Increased cathepsin K and tartrate-resistant acid phosphatase expression in bone of streptozotocin-induced diabetic rats

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Abstract

The effect of insulin-dependent diabetes mellitus (IDDM) on bone metabolism was evaluated using the streptozotocin (STZ)-induced diabetic rat 1 week after the induction of diabetes. The urinary excretion of cross-linked N-telopeptides of type I collagen (NTx) and deoxypyridinoline (Dpd) in diabetic rats increased to 3.6-fold and 1.2-fold the control level, respectively. The amount of hydroxyproline and calcium in the distal femur of diabetic rats significantly decreased to 76% and 90% of the control, respectively. The levels of serum osteocalcin and alkaline phosphatase (ALP) activity in the distal femur of the diabetic rats were significantly reduced to about 40% and 70% of the control levels, respectively. The decrease in the expression osteocalcin was observed in distal femur of the diabetic rats, although the level of ALP mRNA was unchanged. The activity and the mRNA level of tartrate-resistant acid phosphatase (TRAP) increased to 1.5- and 2.3-fold the control level, respectively, in distal femur of the diabetic rats. The activity, protein, and mRNA levels of cathepsin K of diabetic rats also elevated to about 2-, 2.3-, and 2-fold the control levels, respectively. These results suggest that IDDM contributes to bone loss through changes in gene expression of TRAP and cathepsin K in osteoclasts as well as osteocalcin in osteoblasts resulting in increased bone resorptive activity and decreased bone formation.

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Keywords: Diabetes mellitus; Cathepsin K; Tartrate-resistant acid phosphatase (TRAP); Cross-linked N-telopeptides of type I collagen (NTx); Bone resorption

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 [1,2]. Insulin-dependent diabetes mellitus (IDDM) was associated with decreased bone mass [3–7], osteoporosis [7,8] and increased fracture rates [9,10] in human. The lower bone mineral density in IDDM is correlated with decreased markers of bone formation and dysregulation of the osteoblast-stimulating growth factor [11]. Serum concentrations of osteocalcin, a marker of osteoblast activity, were significantly decreased in adults and children with IDDM [12,13]. These pathological findings are also observed in experimental diabetic rats. Streptozotocin-induced diabetes in the rat is a well-recognized model for IDDM in the human [14]. In these rats, there was decreased bone formation and mineralization as well as altered collagen synthesis [15–18] which were corrected to normal levels with insulin treatment [17,18]. The effects of IDDM have generally been attributed to insulin deficient and to an impairment in osteoblast function since insulin has a stimulatory action on osteoblasts. However, the increased markers of osteoclast function such as urine excretions of calcium, hydroxyproline, and deoxypyridinoline were reported in subjects with IDDM [19–23], although normal [24] or low [25] activity of bone resorption was also reported. Bone histology and biochemical markers of bone formation and resorption in the diabetic animal models indicated a decreased osteoblast activity combined with normal [15,16,26,27] or decreased osteoclast activity [17,20,28]. Thus, there is some controversy about the effects of diabetes mellitus on the markers of bone resorption, although reports on bone formation are consistent across studies and markers.

Abbreviations: IDDM, insulin-dependent diabetes mellitus; TRAP, tartrate-resistant acid phosphatase; ALP, alkaline phosphatase; NTx, cross-linked N-telopeptides of type I collagen; Dpd, deoxypyridinoline; STZ, streptozotocin.

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Bone resorption, which is carried out by osteoclasts, involves demineralization, in which osteoclasts release protons and TRAP to solubilize the inorganic salt, followed by the degradation of the organic matrix (mainly Type I collagen) with cysteine proteinases [29,30]. Cathepsin K is a cysteine protease that is selectively and highly expressed by bone-resorbing osteoclasts and has been proposed to play a key role in bone resorption [31]. Immunolocalization [32], in situ hybridization [33], and fluorescence microscopic studies [34] have shown that cathepsin K is abundantly expressed by osteoclasts, especially at the cell surface adjacent to bone [32,33]. Inhibition of cathepsin K activity with synthetic inhibitors or cathepsin K antisense oligonucleotides was shown to inhibit osteoclast-mediated bone resorption in vitro and in vivo [35,36]. In humans [37] and in mice [38], mutations in cathepsin K gene lead to impaired bone resorption. Cathepsin K has a strong potency in digesting native collagen fibers and releases cross-linked N-telopeptides of type I collagen (NTx), which contain the cross-links of mature collagen, pyridinoline and deoxypyridinoline (Dpd) [39]. Urinary levels of NTx and Dpd are elevated when bone resorption is accelerated, for example, in women after menopause [40,41], and lowered by therapies known to inhibit resorption [40,42,43].

In this study, we investigated the effect of STZ-induced diabetes on bone resorption as well as bone formation at an early stage by measuring the mRNA levels of ALP, osteocalcin, TRAP, and cathepsin K, their enzymatic activities, and the contents of calcium and hydroxyproline of the distal femur. To our knowledge, this is the first report that the increase in cathepsin K and TRAP expression is involved in bone loss at an early stage of diabetes.

Materials and methods

Animals and study design

Ten-week-old female rats of 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 commercial laboratory chow (MF, Oriental Yeast, Osaka, Japan) and water. After overnight fasting, rats were divided into two groups, control (n=8) and diabetes group (n=24). Experimental diabetes was induced by a single intra-peritoneal injection of streptozotocin (STZ: 45 mg/kg body weight in 0.05 M citrate buffer, pH 4.5), a pancreatic beta-cell cytotoxin. Control animals received the same volume of the STZ diluent. The diagnosis of diabetes was based on the increase in blood glucose levels, after overnight access to feed (non-fasting). Blood and femoral bones were collected to determine the contents of glucose, deoxypyridinoline (Dpd), and cross-linked N-telopeptides of type I collagen (NTx).

For the biochemical analysis of bone, the femoral bone was sectioned into quarters as previously described [44]. The quarter from the aspect of the knee of the femur (distal femur) was homogenized with 10 volumes of 10 mM triethanolamine buffer, pH 7.5, and was stirred for 1.5 h at 4 °C. After centrifugation, an aliquot of the bone extracts was used for determining the activities of ALP, TRAP, and cathepsin K and for western blot analysis. The insoluble pellets were washed with 6 N HCl at 105 °C for 24 h and analyzed for calcium and hydroxyproline.

Analytical methods

The glucose concentration in serum and urine was assayed by the mutarotase-glucose oxidase method using commercial kits (Wako Diagnostic, Osaka, Japan).

Serum osteocalcin levels were measured using a rat osteocalcin enzyme immunoassay kit (Rat EIA kit; Biomedical Technologies, Stoughton, MA). ALP and TRAP activities were determined using 10 mM p-nitrophenyl phosphate as the substrate in 0.1 M diethanolamine buffer (pH 9.8) and in 50 mM acetate buffer (pH 5.5) containing 20 mM sodium tartrate, respectively, for 30 min at 25 °C, as reported previously [44]. A unit of activity is defined as the release of 1 μmol of p-nitrophenol per minute.

The activity of cathepsin K was determined by a fluorogenic assay according to the method of Kirschke and Wiederanders [45] with slight modifications. Briefly, the bone extract was incubated with the substrate Z-GPR-AMC (80 μM) and the cathepsin B-specific inhibitor CA074 (16 μM) in 50 mM sodium acetate buffer, pH 5.5, containing 2.5 mM EDTA and 2.5 mM DTT at 30 °C and the reaction was terminated by the addition of stopping reagent after 15 min. The enzymatic activity was expressed as pmol of liberated 7-amino-4-methylcoumarin per minute.

The amounts of Ca were determined by an o-cresolphthalein complexone (OCPC) method using commercial kits (Wako Diagnostic, Osaka, Japan). The content of hydroxyproline was measured by the method of Bergman and Loxley [46].

Deoxypyridinoline (Dpd) and cross-linked N-telopeptides of type I collagen (NTx) in urine were determined by an enzyme-linked immunoorbent assay using a Pyrilinks D-assay kit (Metra Biosystems, Palo Alto, CA) and an Osteomark assay kit (Ostex International, Inc., Seattle, WA), respectively. The results were expressed as nmol/mM creatinine (Cre) for Dpd and as nmol of bone collagen equivalent (BCE)/mM creatinine (Cre) for NTx. Creatinine was assayed by the modified Jaffe method [47].

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 in 10 mM Tris–HCl buffer, pH 7.2, containing 0.15 M NaCl, 0.05% Tween 20, and 10% nonfat powdered milk overnight and incubated with a specific antibody to cathepsin K. After incubation with a secondary antibody conjugated to horseradish peroxidase, immunoreactive proteins were detected with the enhanced chemiluminescence system (ECL; Amersham). The equal loading of protein samples was confirmed by the Bicinchoninic acid protein assay and staining of the gel with Coomassie brilliant blue.

Quantitative real-time PCR

Total RNA from the distal femur was prepared using a commercial kit (NucleoSpin RNA II kit, Macherey-Nagel, France) after homogenization in the presence of 0.1 M EDTA. The total RNA was reverse transcribed by 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 Realtime PCR Master Mix plus (Toyobo, Tokyo, Japan) and specific primers (ALP: 5'-GCAACACATCAAGGACATCG-3' and 5'-TGGCTTCTCTATC-CAGTTCA-3'; osteocalcin: 5'-ATAAGACTCCGGCGCTACCT-3' and 5'-GA-GCTACACACCTCCCTGTT; TRAP: 5'-CAGCCCTTTATACGTGGTGTG-3' and 5'-GAATGTCGCACAGACATC-3'; cathepsin K: 5'-TGCTGTA-GAATATGGCTGGTGG-3' and 5'-ATACGCGTAAGCTCTTCAAG-3'; actin: 5'-AGCCATGACGTAGCCATCCA-3' and 5'-TCCGGAGTCCATCA-AATG-3') using a LightCycler 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 s, 60 °C for 15 s, and 72 °C for 30 s. Melting curve and gel analyses were used to verify specific products of the appropriate size. Levels of gene expression were shown relative to the internal standard (actin).
Table 1
Effects of diabetes on glucose levels in serum and urine, body weight, food intake, and bone length and weight of femur and tibia

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetes</td>
<td>Control</td>
</tr>
<tr>
<td>Glucose level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In serum (mg/dl)</td>
<td>161.8±4.6</td>
<td>578.7±59.7 *</td>
<td>139.1±8.2</td>
</tr>
<tr>
<td>In urine (mg/24 h urine)</td>
<td>1.8±0.2</td>
<td>7860.7±390.0 *</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starting (before fasting)</td>
<td>198.0±4.6</td>
<td>199.6±1.8</td>
<td>199.1±2.2</td>
</tr>
<tr>
<td>Before the injection</td>
<td>182.3±4.8</td>
<td>183.8±1.4</td>
<td>181.9±2.1</td>
</tr>
<tr>
<td>Final</td>
<td>200.3±5.1</td>
<td>191.2±1.6</td>
<td>209.8±4.7</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>15.0±0.7</td>
<td>19.5±1.2 *</td>
<td>16.0±1.1</td>
</tr>
<tr>
<td>Bone length (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur</td>
<td>30.9±0.2</td>
<td>31.1±0.2</td>
<td>31.0±0.1</td>
</tr>
<tr>
<td>Tibia</td>
<td>34.7±0.21</td>
<td>35.0±0.2</td>
<td>35.1±0.3</td>
</tr>
<tr>
<td>Bone weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur</td>
<td>0.567±0.015</td>
<td>0.563±0.012</td>
<td>0.590±0.007</td>
</tr>
<tr>
<td>Tibia</td>
<td>0.453±0.006</td>
<td>0.464±0.010</td>
<td>0.491±0.010</td>
</tr>
<tr>
<td>Distal femur</td>
<td>0.199±0.005</td>
<td>0.194±0.004</td>
<td>0.207±0.003</td>
</tr>
</tbody>
</table>

Values are mean±S.E. of 8 rats.
* Significantly different from the corresponding control value (P<0.05).

Statistical analysis

All statistical analyses were performed using the Microsoft Excel data analysis program for Welch’s t-test. Experiments were repeated at least three times. Values are expressed as a mean±S.E.

Results

STZ-induced diabetic rat model

The injection of STZ increased the glucose levels in serum and urine to over 400 mg/dl and 7 g/24 h urine, respectively, within 2 days after the injection and remained elevated throughout the study (Table 1). These hyperglycemia and glycosuria indicated that diabetes was induced in the STZ-injected animals. In the diabetic group, the body weight (final) was significantly lower than that in the control at Day 7, not at day 2 or 4 (Table 1).

The bone lengths and weights of tibia and femur and the weight of the distal femur in the diabetic rats were not significantly different from the control during 1 week of diabetes (Table 1). The food intake in the diabetic rats significantly increased to 1.3-, 1.7-, 2.2-fold the control value at 2, 4 and 7 days of diabetes, respectively (Table 1).

The levels of serum osteocalcin in diabetic group significantly decreased to about 40% of the control values (Table 2). The serum ALP and TRAP activity of the diabetic rats significantly increased to about 5-fold and 2-fold the control level, respectively (Table 2).

The urinary levels of Dpd and NTx significantly increased to 1.2- and 3.6-fold the control, respectively (Table 2).

Table 3
Effects of diabetes on the activities of ALP, TRAP, and cathepsin K and the contents of hydroxyproline and calcium in distal femur

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin (ng/ml)</td>
<td>18.47±0.86</td>
<td>7.82±0.81 *</td>
<td>0.00015</td>
</tr>
<tr>
<td>ALP activity (U/l)</td>
<td>277.6±12.2</td>
<td>1338.8±107.1 *</td>
<td>0.0001</td>
</tr>
<tr>
<td>TRAP activity (U/l)</td>
<td>10.97±1.22</td>
<td>19.44±2.33 *</td>
<td>0.0107</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dpd (nmol/mmol Cre)</td>
<td>152.3±12.9</td>
<td>188.4±6.5 *</td>
<td>0.0388</td>
</tr>
<tr>
<td>NTx (nmolBCE/mmol Cre)</td>
<td>37.8±4.9</td>
<td>134.5±21.1 *</td>
<td>0.0034</td>
</tr>
</tbody>
</table>

At 7 days after the diabetes induction, blood (non-fasting) was collected under diethyl ether anesthesia. The sera were used to determine the levels of osteocalcin and activities of ALP and TRAP as described in Materials and methods. Urine in the 24-h before sacrifice was collected to determine the contents of Dpd and NTx. Values are mean±S.E. of 6–8 rats.
* Significantly different from the corresponding control value (P<0.05).
Diabetic effects on the activities of ALP, TRAP, and cathepsin K, and the contents of hydroxyproline and Ca in the distal femurs

The ALP activity in the distal femur of the diabetic group significantly decreased to about 70% of the control (Table 3). The TRAP activity of the diabetic group significantly increased to 1.5-fold the control level (Table 3). The cathepsin K activity of the diabetic group also significantly increased to about 2.4-fold the control level (Table 3). Consistent with the increase in the cathepsin K activity, the hydroxyproline content in the diabetic group significantly decreased to 76% of the control (Table 3). The content of Ca in the diabetic rats also significantly decreased to 90% of the control (Table 3).

Diabetic effects on protein levels of cathepsin K in the distal femurs

As shown in Fig. 1, the anti-cathepsin K antibody detected two bands, proenzyme (37 kDa) and mature cathepsin K (27 kDa). In the diabetic group, the protein level of mature cathepsin K (27 kDa) significantly increased to 3.2-fold the corresponding control level while the increase in the higher band (37 kDa) was less pronounced (Fig. 1). The total protein level of cathepsin K (37 kDa and 27 kDa) in the diabetic rats increased to 2.3-fold the control (Fig. 1).

Diabetic effects on the mRNA levels of ALP, osteocalcin, TRAP, and cathepsin K of the distal femurs

The expression levels of the ALP, osteocalcin, TRAP, and cathepsin K genes relative to the internal control, actin, in distal femur were shown in Fig. 2. The ALP mRNA level was not significantly changed. The mRNA level of osteocalcin in diabetic group significantly decreased to about 40% of the control level. The mRNA levels of TRAP, and cathepsin K increased to 2.3-fold and 1.9-fold the control level, respectively.

Discussion

This study demonstrated that diabetes increased bone resorption in the distal femur within 1 week. The TRAP and cathepsin K activity in the distal femoral bone increased in fold the control level (Table 3). Consistent with the increase in the cathepsin K activity, the hydroxyproline content in the diabetic group significantly decreased to 76% of the control (Table 3). The content of Ca in the diabetic rats also significantly decreased to 90% of the control (Table 3).
diabetic rats. In accordance with the increases in these activities, the calcium and hydroxyproline contents of the distal femur decreased in the diabetes group. In diabetic rats, the elevated cathepsin K activity induced a stimulation of the degradation of bone matrix, resulting in a decrease in the content of hydroxyproline of the distal femur and an increase in the urinary excretion of Dpd and NTx, which are the degradation products of collagen. These results are consistent with the previous reports that hydroxyproline excretion increased in subjects with Type 1 diabetes [23]. The increase in cathepsin K activity was associated with the up-regulation of protein and also mRNA levels. The mRNA levels of TRAP, another marker of osteoclastic activity, also significantly increased in the distal femur of diabetic rats. These results suggest that osteoclasts exhibited immediate/early acute changes in gene expression in response to diabetes and an increase in osteoclastic activity contributed to the bone loss in diabetes. However, previous studies indicated no change or a decrease in diabetic osteoclast activity based on histology and/or secretion of deoxypyridinoline after 2 weeks or longer of diabetes in rat or mouse [17,20,27,28]. The difference may be accounted for by the duration of diabetic status. Our data supports the hypothesis that the upregulation of gene expression of TRAP and cathepsin K in osteoclasts stimulates bone resorption at an early stage of diabetes. However, the possibility of a decrease in osteoclast activity at later time points can not be excluded.

The ALP activity in the distal femur decreased in diabetic rats, although an increase was observed in serum. An increased concentration of total serum ALP, especially increased level of the intestinal form of ALP was already described in diabetes [48]. The increase in food intake in the diabetic rats observed in this study suggested an elevated level of intestinal form. In fact, the ALP activity of bone in diabetic rats decreased to about 70% of the control. The decreased ALP activity in the distal femur was not associated with a significant change in the mRNA level. In agreement with our results, no decrease in the ALP mRNA levels was reported in diabetic mice or in MC3T3-E1 cells under hyperglycemia [27,49]. These results suggest that ALP was regulated at the post-transcriptional level. Elevated blood glucose level is a key characteristic of IDDM. One of the major consequences of hyperglycemia is an excessive non-enzymatic glycosylation of extracellular proteins such as collagen and ALP [50]. ALP activity was inhibited by the glycosylation of itself [51] and/or collagen [52]. It is possible that the non-enzymatic glycosylation of collagen and/or ALP results in the decrease in ALP activity observed in this study.

The serum levels of osteocalcin, another marker of osteoblast activity, decreased in diabetic rats. The mRNA level of osteocalcin in femoral bone was also reduced. The decreased ALP activity and reduced levels of osteocalcin expression in distal femur indicated the decreased bone formation, which would cause the decreased contents of calcium and hydroxyproline in distal femur of the diabetic rats at 1 week observed in this study.

The decreased levels of serum osteocalcin and tibial osteocalcin mRNA were also described in diabetic mice [27]. The osteocalcin expression, a marker of late-stage osteoblast differentiation, was already reduced within 1 week in this study, although the mRNA level of ALP was not reduced. Gene expressions of ALP and runx2, earlier-stage of osteoblast differentiation, were also reported to be unaltered in diabetic mice [27]. These suggest that diabetes suppresses osteoblast differentiation at late stage, not earlier stage, and leads to osteopenia through a decreased mineralization.

Our paper has provided evidence that osteoclasts as well as osteoblasts exhibit immediate changes in gene expression in response to diabetes at an early stage. We hypothesize that IDDM contributes to bone loss through changes in gene expression of TRAP and cathepsin K in osteoclasts as well as osteocalcin in osteoblasts resulting in increased bone resorptive activity and decreased bone formation. Further study on the molecular mechanisms promoting bone loss under diabetes will contribute to the development of better treatments for diabetes.

References


