osteoblastic differentiation [19–21]. Effects of simvastatin were selectively reversed by either mevalonate or its metabolite geranylgeranyl pyrophosphate (GGPP), but not by cholesterol or farnesyl pyrophosphate [22]. These results suggest that the effects were mainly derived from depletion of intracellular pools of GGPP, the substrate required for the geranylgeranylation. In addition, simvastatin suppressed the ROK activity, and this effect was reversed by addition of GGPP [23]. We also showed that the AMPK activator as well as the ROK inhibitor was able to stimulate the mineralization of osteoblasts through modulating the mevalonate pathway and enhancing endothelial NOS and BMP-2 expression [24]. These findings suggest that PTH might affect BMP-2 expression in osteoblasts through modulating the mevalonate pathway, although there are no studies investigating this possibility until now.

In this study, to clarify this issue, we used osteoblastic MC3T3-E1 cells and examined if PTH affects BMP-2 expression, or if the mevalonate pathway is involved in its process.

Materials and Methods

Materials

Cell culture medium and supplements were purchased from GIBCO-BRL (Rockville, MD, USA). Human PTH-(1–34) was kindly gifted from Asahi-kasei corporation (Tokyo, Japan). Mevalonate and geranylgeranyl pyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO, USA). For Rho kinase activity analysis, a Rho kinase assay ELISA kit was purchased from Cyclex (Nagano, Japan). All other chemicals were of the highest grade available commercially. PCR primers for BMP-2 and mevalonate kinase were obtained from Sigma Aldrich (St. Louis, MO, USA). Vehicle for PTH peptide was 10 mM acetic acid (100 nM final concentration).

Cell Culture

MC3T3-E1 cells, a clonal osteoblastic cell line isolated from calvariae of late stage mouse embryo, were kindly provided by Dr. H. Kodama (Ohu Dental College, Japan). This cell line has been widely used as a cell culture model for osteoblastic differentiation. MC3T3-E1 cells were cultured in α-MEM (containing 50 μg/ml of ascorbic acid) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO-BRL) in 5% CO_2 at 37°C. The medium was changed twice a week for general culture.

Real-time PCR

SYBR green chemistry was used to perform quantitative determinations of the mRNAs for BMP-2, mevalonate kinase (MVK), and a house keeping gene, 36B4, according to an optimized protocol [25,26]. Total RNA was collected from cultured MC3T3-E1 cells using Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer's recommended protocol. Two μg total RNA was employed for the synthesis of single-stranded cDNA (cDNA synthesis kit; Invitrogen). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the QuantiTech SYBR PCR kit (QIAGEN, Valencia, CA) to allow for quantitative detection of the PCR product. The sense and antisense primers were designed using the Primer Express Version 2.0.0 (Applied Biosystems Inc.) based on published cDNA sequences. Real-time PCR was performed using ABI PRISM 7000 (PE Applied Biosystems Inc.). The PCR primers (listed as forward primer and reverse primer) were as follows:

BMP-2, 5′-CGTCAAGCCAAACACAAACAGCG-3′ and 5′-CACCCACA-ACCCTCCACAACCAT-3′; MVK, 5′-GGGACGATGTCTTCCTTGAA-3′ and 5′-GAACTTGGTCAGCCTGCTTC-3′; 36B4, 5′-AAGCGCGTCCT-GGCATTGTCT-3′ and 5′-CCGCAGGGGCAGCAGTGGT-3′.

Rho kinase activity assay

Cells were rinsed with ice-cold PBS and scraped on ice into a lysis buffer (Cell Signaling Technology) that contained 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EGTA, 1 mM Na_2EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin. The cell lysates were then sonicated for 30 s. After the cell lysates were centrifuged at 15 000 × *g* for 10 min, supernatants were collected for assessing Rho kinase activities using the Rho kinase assay kit (Cyclex) as indicated by the manufacturer. Briefly, 10 μl of the supernatants of lysed cells were added into 96-well plates precoated with a substrate corresponding to the C terminus of the recombinant myosin-binding subunit of myosin phosphate (MSB), which contains a threonine residue that may be phosphorylated by Rho kinase. Subsequently, 90 μl of a kinase reaction buffer (containing 0.1 mM ATP) was added, incubated for 30 min at room temperature, washed five times with a washing buffer provided by the kit, and incubated with 100 μl of a horseradish peroxidase-conjugated monoclonal antiphospho-specific MSB antibody, which specifically detects the phosphorylated form of threonine 697 on MSB (provided by the kit). The colored products were developed by incubating with 100 μl of a horseradish peroxidase substrate tetramethylbenzidine at room temperature for 10 min. The reaction was stopped by adding 100 μl of stop solution containing 0.5 M H_2SO_4, and the absorbance was read at 450 nm. Each value was normalized to the protein concentration.

Statistics

Each experiment was repeated at least three times. Data were shown as means ± SEM. Statistical evaluations for differences between groups were carried out using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). We used paired *t*-test to analyze statistical evaluations, if it was applicable. For all statistical tests, a value of $p < 0.05$ was considered to be a statistically significant difference.

Results

Time course of BMP-2 mRNA expression treated by hPTH-(1–34)

Time course of BMP-2 mRNA expression was examined after treatment of osteoblastic MC3T3-E1 cells with 10^{-8} M hPTH-(1–34). The expression of BMP-2 mRNA was increased in a time dependent manner up to 6 h and was decreased thereafter (**Fig. 1A**).

Dose response of PTH-induced upregulation of BMP-2 mRNA expression

Dose response of PTH-induced upregulation of BMP-2 mRNA expression was examined under 6-hour treatment with hPTH-(1–34). BMP-2 mRNA expression was significantly upregulated by 10^{-8} M and 10^{-9} M of hPTH-(1–34), compared with the control treated with vehicle alone (100 nM of acetic acid) (**Fig. 1B**).

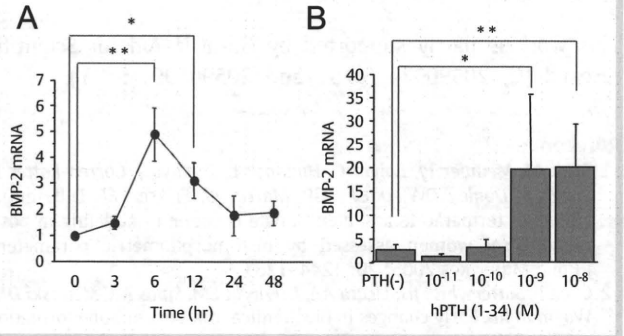

Fig. 1 Effects of PTH on BMP-2 mRNA expression in osteoblastic MC3T3-E1 cells. Time course of BMP-2 mRNA expression was shown after treatment with 10^{-8} M hPTH-(1–34) (**A**), and dose response of PTH-induced upregulation of BMP-2 mRNA expression was demonstrated (**B**). Results were expressed as the mean ± SEM fold increase over control values from more than 3 independent experiments. **p<0.01, *p<0.05.

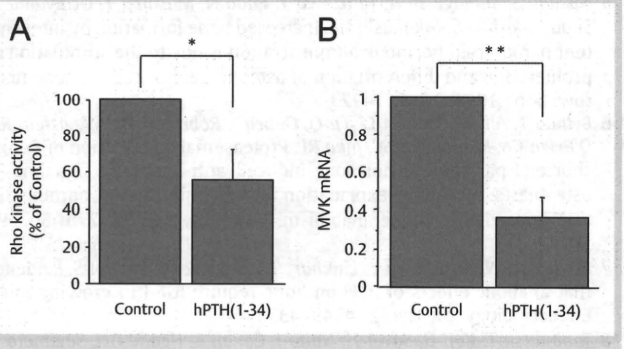

Fig. 2 Effects of PTH on ROK and MVK activities in osteoblastic MC3T3-E1 cells. **A.** ROK activities were measured in whole cell lysate extracted from MC3T3-E1 cells 10 min after treatment of the cells with or without 10^{-8} M hPTH-(1–34). **B.** Total RNA was extracted from MC3T3-E1 cells after 3-h treatment of the cells with or without 10^{-8} M hPTH-(1–34). MVK mRNA expression was quantified using real-time PCR and corrected with 36B4, house-keeping gene expression. Results were expressed as the mean ± SEM-fold increase over control values from more than 3 independent experiments. **p<0.01, *p <0.05.

Rho kinase activity was suppressed by PTH

Next, ROK activity in whole cell lysate was measured to address whether or not PTH would affect the ROK activity. We observed that 10 min treatment of MC3T3-E1 cells with 10^{-8} M hPTH-(1–34) significantly decreased the ROK activity compared with the vehicle-treated control ($p<0.05$) (○ **Fig. 2A**).

Mevalonate kinase mRNA was decreased by PTH

We determined mevalonate kinase (MVK) mRNA expression by real-time PCR, which is located upstream of ROK. MVK mRNA level was significantly decreased by 3-hour treatment of the cells with 10^{-8} M hPTH-(1–34) compared with the control (○ **Fig. 2B**). Significant inhibition of MVK mRNA expression was also observed at 6 and 12 h after PTH treatment (data not shown). These findings indicate that PTH might rapidly suppress the MVK activity.

Pretreatment with GGPP inhibited PTH-induced BMP-2 upregulation

We investigated whether or not additions of GGPP, downstream of MVK, or mevalonate, upstream of MVK, were able to reverse

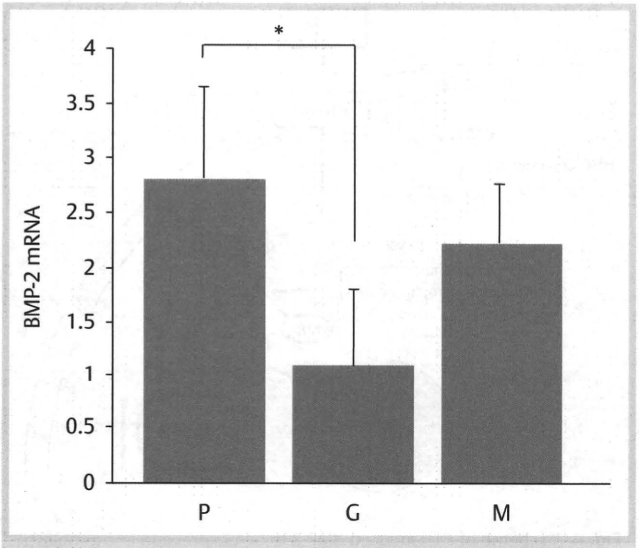

Fig. 3 Pretreatment with GGPP inhibited PTH-induced BMP-2 upregulation in osteoblastic MC3T3-E1 cells. GGPP and mevalonate were preincubated overnight before PTH treatment. Total RNA was extracted from MC3T3-E1 cells after 6-hour treatment of the cells with 10^{-8} M hPTH-(1–34). BMP-2 mRNA expression was quantified using real-time PCR and corrected with 36B4. Results were expressed as the mean ± SEM-fold increase over control values from more than 3 independent experiments. P: 10^{-8} M hPTH-(1–34); G: 5 μM GGPP + 10^{-8} M hPTH-(1–34); M: 1 mM mevalonate + 10^{-8} M hPTH-(1–34). *p<0.05.

the PTH-induced BMP-2 upregulation in MC3T3-E1 cells. PTH-induced upregulation of BMP-2 mRNA in the cells was inhibited by overnight pretreatment with 5 μM GGPP, but not with 1 mM mevalonate (○ **Fig. 3**), indicating that PTH directly interacts with MVK, and not with its upstream molecules.

Taken together, these results suggest that PTH possibly inhibited MVK activity, which in turn suppressed ROK activity and induced BMP-2 expression (○ **Fig. 4**).

Discussion

In this study, we demonstrated for the first time that PTH stimulated BMP-2 mRNA expression via the mevalonate pathway and the ROK activity in osteoblastic MC3T3-E1 cells. This might be one of the mechanisms by which PTH can accelerate bone formation, although it seems necessary to confirm the present observation by using other experimental system and by protein expression of BMP-2.

Mechanisms of the PTH anabolic action on the bone have mainly been explained as follows:

i PTH increases osteoblastic differentiation by suppressing proliferation [4,5].

ii PTH has an effect of anti-apoptotic action in osteoblastic cells [6,7].

iii The replication, differentiation and survival of osteoblast progenitors are controlled by locally produced autocrine/paracrine factors that are supplied by PTH treatment. Members of Wnt and insulin-like growth factor-I (IGF-I) are included in the local factors [8–11]. Hedgehog and bone morphogenetic protein (BMP) families, as well as transforming growth factor-β (TGF-β), fibroblast growth factor-2 (FGF-2), and interleukin-6 (IL-6) type cytokines can be the candidates. Moreover, many of these growth factors are deposited into

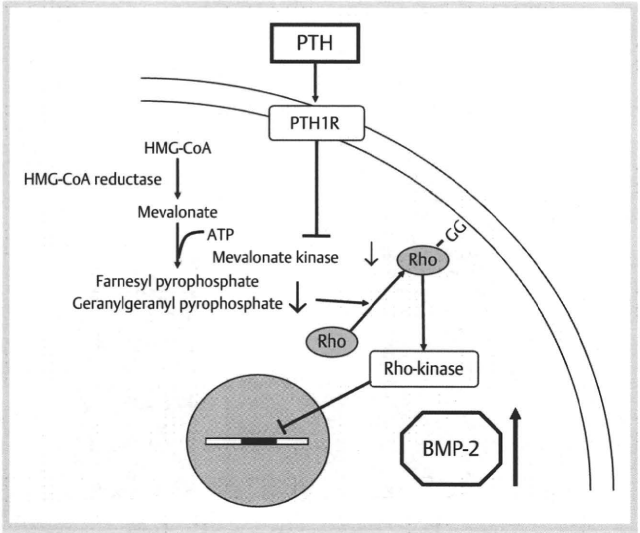

Fig. 4 Scheme of PTH-induced BMP-2 mRNA expression in osteoblasts. PTH binds to a G protein-coupled, 7-transmembrane receptor, PTH1R, located on the cell surface of osteoblasts. PTH1R activation would lead to suppression of MVK activity through undetermined signaling pathways, which results in a decrease in farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP). GGPP deprivation causes inhibition of isoprenylation of Rho protein, which in turn reduces ROK activity [22,23]. Reduction in ROK activity has been reported to stimulate BMP-2 mRNA transcription probably via activation of Akt and stabilization of eNOS mRNA [19–21,24]. In the present study, we have demonstrated for the first time that PTH upregulated BMP-2 mRNA presumably via the mevalonate and Rho-associated kinase pathway.

the bone matrix by osteoblasts and are thought to be released in active form during osteoclastic bone resorption.

iv PTH stimulates activating protein-1 (AP-1), which is a transcription regulatory factor constructed by heterodimer of Fos related proteins (c-fos, fos B, fra-1, fra-2) and Jun related proteins (c-Jun, Jun B, Jun-D) [27].

v PTH inhibits the production of sclerostin, which is made exclusively by osteocytes and prevents binding of Wnt ligands to their receptors and plays an important role in the regulation of bone formation [28].

Our present findings seem to be related to the category (iii), an enhancement of local factor activities. In addition, we have newly clarified the participation of the mevalonate pathway in PTH-induced BMP-2 mRNA upregulation.

In the present study, when MVK mRNA level was quantified by real-time PCR, a significant decrease was observed. This finding was consistent with microarray data reported by Qin et al., where 3-hour treatment of osteoblastic cells with PTH downregulated MVK mRNA expression by approximately 2.5-fold [29]. In this study, addition of GGPP selectively inhibited the BMP-2 upregulation, while that of mevalonate did not. Thus, PTH may suppress the activity of the mevalonate pathway at the level of MVK, but not at the levels of its upstream molecules. Further studies on the interaction between PTH and MVK would more clearly disclose one of the mechanisms by which PTH exerts anabolic action in osteoblasts, and would lead to the discovery of candidate drugs that promote bone formation for the treatment of osteoporosis.

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Serum Osteocalcin/Bone-Specific Alkaline Phosphatase Ratio Is a Predictor for the Presence of Vertebral Fractures in Men with Type 2 Diabetes

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Abstract We examined whether or not BMD or bone markers were useful for assessing the risk of vertebral fractures in 248 Japanese men with type 2 diabetes. We analyzed the relationships between bone markers (osteocalcin [OC], bone-specific alkaline phosphatase [BAP], urinary N-terminal cross-linked telopeptide of type-I collagen) or BMD and HbA_{1c}, urinary C-peptide, insulin-like growth factor-I (IGF-I), parathyroid hormone, $1,25(OH)_2$ vitamin D, and the presence of prevalent vertebral fractures. Multiple regression analysis adjusted for age, body height, weight, duration of diabetes, and serum creatinine showed that serum OC and OC/BAP ratio were correlated negatively with HbA_{1c} ($P < 0.01$) and positively with IGF-I ($P < 0.01$). Multivariate logistic regression analysis adjusted for the above parameters showed that serum OC/BAP ratio was inversely associated with the presence of vertebral fractures (odds ratio = 0.695, $P < 0.05$). This association was still significant after additional adjustment for lumbar or femoral neck BMD. Our results suggest that poor diabetic control and lower IGF-I level are linked to impaired bone formation and resultant reduction in OC/BAP ratio in men with type 2 diabetes. The OC/BAP ratio could be clinically useful for assessing the risk of vertebral fractures independent of BMD in diabetic men.

Keywords Osteocalcin · Bone-specific alkaline phosphatase · Type 2 diabetes mellitus · Vertebral fracture · Bone fragility

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The number of patients with diabetes mellitus and osteoporosis is rapidly increasing in industrialized countries, where Western-style aging societies are prevalent. A relationship between diabetes and osteoporotic fractures is becoming increasingly recognized [1]. Vertebral and hip fractures are the most important osteoporotic fractures because they frequently occur and increase the mortality of elderly people as high as six- to ninefold [2, 3]. Although patients with type 2 diabetes show no bone mineral density (BMD) reduction, fracture risks are known to increase approximately up to 1.5-fold at the hip, proximal humerus, forearm, and foot [4–6]. Moreover, our recent study revealed that Japanese patients with type 2 diabetes have an increased risk of vertebral fractures independent of BMD [7].

Bone fragility in patients with type 2 diabetes may be caused by low bone turnover [8]. Hyperglycemia in type 2 diabetes might be associated with factors that influence bone strength and quality independently of BMD [9–11]. Several studies have indicated that hyperglycemia induces a low turnover bone with osteoblast dysfunction [12, 13]. Hyperglycemia and advanced glycation end products (AGEs) promote the apoptosis of osteoblastic cells [14, 15] and restrain the differentiation of cells [16–19]. These findings suggest that hyperglycemia may cause diminished

bone formation. A previous clinical study has indicated that serum osteocalcin (OC) was low before treatments and elevated after treatments of diabetes, while bone-specific alkaline phosphatase (BAP) was reduced [20]. Previous in vitro studies have shown that chronic hyperglycemia increased the activity and expression of BAP and decreased OC expression and cellular calcium uptake [10]. It is well-known that BAP is expressed in the early period of osteoblastic differentiation, whereas OC is expressed in the later period [21]. Thus, hyperglycemia could cause impaired osteoblastic maturation, resulting in bone fragility in patients with type 2 diabetes.

It is thought that bone metabolism in type 2 diabetes is affected by abnormal hormonal actions. Patients with type 2 diabetes appear to have increased BMD, possibly due in part to an anabolic effect of hyperinsulinemia [22, 23] and in part to obesity [24]. In addition, patients with type 2 diabetes have reduced bone turnover and may have reduced levels of parathyroid hormone (PTH) [25]. These factors may protect patients from reduction of BMD and fracture risks. On the other hand, insulin-like growth factor-I (IGF-I), which is anabolic for bone, may also be reduced in patients with type 2 diabetes [26, 27]. However, it is still unclear how these factors are associated with BMD, bone markers, or bone fragility in patients with type 2 diabetes.

In this study, to examine these issues, we investigated the relationships between bone markers (OC, BAP, and urinary N-terminal cross-linked telopeptide of type-I collagen [uNTX]) or BMD and HbA_{1c}, urinary C-peptide (uC-peptide), IGF-I, PTH, $1,25(OH)_2$ vitamin D, and the presence of vertebral fractures in Japanese men with type 2 diabetes.

Subjects and Methods

Subjects

The subjects in this study were 248 Japanese men with type 2 diabetes aged 20–83 years (mean 59.0). We consecutively recruited subjects who visited Shimane University Hospital for education, evaluation, or treatment of diabetes. Subjects agreed to participate in this study and gave informed consent. This study was approved by the institutional review board of our institution. None had hepatic or renal dysfunction or nutritional derangements that might cause changes in bone metabolism. We excluded patients with histories of falls and traffic accidents in order to eliminate the possibility of injury-associated fractures. Forty-two patients had received insulin treatment, 95 patients had taken oral hypoglycemic agents (sulfonylurea, 82; metformin, 28; alpha-glucosidase inhibitor, 28), and 121 patients had not previously been under any medications for diabetes. All subjects were free of drugs known to influence bone and calcium metabolism like vitamin D and bisphosphonate as well as thiazolidinedione until the time of the present study.

Radiography

Lateral X-ray films of the thoracic and lumbar spine were taken in the same week as the serum collection. The anterior, central, and posterior heights of each of the 13 vertebral bodies from Th4 to L4 were measured. A vertebral fracture was diagnosed if at least one of three height measurements along the length of the same vertebrae had decreased by >20% compared to the height of the nearest uncompressed vertebral body [28]. None of the subjects had a history of serious trauma.

BMD and Biochemical Measurements

BMD values of the lumbar spine (L), femoral neck (F), and one-third of the radius (1/3R) were measured by dual-energy X-ray absorptiometry (QDR-4500; Hologic, Waltham, MA). The same operator tested all of the subjects during the study to eliminate operator discrepancies. The coefficients of variation (precision) of measurements of the lumbar spine, femoral neck, and mid-radius by our methods were 0.9, 1.7, and 1.9%, respectively. Values were also expressed relative to the standard deviation (SD) of age- and sex-matched normal Japanese mean values provided by the manufacturer (Z score).

After overnight fasting, serum and first void urine samples were collected. Biochemical markers were measured by standard biochemical methods, as previously described [29, 30]. Hemoglobin A_{1c} (HbA_{1c}) was determined by high-performance liquid chromatography (HPLC). BAP in serum and uC-peptide pooled for 24 h were measured by enzyme immunoassay and chemiluminescent enzyme immunoassay, respectively. Intact PTH was measured by electrochemiluminescent immunoassay. $1,25(OH)_2$ vitamin D, OC, and IGF-I were measured by radioimmunoassay. uNTX was measured by enzyme linked immunosorbent assay.

Statistical Analysis

Data were expressed as mean ± SD. Because uC-peptide and intact PTH showed a markedly skewed distribution, logarithmic (log) transformation of these values was carried out before performing correlation and regression analyses. Statistical significance between the groups was determined using Student's *t*-test. Simple, multiple, and logistic regression analyses were performed using the statistical computer program StatView (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant.

Results

Relationships between BMD or Bone Markers Versus HbA_{1c}, uC-Peptide, IGF-I, Intact PTH, and $1,25(OH)_2$ Vitamin D

Baseline characteristics of subjects are shown in Table 1. Since our simple regression analysis showed that HbA_{1c}, uC-peptide, IGF-I, intact PTH, and $1,25(OH)_2$ vitamin D were affected by age, body stature, and renal function (data not shown), multiple regression analyses were performed with each of these parameters adjusted for age, body height, weight, duration of diabetes, and serum creatinine as an independent variable versus BMD at each skeletal site or bone markers as a dependent variable (Table 2). OC and OC/BAP ratio were correlated significantly and negatively with HbA_{1c} ($P = 0.0057$ and $P < 0.0001$, respectively) and positively with IGF-I ($P = 0.0095$). BAP was correlated significantly and negatively with IGF-I ($P = 0.0304$) and positively with log(intact PTH) ($P = 0.0247$). Although L- and F-BMD were not significantly correlated with HbA_{1c} or any hormonal parameters, 1/3R-BMD was correlated positively with HbA_{1c} ($P = 0.0416$) and negatively with log(intact PTH) ($P = 0.0324$).

Table 1 Baseline characteristics of subjects

Characteristic	Normal range	
Number of subjects		248
Age (years)		59.0 ± 13.7
Duration of diabetes (years)		10.7 ± 9.1
Body height (cm)		165.4 ± 7.0
Body weight (kg)		64.9 ± 16.0
BMI (kg/m^2)		23.6 ± 4.7
Serum creatinine (mg/dl)	0.44–1.23	0.77 ± 0.15
Fasting plasma glucose (mg/dl)	60–110	171 ± 60
HbA_{1c} (%)	4.3–5.8	9.1 ± 2.5
uC-peptide (μg/day)	60–120	70.9 ± 49.6
IGF-I (ng/ml)	59–215	151 ± 60
Intact PTH (pg/ml)	10–65	38.4 ± 16.2
$1,25(OH)_2$ vitamin D (pg/ml)	20–60	49.2 ± 19.4
BAP (U/L)	9.6–35.4	26.3 ± 9.4
OC (ng/ml)	2.5–13.0	5.1 ± 2.4
uNTX (nMBCE/mM-Cr)	13.0–66.2	34.8 ± 24.3
L2–L4 BMD (g/cm^2)		1.042 ± 0.181
T score		−0.04 ± 1.152
Z score		0.47 ± 1.12
F-BMD (g/cm^2)		0.776 ± 0.132
T score		−0.69 ± 1.06
Z score		0.25 ± 1.05
1/3R-BMD (g/cm^2)		0.711 ± 0.070
T score		−1.62 ± 1.32
Z score		−0.66 ± 1.14
Vertebral fracture (yes/no)		76/172 (30.6%)

BMI body mass index, *PTH* parathyroid hormone, *NTX* N-terminal cross-linked telopeptide of type-I collagen, *L* lumbar, *F* femoral neck, *1/3R* one-third of the radius

Comparison of Demographic and Biochemical Parameters, Bone Markers, and BMD Between Patients with and Without Vertebral Fractures

Next, we compared various parameters including HbA_{1c}, uC-peptide, IGF-I, intact PTH, $1,25(OH)_2$ vitamin D, bone markers, and BMD values at each site between patients with and without vertebral fractures (Table 3). Patients with vertebral fractures were significantly older ($P = 0.0071$), were shorter ($P = 0.0203$), and had lower absolute L-BMD ($P = 0.0441$) than patients without vertebral fractures. IGF-I and OC/BAP ratio in patients with vertebral fractures tended to be lower than in patients without them ($P = 0.0620$ and $P = 0.0940$, respectively). On the other hand, no significant differences in the levels of HbA_{1c}, uC-peptide, IGF-I, intact PTH, $1,25(OH)_2$ vitamin D, or bone markers were observed between subjects with and without fractures.

When multivariate logistic regression analysis was performed with the presence of vertebral fractures as a dependent variable and levels of HbA_{1c}, uC-peptide, IGF-I, intact PTH, $1,25(OH)_2$ vitamin D, bone markers, and BMD adjusted for age, body weight, height, duration of diabetes,

Table 2 Correlations between bone markers or BMD versus HbA_{1c}, uC-peptide, IGF-I, intact PTH, and $1,25(OH)_2$ vitamin D

	HbA_{1c}		Log (uC-peptide)		IGF-I		Log (intact PTH)		$1,25(OH)_2D$	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
BAP	0.063	0.3365	0.011	0.8776	−0.154	0.0304	0.154	0.0247	−0.092	0.2267
OC	−0.184	0.0057	–	0.9984	0.180	0.0095	0.033	0.6379	−0.098	0.2241
OC/BAP ratio	−0.250	<0.0001	−0.135	0.0618	0.255	0.0002	−0.042	0.5385	−0.049	0.5289
uNTX	0.040	0.5437	0.088	0.2165	−0.042	0.5487	–	0.9954	−0.095	0.2133
L2–L4 BMD	−0.074	0.2650	−0.005	0.9409	−0.002	0.9757	−0.134	0.0513	0.005	0.9499
F-BMD	−0.075	0.1973	0.071	0.2498	−0.002	0.9798	−0.101	0.0877	−0.031	0.6316
1/3R-BMD	0.127	0.0416	0.032	0.6437	0.062	0.2392	−0.142	0.0324	0.072	0.3392

Multiple regression analysis adjusted for age, body height, weight, duration of diabetes, and serum creatinine

PTH parathyroid hormone, *NTX* N-terminal cross-linked telopeptide of type-I collagen, *L* lumber, *F* femoral neck, *1/3R* one-third of the radius

Table 3 Comparison of demographic and biochemical parameters, bone markers, and BMD between those with and without vertebral fractures

	Vertebral fractures		*P*
	Yes	No	
Number of subjects	76	172	
Age (years)	62.5 ± 13.0	57.5 ± 13.7	0.0071
Duration (years)	11.7 ± 8.4	10.2 ± 9.4	0.2585
Body height (cm)	163.8 ± 6.7	166.1 ± 7.0	0.0203
Body weight (kg)	62.1 ± 12.2	66.1 ± 17.3	0.0647
BMI (kg/m^2)	23.0 ± 3.7	23.8 ± 5.1	0.2230
Creatinine (mg/dl)	0.77 ± 0.16	0.77 ± 0.15	0.8471
Fasting plasma glucose (mg/dl)	169 ± 51	172 ± 65	0.6965
HbA_{1c} (%)	9.0 ± 2.0	9.1 ± 2.7	0.6404
uC-peptide (μg/day)	71.8 ± 44.4	70.5 ± 51.9	0.8562
IGF-I (ng/ml)	140.8 ± 52.3	156.3 ± 62.7	0.0620
Intact PTH (pg/ml)	38.7 ± 13.5	38.3 ± 17.3	0.8609
1,25$(OH)_2$ vitamin D (pg/ml)	46.8 ± 15.9	50.2 ± 20.7	0.2976
BAP (U/l)	27.2 ± 8.9	26.3 ± 9.7	0.3651
OC (ng/ml)	4.9 ± 2.4	5.1 ± 2.4	0.4369
OC/BAP ratio	0.19 ± 0.10	0.22 ± 0.11	0.0940
uNTX (nMBCE/mM-Cr)	34.8 ± 15.7	34.9 ± 27.4	0.9848
L2–L4 BMD (g/cm^2)	1.006 ± 0.150	1.057 ± 0.192	0.0441
Z score	0.31 ± 0.92	0.54 ± 1.19	0.1349
F-BMD (g/cm^2)	0.754 ± 0.121	0.786 ± 0.137	0.0898
Z score	0.16 ± 0.91	0.30 ± 1.11	0.3368
1/3R-BMD (g/cm^2)	0.707 ± 0.062	0.712 ± 0.074	0.6487
Z score	−0.63 ± 1.08	−0.67 ± 1.17	0.7990

BMI body mass index, *PTH* parathyroid hormone, *NTX* N-terminal cross-linked telopeptide of type-I collagen, *L* lumbar, *F* femoral neck, *1/3R* one-third of the radius

Table 4 Associations between the presence of vertebral fractures and HbA_{1c}, uC-peptide, IGF-I, intact PTH, 1,25 $(OH)_2$ vitamin D, bone markers, and BMD

	Presence of vertebral fractures, OR (95% CI)	*P*
HbA_{1c}	1.021 (0.755–1.382)	0.8917
uC-peptide	1.215 (0.833–1.673)	0.2321
IGF-I	0.892 (0.634–1.256)	0.5132
Intact PTH	1.052 (0.776–1.426)	0.7435
1,25$(OH)_2$ vitamin D	0.824 (0.563–1.206)	0.3186
BAP	1.217 (0.922–1.605)	0.1654
OC	0.868 (0.644–1.168)	0.3493
OC/BAP ratio	0.695 (0.496–0.974)	0.0345
uNTX	0.984 (0.714–1.357)	0.9219
L2–L4 BMD	0.744 (0.549–1.007)	0.0559
F-BMD	0.899 (0.635–1.245)	0.4943
1/3R-BMD	1.174 (0.833–1.655)	0.3602

Multivariate logistic regression analysis was performed with the presence of vertebral fractures as a dependent variable and HbA_{1c}, uC-peptide, IGF-I, intact PTH, 1,25$(OH)_2$ vitamin D, BMD at each site, and bone markers adjusted for age, body height, weight, duration of diabetes, and serum creatinine as independent variables

PTH parathyroid hormone, *NTX* N-terminal cross-linked telopeptide of type-I collagen, *L* lumbar, *F* femoral neck, *1/3R* one-third of the radius, *OR* odds ratio, *CI* confidence interval

Table 5 Associations between the presence of vertebral fractures and OC/BAP ratio

	Presence of vertebral fractures, OR (95% CI)	*P*
OC/BAP ratio	0.695 (0.496–0.974)	0.0345
OC/BAP ratio[a]	0.682 (0.481–0.966)	0.0310
OC/BAP ratio[b]	0.707 (0.502–0.995)	0.0465
OC/BAP ratio[c]	0.687 (0.485–0.974)	0.0346
OC/BAP ratio[d]	0.708 (0.501–0.999)	0.0493
OC/BAP ratio[e]	0.704 (0.493–1.005)	0.0533

Multivariate logistic regression analysis was performed with the presence of vertebral fractures as a dependent variable and OC/BAP ratio as an independent variable adjusted for age, body height, weight, duration of diabetes, and serum creatinine

[a] Additionally adjusted for L-BMD

[b] Additionally adjusted for F-BMD

[c] Additionally adjusted for HbA_{1c}

[d] Additionally adjusted for IGF-I

[e] Additionally adjusted for HbA_{1C} and IGF-I

OR odds ratio, *CI* confidence interval

and serum creatinine as independent variables (Table 4), OC/BAP ratio was selected as an index affecting the presence of vertebral fractures ($P = 0.0345$). L-BMD tended to affect the presence of vertebral fractures ($P = 0.0559$) but was not significant. In contrast, F-BMD, 1/3R-BMD, and any other bone markers or hormones were not associated with the presence of vertebral fractures. OC/BAP ratio was still significantly and inversely associated with the presence of vertebral fractures after additional adjustment for L- or F-BMD, HbA_{1c}, or IGF-I (Table 5).

Discussion

In this study, OC/BAP ratio was correlated negatively with HbA_{1c} and positively with IGF-I in men with type 2 diabetes. Moreover, OC/BAP ratio was significantly and inversely associated with the presence of vertebral fractures independently of BMD. These findings suggest that poor glycemic control and lower IGF-I level may cause impaired osteoblastic differentiation and resultant reduction in OC/BAP ratio, which in turn may cause bone fragility and vertebral fractures independently of BMD in diabetic men. Thus, our findings seem to support the previous observations that hyperglycemia and reduced IGF-I are involved in bone fragility in type 2 diabetes [8–19, 26, 27]. However, multivariate logistic regression analysis showed that OC/BAP ratio was associated with the presence of vertebral fractures independently of HbA_{1c} or IGF-I (Table 5). This result as well as no association of HbA_{1c} or IGF-I with the presence

of vertebral fractures (Table 4) suggest that hyerglycemia or reduced IGF-I themselves are not directly linked to bone fragility but indirectly related to it by causing osteoblast dysfunction.

A recent meta-analysis showed that patients with type 2 diabetes had higher hip BMD than nondiabetic controls, despite an increased risk of hip fracture [4], suggesting that BMD values may not reflect bone fragility in type 2 diabetes. Recently, we also reported that L-BMD was not associated with the presence of prevalent vertebral fractures in women with type 2 diabetes, suggesting that L-BMD was not sensitive enough to assess the risk of vertebral fractures in this group [31]. In this study, we found that BMD at any site was not associated with the presence of vertebral fractures in men with type 2 diabetes, although L-BMD showed a tendency ($P = 0.0559$). Therefore, BMD, which is considered the gold standard for evaluating fracture risk in primary osteoporosis, seems to be not useful for assessing the risk of vertebral fractures in both men and women with type 2 diabetes. In postmenopausal women with type 2 diabetes, we have recently shown that serum IGF-I and pentosidine levels were associated with the presence of vertebral fractures independently of BMD, suggesting that they become surrogate markers for assessing the risk of vertebral fractures [29, 32]. In this study, we have shown that serum OC/BAP ratio could predict the presence of vertebral fractures in men with type 2 diabetes and could compensate for the insensitivity of BMD in the population.

IGFs are thought to be linked to the pathogenesis of diabetes-related complications [33]. Impaired production of IGFs could also cause bone complication in diabetes because IGFs are among the most important regulators of bone cell function [34]. Indeed, we previously found that serum IGF-I level was inversely associated with the risk of vertebral fractures in nondiabetic postmenopausal women [35, 36] as well as in their type 2 diabetic counterparts [29]. However, in men with type 2 diabetes, the relationship between serum IGF-I level and bone metabolism has been little documented. In this study, serum IGF-I level was correlated negatively with OC and OC/BAP ratio and positively with BAP, while the hormone was not significantly associated with BMD or the presence of vertebral fractures. Thus, in patients with type 2 diabetes, serum IGF-I level could predict the presence of vertebral fractures in postmenopausal women but not in men, although the significant positive correlation between IGF-I and OC/BAP ratio (Table 2) suggests that its reduction in the circulation was associated with impaired osteoblast function in men.

Several studies have shown that hyperglycemia causes hypercalciuria [37], which might result in enhancement of PTH secretion, while hyperglycemia could also cause suppressed PTH secretion from the parathyroid [25, 38]. Thus, impaired PTH and vitamin D metabolism might be involved in diabetic bone fragility. However, our present findings show that intact PTH and $1,25(OH)_2$ vitamin D are not associated with any bone markers or the presence of vertebral fractures in men with type 2 diabetes.

Although circulating insulin is considered to stimulate osteoblastogenesis and enhance bone formation [22, 39], the present study shows that uC-peptide, as a surrogate marker for residual insulin secretion, was not significantly associated with BMD or bone markers in men with type 2 diabetes. We also found that its level was not different between patients with and those without vertebral fractures. These findings are consistent with our previous ones in patients with type 2 diabetes, in which there were no associations between serum fasting C-peptide and BMD, bone metabolic markers, or vertebral fractures [29, 30]. However, subjects in our studies had received several treatments including insulin administration. Therefore, we should be cautious about the relationship between the capacity of residual insulin secretion and bone metabolism.

This study has some limitations. First, the sample size was not large enough to make definite conclusions. Second, we analyzed only subjects who visited Shimane University Hospital, a tertiary center, for evaluation or treatment of diabetes mellitus and osteoporosis. Therefore, the patients enrolled in this study might have relatively severe states of the disorders and might not be representative of Japanese men with the disorders. Third, the subjects in this study were only Japanese. The capacity of insulin secretion and degree of obesity in Asians are known to be different compared to Western people [40]. Therefore, it needs to be clarified whether or not our findings are universal. Fourth, we did not measure the fraction of undercarboxylated OC in men with and without fractures compared with healthy age-matched men. Increased metabolic bioactivity of undercarboxylated OC increased pancreatic β-cell proliferation, energy expenditure, insulin sensitivity, and adiponectin production and decreased adiposity [41, 42]. Thus, the undercarboxylated form of OC appears to regulate glucose homeostasis and to be one of the important bone markers when diabetes is studied. Finally, the conclusions of this study are weakened by its cross-sectional design and absence of age-matched healthy controls. Moreover, several other important variables were missing, such as 25-hydroxyvitamin D, estradiol, sex hormone binding globulin, and free testosterone. More than 50% of subjects were treated.

In conclusion, we found that serum OC/BAP ratio was more potently associated with the presence of vertebral fractures than BMD or other bone markers in men with type 2 diabetes, and it could be used as a surrogate marker for assessing the risk of vertebral fractures in that population. Thus, our previous and current studies together suggest that serum IGF-I and pentosidine levels in postmenopausal women [29, 32] and serum OC/BAP ratio in men may

compensate for the ineffectiveness of BMD in evaluating the risk of vertebral fractures in type 2 diabetes. We need to determine their cut-off values that most effectively detect incident vertebral fractures by conducting a prospective study on larger populations in future.

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Adiponectin Is Associated with Changes in Bone Markers during Glycemic Control in Type 2 Diabetes Mellitus

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Adiponectin Is Associated with Changes in Bone Markers during Glycemic Control in Type 2 Diabetes Mellitus

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Objective: Although several experiments show that adiponectin is associated with bone metabolism, a relationship between adiponectin and bone markers is still unclear. We monitored chronological changes in hyperglycemia, serum adiponectin, and bone markers during glycemic control in type 2 diabetes and analyzed relationships among these parameters.

Subjects and Results: A total of 50 Japanese patients with poorly controlled type 2 diabetes [initial hemoglobin A_{1c} (HbA_{1c}) = 10.0 ± 2.5%] were recruited, and biochemical data were collected before and after glycemic control for a month. Of bone formation markers, bone-specific alkaline phosphatase was decreased with a mean change of −3.11 [95% confidence interval (CI), −5.03 to −1.20; $P < 0.01$], whereas osteocalcin (OC) was increased with a mean change of 1.94 (95% CI, 1.45–2.42; $P < 0.001$) and undercarboxylated OC (ucOC)/OC ratio was decreased with a mean change of −0.15 (95% CI, −0.27 to −0.03; $P < 0.01$). Although adiponectin level was not significantly different before and after glycemic control, baseline adiponectin level, but not HbA_{1c}, was positively correlated with changes in OC, ucOC, and urinary N-terminal cross-linked telopeptide of type I collagen (uNTX) ($r = 0.30$, $P = 0.04$; $r = 0.32$, $P = 0.03$; and $r = 0.36$, $P = 0.01$, respectively). Changes in adiponectin were also negatively correlated with changes in OC and uNTX ($r = -0.42$, $P < 0.01$; and $r = -0.38$, $P < 0.01$, respectively). Changes in HbA_{1c} were negatively correlated with changes in OC ($r = -0.30$, $P = 0.03$).

Conclusion: These findings show that treatments for hyperglycemia enhance OC level and suggest that serum adiponectin level before starting to compensate poorly controlled diabetics could predict the subsequent improvement of bone remodeling markers during glycemic control. (***J Clin Endocrinol Metab*** **94: 3031–3037, 2009)**

The number of patients with diabetes mellitus and osteoporosis is rapidly increasing in industrialized countries where Western-style aging societies are prevalent. Recently, a relationship between diabetes and osteoporotic fractures is becoming increasingly recognized (1). Previous studies have shown that type 1 diabetes is associated with a decrease in bone mineral density (BMD) and an increased risk of osteoporotic hip and other fractures (2, 3). In contrast, although patients with type 2 diabetes show no BMD reduction, fracture risks are known to increase approximately up to 1.5-fold at the hip, proximal humerus, forearm, and foot (3–5), suggesting that they might have bone fragility that is not defined by BMD. However, it is still unclear why patients with type 2 diabetes have an increased risk of fracture despite normal BMD.

Bone mass is determined by a long-term net balance between bone formation and bone resorption. Bone fragility in patients with type 2 diabetes, if any, may be caused by low bone turnover (6, 7). Several studies indicated that hyperglycemia induced a low turnover of bone with osteoblast dysfunction and caused suppression of serum osteocalcin (OC) level (7, 8). Hyperglycemia and advanced glycation endproducts promote the apoptosis of osteoblastic cells (9, 10) and restrain the differentiation of the

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Abbreviations: BAP, Bone-specific alkaline phosphatase; BCE, bone collagen equivalents; BMD, bone mineral density; BMI, body mass index; CI, confidence interval; Cr, creatinine; FPG, fasting plasma glucose; HbA_{1c}, hemoglobin A_{1c}; OC, osteocalcin; ucOC, undercarboxylated OC; uNTX, urinary N-terminal cross-linked telopeptide of type I collagen.

cells (11–14). These findings suggest that hyperglycemia may cause diminished bone formation.

OC is a bone-specific protein of 49 amino acids that is synthesized by osteoblasts. Because a fraction of newly synthesized OC is released into the circulation, its serum concentration generally reflects mature osteoblastic activity and bone formation. OC contains three γ-carboxyglutamic acid residues derived from the vitamin K-dependent posttranslational modification of glutamic acid residues (15, 16). Serum undercarboxylated OC (ucOC) level has been reported to be increased in elderly women (17), particularly those with hip fracture (18), and to be negatively correlated with BMD (19). Although total OC level increases after improved glycemic control in type 2 diabetes (20, 21), it is still unknown how glycemic control affects serum ucOC level.

Adiponectin is one of the adipocytokines specifically and highly expressed in visceral, sc, and bone marrow fat depots (22). We and other researchers have shown that osteoblasts have an adiponectin receptor and that the proliferation, differentiation, and mineralization of osteoblastic cells are enhanced by adiponectin, suggesting that adiponectin could also influence bone metabolism (23, 24). Luo *et al.* (25) have shown that adiponectin regulated bone turnover via enhancing the receptor activator of nuclear factor-κB ligand (RANKL) expression and suppressing its decoy receptor, osteoprotegerin (OPG), although osteoclasts were not directly influenced by adiponectin. Thus, serum adiponectin could improve not only osteoblastic dysfunction but also low bone turnover, which is typically seen in diabetic patients and may cause bone fragility. However, only a few cross-sectional studies were performed on an association between serum adiponectin and bone markers in humans (26–28).

In this study, to clarify these issues, we investigated longitudinal changes in bone markers and serum adiponectin level before and after glycemic control in patients with type 2 diabetes for a month and statistically analyzed their relationships. We found that glycemic control might improve impaired bone formation and that serum adiponectin level could be clinically useful for predicting the beneficial bone reaction ahead of treatments.

Subjects and Methods

Subjects

From November 1, 2006, to April 30, 2008, type 2 diabetes patients with hemoglobin A_{1c} (HbA_{1c}) above 6.5% were enrolled and admitted to Shimane University Hospital. The subjects in this study were 50 Japanese patients with type 2 diabetes (31 men and 19 women) aged 28–87 yr (mean 63.6). Of the 19 female patients, 18 were postmenopausal. Nobody had hepatic or renal dysfunction or nutritional derangements that might cause changes in bone metabolism. All subjects were free of drugs known to influence bone and calcium metabolism like sex steroids, corticosteroids, vitamin D, vitamin K, calcitonin, or bisphosphonate as well as thiazolidinedione until the time of the present study. On admission, all subjects were put on a diet program; the calcium, vitamin D, and vitamin K content of the diet was 650 mg, 12 μg, and 242 μg/d depending on the caloric intake, which was 25–30 kcal/kg ideal body weight. At admission, 24 patients had previously not been under any medications for diabetes. Among the remaining 26 patients, 16 were on sulfonylurea agents (including four combined with metformin, two with α-glucosidase inhibitor, and two with insulin), 10 were on insulin (including one combined with metformin, two with sulfonylurea, and one with α-glucosidase inhibitor). At discharge, five patients were on diet alone, nine on sulfonylurea agents (including two combined with metformin, two with α-glucosidase inhibitor, two with both metformin and α-glucosidase inhibitor, and one with insulin), one on α-glucosidase inhibitors, three on metformin, and 32 on insulin (including one combined with α-glucosidase inhibitors and four with metformin). This study was approved by the ethical review board of our institution and complied with the Helsinki Declaration. All patients agreed to participate in the study and provided informed consent.

Biochemical measurements

On the second day after admission and the day before discharge, serum was collected after overnight fasting. The interval between the two determinations was 27.9 ± 6.3 d. Biochemical markers were measured by standard biochemical methods. HbA_{1c} was determined by HPLC. Bone-specific alkaline phosphatase (BAP) and serum OC were measured by enzyme immunoassay and RIA, respectively, as previously described (29, 30). ucOC was measured by electrochemiluminescence immunoassay, as previously described (31). Serum C-peptide and urinary N-terminal cross-linked telopeptide of type-I collagen (uNTX) were measured by ELISA, as previously described (29, 30). Total adiponectin was measured by an ELISA kit (Otsuka Pharmaceuticals, Tokyo, Japan) as indicated by the manufacturer. The coefficients of variation of measurements of total adiponectin by the ELISA kit was 3.1%.

Statistical analysis

Data are expressed as mean ± SD. We calculated changes in parameters by subtracting the baseline measurement from the second measurement. Statistical significance before and after glycemic control was determined using the Wilcoxon signed rank test, because serum adiponectin and bone markers showed a markedly skewed distribution [serum adiponectin: median value (med) = 5.7 μg/ml, minimum value (min) = 2.1 μg/ml, and maximum value (max) = 30.8 μg/ml; BAP: med = 26.6 U/liter, min = 9.1 U/liter, and max = 64.7 U/liter; OC: med = 4.4 ng/ml, min = 1.0 ng/ml, and max = 12.0 ng/ml; ucOC: med = 2.00 ng/ml, min = 0.39 ng/ml, and max = 10.10 ng/ml; uNTX: med = 32.6 nM bone collagen equivalents (BCE)/mM creatinine (Cr), min = 9.9 nM BCE/mM Cr, and max = 172.9 nM BCE/mM Cr]. Logarithmic (log) transformation of these values was carried out before performing correlation and regression analysis. Multiple regression analysis was performed after being adjusted for age, gender, duration of diabetes, body mass index (BMI), and serum Cr. Correlation analysis and multiple regression analysis were performed using the statistical computer program StatView (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered to be significant.

Results

Changes in markers for glucose and bone metabolism before and after glycemic control

Changes in markers for glucose and bone metabolism are summarized in Table 1. Diabetic control of the enrolled patients was poor on admission (HbA_{1c} = 10.0 ± 2.5%). Both fasting plasma glucose (FPG) and HbA_{1c} were significantly decreased after treatments with mean changes of −50.10 [95% confidential interval (CI), −74.65 to −25.55, $P < 0.001$] and −1.23 (95% CI, −1.56 to −0.89, $P < 0.001$), respectively, showing that hyperglycemia was markedly improved. Although body

TABLE 1. Changes in markers for glucose and bone metabolism

	Before	After	Mean change	95% CI	P
Subjects (male/female)	50 (31/19)				
Age (yr)	63.6 ± 13.7				
Duration of diabetes (yr)	12.2 ± 9.7				
Body height (cm)	160.0 ± 10.1				
Body weight (kg)	62.0 ± 19.1	61.0 ± 17.8	−1.75	−2.67 to −0.83	<0.001
BMI (kg/m^2)	24.0 ± 5.3	23.5 ± 4.9	−0.66	−1.00 to −0.32	<0.001
Serum Cr (mg/dl)	0.71 ± 0.25				
Serum C-peptide (ng/ml)	1.8 ± 1.0				
FPG (mg/dl)	197 ± 71	147 ± 53	−50.10	−74.65 to −25.55	<0.001
HbA_{1c} (%)	10.0 ± 2.5	8.8 ± 1.9	−1.23	−1.56 to −0.89	<0.001
Adiponectin (μg/ml)	8.5 ± 6.9	8.6 ± 6.5	0.05	−1.21 – 1.12	0.81
BAP (U/liter)	29.6 ± 12.5	26.6 ± 10.2	−3.11	−5.03 to −1.20	<0.01
OC (ng/ml)	4.8 ± 2.7	6.7 ± 3.4	1.94	1.45 – 2.42	<0.001
ucOC (ng/ml)	2.86 ± 2.44	3.37 ± 3.12	0.43	−0.26 – 1.13	0.42
ucOC/OC ratio	0.58 ± 0.38	0.44 ± 0.25	−0.15	−0.27 to −0.03	<0.01
uNTX (nM BCE/mM Cr)	39.9 ± 29.8	43.5 ± 33.8	3.59	−1.21 – 8.40	0.10
No medications [n (%)]	24 (48%)	5 (10%)			
Insulin [n (%)]	10 (20%)	32 (64%)			
Sulfonylurea [n (%)]	16 (32%)	9 (18%)			
Metformin [n (%)]	5 (10%)	11 (22%)			
α-Glucosidase inhibitor [n (%)]	3 (6%)	6 (12%)			

Statistical significance was determined using the Wilcoxon signed rank test. Normal range for serum Cr is 0.44–1.23 mg/dl; serum C-peptide, 0.6–28 ng/ml; FPG, 60–110 mg/dl; HbA_{1c}, 4.3–5.8%; adiponectin, 4.1–18.9 μg/ml; BAP, 9.6–35.4 U/liter; OC, 2.5–13.0 ng/ml; ucOC, <4.5 ng/ml; uNTX male, 13.0–66.2 nM BCE/mM Cr; and uNTX female, 14.3–89.0 nM BCE/mM Cr.

weight and BMI were significantly decreased with mean changes of −1.75 (95% CI, −2.67 to −0.83, $P < 0.001$) and −0.66 (95% CI, −1.00 to −0.32, $P < 0.001$), respectively, serum adiponectin level was not significantly different. BAP was decreased with a mean change of −3.11 (95% CI, −5.03 to −1.20, $P < 0.01$), whereas serum OC was increased with a mean change of 1.94 (95% CI, 1.45–2.42, $P < 0.001$). Although serum ucOC was not significantly changed, ucOC/OC ratio was decreased after treatments with a mean change of −0.15 (95% CI, −0.27 to −0.03, $P < 0.01$). On the other hand, uNTX was not significantly changed.

We have analyzed the difference in baseline data between the insulin treatment group and the noninsulin treatment group. However, we could not find any statistically significant difference in any variable except for serum C-peptide (insulin treatment, 1.29 ± 0.86 ng/ml, *vs.* noninsulin treatment, 1.94 ± 0.96 ng/ml, $P = 0.0498$). We have also reanalyzed changes in serum adiponectin and bone markers after separating between the insulin treatment group and the noninsulin treatment group. We found that BAP decreased and OC increased after treatments for diabetes regardless of insulin or noninsulin treatments (the data not shown).

Relationships between baseline values of demographic and biochemical markers *vs.* changes in bone markers during glycemic control

Next, we investigated whether or not baseline values of demographic and biochemical markers could be useful for predicting changes in bone markers during glycemic control (Table 2). Age was significantly and positively correlated with changes in BAP and ucOC/OC ratio ($r = 0.32$, $P = 0.03$; and $r = 0.42$, $P < 0.01$, respectively). Duration of diabetes was significantly and positively correlated with changes in OC ($r = 0.33$, $P = 0.02$). Changes in ucOC/OC ratio were significantly and negatively correlated with baseline body height, body weight, and BMI ($r = -0.30$, $P = 0.04$; $r = -0.38$, $P = 0.01$; and $r = -0.31$, $P = 0.04$, respectively). Baseline log(adiponectin) was significantly and positively correlated with changes in OC, ucOC, and uNTX ($r = 0.30$, $P = 0.04$; $r = 0.32$, $P = 0.03$; and $r = 0.36$, $P = 0.01$, respectively). Baseline log(BAP), log(OC), log(ucOC), and ucOC/OC ratio were significantly and negatively correlated with changes in BAP ($r = -0.58$, $P < 0.001$; $r = -0.28$, $P = 0.04$; $r = -0.40$, $P < 0.01$; and $r = -0.29$, $P = 0.04$, respectively). Baseline ucOC/OC ratio was significantly and negatively correlated with changes in ucOC ($r = -0.30$, $P = 0.04$). Baseline log(OC) was significantly and positively correlated with changes in ucOC/OC ratio ($r = 0.31$, $P = 0.04$), and baseline ucOC/OC ratio was significantly and negatively correlated with changes in ucOC/OC ratio ($r = -0.81$, $P < 0.001$). However, baseline serum C-peptide, FPG, or HbA_{1c} were not correlated with changes in any bone markers.

Next, to investigate whether baseline adiponectin or HbA_{1c} were related to changes in bone markers independent of age, gender, duration of diabetes, BMI, and serum Cr, multiple regression analysis was performed between baseline adiponectin and HbA_{1c} *vs.* changes in bone markers adjusted for these confounders. Baseline adiponectin was still significantly and positively correlated with changes in OC and uNTX ($r = 0.40$, $P = 0.04$; and $r = 0.48$, $P = 0.02$, respectively). On the other hand, baseline HbA_{1c} was not significantly correlated with changes in any bone markers.

TABLE 2. Correlations between changes in bone markers *vs.* baseline values of demographic and biochemical parameters

	ΔBAP		ΔOC		ΔucOC		ΔucOC/OC		ΔuNTX	
	r	P	r	P	r	P	r	P	r	P
Age	0.32	0.03	−0.04	0.77	0.23	0.14	0.42	<0.01	0.12	0.42
Duration of diabetes	0.01	0.94	0.33	0.02	0.24	0.11	0.00	0.99	0.24	0.09
Body height	−0.24	0.10	0.10	0.48	−0.13	0.40	−0.30	0.04	−0.25	0.08
Body weight	−0.10	0.49	0.10	0.81	−0.23	0.13	−0.38	0.01	−0.21	0.15
BMI	0.04	0.79	−0.02	0.91	−0.23	0.13	−0.31	0.04	−0.13	0.38
Serum Cr	0.07	0.61	−0.14	0.35	−0.19	0.22	−0.06	0.69	−0.04	0.80
Serum C−peptide	0.08	0.58	0.13	0.35	−0.11	0.49	−0.11	0.46	0.01	0.96
FPG	−0.14	0.35	0.19	0.18	0.17	0.28	0.19	0.21	−0.04	0.79
HbA_{1c}	−0.15	0.30	0.15	0.30	0.30	0.05	0.28	0.06	0.03	0.86
Log (adiponectin)	0.04	0.77	0.30	0.04	0.32	0.03	0.21	0.17	0.36	0.01
Log (BAP)	−0.58	<0.001	0.14	0.33	−0.09	0.58	−0.20	0.18	0.07	0.63
Log (OC)	−0.28	0.04	0.05	0.74	0.11	0.49	0.31	0.04	0.01	0.94
Log (ucOC)	−0.40	<0.01	0.11	0.45	−0.10	0.51	−0.24	0.11	−0.04	0.81
ucOC/OC ratio	−0.29	0.04	0.10	0.50	−0.30	0.04	−0.81	<0.001	−0.15	0.31
Log (uNTX)	−0.24	0.10	0.23	0.11	0.13	0.38	0.04	0.80	−0.04	0.81

Numbers in each cell describe a correlation coefficient. Logarithmic (log) transformation of adiponectin, BAP, OC, ucOC, and uNTX was carried out. Δ, Differences between after and before treatments.

Relationships between changes in body weight, hyperglycemia, and serum adiponectin *vs.* changes in bone markers during glycemic control

Next, we investigated relationships between changes in body weight, BMI, FPG, HbA_{1c}, and serum adiponectin level *vs.* changes in bone markers during glycemic control (Table 3). Changes in serum adiponectin were significantly and negatively correlated with changes in OC and uNTX ($r = -0.42$, $P < 0.01$; and $r = -0.38$, $P < 0.01$, respectively). Changes in HbA_{1c} were significantly and negatively correlated with changes in OC ($r = -0.30$, $P = 0.03$). Changes in body weight and BMI were significantly and positively correlated with changes in ucOC/OC ratio ($r = 0.41$, $P < 0.01$; and $r = 0.39$, $P = 0.01$, respectively). Changes in serum adiponectin were still significantly and negatively correlated with changes in OC and uNTX after being adjusted for changes in HbA_{1c} ($r = -0.42$, $P < 0.01$; and $r = -0.38$, $P < 0.01$, respectively). Changes in HbA_{1c} were also still significantly and negatively correlated with changes in OC after being adjusted for changes in serum adiponectin ($r = -0.30$, $P = 0.02$). These results suggest that serum adiponectin and HbA_{1c} were independently associated with bone markers.

Discussion

In this study, improvement of hyperglycemia in patients with type 2 diabetes was associated with a decrease in BAP as well as an increase in OC. Changes in HbA_{1c} were also significantly and inversely associated with changes in OC. Although ucOC was not significantly changed during treatments, the ucOC/OC ratio was decreased. It is known that OC reflects mature osteoblast function, whereas BAP and ucOC reflect an immature one (32, 33). Thus, these findings suggest that glycemic control may stimulate osteoblastic differentiation and enhance bone formation. On the other hand, baseline serum adiponectin level was positively associated with changes in bone markers during glycemic control. Changes in adiponectin were also negatively associated with changes in bone markers. These findings suggest that serum adiponectin could predict the degree of subsequent changes in bone markers during glycemic control and that bone metabolism could be linked to fat metabolism.

Several studies indicated that hyperglycemia induced a low-turnover bone with osteoblast dysfunction and caused suppression of serum OC level (7, 8, 21). Gerdhem *et al.* (7) have shown that serum OC, but not BAP, was lower in the diabetic women after correction for covariance of body weight and serum Cr. Okazaki *et al.* (20) have shown that serum OC was low before treatments and was elevated after treatments of diabetes, whereas BAP was reduced. On the other hand, Gregorio *et al.* (28) reported that improvement of metabolic control in non-insulin-dependent diabetes reduced serum OC level and increased bone mineral content. Our present findings seem to accord well with these former two studies. Previous *in vitro* studies have shown that chronic hyperglycemia increased the activity

TABLE 3. Correlations between changes in bone markers *vs.* changes in body weight, BMI, FPG, HbA_{1c}, and serum adiponectin

	ΔBAP		ΔOC		ΔucOC		ΔucOC/OC		ΔuNTX	
	r	P	r	P	r	P	r	P	r	P
ΔBody weight	−0.03	0.86	0.01	0.94	0.23	0.14	0.41	<0.01	0.20	0.17
ΔBMI	−0.09	0.53	0.05	0.76	0.25	0.11	0.39	0.01	0.19	0.21
ΔFPG	0.20	0.17	−0.26	0.07	−0.15	0.32	−0.09	0.55	−0.04	0.80
ΔHbA_{1c}	0.11	0.44	−0.30	0.03	−0.28	0.07	0.00	0.99	0.00	0.99
ΔAdiponectin	−0.07	0.61	−0.42	<0.01	−0.21	0.16	−0.01	0.97	−0.38	<0.01

Numbers in each cell describe a correlation coefficient. Δ, Differences between after and before treatments.

and expression of alkaline phosphatase, whereas it decreased OC expression and cellular calcium uptake (34). These findings explain the discrepancy in serum levels of OC and BAP in the clinical studies and suggest that hyperglycemia may directly impair osteoblastic maturation, which might result in impairment of bone quality as well as higher fracture rates in patients with type 2 diabetes despite no reduction in BMD.

Circulating ucOC is a valuable nutrition marker reflecting skeletal provision with vitamins K and D (35, 36). Vergnaud *et al.* (36) have shown that ucOC, but not total OC, predicted hip fracture risk independent of femoral neck BMD in elderly women, suggesting that ucOC could be clinically useful for assessing the risk of hip fracture independent of BMD. Although the levels of ucOC and ucOC/OC ratio are thought to be surrogate markers for bone quality as well as bone fragility (18, 37, 38), it was unclear how glycemic control affected ucOC and ucOC/OC ratio in patients with type 2 diabetes. Our present findings showed that OC was increased, whereas ucOC/OC ratio was decreased after glycemic control, suggesting that impaired bone formation in diabetes could be improved by treatments.

Studies on bone resorption status in diabetes are limited, and the results are conflicting. Some previous studies showed that hyperglycemia might activate osteoclasts, resulting in enhancement in bone resorption in diabetes (39). However, histomorphometric analysis of BB rats, which had long-term diabetes, showed that bone resorption was depressed (40). Clinically, alteration of bone resorption markers during glycemic control in type 2 diabetes seems to be controversial as well. Rosato *et al.* (21) indicated that pyridinoline (PYD) and deoxypyridinoline were increased after improved glycemic control, whereas Okazaki *et al.* (20) showed that deoxypyridinoline and type I collagen carboxy-terminal telopeptide were decreased. In this study, uNTX was not significantly changed during glycemic control, whereas serum OC and BAP were significantly changed. Changes in HbA_{1c} were also not significantly correlated with changes in uNTX, although they were significantly and negatively correlated with changes in OC. These findings indicate that glycemic control strongly affects bone formation markers, but not a bone resorption marker, and suggest that processes of bone formation and resorption might be uncoupled in type 2 diabetes and that treatments for hyperglycemia could improve impaired bone formation and bone remodeling.

Several recent experiments have shown that adiponectin could stimulate bone formation (23, 41) and regulate bone turnover (25). Several studies also documented a significant positive relationship between serum adiponectin and bone markers (26–28). Thus, adiponectin appears to mediate bone formation and bone remodeling, and serum hypoadiponectinemia may cause low turnover and lead to bone fragility in patients with type 2 diabetes (42), although the relationship between serum adiponectin level and BMD was still controversial (43–46). In this study, we observed that baseline serum adiponectin level was positively correlated with changes in OC, ucOC, and uNTX during glycemic control. We also found that changes in adiponectin were negatively associated with changes in OC and uNTX, which is independent of changes in HbA_{1c}. These findings indicate that baseline serum adiponectin value could be useful for predicting augmentation in bone markers during glycemic control and confirm the previous observations that serum adiponectin was clinically associated with bone markers in humans.

Fat mass is known to influence bone metabolism through adipocytokines, which are secreted from adipocytes (47). On the other hand, two recent animal studies have shown that OC derived from osteoblasts functions as a hormone regulating glucose metabolism and fat mass (48, 49). Moreover, it has been reported that bone marrow-derived circulating progenitor cells might transdifferentiate into adipocytes (50). Recently, we have shown that serum OC level is associated with glucose metabolism and atherosclerosis parameters in patients with type 2 diabetes (51). These findings suggest that bone metabolism and glucose/fat metabolism might be associated with each other. The association between bone markers *vs.* serum adiponectin and hyperglycemia found in this study might also suggest the interaction between bone metabolism and fat/glucose metabolism in a clinical setting.

This study has some limitations. First, the sample size was not large enough to make definite conclusions. Second, the subjects in this study were only Japanese, and BMIs in the present populations (mean, 24.0 kg/m^2) were lower than those observed in Caucasians. Capacity of insulin secretion and degree of obesity in Asians are known to be different from Western people (52), and thus our findings might not be applicable to Caucasians. Third, our study group was heterogeneous in treatments for diabetes. All diabetic medications may not be expected to have similar effects on bone turnover or adiponectin levels in the circulation. Although we found that changes in adiponectin or bone markers were not significantly different between the insulin treatment group and the non-insulin treatment group in the current study, we need to investigate this issue in future studies. Fourth, we should note the influence of caloric restriction on bone markers, although changes in body weight and BMI were not significantly associated with changes in bone markers except for that in ucOC/OC. Finally, a previous genetic study has shown that low serum adiponectin level might be influenced by genetic factors (53), and thus it is possible that genes for adiponectin may predetermine its serum levels independent of bone status, and the hormone levels may not reflect the bone microenvironment.

In conclusion, we found decreases in BAP and ucOC/OC ratio as well as an increase in OC after treatments for hyperglycemia in type 2 diabetes. Serum adiponectin level could predict these beneficial bone reactions ahead of glycemic control. These clinical observations might support the concept that fat/glucose metabolism and bone metabolism interact with each other.

Acknowledgments

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骨質マーカーとしてのペントシジンと骨折リスク

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要旨 骨強度は「骨密度」と「骨質」の要因から構成され，骨質は構造特性と材質特性から成る．骨コラーゲン内ペントシジン含有量が増加すると骨強度が低下することから，ペントシジンは骨の材質特性を変化させ骨脆弱性を招く．この骨内のペントシジン含有量は血液中の濃度と正相関し，その血液や尿中濃度の増加がそれぞれ2型糖尿病および原発性骨粗鬆症患者において，椎体骨折の増加と関係することが明らかにされた．これらの結果から血液および尿中ペントシジン濃度が骨質を反映した椎体骨折予測マーカーとして有用であることが示唆された．

〈Key point〉

はじめに

2000年の米国国立衛生研究所（NIH）のコンセンサス会議において，骨粗鬆症は「骨強度の低下を特徴とし，骨折のリスクが増大しやすくなる骨格疾患」と定義され，骨強度は「骨密度」と「骨質」の要因から構成されると報告された[1]．骨密度は骨強度の約70%を説明する因子である[1]．しかし続発性骨粗鬆症であるステロイド骨粗鬆症や2型糖尿病では，骨密度が保たれているにもかかわらず脆弱性骨折が増加し，その骨強度の低下は骨密度では説明できないことが明らかとなった．このことより骨質の低下は骨強度に対し強い影響を与えていると考えられ，骨質を評価することが重要視されるようになった．

骨質の低下

本稿では2型糖尿病の骨密度非依存性脆弱性骨折とペントシジンの関連性に着目し，骨質を反映する生化学マーカーとしてのペントシジンの可能性について概説する．

Key words : 骨質，ペントシジン，2型糖尿病，椎体骨折，骨密度

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