*Kidney International, Vol. 63, Supplement 85 (2003), pp. S23–S27*

# Effect of VDR gene polymorphisms on osteocalcin secretion in calcitriol-stimulated human osteoblasts

**Manuel Naves, Daniel Álvarez-Hernández, José L. Fernández-Martín, José Paz-Jiménez, Pedro García-Prado, Teresa Fernández-Coto, Íñigo Santamaría, and Jorge Cannata-Andía**

*Bone and Mineral Research Unit, Instituto Reina Sofía de Investigación; and Orthopaedic Unit I, Orthopaedic Unit II, Biochemistry Unit, Hospital Central de Asturias, Oviedo, Spain*

**Effect of VDR gene polymorphisms on osteocalcin secretion in calcitriol-stimulated human osteoblasts.**

*Background.* The impact of vitamin D receptor (VDR) gene polymorphisms in bone metabolism remains controversial. Some authors have found a beneficial effect of some VDR gene polymorphisms, while others found no differences, or even a lower bone mass in subjects with the same type of polymorphisms. The aim of this study was to assess if the VDR gene polymorphisms could have an effect on the calcitriol-stimulated osteocalcin in human osteoblasts.

*Methods.* Osteoblasts were obtained from human femoral necks replaced because of osteoarthritis. Bones were cut into pieces of 1 to 2 mm and placed in a nylon mesh. After the migration of osteoblasts, the pieces were collected and cultured with different concentrations of calcitriol ($10^{-8}$, $10^{-9}$, and $10^{-10}$ mol/L). After 48 hours of incubation with calcitriol, the osteocalcin secreted into the medium (corrected by either total proteins or total DNA content) was measured. The DNA was extracted from the osteoblasts, amplified by polymerase chain reaction (PCR), and analyzed for target sequences sites of the *Bsm*I, *Apa*I, *Taq*I, and *Fok*I restriction enzymes.

*Results.* The response observed in osteocalcin secretion in the bb or TT genotypes doubled the response observed in the BB or tt genotypes (calcitriol $10^{-8}$ and $10^{-9}$ mol/L). A slight trend was also observed with the aa genotype. Men showed higher levels of osteocalcin secretion than women. Age did not show any influence in osteocalcin secretion.

*Conclusion.* VDR alleles and gender demonstrated an effect on the osteocalcin secretion. BB or tt genotypes, and also the "A" allele, showed the lowest calcitriol-stimulated osteocalcin secretion.

The identification of bone metabolism and bone mass–regulating genes opens new perspectives in the pathogenesis and treatment of metabolic bone disorders. The study of different gene polymorphisms is a useful tool for assessing the genetic influence on bone metabolism. Vitamin D receptor (VDR), estrogen receptor, and collagen type I gene polymorphisms, in addition to the TGFβ1-regulating genes, have shown influence on either bone mass or bone turnover markers [1–4].

Although the VDR gene polymorphisms have been widely studied, their clinical impact remains controversial. Some authors have found a beneficial effect of the *bb* genotype [4], whereas others have found no differences [5], or even lower bone mass in subjects with the b allele [6] in the analysis of *Bsm*I polymorphisms, one of the four VDR gene polymorphisms described to date. These differences could be due to several reasons, such as differences in the selection criteria, sample size, allelic heterogeneity, and environmental factors of the different populations studied [7], in addition to the allelic influence itself. Moreover, in our own population, we have found a different behavior between genders [8].

The synthesis of the VDR, a transcription factor of osteocalcin, is stimulated by calcitriol [9]. Recent clinical studies have reported that osteocalcin levels might be influenced by the different VDR gene polymorphisms [10–13]. Due to the heterogeneity of the in vivo results, the aim of our study was to investigate ex vivo if VDR gene polymorphisms could have an effect on the calcitriol-stimulated osteocalcin secretion in isolated human osteoblasts.

## METHODS

### Primary human osteoblast culture

Osteoblasts were obtained from human femoral necks replaced because of osteoarthritis. The donors (13 men, 11 women) had no metabolic disorders and the mean age was 69 ± 8 years (range, 50–80 years). The methods used to obtain the osteoblasts were based on previous reports by Marie et al [14] and Nàcher et al [15]. Briefly, trabecular bone was removed from the femoral neck and minced into small pieces of about 1 to 2 mm. The fragments were placed on a nylon mesh and incubated with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin/streptomycin (100 U/mL

**Key words:** VDR genotypes, vitamin D, bone cells.

and 100 μg/mL, respectively), nonessential aminoacids (1x), sodium piruvate (1 mmol/L), and 20% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% of $CO_2$. All the reagents used were purchased from Biochrom AG (Berlin, Germany). After one week, the osteoblasts grew from the bone pieces to the nylon mesh, surrounding the bone. The nylon mesh was then treated with a trypsin/EDTA solution (0.25/0.02%) and the osteoblasts were subcultured in 75 $cm^2$ flasks (Nunc, Copenhagen, Denmark) with DMEM, supplemented with antibiotics and 10% FCS to confluency. Cells obtained between the first and second subcultures were used for the experiments. The presence of osteoblasts was confirmed by histochemical staining for alkaline phosphatase, and by stimulation with calcitriol and measurement of the calcitriol-stimulated osteocalcin synthesized.

### VDR gene polymorphisms

DNA was extracted from the osteoblasts following Miller's method [16]. Osteoblasts were treated with a trypsin/EDTA solution (0.25/0.02%), washed thoroughly with phosphate buffer saline (PBS) (Sigma Chemical Co., St. Louis, MO, USA), and then lysed using a solution containing 10% of sodium dodecyl sulfate and proteinase K for 24 hours at 37°C. Afterwards, 6 mol/L of NaCl was added to precipitate DNA with 96% of ethanol. A fragment of 1365 bp of the VDR gene containing *Bsm*I, *Apa*I, and *Taq*I target sites was then amplified by PCR using the following primers: h-VDR1: GCAGAGTGTG CAGGCGATTCG and h-VDR2: GTGAAGCTCGTG TTCC-CCGCAAT in a 30 μL reaction volume. PCR conditions were 30 seconds at 95°C, 15 seconds at 64°C, and 25 seconds at 72°C for 30 cycles. The PCR product was divided into three different aliquots, digested with the *Bsm*I, *Apa*I, and *Taq*I restriction enzymes (Stratagene, La Jolla, CA, USA), and electrophoresed in a 2% agarose gel. The absence of the target site for the three endonucleases produced a single band of 1365 bp (alleles B, A, and T for *Bsm*I, *Apa*I, and *Taq*I, respectively). The allele "b" (*Bsm*I) produced two bands of 113 and 1252 bp, the allele "a" (*Apa*I) 284 and 1081 bp, and the allele "t" (*Taq*I) 208 and 1157 bp.

The genotyping of the *Fok*I polymorphism was performed at a different PCR amplification using the following primers: h-VDR3: GCTCCGAAGGCACTGTGC TCA and h-VDR4: AGAAGATACCACTCACCAAG ACC in a 25 μL reaction volume. PCR conditions were 15 seconds at 95°C, 15 seconds at 56°C, and 40 seconds at 72°C for 40 cycles. The PCR product was digested with the *Fok*I restriction enzyme (New England Biolabs, Beverly, MA, USA) and electrophoresed in 2% agarose. The presence of the target site produced two fragments of DNA of 191 and 302 bp (allele "f").

### Osteocalcin (OC) secretion by the osteoblast

The osteoblasts were seeded in 96-well plates (Nunc) at a density of 15,000 cells/$cm^2$. The cells were incubated with 100 μL of DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. After three days, the medium was replaced by DMEM supplemented with 0.1% bovine serum albumin (BSA) (Sigma Chemical Co.) and incubated for an additional 24 hours. The medium was then replaced by a similar one containing three different concentrations of calcitriol ($10^{-10}$ mol/L, $10^{-9}$ mol/L, and $10^{-8}$ mol/L) and incubated for a 48-hour period. The supernatants were collected for the measurement of osteocalcin (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Cells were washed twice with PBS and lysed by freezing and thawing three times in 100 μL of 0.05% Triton X-100. The total protein content and DNA were measured in the lysate. Total cellular protein was measured by the Bradford method (BioRad, Richmond, CA, USA) [17]. To determine the DNA content, cellular lysate was incubated for 30 minutes at 100°C in a steam bath to solubilize the DNA that was measured by a microfluorimetric method [18]. The osteocalcin secretion was corrected using the DNA content and total cellular protein (Table 1).

### Statistical analysis

All data were expressed as mean ± standard deviation (SD). Differences in the corrected osteocalcin secretion between VDR gene polymorphisms were expressed as fold increase in osteocalcin secreted at the lowest concentration of calcitriol ($10^{-10}$ mol/L). It was used at the lowest concentration because, under no stimulation, the secretion of osteocalcin is almost undetectable [19]. The Wilcoxon/Mann-Whitney test was used to compare the different VDR polymorphisms.

A multivariate analysis was also performed using osteocalcin secretion as dependent variables, and age, gender, and genotype as independent variables.

## RESULTS

The distribution of genotypes found in the primary osteoblast cultures from the 24 different patients studied are shown in Table 1. This table also shows a fold increase over control ($10^{-10}$ mol/L of calcitriol) in osteocalcin secretion corrected by proteins at $10^{-9}$ mol/L and $10^{-8}$ mol/L of calcitriol. The bb homocygote genotype showed a significantly higher osteocalcin secretion than the BB homocygote genotype with both concentrations of calcitriol ($10^{-9}$ mol/L and $10^{-8}$ mol/L). The TT homocygote genotype showed the same pattern but it was only significant with $10^{-9}$ mol/L of calcitriol. The Bb or Tt heterozygotes showed insignificant increments. The osteocalcin secretion in the bb or TT genotypes was double that of the BB or tt genotypes. A similar pattern was seen when

**Table 1.** Fold increase in osteocalcin levels (OC) using $10^{-9}$ mol/L and $10^{-8}$ mol/L concentrations of calcitriol and control ($10^{-10}$ mol/L of calcitriol)

| Genotypes | *N* (%) | OC/protein control group | Fold increase ($10^{-9}$ mol/L/$10^{-10}$ mol/L) | Fold increase ($10^{-8}$ mol/L/$10^{-10}$ mol/L) |
|---|---|---|---|---|
| *BB* | 5 (20.8) | 50 ± 29 | 2.5 ± 1.7 | 4.0 ± 1.9 |
| *Bb* | 10 (41.7) | 33 ± 15 | 4.7 ± 1.7 | 7.5 ± 3.6 |
| *bb* | 9 (37.5) | 37 ± 34 | 5.8 ± 2.5[a] | 8.3 ± 4.7[a] |
| *TT* | 9 (37.5) | 37 ± 34 | 5.8 ± 2.6[a] | 8.3 ± 4.9 |
| *Tt* | 9 (37.5) | 35 ± 16 | 4.5 ± 1.7 | 6.8 ± 3.1 |
| *tt* | 6 (25.0) | 46 ± 29 | 3.0 ± 2.1 | 5.6 ± 4.2 |
| *AA* | 5 (20.8) | 41 ± 29 | 4.4 ± 1.5 | 7.4 ± 3.7 |
| *Aa* | 15 (62.5) | 40 ± 28 | 4.0 ± 2.0 | 6.4 ± 3.2 |
| *aa* | 4 (16.7) | 28 ± 16 | 7.4 ± 2.8 | 9.3 ± 2.8 |
| *FF* | 8 (33.3) | 45 ± 24 | 4.2 ± 1.9 | 6.8 ± 4.4 |
| *Ff* | 10 (41.7) | 31 ± 32 | 5.7 ± 2.6 | 8.2 ± 4.2 |
| *ff* | 6 (25.0) | 41 ± 17 | 3.5 ± 2.0 | 5.6 ± 3.2 |

Table shows the raw data of osteocalcin corrected by protein in the control group (ng OC/mg protein).
[a] $P < 0.05$ compared with the *BB* or *tt* genotypes

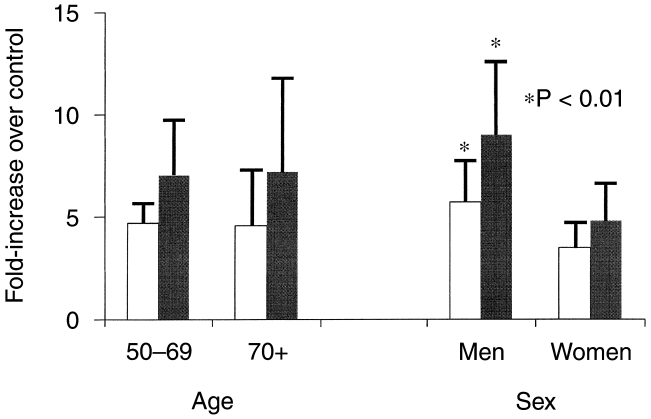


**Fig. 1. Fold increase in osteocalcin levels using $10^{-8}$ mol/L and $10^{-9}$ mol/L concentrations of calcitriol and the control ($10^{-10}$ mol/L of calcitriol) by age and by gender.** * $P < 0.01$ men compared with women.

osteocalcin secretion was corrected by DNA content instead of proteins. In contrast, no differences were found between the different *Apa*I and *Fok*I polymorphisms, although the increase in osteocalcin secretion in the aa homocygote genotype with calcitriol $10^{-9}$ mol/L was significant and almost double the other combined genotypes (AA and Aa) (Table 1).

The age of the donors did not show any significant influence in the osteocalcin secretion when the sample was stratified according to the median age (70 years) (Fig. 1). However, osteoblasts in men showed a higher osteocalcin secretion than in women (Fig. 1).

Furthermore, we performed a multiple linear regression analysis using the fold increase over the control of osteocalcin secretion as the dependent variable and age, gender, and genotype (*Bsm*I and *Taq*I) as independent variables. Only gender correlated with the osteocalcin fold-increase over the control at $10^{-8}$ mol/L of calcitriol ($\beta = -0.528$; $P = 0.008$). However, at $10^{-9}$ mol/L of calcitriol, the osteocalcin fold increase was influenced by the genotype only ($\beta = 0.522$; $P = 0.009$ for *Bsm*I and $\beta = 0.461$; $P = 0.014$ for *Taq*I). In a different analysis, grouping genotypes, the absence of the "A" allele, and gender correlated with the osteocalcin fold increase with $10^{-9}$ mol/L of calcitriol ($\beta = 0.440$; $P = 0.018$ for aa genotype; $\beta = 0.381$; $P = 0.037$ for gender).

## DISCUSSION

In our study, the distribution of some VDR gene genotypes was similar to previously published results carried out in a nonselected population of our region [8] and also other studies from our country [20, 21]. The most common genotype was Bb (42%), followed by bb (37%) and BB (21%).

In the sample studied, the different VDR gene genotypes and gender showed influence on osteocalcin secretion. The fact that the results have been consistent, either expressed as osteocalcin secretion corrected by total proteins or by DNA content, excludes in our study any likely influence of apoptosis on bone cells, as it has been recently suggested [22]. Osteocalcin (bone gla-protein) is synthesized by osteoblasts and may modify the activity of the cells and their precursors. It marks the turning point between bone formation and resorption and it may regulate mineral maturation [23].

Taking into account the known effects of the VDR gene on the regulation of the osteocalcin gene expression, it is reasonable to hypothesize that, among other things, different VDR gene polymorphisms may influence osteocalcin secretion. Our results support this hypothesis, as we found differences in the osteocalcin secreted by human osteoblasts in three out of the four VDR gene polymorphisms studied. The presence of the "b" or "T" alleles, and the aa homocygote genotype, was always associated with a higher osteocalcin secretion

in the osteoblasts under calcitriol stimulation. Accordingly, we have found the greater values in osteocalcin secretion in the bb, TT, or aa patterns. On the contrary, the lowest secretion of osteocalcin was seen in the BB and tt patterns, opposite haplotypes of bb and TT, which showed the positive significant results.

The favorable effect of some VDR alleles is in agreement with several previous clinical studies. Howard et al [10] observed an increment in serum osteocalcin in patients with the bb genotype who received calcitriol, and others [4, 8, 11, 24] have also found a positive effect of the "b" allele on bone mass and prevalence of vertebral fractures [8]. In addition, our group confirmed in a clinical study that the favorable bb, aa, and TT homocygote genotypes showed higher and more significant osteocalcin levels than any other haplotypes having "B," "A," and "t" when the 25-hydroxyvitamin D levels were adequate (>35 ng/mL) (data not yet published). The population studied is the same in which we found a slightly positive association between the favorable bb genotype and lower prevalence of vertebral fractures [8].

The *Fok*I polymorphisms showed absence of effect but this finding could be explained by the fact that *Bsm*I, *Taq*I, and *Apa*I polymorphisms are in linkage disequilibrium, while the *Fok*I site is in a region far from the rest of VDR gene polymorphisms.

There has also been described an influence of gender on VDR gene polymorphisms [8, 11]. In clinical studies, VDR gene polymorphisms were related to bone mass and bone loss in women [24], and also to a trend in the prevalence of vertebral fractures in postmenopausal women [8], but not men. In our study, the multivariate analysis also showed some gender effect on the osteocalcin secretion by human osteoblasts. The stimulation with calcitriol $10^{-9}$ mol/L did not show any differences by gender. Furthermore, using calcitriol $10^{-8}$ mol/L, osteoblasts from men ($N = 13$) showed a significant higher osteocalcin secretion. These differences cannot be explained by age. In contrast to another recent study, which has found an age effect [25, 26], we have not found any influence of age on the osteocalcin secreted by human osteoblasts obtained from the femoral neck.

In summary, the osteocalcin secretion showed a different pattern according to the VDR gene polymorphisms in human osteoblasts stimulated with calcitriol. The BB and tt genotypes, and also the "A" allele, showed the lowest osteocalcin secretion under stimulation with calcitriol. The effect of VDR polymorphisms was more pronounced with $10^{-9}$ mol/L of calcitriol.

## ACKNOWLEDGMENTS

We would like to thank Alejandra Canteros for her contribution to this work. The Fundación Renal Iñigo Alvárez de Toledo supported this study. Dr. Iñigo Santamaria is a recipient of a research contract from Fondo Investigaciones Sanitarias (FIS 00/3161). Daniel Álvarez-Hernández was supported by the Fundación Renal Íñigo Álvarez de Toledo, Instituto Reina Sofía de Investigación, and by the FICYT (Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y la Tecnología). Language consultants were Covadonga Díaz Díaz and Francesca Pieraccini.

*Reprint requests to Dr. Jorge Cannata Andía, Bone and Mineral Research Unit, Instituto Reina Sofía de Investigación, Hospital Central de Asturias, C/Julián Clavería s/n, 33006 Oviedo, Spain.*
*E-mail: metoseo@hca.es*

## REFERENCES

1. Eriksen EF, Langdahl B. The pathogenesis of osteoporosis. *Horm Res* 48(Suppl 5):78–82, 1997
2. Grant SFA, Reid DM, Blake G, *et al*: Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I a 1gene. *Nat Genet* 14:203–205, 1996
3. Kobayashi S, Inoue S, Hosoi T, *et al*: Association of bone mineral density with polymorphism of the estrogen receptor gene. *J Bone Miner Res* 11:306–311, 1996
4. Morrison NA, Qi JC, Tokita A, *et al*: Prediction of bone density from vitamin D receptor alleles. *Nature* 367:284–287, 1994
5. Garnero P, Borel O, Sornay-Rendu E, *et al*: Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss and bone mass in postmenopausal women: The OFELY study. *J Bone Miner Res* 11:827–834, 1996
6. Salamone LM, Ferrell R, Black DM, *et al*: The association between vitamin D receptor gene polymorphisms and bone mineral density at the spine, hip and whole-body in premenopausal women. *Osteoporosis Int* 6:63–68, 1996
7. Gómez-Alonso C, Naves-Díaz ML, Díaz-Corte C, *et al*: Vitamin D receptor gene (VDR) polymorphisms: Effect on bone mass, bone loss and on parathyroid hormone regulation. *Nephrol Dial Transpl* 13(Suppl 3):73–77; 1998
8. Gómez C, Naves ML, Barrios Y, *et al*: Vitamin D receptor gene polymorphisms, bone mass, bone loss and prevalence of vertebral fractures: Differences in postmenopausal women and men. *Osteoporosis Int* 10:175–182, 1999
9. Lian JB, Staal A, van Wijnen A, *et al*: Biological and molecular effects of vitamin D on bone, in *Vitamin D: Physiology, Molecular Biology and Clinical Applications*, edited by Holick MF. Totowa, NJ, Humana Press, Inc., 1998 pp 175–193
10. Howard G, Nguyen T, Morrison N, *et al*: Genetic influences on bone density: Physiological correlates of vitamin D receptor gene alleles in premenopausal women. *J Clin Endocrinol Metab* 80:2800–2805, 1995
11. Howard G, Nguyen T, Morrison N, *et al*: Genetic influences on bone density: Physiological correlates of vitamin D receptor gene alleles in premenopausal women. Notification of genotype corrections. *J Clin Endocrinol Metab* 83:1043, 1998
12. Morrison NA, Yeoman R, Kelly PJ, Eisman JA: Contribution of trans-acting factor alleles to normal physiological variability: Vitamin D receptor gene polymorphisms and circulating osteocalcin. *Proc Natl Acad Sci USA* 89:6665–6669, 1991
13. Tokita A, Matsumoto H, Morrison N, *et al*: Vitamin D receptor alleles, bone mineral density and turnover in premenopausal Japanese women. *J Bone Miner Res* 11:1003–1009, 1996
14. Marie PJ, Lomri A, Sabbagh A, Basle M: Culture and behaviour of osteoblastic cells isolated from normal trabecular bone surfaces. *In Vitro Cell Dev Biol* 25:373–380, 1989
15. Nàcher M, Aubia J, Serrano S, *et al*: Effect of cyclosporine A on normal human osteoblasts in vitro. *Bone Miner* 26:231–243, 1994
16. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215, 1988
17. Bradford MA: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
18. Cesarone CF, Bolognesi C, Santi L: Improved microfluorimetric DNA determination in biological material using 33258 Hoechst. *Anal Biochem* 100:188–197, 1979

19. Ohtera K, Ishii S, Matsuyama T: Influence of the vitamin D receptor alleles on human osteoblast-like cells. *J Bone Joint Surg Br* 83:134–138, 2001
20. Bernard M, Jaramillo MC, Aguado P, *et al*: Polymorphisms of the gene of vitamin D receptor and bone mineral density in postmenopausal women. *Med Clin* (*Barc*) 112:651–656, 1999
21. Fernández E, Fibla J, Betriu A, *et al*: Association between vitamin D receptor gene polymorphism and relative hypoparathyroidism in patients with chronic renal failure. *J Am Soc Nephrol* 8:1546–1552, 1997
22. Jilka RL, Weinstein RS, Bellido T, *et al*: Osteoblast programmed cell death (apoptosis): Modulation by growth factors and cytokines. *J Bone Miner Res* 13:793–802, 1998
23. Lian JB, Stein GS, Canalis E, *et al*: Bone formation: Osteoblast lineage cells, growth factors, matrix proteins, and the mineralization process, in *Primer on the Metabolic Bone Disease and Disorders of Mineral Metabolism*, edited by Favus MJ, Philadelphia, Lippincott-Williams & Wilkins, 1999, pp 14–29
24. Cooper GS, Umbach DM: Are vitamin D receptor polymorphisms associated with bone mineral density? A meta-analysis. *J Bone Miner Res* 11:1841–1849, 1996
25. Martinez ME, Medina S, Sánchez M, *et al*: Influence of skeletal site of origin and donor age on 1,25 (OH)2D3-induced response of various osteoblastic markers in human osteoblastic cells. *Bone* 24:203–209, 1999
26. Martínez P, Moreno I, de Miguel F, *et al*: Changes in osteocalcin response to 1,25-dihydroxyvitamin D3 stimulation and basal vitamin D receptor expression in human osteoblastic cells according to donor age and skeletal origin. *Bone* 29:35–41, 2001