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Association of Bone Mineral Density with a Polymorphism of the Peroxisome Proliferator-Activated Receptor γ Gene: PPARγ Expression in Osteoblasts

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The peroxisome proliferator-activated receptor γ (PPARγ) protein as well as its transcript was detected in primary osteoblasts derived from rat calvariae. To analyze the possible involvement of PPARγ in the human bone metabolism, association between bone mineral density (BMD) and a polymorphism of PPARγ gene was investigated in Japanese postmenopausal women. We examined a polymorphism corresponding to a silent C → T transition located in exon 6 of the PPARγ gene, that was previously reported to be associated with plasma leptin levels in the obese. The frequencies of the C and T alleles in the population studied here were 0.851 and 0.149, respectively. When we separated the subjects into two groups, one bearing at least one T allele (CT + TT) and the other which did not (CC), the former subjects had lower BMD (Z score of total body; 0.056 ± 1.00. L2-4; −0.25 ± 1.26, mean ± standard deviation). These data suggest that there is an association between the restriction fragment length polymorphism (RFLP) of PPARγ gene and BMD and the possible involvement of this single nucleotide polymorphism (SNP) in the cause of postmenopausal osteoporosis in Japanese women.

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily of ligand-dependent transcription factors that is expressed predominantly in adipose tissue (1, 2) and the immune system (3), controls adipocyte differentiation and regulates glucose and lipid homeostasis (4, 5). Cumulative evidence indicates that osteoblasts and adipocytes share a common mesenchymal precursor (6, 7). PPARγ mRNA is expressed in some cells derived from osteosarcoma such as MG63 and SaOS-2, and its mRNA expression is significantly increased during the switch from osteoblastic lineage to adipocytic lineage in response to treatment with several PPARγ activators (8).

Osteoporosis is characterized by low bone mineral density (BMD) and by microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (9, 10). The most important predictor of fracture is BMD, which is determined by genetic as well as environmental factors. Importance of genetic factors has been well supported from twin (11–13) and family studies (14, 15). People having genetic risk factors are considered more susceptible to life-style factors. Therefore, it is required to clarify these genetic risk factors for diagnosis, prevention, and early effective treatment of osteoporosis. In addition, if the relevant genes were identified, the pathogenesis of osteoporosis would be explained by the variation of those genes or the loci adjacent to those genes.

Since the association of vitamin D receptor (VDR) genotype and BMD was reported (16), several genes have been investigated as genetic risk factors (17). These include estrogen receptor (ER) gene polymorphism (18, 19), collagen type Iα1 (COLIA1) gene polymorphism (20) and parathyroid hormone (PTH) gene polymorphism (21). It is a matter of concern that the panel of candidate genes should be expanded to elucidate the whole mechanism of the genetic aspect of osteoporosis, considering the polygenic nature of BMD distribution and many endocrinological factors known to regulate bone mass and bone turnover.

In the present study, we have chosen PPARγ gene as a candidate gene for a genetic marker of osteoporosis. We examined the expression of PPARγ gene in primary osteoblasts and investigated the association between a

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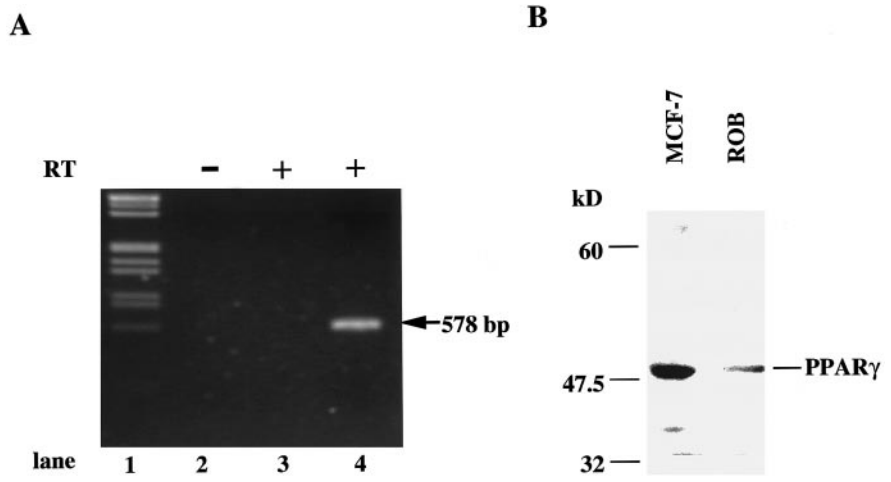

FIG. 1. Expression of PPARγ in primary osteoblasts. (A) Detection of rat PPARγ in poly(A)$^+$RNA from rat primary osteoblasts by RT-PCR with specific primers for PPARγ. There was no band in the sample without reverse transcriptase (RT) (lane 2) or template RNA (lane 3). A single band with predicted size (578 bp, indicated by an arrow) was detected in the lane with RT (lane 4). Lane 1 shows the molecular weight marker. (B) Immunoblot analysis of PPARγ. Twenty μg of MCF-7 cell and ROB extracts were resolved by SDS–polyacrylamide gel-electrophoresis, transferred to nitrocellulose filter and blots were probed with an anti-PPARγ antibody.

PPARγ gene polymorphism and BMD in postmenopausal Japanese women.

MATERIALS AND METHODS

Subjects. Genotype analysis was done using the samples obtained from 404 healthy postmenopausal Japanese women (ages 46–91 years, mean age ± S.D.; 66.3 ± 9.3 years) living in Nagano prefecture. Exclusion criteria included endocrinological disorders (such as hyperthyroidism, hyperparathyroidism, diabetes mellitus), liver disease, renal disease, use of medications that were known to affect bone metabolism (such as corticosteroids, anticonvulsants, heparin), or unusual gynecological history. All were non-related volunteers and we obtained informed consent from them before the study.

Cell culture. Rat primary osteoblasts were isolated by three sequential enzymatic digestions as described previously (22, 23). Briefly, calvariae of 21-day-old rat embryo were incubated at room temperature for 20 min with gentle shaking in an enzyme solution containing 0.1% collagenase, 0.05% trypsin, and 4 mM EDTA in phosphate-buffered saline (PBS(−)). Only the cells released from the fourth to sixth consecutive digests were cultured separately in α-modified minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS) and with antibiotics (100 μg/ml of streptomycin and 100 IU/ml of penicillin G) and the cells from the second passage were used for experimental determinations. Whole cell extracts were prepared by freeze-thawing and diluted in 100 μl of TEG buffer (10 mM Tris, pH 7.5, 1.5 mM EDTA, 10% glycerol).

Reverse-transcription PCR (RT-PCR). Poly(A)$^+$RNA was prepared from rat primary osteoblasts as described elsewhere (24). cDNA was synthesized from 0.1 μg of rat poly(A)$^+$RNA of primary osteoblasts using random 9 mers and AMV reverse transcriptase (Takara, Kyoto, Japan). Subsequent PCR amplification was carried out by the RNA PCR kit (Takara, Kyoto, Japan) for 30 cycles using an annealing temperature of 55°C in a Perkin–Elmer thermalcycler (Perkin–Elmer Cetus, Norwalk, CT). The oligonucleotides, 5′-GCC-AGTTTCGATCCGTGGAA-3′ and 5′-ATACAAGTCCTTGTAGATCT-3′ were used for amplification of 578 bp fragment of PPARγ mRNA (accession number:AB011365). Amplified PCR products from rat primary osteoblasts were enzymatically confirmed by digestion with Pst I followed by gel-electrophoresis.

Western blot analysis. Whole cell extracts were fractionated on 7.5% polyacrylamide SDS gels under reducing conditions. Twenty μg of proteins were then subjected to Western blot analysis using a monoclonal anti-PPARγ antibody which cross-reacts with human, rat and mouse PPARγ (Santa Cruz, CA) as described (23).

Measurement of bone mineral density (BMD) and biochemical markers. Lumber spine BMD and total body BMD (BMD in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast scan mode (DPX-L, Lunar Co., Madison, WI). The following biochemical parameters were measured; serum concentration of calcium (Ca), phosphate (P), alkaline phosphatase (AL-P), serum intact-osteocalcin (I-OC, ELISA, Teijin, Tokyo, Japan), serum 1,25(OH)2D3, parathyroid hormone (PTH) and calcitonin (CT). Calcium/creatinine ratio, phosphate/creatinine ratio, pyridinoline (Pyr, HPLC method) and deoxypyridinoline (Dpyr, HPLC method) in urine were also measured. Z score was used to analyze the data of BMD, which is a deviation from the weight-adjusted average BMD of each age. Z scores were calculated using the installed software of Lunar DPX-L based on 20,000 Japanese women's data.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). PCR-RFLP was performed as previously described (19). PCR was performed using oligonucleotide primers designed to amplify part of exon 6 of the PPARγ gene. The reaction was carried out in a final volume of 25 μl containing 100 ng of genomic DNA obtained from peripheral white blood cells, 10 pmol of each primer (primer 1: 5′-GCCCAGGTTTGCTGAATG-3′, primer 2: 5′-TGA-AGACTCATGTCTCTC-3′), 200 mM dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 0.1U Taq DNA polymerase (Takara, Kyoto, Japan). Thirty PCR cycles (each for 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C) were performed. PCR product was electrophoresed in a 2% agarose gel to verify the reaction. After amplification, the PCR product was digested with Pml I and electrophoresed in a 3.0% agarose gel. The polymorphic Pml I site was detected in this 208 bp fragment.

Statistical analysis. Comparisons of BMD by Z scores and biochemical markers between the group of individuals possessing one or two alleles of the genotype and the group without that genotype were

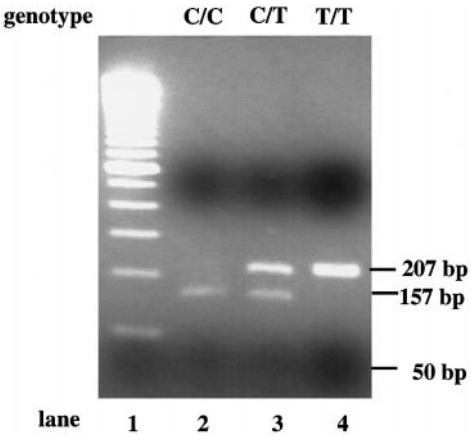

FIG. 2. Electrophoresis results of the PCR screening for polymorphism in PPARγ. PCR amplification of DNA followed by Pml I digestion was described in 'Materials and Methods'. When applied to a recombinant DNA sample, a single 208 bp product was derived from T/T homozygotes (lane 4); 208, 158, and 50 bp products were derived from C/T heterozygotes (lane 3); and 158 and 50 bp products were derived from C/C homozygotes (lane 2). Lane 1 shows the molecular weight marker.

performed using non parametrical analysis (Student's *t*-test, StatView-J 4.5). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Expression of PPARγ in primary osteoblasts derived from rat calvariae was shown by RT-PCR with specific primers for PPARγ (Fig. 1A). There was no band in the sample without reverse transcriptase (lane 2) or template RNA (lane 3). A single band with the predicted size (578 bp, indicated by an arrow) was detected in the lane with reverse transcriptase (lane 4). Endogenous PPARγ protein was also detected in rat primary osteoblasts (ROB) (Fig. 1B, lane 2). The size of the band corresponded to that of the band detected in MCF-7 cells (lane 1).

The CT transition located in exon 6 of the PPARγ gene was detected in Japanese women by the PCR-RFLP method (Fig. 2). As predicted, digestion of the 208 bp PCR product with Pml I produced three distinct patterns. A single 208 bp product was derived from T/T homozygotes (lane 4); 208, 158, and 50 bp products were derived from C/T heterozygotes (lane 3); and 158 and 50 bp products were derived from C/C homozygotes (lane 2).

In the 404 healthy postmenopausal volunteers who were not taking any medication, 291 C/C homozygotes, 106 C/T heterozygotes and 7 T/T homozygotes were detected. Allelic frequencies were 0.851 for the wild-type C allele and 0.149 for the rare T allele in Japanese postmenopausal women. Because there was only 7 T/T homozygotes, these subjects were included in C/T heterozygotes for all association studies.

We compared Z scores of BMD of lumbar and total body between the subjects bearing at least one T allele (C/T+T/T) and subjects without the T allele (C/C). Comparison of the Z scores of the lumber BMD be-

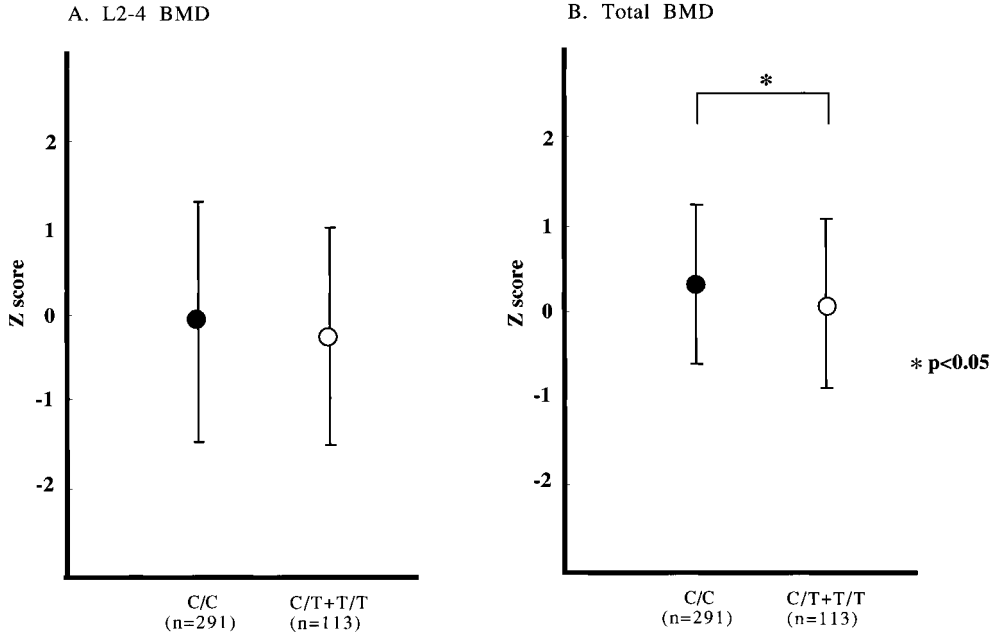

FIG. 3. The Z score values of lumbar and total body BMD in the groups with each genotype of PPARγ RFLP. (A) Z score value of lumber BMD is shown as the solid circle for genotype C/C and as the open circle for genotype CT + TT. Values are expressed as mean ± S.D. Number of subjects in parentheses. (B) Z score values of total body BMD as shown in the same manner as (A).

TABLE 1

Comparison of Background and Biochemical Data of the Subjects between the Two Groups of Genotypes

Items	Genotype		
	C/C	C/T + T/T	*p* value
Number of subjects	281	113	—
Age (years)	64.7 (9.7)	64.9 (9.5)	n.s.
Height (cm)	150.0 (6.4)	150.8 (5.9)	n.s.
Body weight (kg)	50.6 (8.1)	51.4 (7.9)	n.s.
Years since menopause	15.3 (9.9)	15.9 (9.4)	n.s.
Lumber spine BMD (Z score)	−0.076 (1.42)	−0.25 (1.26)	n.s.
Total body BMD (Z score)	0.29 (0.95)	0.056 (1.00)	0.044
Ca (mg/dl)	9.13 (0.39)	9.12 (0.42)	n.s.
P (mg/dl)	3.52 (0.45)	3.47 (0.43)	n.s.
AL-P (IU/l)	177.3 (57.2)	170.1 (43.5)	n.s.
I-OC (ng/ml)	7.47 (3.52)	8.1 (4.71)	n.s.
Pyr (pmol/μmol of Cr)	33.7 (11.3)	34.7 (10.4)	n.s.
Dpyr (pmol/μmol of Cr)	7.43 (2.77)	7.34 (2.15)	n.s.
intact PTH (pg/ml)	37.4 (13.7)	34.5 (13.3)	n.s.
CT (pg/ml)	22.9 (9.9)	23.2 (9.3)	n.s.
1,25-$(OH)_2D_3$ (pg/ml)	33.7 (12.5)	33.6 (11.0)	n.s.
TC (mg/dl)	197.8 (34.6)	195.2 (33.6)	n.s.
TG (mg/dl)	150.6 (89.1)	144.5 (78.0)	n.s.
%FAT	32.0 (7.9)	32.6 (8.2)	n.s.
BMI	22.5 (3.4)	22.6 (3.2)	n.s.

Note. Statistical analysis was done according to the method described in the text. Mean (±SD).

tween those with and without T allele showed a slightly higher average value, but its difference was not statistically significant (Fig. 3A). In contrast, Z scores of the total body BMD in C/C homozygote group was significantly higher than the other group (Fig. 3B). As shown in Table 1, there is no difference in the background data: values of mean age, height, weight, years since menopause, and biochemical markers (AL-P, I-OC, Pyr, and Dpyr) of each group. Thus, there was no statistically significant association between the C/T exon 6 PPARγ polymorphism and the other subject's profiles.

DISCUSSION

In the present study, endogenous expression of PPARγ was clearly demonstrated in primary osteoblasts, suggesting some roles of PPARγ in the bone metabolism. The rat and human predicted PPARγ proteins consist of 475 and 474 amino acids, with a calculated relative molecular mass (Mr) of 54.5 kDa and 54.3 kDa, respectively (25). Western blotting detected the band in rat osteoblasts of which the size corresponded to predicted Mr of the rat PPARγ. The immunoreactive band in MCF-7 cells (26) was also detected, confirming the specificity of the antibody used here. In addition to the induction of the differentiation of preadipocytes into adipose tissues, the ligands for PPARγ, such as prostaglandin J2, fatty acids and synthetic ligands (27–29), might modulate the osteoblast differentiation and/or switch from osteoblastic lineage to adipocytic lineage, via the receptor expressed in osteoblasts. The function of PPARγ *in vivo* is recently focused on the pathogenesis of atherosclerosis and on monocyte/macrophage differentiation (30–33). More functional studies should be required in diverse target tissues including the skeletal systems at physiological and pathophysiologocal conditions.

This study is the first to investigate the influence of a polymorphism of the PPARγ gene on the bone mineral property. Allele frequency of the C → T substitution in exon 6 of the PPARγ gene in Japanese postmenopausal women was in Hardy-Weinberg equilibrium and similar to the northern French population (34). We demonstrated that the Japanese postmenopausal women who had one or two allele(s) of a silent C → T transition showed lower total body BMD. Lower BMD in postmenopausal women can be considered as a result of abnormally rapid bone loss and/or lower peak bone mass (bone mass of the young adult). The data presented here showed no association between the bone metabolic markers and PPARγ gene polymorphism. This suggests that the effects of this locus on BMD may be related to the bone metabolism in earlier period of life, for example in the growing phase. Studies in different age groups could help understanding the mechanism of PPARγ gene effects.

Although it is still unclear how BMD is affected by this seemingly silent single nucleotide polymorphism (SNP) as revealed by an RFLP of the PPARγ gene, two hypotheses could be proposed. (i) These silent polymorphism may be linked with other exon mutation and contribute to the change of the PPARγ protein function or may be linked with mutation of other regulatory elements affecting the levels of expression through transcriptional regulation. (ii) The polymorphism in the PPARγ gene may be linked with mutation of another unidentified gene adjacent to the PPARγ gene which causes low BMD directly or indirectly.

It is reported that the C → T transition in exon 6 of the PPARγ gene is associated with plasma leptin levels in obese French subjects, suggesting that the polymorphism may influence the relationship between leptin concentration and adipose tissue mass in the obese (34). In practice, obese CT + TT subjects (BMI > 30 kg/m^2) had higher plasma leptin levels than C/C subjects. On the other hand, no statistically significant difference was found in the non-obese group. Leptin is recently reported to act on bone marrow stromal cells to enhance osteoblastic differentiation and to inhibit adipocytic differentiation (7). Moreover, recent genetic studies revealed that the several mutations in PPARγ gene may be involved in obesity (35) and insulin insensitivity (36, 37). It is possible that the mutation of the

PPARγ gene also modify the bone mineral property. For example, PPARγ gene variations might be involved in dysfunction of osteoblasts, leading to the decreased bone mineral property. Further studies are required to clarify the role of PPARγ in the bone metabolism.

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