx2 to induce osteoblastic differentiation in bipotential chondro-osteoprogenitor cells, directing the pre-osteoblasts to become immature osteoblasts (15). BMP-2 also induces the

Table I. Primers used for quantitative real-time PCR.

Target	Forward primer sequence	Reverse primer sequence
Actin	AGCCATGTACGTAGCCATCCA	TCTCCGGAGTCCATCACAATG
ALP	TCCCAAAGGCTTCTTCTTGC	ATGGCCTCATCCATCTCCAC
BMP2	TGAACACAGCTGGTCTCAGG	ACCCCACATCACTGAAGTCC
β-catenin	CATCACCACGCTGCATAATC	GAGCTTGCTTTCCTGATTGC
Col1α1	GCCTCCCAGAACATCACCTA	GCAGGGACTTCTTGAGGTTG
Dkk1	AATCGAGGAAGGCATCATTG	CTGTGCTTGGTGCATACCTG
Dlx5	TGACAGAAGAGTCCCAAGCA	GCTTTGCGGTAAGAAGCAGA
GSK3β	CAAGCAGACACTCCCTGTGA	GTGGCTCCAAAGATCAGCTC
IGF-I	CAGTTCGTGTGTGGACCAAG	TCAGCGGAGCACAGTACATC
IGF-IR	GTGCTGTACGCCTCTGTGAA	TTGCAGCCTCATTCACTGTC
InsR	ACATTGCCCTGAAGACCAAC	AACAGCATGAATCCCAGGAG
LRP5	CTGTCATTGGCATCATCCTG	CACCAGGCTCACAGAACTCA
Osteocalcin	AATAGACTCCGGCGCTACCT	GAGCTCACACACCTCCCTGT
Osx	AAGGCAGTTGGCAATAGTGG	TGAATGGGCTTCTTCCTCAG
Runx2	GCCGGGAATGATGAGAACTA	TGGGGAGGATTTGTGAAGAC
Sost	CCTTCAAGAATGATGCCACA	ACTCGGACACGTCTTTGGTG
Wnt3a	AACGACCATCCTGGATCAC	GTGGGTGGCTTAAAGAGTG

expression of the homeobox-containing gene, distal-less 5 (Dlx5), which is a common upstream regulator for both Runx2 and Osx (16,17).

Recently, canonical Wnt signaling has been shown to play a significant role in the control of osteoblastogenesis and bone formation (18). The signaling involves the formation of a complex between Wnt protein, frizzled, and low-density lipoprotein receptor-related protein (LRP) 5 or 6 receptors. These complexes promote the phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β), inhibition of β-catenin's degradation, and subsequent cytoplasmic accumulation and translocation of β-catenin to the nucleus. The nuclear β-catenin interacts with transcription factors and regulates the expression of certain transcription factors such as Runx2, Osx and Dlx5, and strongly stimulates osteoblastogenesis (19). Wnt signal transduction through β-catenin is thought to require the inhibition of GSK3β. On the other hand, Wnt signaling is inhibited by Sclerostin, the product of the sclerosteosis (Sost) or Dickkopf (Dkk) genes. Sost or Dkk inhibit the formation of the complex between Wnt protein, frizzled and LRP5/6 by binding to LRP5/6 (20-22), resulting in the inhibition of GSK3β phosphorylation and the subsequent degradation of β-catenin. In addition to Wnt signaling, GSK3β is inhibited via phosphorylation by Akt upon insulin or insulin-like growth factor-I (IGF-I) signaling. Insulin and IGF-I, through their receptors, insulin receptor (IR) and IGF-I receptor (IGF-IR), respectively, share common components in their signal transduction pathways, IRS-1/PI3K/Akt/GSK3β.

Rats with STZ-induced diabetes show low circulating levels of IGF-I as well as insulin (23-25). In IDDM children, inadequate control of blood sugar levels correlates with low plasma IGF-I levels and sluggish growth (26). Locally and systemically delivered IGF-I produced significant increases in new bone formed in aged mice (27). These results suggest the involvement of IGF-I as well as insulin in diabetic bone loss through inhibition of Wnt activation via Akt/GSK3β.

In this study, we investigated the effect of IDDM on bone metabolism and osteoblastogenesis, especially on the Wnt signaling pathway, using diabetic rats at 4 weeks after the injection of STZ. The decreased osteoblastic activity in IDDM was shown to be associated with decreased osteoblastogenesis through the inhibition of Wnt/LRP5/6 receptor-dependent and Akt-dependent activation of β-catenin signaling.

Materials and methods

Animals and study design. Ten-week-old female rats of the Wistar/ST strain were purchased from Japan SLC (Shizuoka, Japan) and housed individually in a temperature-controlled room with a 12-h light cycle. The animals were allowed free access to a standard diet (AIN 76A; Research Diets, Inc., NJ) and water which were provided fresh daily. After an overnight fast, rats were divided into a control group (n=8) and a diabetic group (n=24). Experimental diabetes was induced by a single intraperitoneal injection of STZ (30 mg/kg body weight in 0.05 M citrate buffer, pH 4.5), a pancreatic β-cell cytotoxin. Control animals received the same volume of the STZ diluent. Diabetes was confirmed by the development of glycosuria and hyperglycemia (non-fasting blood glucose >300 mg/dl). All rats subjected to the STZ-injection were rendered diabetic. Forty-eight hours post STZ-injection, the diabetic rats were further divided into three groups, an untreated group (Diabetes), an insulin-treated group (Insulin), and an IGF-I-treated group (IGF-I). The latter two groups were treated with insulin (from bovine pancreas, Sigma -Aldrich; 1.6 U/day) or IGF-I (from human recombinant, Bachem, Inc.; 50 ng/day) by continuous infusion through a subcutaneously implanted osmotic pump (Alzet 2002, DURECT Corp., Cupertino, CA). At 4 weeks after

Table II. Clinical characteristics.

	Control	Diabetes	Insulin	IGF-I
Serum				
Glucose (mg/dl)	161.9±5.9	638.3±26.9[a]	309.8±7.8[a,b]	611.2±27.8[a]
Insulin (ng/ml)	5.74±0.40	0.09±0.02[a]	2.86±0.37[a,b]	0.08±0.02[a]
PTH (pg/ml)	78.9±14.4	77.0±10.7	72.1±9.9	80.0±11.8
IGF-I (mg/ml)	2.09±0.23	0.62±0.17[a]	1.93±0.16[b]	1.90±0.16[b]
Body weight (g)				
Start (before fasting)	203.6±1.6	203.1±1.9	203.5±2.1	202.7±1.7
Before the injection	191.3±1.6	192.1±2.2	193.2±1.6	190.6±1.7
Final	249.4±3.5	188.1±3.6[a]	232.6±4.5[a,b]	182.4±5.4[a]
Bone length (mm)				
Femur	34.1±0.4	32.6±0.2[a]	33.3±0.3[b]	32.3±0.2[a]
Tibia	37.7±0.2	35.9±0.2[a]	37.4±0.2[b]	36.5±0.2[a]
Bone weight (g)				
Femur	0.710±0.013	0.632±0.005[a]	0.679±0.013[b]	0.618±0.010[a]
Tibia	0.536±0.014	0.490±0.005[a]	0.531±0.003[b]	0.484±0.005[a]
Proximal tibia	0.260±0.008	0.205±0.002[a]	0.248±0.010[b]	0.201±0.011[a]

Values are the mean ± SEM from 8 rats. [a]Significantly different from the control value (P<0.05). [b]Significantly different from the diabetes value (P<0.05).

the injection of STZ or the diluent, blood and the femoral and tibial bones were collected under anesthesia with sodium pentobarbital (25 mg/kg bw) after overnight access to food (non-fasting). Urine was collected over 24 h to determine the amount of deoxypyridinoline (Dpd) excreted. Serum samples were used to determine the concentrations of glucose, insulin, parathyroid hormone (PTH), IGF-I, and osteocalcin. After the removal of muscle and tendons, the tibia was used for biochemical and histological analyses. Animal experiments were performed in accordance with protocols approved by the Animal Care Research Committee of Nara Women's University.

Biochemical analysis. The bone of the proximal tibia (the quarter from the knee aspect of the tibia) was homogenized and the bone extract was prepared as described (28). The activities of ALP, TRAP, and cathepsin K of the bone extract and the amounts of Ca and hydroxyproline (Hyp) in the proximal tibia were determined as previously described (8).

Serum levels of glucose, insulin, PTH, IGF-I and osteocalcin and the urinary concentration of Dpd were measured using a Glucose C-II-test Wako (Wako Diagnostics, Osaka, Japan), an Ultra Sensitive Rat Insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Japan), a Rat Intact PTH ELISA kit (Immunotopics, San Clemente, CA), a Mouse/Rat IGF-I Quantikine ELISA kit (R&D Systems, Minneapolis, MN), a Rat Osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA), and a Pyrilinks-D assay kit (Metra Biosystems, Palo Alto, CA), respectively.

Histological analysis. The tibia was fixed in 4% para-formaldehyde, decalcified in 10% EDTA, and embedded in paraffin. Sections (4 μm) were stained for TRAP activity using a leukocyte acid phosphatase kit (Sigma 387-A) as previously described (28). Morphometric measurements of trabecular structure (trabecular bone volume, bone surface, thickness, and number), and the number of osteoblasts (cuboidal cells on the trabecular surfaces) and osteoclasts (TRAP-stained cells with more than 3 nuclei) were carried out at standardized sites (300x300 μm) under growth plates in the metaphysis of the proximal tibia (29).

Quantitative real-time PCR. Total-RNA from the proximal tibia was prepared using a commercial kit ('NucleoSpin RNA II kit', Macherey-Nagel, France) after washing out bone marrow cells and homogenizing in the presence of 0.1 M EDTA. The total-RNA was reverse transcribed with a first-strand cDNA synthesis kit (Toyobo, Tokyo, Japan). Real-time PCR was performed using the cDNA, or total-RNA for the negative control, with SYBR-Green real-time PCR Master Mix plus (Toyobo) and specific primers (Table I) using a Light Cycler real-time PCR detection system (Toyobo) as previously described (9). Levels of gene expression were determined relative to an internal standard (actin).

Western blot analysis. An equal amount of the bone extract from each rat was electrophoresed in SDS-polyacrylamide gels and transferred to membranes. The membranes were blocked, incubated with the corresponding antibodies, and detected with the enhanced chemiluminescense system (ECL; Amersham) after incubation with a secondary antibody as described (9). For reprobing, membranes were stripped with STRIP reagent (Nacalai Tesque, Inc., Japan) according to the manufacturer's directions. Antibodies recognizing actin (H-300), Runx2 (M-70), Ser9 phospho GSK3β, GSK3β (H-76),

Table III. Effect of diabetes on bone biochemical markers.

	Control	Diabetes	Insulin	IGF-I
Proximal tibia				
ALP activity (U/g)	16.48±0.44	10.57±0.68[a]	17.91±0.69[b]	9.23±1.67[a]
TRAP activity (U/g)	0.312±0.013	0.295±0.012	0.294±0.027	0.271±0.043
Cathepsin K activity (U/g)	605.2±48.8	593.4±43.9	567.0±32.6	585.8±40.9
Ca (mg/g)	137.3±6.7	100.9±5.8[a]	144.2±3.9[b]	107.5±7.8[a]
Hydroxyproline (mol/g)	113.7±5.9	78.4±0.5[a]	121.5±6.5[b]	80.4±3.1[a]
Serum osteocalcin (ng/ml)	9.80±0.36	1.24±0.04[a]	9.21±0.43[b]	1.86±0.39[a]
Urinary Dpd (nmol/mM Cr)	174.6±9.9	191.9±22.6	185.8±10.7	191.8±13.1

Values are the mean ± SEM from 8 rats. [a]Significantly different from the control value ($P<0.05$). [b]Significantly different from the diabetes value ($P<0.05$).

β-catenin (H-102) and Dkk1 (H-120) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Akt, anti-Ser473 phospho-Akt and anti-IGF-I-R antibody were obtained from Cell Signaling Technology (Hitchin, UK). The anti-Sost and anti-active-β-catenin antibody were from R&D Systems and Upstate Signaling (Dundee, Scotland, UK), respectively. The equal loading of protein samples was confirmed by the bicinchoninic acid protein assay and the reprobing with the actin antibody. The molecular sizes of the developed proteins were determined by comparison with pre-stained markers (New England Biolabs).

Statistical analysis. Data are presented as the mean ± SEM All statistical analyses were performed with a one-way analysis of variance with pairwise comparison by the Bonferroni method using the Microsoft Excel data analysis program. Values of $P<0.05$ were considered statistically significant.

Results

Clinical characteristics. The injection of STZ increased the serum glucose level to over 500 mg/dl and decreased the serum insulin level (Table II). The hyperglycemia and hypoinsulinemia indicated that diabetes was induced in the STZ-injected animals. The serum IGF-I level in the diabetic rats decreased to about 30% of the control value, while a similar concentration of PTH was observed. Treatment with insulin significantly restored the serum levels of glucose, insulin and IGF-I, but glucose and insulin levels were still significantly different from the control values. The treatment with IGF-I did not recover these changes except the serum IGF-I level.

Body weight and the length and weight of the femur and tibia were significantly lower in the diabetic rats than the control. The administration of insulin, but not IGF-I, to diabetic animals restored these parameters to levels not significantly different from the control values, although body weight was still significantly lower than the control.

Bone biochemical markers. The ALP activity in the proximal tibia and serum level of osteocalcin in the diabetic group
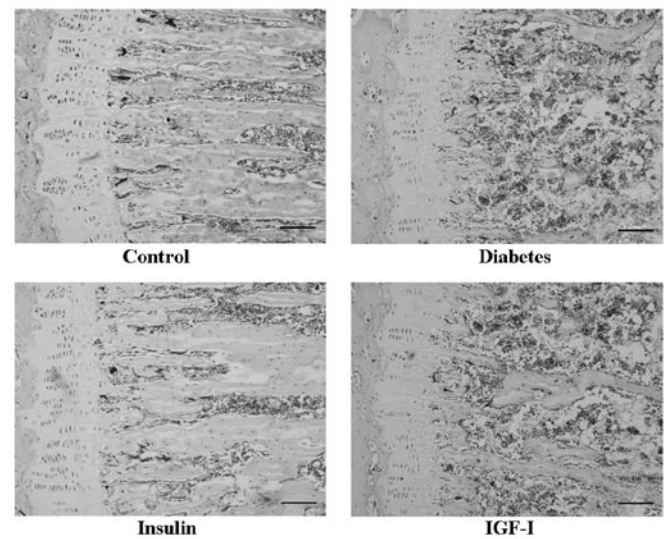

Figure 1. TRAP staining of the proximal tibia in control, diabetic, insulin-treated diabetic and IGF-I-treated diabetic rats. Paraformaldehyde-fixed, decalcified, and paraffin-embedded tibiae obtained from control, diabetic, insulin-treated diabetic (Insulin), and IGF-I-treated diabetic (IGF-I) rats were processed for TRAP staining as described in Materials and methods. The results presented here are typical of 4 rats of each group. TRAP-positive cells appear red. Hematoxylin counterstaining. x200 magnification. Scale bar, 100 μm.

decreased significantly to about 60 and 10% of the control value, respectively (Table III). The activities of TRAP and cathepsin K in the diabetic group were similar to the control levels. The urinary amounts of Dpd in the diabetic rats were also similar to those in the controls. The amounts of Ca and hydroxyproline in the diabetic rats decreased significantly to about 70% of the control levels. The injection of insulin restored these levels to the control values. However, the treatment with IGF-I did not reverse these changes at all.

Histological analysis. Trabecular bone volume, surface, thickness and number were significantly reduced in the diabetic rats (Fig. 1 and Table IV). The numbers of osteoblasts were significantly decreased to about 60% of the control values, although no significant difference was observed in the

Table IV. Bone histomorphometry.

	Control	Diabetes	Insulin	IGF-I
Trabecular bone volume (%)	64.0±2.7	21.2±3.4[a]	60.0±2.8[b]	18.6±2.4[a]
Trabecular bone surface (mm/mm²)	36.7±1.6	23.2±1.9[a]	37.1±1.6[b]	24.4±3.1[a]
Trabecular thickness (μm)	35.5±2.9	18.0±0.2[a]	32.7±1.6[b]	18.5±3.1[a]
Trabecular number/mm	18.3±0.8	11.6±0.9[a]	18.5±0.8[b]	10.4±1.0[a]
Osteoblast index (N of Ob/mm trabecular bone length)	36.2±1.6	20.1±1.2[a]	36.2±2.3[b]	21.6±3.4[a]
Osteoclast index (N of Oc/mm trabecular bone length)	5.81±0.71	6.10±0.64	5.97±0.35	6.19±0.53

Values are the mean ± SEM from 8 rats. [a]Significantly different from the control value (P<0.05). [b]Significantly different from the diabetes value (P<0.05). N, number; Ob, osteoblast; Oc, osteoclast.
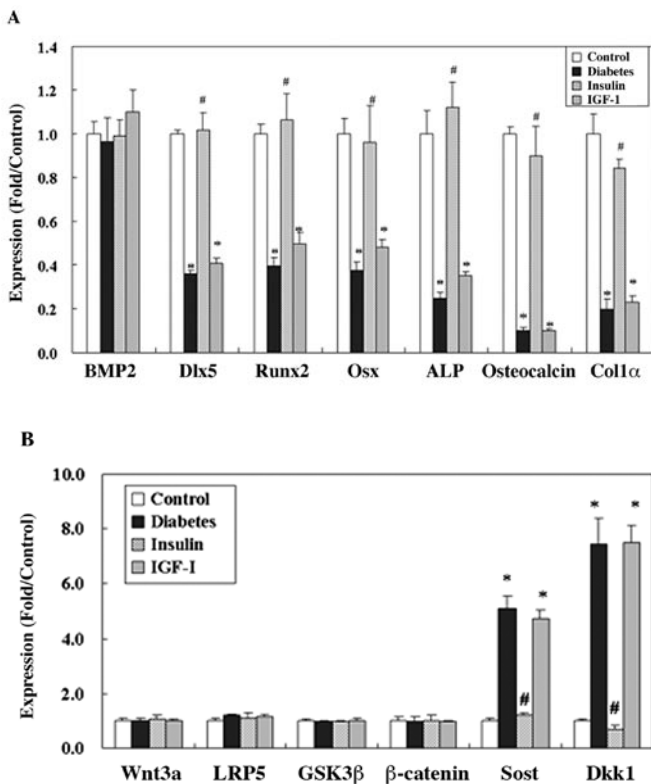

Figure 2. Expression of genes involved in osteoblastic differentiation in the proximal tibia. Total-RNA was extracted from the proximal tibia of control, diabetic, insulin-treated diabetic, and IGF-I-treated diabetic rats and the mRNA levels of (A) BMP2, Dlx5, Runx2, Osx, ALP, osteocalcin, Col1α, (B) Wnt3a, LRP5, GSK3β, β-catenin, Sost and Dkk1 were assessed by quantitative real-time PCR as described in Materials and methods. Values are the mean ± SEM from 8 rats. Levels are expressed relative to the control value. *Significantly different from the control value (P<0.05). #Significantly different from the diabetic group (P<0.05).

numbers of osteoclasts. The treatment with insulin, but not IGF-I, restored the trabecular bone index and the numbers of osteoblasts to control levels.

Expression of genes involved in osteoblastic differentiation in the proximal tibia. The gene expression levels of the osteo-blastogenesis-related factors, BMP2, Dlx5, Runx2 and Osx, and osteoblast-specific proteins, ALP, osteocalcin and Col1α, are shown in Fig. 2A. The expression of BMP2 mRNA in diabetic rats was similar to the control level. However, the levels of Dlx5, Runx2 and Osx, were significantly decreased to about 34, 40 and 38% of the control value, respectively. The mRNA levels of ALP, osteocalcin and Col1α significantly decreased to about 25, 10 and 20% of the control level, respectively. The expression levels of Wnt3a, LRP5, GSK3β, and β-catenin were unchanged (Fig. 2B). However, the expression of Wnt-signaling inhibitors, Sost and Dkk1, significantly increased to about 5- and 7-fold the control level, respectively. The altered expression of the genes observed in the diabetic rats was recovered by the injection of insulin, but not IGF-I.

The protein levels of Runx2, β-catenin, Akt, GSK-3β, Sost and Dkk1 in the proximal tibia. The results of the Western blot analysis are shown in Fig. 3. The levels of Runx2 and β-catenin, decreased to about 15% of the control value. The active β-catenin was undetectable in diabetic rats. The phosphorylated GSK3β decreased to 40% of the control level and the phosphorylated Akt was barely detectable, although the protein levels of GSK3β and Akt were unchanged. The levels of Sost and Dkk1 protein in diabetic rats increased to about 4- and 5-fold the control value, respectively. These changes were reversed by treatment with insulin, but not IGF-I.

The levels of InsR, IGF-I, and IGF-IR mRNA and IGF-IR protein in the proximal tibia. The mRNA levels of IGF-I and IGF-IR in the proximal tibia of diabetic rats decreased to about 20 and 60% of the control value, respectively, although InsR expression was unaltered (Fig. 4A). The IGF-IR protein decreased to 40% of the control value (Fig. 4B). These decreased levels recovered to the control values on the injection of insulin, but not on treatment with IGF-I.

Discussion

This study clearly demonstrated that diabetes decreased osteoblastogenesis through the inhibition of Wnt signaling. The markers of osteoblastic activity, bone ALP activity and
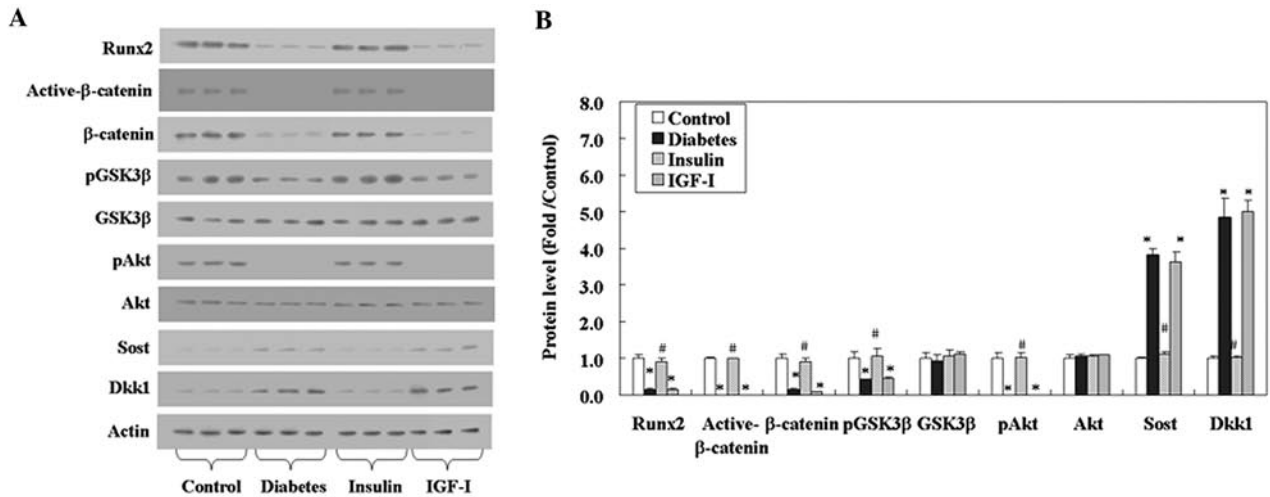
Figure 3. Protein levels of Runx2, β-catenin, GSK-3β, Akt, Sost and Dkk1 in the proximal tibia. (A) Western blot analyses. Bone extracts from the proximal tibiae of control, diabetic, insulin-treated diabetic (Insulin), and IGF-I-treated diabetic (IGF-I) rats were resolved by SDS-PAGE. After transfer, the blot was probed or reprobed with antibody and detected by ECL as described in Materials and methods. (B) The protein levels were quantified by densitometry and represented graphically. Data represent the mean ± SEM from 8 rats. *Significantly different from the control value (P<0.05). #Significantly different from the diabetic group (P<0.05).
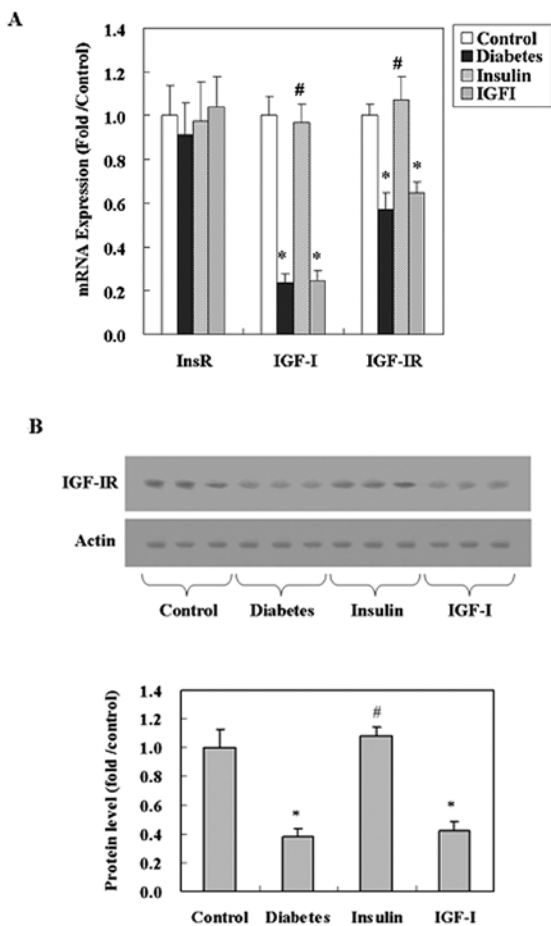

Figure 4. Levels of insulin receptor (IR), IGF-I, and IGF-IR mRNA and IGF-IR protein in the proximal tibia. (A) The expression of IR, IGF-I and IGF-IR in the proximal tibia was assessed by quantitative real-time PCR as described in Materials and methods. Expression levels are expressed relative to the control value. (B) Western blot analysis of IGF-IR. The protein levels of IGF-IR were quantified by densitometry and represented graphically. Values are the mean ± SEM from 8 rats. *Significantly different from the control value (P<0.05). #Significantly different from the diabetic group (P<0.05).

serum osteocalcin levels, decreased without a change in osteoclastic activity in the diabetic rats at 4 weeks. Data from our laboratory have shown an increase in the osteoclastic activity and in the number of osteoclasts in short-term diabetes (at 1 and 2 weeks after the injection of STZ) (8,10). However, in the model of long-term (4 weeks) diabetes used here, the diabetic rats showed normal activities and numbers of osteo-clasts. These results suggest that the bone resorptive activity fluctuates during the diabetic term, increasing early on and normalizing later. The controversy over the effects of diabetic mellitus on bone resorptive activity may be explained by the duration of diabetes. However, a decrease in osteoblastic activity was observed in the long-term as well as short-term diabetes. The decrease in osteoblastic activity was found to be associated with decreases in the number of osteoblasts and osteoblastogenesis. In osteoblastic differentiation, Runx2 is considered a 'master regulator', and its expression is essential for normal bone formation (30). Previous studies have presented conflicting data regarding Runx2 expression, suggesting a reduction (31-33) or no change (5,34) in skeletal tissues of diabetic animals. Our study clearly showed a decrease in the mRNA and protein levels of Runx2 in diabetic bone. Consistent with the reduction in Runx2, the expression of the other BMP-induced transcription factors, Dlx5 and Osx, was also suppressed in diabetic rats. However, consistent with previous reports (5,34), the expression of BMP2, a critical regulator of osteoblastogenesis, was not altered. These results suggest that Runx2 was suppressed by a BMP2-independent mechanism.

The Wnt pathway, through the elaborate interaction of various components, regulates the expression of certain transcription factors, such as Runx2, Osx and Dlx, and strongly stimulates osteoblastogenesis (18,19). A deficiency of insulin in IDDM is considered to lead to decreased phosphorylation of GSK3β through inhibition of the signaling pathway IRS-1/ PI3K/Akt and to reduced levels of β-catenin, the key mediator of the Wnt pathway. Indeed, the present study has shown the

inhibition of Akt activation, and that the decreased levels of phosphorylated Akt are associated with the decreased levels of phosphorylated GSK3β, β-catenin and active β-catenin in the proximal tibia of diabetic rats. The decreased expression of Runx2 may be explained by the lower level of β-catenin through the inhibition of Akt phosphorylation by insulin deficiency, suggesting that Runx2 is suppressed via an Akt-dependent mechanism.

The insulin and IGF-I signaling pathways share the components, IRS-1/PI3K/Akt/GSK3β. The serum concentration of IGF-I and the expression levels of IGF-I and IGF-IR in the bone decreased in diabetic rats. The reduced levels of IGF-I and IGF-IR, in addition to insulin-deficiency, would contribute to inhibition of the phosphorylation of Akt and subsequent decreases in phosphorylated GSK3β and β-catenin proteins. To examine the contribution of IGF-I to the inhibition of Akt activation and GSK3β phosphorylation, diabetic rats were treated with IGF-I. The treatment did not restore the phosphorylated levels of Akt and GSK3β or the β-catenin protein levels, although the serum concentration of IGF-I returned to the control level. Systemic IGF-I is synthesized primarily in the liver, but IGF-I is also produced in multiple extrahepatic tissues including the bone, where it acts locally as an autocrine/paracrine growth factor (35,36). Although debate remains as to the relative roles of systemic versus local IGF-I in bone formation, both of IGF-I produced significant increases in new bone formation (27). However, in this study, the restoration of systemic IGF-I level did not affect the expression levels of IGF-IR and IGF-I in the bone, or the osteoblastic activity and osteoblastogenesis of diabetic rats. These results indicate that the normalization of circulating IGF-I levels did not have a favorable effect on the bone in IDDM.

Unexpectedly, increased levels of mRNA and protein for Sost and Dkk1 were observed in the bone of the diabetic rats. Although the regulatory mechanism for the expression of Sost and Dkk1 remains unknown, Sost and Dkk1 inhibit the Wnt pathway by binding to LRP5 and LRP6 (20,22,37-39). The inhibition of the formation of the complex between Wnt protein, frizzled and LRP5 or LRP6 receptors leads to inhibition of the phosphorylation and inactivation of GSK3β and subsequently results in the destruction of β-catenin. The increased expression of Sost and Dkk1 as well as the inhibition of Akt activation may contribute to the reduced level of β-catenin through GSK3β. These results suggest that osteoblastogenesis in diabetic rats is suppressed by the inhibition of Wnt/LRP5/6 receptor-dependent activation of β-catenin signaling.

This study has provided evidence that IDDM decreases osteoblastogenesis associated with inhibition of the Wnt signaling through the increased expression of Sost and Dkk1 and inhibition of Akt activation. Further study of the relation of genes involved in osteoblastogenesis is needed for the development of better treatments for diabetic patients.

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