

# Increased expression of the receptor for activation of NF- $\kappa$ B and decreased runt-related transcription factor 2 expression in bone of rats with streptozotocin-induced diabetes

MAMIKO HIE and IKUYO TSUKAMOTO

Department of Food Science and Nutrition, Nara Women's University, Kita-uoya nishi-machi, Nara 630-8506, Japan

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**Abstract.** Insulin-dependent diabetes mellitus (IDDM) is associated with an increased risk of osteopenia/osteoporosis in humans. The effects of IDDM on osteoblastogenesis and osteoclastogenesis were investigated using diabetic rats at 2 weeks after the streptozotocin (STZ) injection. The weight of the tibia and proximal tibia and the amount of hydroxyproline and calcium in the proximal tibia were significantly lower in diabetic rats than control rats. Markers of bone formation, alkaline phosphatase (ALP) activity and the number of osteoblasts in the proximal tibia and the serum osteocalcin level, were significantly lower. Markers of bone resorption, activity of tartrate-resistant acid phosphatase (TRAP) and cathepsin K and the number of osteoclasts in the proximal tibia and urinary excretion of deoxypyridinoline, were higher in diabetic rats than control rats. mRNA levels of receptor for activation of NF- $\kappa$ B (RANK), c-fos, c-jun, TRAP and cathepsin K were significantly increased in diabetic rats, although RANK ligand, osteoprotegerin, macrophage colony-stimulating factor and c-fms levels were similar to the control value. The decreased expression of ALP, osteocalcin and collagen mRNA in diabetic rats was associated with decreases in the expression of Runx2, Dlx5 and osterix and an unaltered expression of bone morphogenetic protein-2. The level of RANK protein increased and Runx2 protein decreased in diabetic rats. These changes in the bone of STZ-induced diabetic rats were reversed by insulin-

treatment. These suggested that short-term IDDM induced upregulation of osteoclastogenesis with an increase in RANK and downregulation of osteoblastogenesis with a decrease in Runx2 in bone.

## Introduction

The coexistence of diabetes mellitus and altered bone and mineral metabolism has been established by a number of investigations both in diabetic patients and in animals with experimentally induced insulin deficiency syndromes (1-3). STZ-induced diabetes in rats is a well-recognized model for insulin-dependent diabetes mellitus (IDDM) (4,5). Bone histology and biochemical markers of bone formation and resorption in diabetic animal models indicate decreased osteoblastic activity with normal animals (5) or decreased osteoclastic activity (6,7). However, data from our laboratory examining bone metabolism in animal models of IDDM have clearly demonstrated an increase in bone resorption as well as a decrease in bone formation at 1 or 2 weeks after STZ injection (8,9). 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 the bone formation and bone resorption in IDDM.

Bone resorption is carried out by hematopoietically derived osteoclasts (10), whose number and activity are determined by cell lineage allocation, proliferation and differentiation of osteoclast precursors, and resorptive efficacy of mature osteoclasts (11). Osteoclastic differentiation which requires macrophage colony-stimulating factor (M-CSF) and receptor for activation of NF- $\kappa$ B ligand (RANKL) is a multi-step process that eventually leads to the expression of tartrate-resistant acid phosphatase (TRAP), multinucleation, and bone-resorbing activity (12). The binding of M-CSF to c-fms stimulates the expression of RANK in the hematopoietic osteoclast precursor cells. The binding of RANKL to its receptor RANK activates NF- $\kappa$ B and activator protein-1 (AP-1) and induces osteoclastic differentiation (12). In IDDM, change in expression of these osteoclastogenesis-related genes remains unclear.

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 expressing high levels of alkaline phosphatase (ALP), osteocalcin, and collagen.

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*Correspondence to:* Professor Ikuyo Tsukamoto, Department of Food Science and Nutrition, Nara Women's University, Kita-uoya nishi-machi, Nara 630-8506, Japan  
E-mail: itsuka@cc.nara-wu.ac.jp

*Abbreviations:* ALP, alkaline phosphatase; AP-1, activator protein-1; BMP, bone morphogenetic protein; Col1 $\alpha$ 1, collagen type I  $\alpha$ 1; Dlx5, distal-less homeobox 5; IDDM, insulin-dependent diabetes mellitus; M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; Osx, osterix; RANK, receptor for activation of NF- $\kappa$ B; RANKL, receptor for activation of NF- $\kappa$ B ligand; Runx2, runt-related transcription factor 2; STZ, streptozotocin; TRAP, tartrate-resistant acid phosphatase

*Key words:* diabetes mellitus, receptor for activation of nuclear factor- $\kappa$ B, runt-related transcription factor 2, osteoclastogenesis, osteoblastogenesis

Osteoblast commitment, differentiation and function are all governed by several transcription factors, resulting in the expression of phenotypic genes and acquisition of the osteoblast phenotype (13). The only factors capable of initiating osteoblastogenesis from uncommitted progenitors are bone morphogenetic proteins (BMPs) (14). BMPs stimulate the transcription of the gene encoding an osteoblast-specific transcription factor, runt-related transcription factor 2 (Runx2) (15). Runx2 is essential for the differentiation of mesenchymal cells into osteoblasts and inhibits differentiation into adipocytes and chondrocytes (16). Osterix (Osx) acts downstream of Runx2 to induce osteoblastic differentiation in bipotential chondro-osteoprogenitor cells, directing the pre-osteoblasts towards becoming immature osteoblasts (11). BMP-2 also induces the expression of a homeobox-containing gene, distal-less 5 (Dlx5) (17). Dlx5 has been shown to be a direct transcriptional activator of Runx2 *in vitro* by binding to its P1 promoter which regulates transcription of the Runx2-II isoform (18). Dlx5 regulates the expression of osteoblast-specific genes such as osteocalcin and ALP, as well as mineralization (19). Twist-1 and Twist-2 inhibit osteoblastic differentiation by inhibiting Runx2 function (20). However, the regulation of these genes involved in osteoblastic differentiation has not been fully elucidated in IDDM.

In this study, we corroborated previous findings that IDDM increased bone resorption and decreased bone formation at the early stage of STZ-induced diabetic rats. Next, we investigated the effect of IDDM on osteoblastogenesis and osteoclastogenesis by measuring the gene expression in bone. We demonstrated that IDDM at the early stage induced an increase in RANK expression and a decrease in Runx2 expression in bone.

## 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. Rats were allowed free access to a standard diet (AIN 76A; Research Diets Inc., NJ) and water, both which were provided fresh daily. After an overnight fast, rats were divided into a control group (n=8) and a diabetic group (n=16). Experimental diabetes was induced by a single intraperitoneal injection of streptozotocin [STZ, 30 mg/kg body weight (bw) in 0.05 M citrate buffer, pH 4.5], a pancreatic  $\beta$ -cell cytotoxin. Control animals received the same volume of the STZ diluent. Diabetes was confirmed by the development of glycosuria and hyperglycemia [blood glucose (non-fasting) >300 mg/dl]. All rats subjected to STZ injection were rendered diabetic. After 48 h from STZ injection, diabetic rats were further divided into untreated group (Diabetes) and insulin-treated group (Insulin). The latter group was treated daily with insulin (8 U/kg bw). At 14 days after injection of STZ or diluent, blood and the femoral and tibial bones were collected under anaesthesia with sodium pentobarbital (25 mg/kg bw) after overnight access to food (non-fasting). Urine was collected over 24 h to determine the level of deoxypyridinoline. Sera were used to determine the concentrations of glucose, insulin, parathyroid hormone (PTH), osteocalcin, Ca, and P. 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 serum glucose, Ca and P concentration were assayed by the mutarotase-glucose oxidase method, 0-cresolphthalein complexone and p-methylaminophenol reduction methods, respectively, using commercial kits (Wako Diagnostic, Osaka, Japan). Serum levels of insulin, PTH and osteocalcin and the urinary concentration of deoxypyridinoline were measured using an Ultra Sensitive Rat Insulin ELISA kit (Morinaga Institute of Biological Science, Japan), Rat intact PTH ELISA kit (Immunotopics, San Clemente, CA), Rat osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA), and Pylrilinks-D assay kit (Metra Biosystems, Palo Alto, CA), respectively.

The bone of the proximal tibia (the quarter from the aspect of the knee of the tibia) was homogenized and bone extract was prepared as described (21). Activity of ALP, TRAP and cathepsin K and amounts of Ca and hydroxyproline (Hyp) in proximal tibia were determined as reported previously (8).

**Histomorphometric analysis.** The tibia was fixed in 4% paraformaldehyde, decalcified in 10% EDTA, and embedded in paraffin. Sections (4  $\mu$ m) were stained for TRAP activity using a leukocyte acid phosphatase kit (sigma 387-A) as described previously (21). Morphometric measurements of trabecular structure (trabecular bone volume, bone surface, thickness, and number), and the number of osteoblast and osteoclast (TRAP-stained cells with >3 nuclei) were carried out at standardized sites (300x300  $\mu$ m) under growth plate in the metaphysis of the proximal tibia (22).

**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. 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, Tokyo, Japan) and specific primers (Table I) using a Light Cycler real-time PCR detection system (Toyobo, Tokyo, Japan). The amplification program consisted of 1 cycle for 1 min at 95°C followed by 45 cycles of 94°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec. Melting curve and gel analyses were used to verify specific products of appropriate size. Levels of gene expression were shown relative to an internal standard (actin).

**Western blot analysis.** Bone extract was used for Western blot analysis. Protein concentrations were measured using the BCA protein assay kit. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes. Western blotting and reprobing were performed and the chemiluminescent signals were quantified by a densitometer as reported previously (8).

**Statistical analysis.** Data are presented as the mean  $\pm$  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.

 SPANDIDOS  
PUBLICATIONS Primers used for quantitative real-time PCR.

Target	Forward primer sequence	Reverse primer sequence
Actin	AGCCATGTACGTAGCCATCCA	TCTCCGGAGTCCATCACAATG
ALP	TCCCAAAGGCTTCTTCTTG	ATGGCCTCATCCATCTCCAC
BMP2	TGAACACAGCTGGTCTCAGG	ACCCACATCACTGAAGTCC
Cathepsin K	TGTCTGAGAACTATGGCTGTGG	ATACGGGTAACGTCTTCAGAG
c-fms	TAGAGCCAGGTGCAACAGTG	CGCATAGGGTCTTCAAGCTC
c-fos	CTTCACCCTGCCTCTTCTCA	TCAAAGGGTTCAGCCTTCAG
c-jun	TGAAGCAGAGCATGACCTTG	TAGTGGTGATGTGCCCATTG
Col1 $\alpha$ 1	GCCTCCCAGAACATCACCTA	GCAGGGACTTCTTGAGGTTG
Dlx5	TGACAGAAGAGTCCCAAGCA	GCTTTGCGGTAAGAAGCAGA
M-CSF	CATCCAGGCAGAGACTGACA	TTCGCGCAGTGTAGATGAAC
OPG	TGTTCTGGTGGACAGTTTGC	GCTGGAAAGTTTGCTCTTGC
Osteocalcin	AATAGACTCCGGCGCTACCT	GAGCTCACACACCTCCCTGT
Osterix	AAGGCAGTTGGCAATAGTGG	TGAATGGGCTTCTTCCTCAG
RANK	ATATGCCTGCATCCCCTGAA	TAGCCATCCGTTGAGTTGGA
RANKL	AGCGCAGATGGATCCTAACA	TCGAGTCCTGCAAACCTGTA
Runx2	GCCGGGAATGATGAGAACTA	TGGGGAGGATTTGTGAAGAC
TRAP	CAGCCCTTATTACCGTTTGC	GAATTGCCACACAGCATCAC
Twist2	CAGCAAGAAATCGAGCGAAG	TGAGCGTCTGGATCTTGCTA

Primer sets for actin, ALP, BMP2, cathepsin K, c-fms, c-fos, c-jun, Col1 $\alpha$ 1, Dlx5, M-CSF, OPG, osteocalcin, osterix, RANK, RANKL, Runx2, TRAP and Twist2 were used for real-time RT-PCR.

Table II. Effects of diabetes on serum levels of glucose, insulin, PTH, Ca and P, body weight, and length and weight of femur and tibia.

	Control	Diabetes	Insulin
<b>Serum</b>			
Glucose (mg/dl)	150.6 $\pm$ 8.3	518.6 $\pm$ 25.2 <sup>a</sup>	233.8 $\pm$ 13.4 <sup>ab</sup>
Insulin (ng/ml)	4.87 $\pm$ 0.44	0.08 $\pm$ 0.04 <sup>a</sup>	2.74 $\pm$ 0.73 <sup>ab</sup>
PTH (pg/ml)	63.13 $\pm$ 8.66	59.75 $\pm$ 7.58	60.42 $\pm$ 12.72
Ca (mg/dl)	10.31 $\pm$ 0.32	10.70 $\pm$ 0.49	10.84 $\pm$ 0.31
P (mg/dl)	6.50 $\pm$ 0.38	7.71 $\pm$ 0.18	6.76 $\pm$ 0.42
<b>Body weight (g)</b>			
Before fasting	200.4 $\pm$ 1.9	204.4 $\pm$ 0.9	198.7 $\pm$ 2.5
Before injection	189.0 $\pm$ 2.4	192.8 $\pm$ 1.1	188.0 $\pm$ 1.0
Final	229.5 $\pm$ 3.3	189.9 $\pm$ 1.9 <sup>a</sup>	205.1 $\pm$ 3.2 <sup>ab</sup>
<b>Bone length (mm)</b>			
Femur	33.1 $\pm$ 0.2	32.5 $\pm$ 0.2	32.9 $\pm$ 0.4
Tibia	36.5 $\pm$ 0.2	36.1 $\pm$ 0.1	36.0 $\pm$ 0.1
<b>Bone weight (g)</b>			
Femur	0.674 $\pm$ 0.012	0.623 $\pm$ 0.013 <sup>a</sup>	0.678 $\pm$ 0.006 <sup>b</sup>
Tibia	0.532 $\pm$ 0.013	0.479 $\pm$ 0.010 <sup>a</sup>	0.512 $\pm$ 0.004 <sup>b</sup>
Proximal tibia	0.248 $\pm$ 0.009	0.206 $\pm$ 0.006 <sup>a</sup>	0.238 $\pm$ 0.008 <sup>b</sup>

At 2 weeks after the STZ-injection, blood (non-fasting) and femoral and tibial bone were collected. Sera were used to determine the levels of glucose, insulin, PTH, Ca, and P. Length and weight of the femur and tibia and the weight of the proximal tibia were measured. Values are the mean  $\pm$  SEM for 8 rats. <sup>a</sup>Significantly different from control value ( $P < 0.05$ ); <sup>b</sup>Significantly different from diabetes ( $P < 0.05$ ).

## Results

**Clinical characteristics.** The injection of STZ increased the serum glucose level to  $>500$  mg/dl and decreased the serum

insulin level (Table II). The hyperglycemia and hypo-insulinemia indicated that diabetes was induced in STZ-injected rats. Serum levels of PTH, Ca and P in the diabetic rats were similar to the control. Body weight (final) was significantly

Table III. Effects of diabetes on biochemical bone markers.

	Control	Diabetes	Insulin
Serum osteocalcin (ng/ml)	11.34±0.29	1.19±0.05 <sup>a</sup>	11.07±0.76 <sup>b</sup>
Urinary deoxypyridinoline (nmol/24 h urine)	8.76±0.48	12.18±0.52 <sup>a</sup>	8.68±1.18 <sup>b</sup>
Proximal tibia			
ALP activity (U/g proximal tibia)	12.70±0.41	6.69±0.57 <sup>a</sup>	11.07±0.52 <sup>b</sup>
TRAP activity (U/g proximal tibia)	0.480±0.033	0.978±0.060 <sup>a</sup>	0.354±0.014 <sup>b</sup>
Cathepsin K activity (U/g proximal tibia)	505.9±72.6	1,354.1±127.9 <sup>a</sup>	622.8±46.3 <sup>b</sup>
Ca (mg/g proximal tibia)	134.1±4.6	108.6±4.7 <sup>a</sup>	137.4±4.6 <sup>b</sup>
Hyp (μmol/g proximal tibia)	110.7±4.1	90.1±5.1 <sup>a</sup>	121.1±6.0 <sup>b</sup>

At 2 weeks after the STZ-injection, serum osteocalcin, urinary deoxypyridinoline, the activities of ALP, TRAP, and cathepsin K, and levels of hydroxyproline and Ca in the proximal tibia were determined as described in Materials and methods. Values are the mean ± SEM for 8 rats in biochemical markers and for 4 rats in histological markers. <sup>a</sup>Significantly different from control value (P<0.05); <sup>b</sup>Significantly different from diabetic group (P<0.05).

lower in the diabetic group than control group. The treatment with insulin significantly restored the serum levels of glucose and insulin and body weight in the diabetic rats, but values were still significantly different from those for the control group. The weights of the femur, tibia and proximal tibia were significantly lower in diabetic rats, although no significant difference was observed in the length of the femur or tibia. Administration of insulin to diabetic animals significantly restored these parameters to levels not significantly different from the control.

**Biochemical bone markers.** Serum osteocalcin levels in the diabetic group decreased significantly to ~10% of the control values (Table III). The urinary concentration of deoxypyridinoline significantly increased to 1.4-fold the control level. These values recovered to control levels on treatment with insulin.

ALP activity in the proximal tibia of the diabetic group decreased significantly to ~50% of the control value (Table III). The activity of TRAP and a cathepsin K in the diabetic group increased significantly to ~2- and 2.7-fold the control level, respectively. Consistent with the increase in cathepsin K activity, hydroxyproline content in the diabetic group decreased significantly to 80% of the control value. The amount of Ca in the diabetic rats also decreased significantly to 80%. The injection of insulin restored these levels to the control values.

**Histomorphometry.** The histochemical staining of the tibia for TRAP, a marker of osteoclasts, showed that diabetic rats had more TRAP-positive cells and less osteoblasts at metaphysis of the proximal tibia than the controls as shown in Fig. 1. Trabecular bone volume and thickness were also significantly lower in the diabetic than the control rats, although the significant difference was not observed in trabecular bone surface and number (Table IV). The number of osteoclasts in the diabetic rats were significantly increased to ~2-fold the control value, whereas the number of osteoblasts decreased to

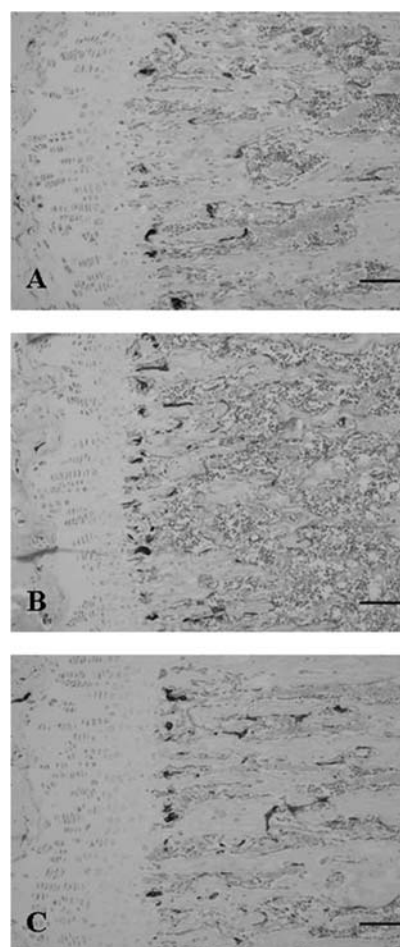


Figure 1. TRAP staining of the proximal tibia in control, diabetic, and insulin-treated diabetic rats. Paraformaldehyde-fixed, decalcified and paraffin-embedded tibiae obtained from control (A), diabetic (B), and insulin-treated diabetic (C) rats were processed for TRAP staining as described in Materials and methods. TRAP-positive cells appear red (black in this photograph). Hematoxylin counterstaining. x200 magnification. Bar indicates 100 μm. The results presented here are typical of four separate experiments.

	Control	Diabetes	Insulin
Trabecular bone volume (%)	63.5±4.6	44.4±1.8 <sup>a</sup>	57.4±3.0 <sup>b</sup>
Trabecular bone surface (mm/mm <sup>2</sup> )	34.1±2.6	33.9±1.7	32.9±3.3
Trabecular thickness (μm)	35.5±0.9	26.7±1.9 <sup>a</sup>	35.3±2.1 <sup>b</sup>
Trabecular number (/mm)	17.0±1.3	16.9±0.9	16.4±1.7
Osteoclast index (N Oc/mm trabecular bone length)	5.51±0.71	10.85±0.82 <sup>a</sup>	6.24±0.78 <sup>b</sup>
Osteoblast index (N Ob/mm trabecular bone length)	26.0±3.1	12.6±1.3 <sup>a</sup>	23.5±3.8 <sup>b</sup>

Paraformaldehyde-fixed, decalcified and paraffin-embedded tibiae obtained from control, diabetic, and insulin-treated diabetic rats were analyzed as described in Materials and methods. Values are the mean ± SEM for 4 rats. <sup>a</sup>Significantly different from control value (P<0.05); <sup>b</sup>significantly different from diabetic group (P<0.05).

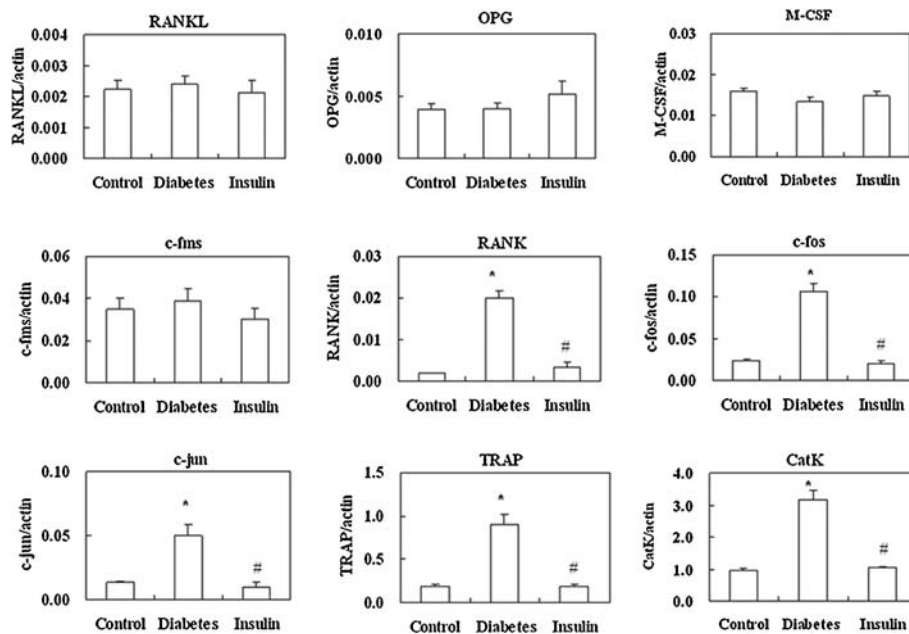


Figure 2. Expression of genes involved in osteoclastic differentiation in proximal tibia. Total RNA was extracted from proximal tibia of control, diabetic and insulin-treated diabetic rats and the mRNA levels for RANKL, OPG, M-CSF, c-fms, RANK, c-fos, c-jun, TRAP and cathepsin K were assessed by real-time PCR as described in Materials and methods. Values are the mean ± SEM for 4 rats. \*Significantly different from control value (P<0.05); #significantly different from diabetic group (P<0.05).

50% of the control level. The treatment with insulin restored the trabecular bone index and the number of osteoclasts and osteoblasts in diabetic rats to the control level.

**Expression of genes involved in osteoclastic differentiation in the proximal tibia.** The gene expression levels of the osteoclastogenesis-related factors, RANKL, OPG, M-CSF, c-fms, c-fos, and c-jun, and osteoclast-specific proteins, TRAP and cathepsin K, relative to the internal control, actin, are shown in Fig. 2. The mRNA levels of RANKL, OPG, M-CSF, and c-fms in the diabetic and insulin-injected rats were similar to control values. However, those of RANK, c-fos and c-jun in diabetic rats were ~10-, 4-, and 4-fold the control values, respectively. The expression of TRAP and cathepsin K mRNA also increased to 4.7- and 3.3-fold the control values in the

diabetic rats, respectively. In insulin-injected diabetic rats (Insulin), the increased expression was restored to the control level.

**Expression of genes involved in osteoblastic differentiation in the proximal tibia.** The gene expression levels of the osteoblastogenesis-related factors, BMP2, Runx2, Osx, Dlx5, and Twist2, and osteoblast-specific proteins, ALP, osteocalcin, and Col1α relative to actin are shown in Fig. 3. The expression of BMP2 mRNA in diabetic rats was similar to the control level. However, the levels of Runx2, Osx, and Dlx5 were significantly decreased to ~18, 16 and 10% of the control value, respectively. Twist2 expression in diabetic rats increased to 10-fold the control value. The mRNA levels of ALP, osteocalcin and Col1α in the diabetic group decreased significantly

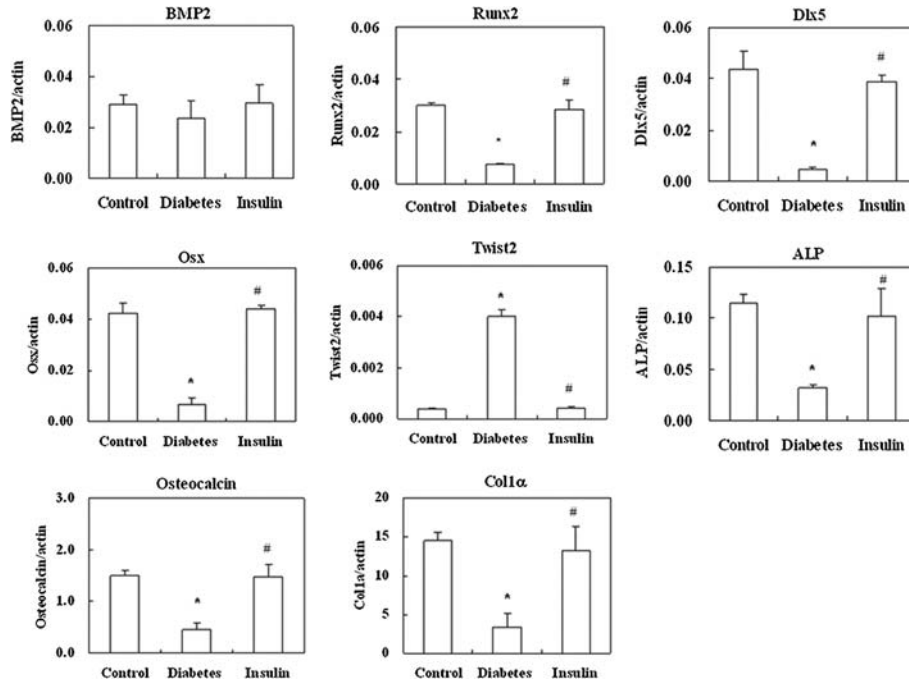


Figure 3. Expression of genes involved in osteoblastic differentiation in the proximal tibia. Total RNA was extracted from the proximal tibia of control, diabetic and insulin-treated diabetic rats and the mRNA levels of BMP2, Runx2, Osx, Twist2, Dlx5, ALP, Osteocalcin and Col1 $\alpha$  were assessed by quantitative real-time PCR as described in Materials and methods. Values are the mean  $\pm$  SEM for 4 rats. \*Significantly different from control value ( $P<0.05$ ); #significantly different from the diabetic group ( $P<0.05$ ).

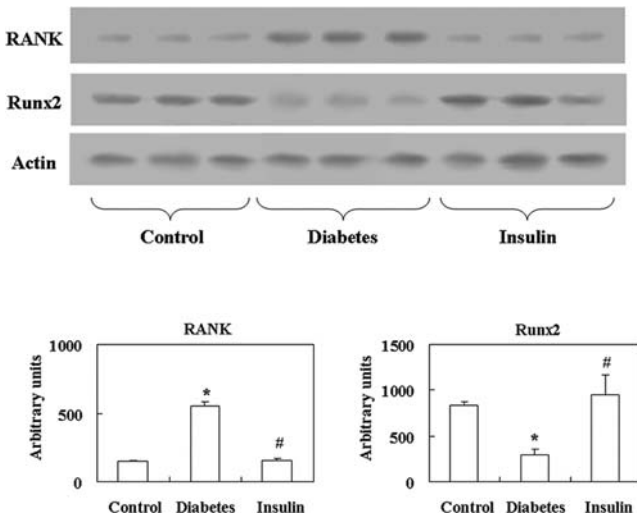


Figure 4. Levels of RANK and Runx2 protein in the proximal tibia. (A) Western blot analyses of RANK and Runx2. Bone extracts from the proximal tibiae of control, diabetic and insulin-treated diabetic rats were resolved by SDS-PAGE. After transfer, the blot was probed or re-probed with antibody and detected by ECL as described in Materials and methods. (B) Levels of RANK and Runx2 protein were quantified by densitometry and represented graphically. Data represent the mean  $\pm$  SEM for 4 independent experiments. \*Significantly different from the control value ( $P<0.05$ ). #significantly different from the diabetic group ( $P<0.05$ ).

to ~30, 20, and 20% of the control level, respectively. The altered expression of the genes observed in diabetic rats was recovered by the injection of insulin.

*Levels of RANK and Runx2 protein in the proximal tibia.* The results of the Western blot analysis are shown in Fig. 4. The

level of RANK protein in diabetic rats increased to ~3.7-fold the control value. The level of Runx2 protein decreased to 35% of the control value. These changes were reversed by treatment with insulin.

## Discussion

This study confirmed that diabetes increased bone resorption and decreased bone formation in short-term diabetic rats. Consistent with previous findings in the distal femur at 1 or 2 weeks after the injection of STZ (8,9), increases in TRAP and cathepsin K activity as well as decreases in ALP activity in the proximal tibia and the serum osteocalcin concentration were observed at 2 weeks. Accompanying these changes, levels of calcium and hydroxyproline in the proximal tibia decreased in the diabetic group. The increase in cathepsin K activity stimulated the degradation of the bone matrix, resulting in a decrease in the amount of hydroxyproline in the proximal tibia and an increase in the urinary excretion of deoxypyridinoline which is a product of the degradation of collagen. These results are consistent with reports that the excretion of hydroxyproline increased in subjects with Type 1 diabetes (23) and rats with STZ-induced diabetes (8,9). The increase in TRAP and cathepsin K activity was associated with an upregulation of its mRNA expression. Furthermore, histological analysis showed an increase in the number of osteoclasts in diabetic rats. Although there is a debate about the effects of IDDM on osteoclastic activity, the current study showed an increased osteoclast-mediated bone resorption in short-term diabetic rats induced by a single STZ injection. To examine the involvement of osteoclastogenesis in the increase of the number of osteoclasts, effects of diabetes on osteoclastic differentiation were investigated. The precursors of



are hematopoietic cells of the monocyte/macrophage originating in the bone marrow. Their differentiation into osteoclasts, however, occurs on the bone surface (24). Therefore, the gene expression of factors involved in osteoclastogenesis, RANKL, OPG, M-CSF, c-fms, RANK, c-fos and c-jun, was examined in the bone. RANKL plays a critical role in osteoclastogenesis. Osteoclastic differentiation is principally stimulated by an increase in the biological availability of RANKL assessed by the ratio of RANKL to its decoy receptor, OPG (25,26). To evaluate its importance in the bone metabolism of diabetic rats, the expression of RANKL and OPG in diabetic rats did not differ from the control values. These results suggest that the increase in the number of osteoclasts was not associated with an upregulation of RANKL expression or its biological availability in the bone of diabetic rats. In addition to RANKL, M-CSF is required for osteoclastic differentiation. M-CSF induces the proliferation of osteoclast precursor cells, supports their survival, and upregulates RANK expression, which is a prerequisite for osteoclastogenesis (27). The binding of M-CSF to its receptor, c-fms, induces the expression of RANK on the osteoclast precursor cells. The binding of RANKL to RANK activates the transcription factor AP-1, a heterodimer of c-fos and c-jun. The mRNA levels of M-CSF and c-fms in diabetic rats were similar to the control values. However, the expression of RANK significantly increased in diabetic rats. These results suggest that the expression of RANK increased without the upregulation of the signal required for RANK expression, M-CSF, and its receptor c-fms in the bone of diabetic rats. The level of RANK protein also increased in diabetic rats. This is the first report demonstrating that diabetes increases RANK protein *in vivo*. Furthermore, the expression of c-fos and c-jun also increased. AP-1 (c-fos and c-jun) is required in the RANK-RANKL signaling pathway for differentiation into osteoclasts (12). These results suggest that diabetes stimulated osteoclastogenesis through the increased expression of RANK followed by the upregulation of c-fos and c-jun expression.

The gene expression of ALP, osteocalcin, and collagen decreased in diabetic rats at 2 weeks. The association of this downregulation with osteoblastic differentiation was investigated. Runx2 is considered a 'master regulator' of osteoblastic differentiation, and its expression is essential for the normal bone formation (28). Our study clearly showed a decrease in the levels of mRNA and protein of Runx2 in diabetes. A previous study reported no change in Runx2 mRNA in 2 week-diabetic mice (5). This may be explained by the differences of the species, the STZ-injection method, or the examined site of bone, the whole tibia or the proximal tibia in our study. The analyses were at 2 weeks to examine changes in gene expression that occur before major bone loss, so a decrease in the mRNA levels may have been detected not in whole bone but in proximal tibia. Consistent with the reduction in Runx2, the expression of Osx and Dlx5 was also suppressed in diabetic rats. BMP-2 induces Dlx5 expression which activates the expression of Runx2 and mediates the BMP-2-dependent induction of Osx expression through a specific homeodomain responsive element (18,29). In this study, a decrease in the expression of the BMP-induced transcription factors, Runx2, Dlx5 and Osx, was observed in diabetic rats.

However, BMP-2, a critical regulator of osteoblastogenesis, was not altered in its expression. On the other hand, the expression of Twist2, a cosuppressor of Runx2, increased in the diabetic state. Runx2 expression was reported to be associated with downregulation of Twist expression (20). Indeed, expression of Twist2 was upregulated and upregulation was associated with a decrease in the expression of ALP and collagen in osteoporotic patients (30). The decreased expression of Runx2 might be associated with the increased Twist2 expression. The decrease in the expression of Runx2, Dlx5 and Osx as well as the increase in Twist2 induced by diabetes was reversed with insulin treatment. These results suggest that the decreased expression of Runx2 and Dlx5 but not BMP-2 resulted in the decrease in osteoblastogenesis, number of osteoblasts, and bone formation under insulin-deficient conditions.

This study has provided evidence that IDDM increased the expression of RANK and decreased the expression of Runx2 in bone of short-term diabetic rats. The findings suggest an increase in osteoclastogenesis with a decrease in osteoblastogenesis to be involved in IDDM-induced bone loss at the early stage. Further study on the relation of genes involved in osteoclastogenesis and osteoblastogenesis is needed for the development of better treatments for diabetic patients.

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