Influence of the vitamin D receptor alleles on human osteoblast-like cells

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We investigated the effect of vitamin D receptor gene (VDRG) polymorphism on the responsiveness to 1,25(OH)\(_2\)D\(_3\) in human osteoblast-like cells. The cells were obtained from the femoral heads of 18 women with osteoarthritis of the hip. Three different restriction enzymes, BsmI, ApaI, and TaqI, were used to analyse the polymorphism. The genotypes of the 18 patients were bbAaTT (8), bbaaTT (6), BbAaTt (3), and BbAAI t (1).

Our findings showed that there were no differences according to the VDR genotype, but there was a statistically significant difference in the production of osteocalcin between BbAaTt and bbAaTT, and between BbAaTt and bbaaTT. Northern blot analysis of osteocalcin and VDR mRNA showed no significant differences among the three VDR genotypes. These findings suggest that VDR gene polymorphism affects the individual responsiveness of 1,25(OH)\(_2\)D\(_3\).

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Morrison et al\(^1\) reported that vitamin D receptor gene (VDRG) alleles affect bone mineral density (BMD). Their study, which used a molecular biological approach, was the first to clarify that genetic agents affect BMD. Subsequent studies have shown that VDRG alleles are involved in the decrease in postmenopausal BMD and that they affect absorption of calcium in the small intestine.\(^2\) It has also been reported that in osteoporosis VDRG alleles affect the additive effect of the BMD as a result of treatment with vitamin D,\(^3\) but Hustmyer et al\(^4\) and Garnero et al\(^5\) reported no relationship between BMD and VDRG alleles.

Materials and Methods

Isolation and culture of cells. We obtained bone specimens during hip replacement surgery for osteoarthritis from 18 women with a mean age of 63.22 years (sd 14.3). Clinical data obtained from measurement of the blood chemistry before surgery confirmed that the patients had no disorder of bone metabolism. The bone fragments were washed extensively in sterile phosphate-buffered saline (PBS) to remove blood and marrow tissue and were then dissected into particles 3 to 5 mm in diameter for seeding as explants onto 10 cm culture dishes (Falcon, Oxford, UK). These explants were cultured in 1 ml of Dulbecco’s modified medium (DMEM) containing 10% (v/v) of fetal bovine serum (FBS), 40 units/ml of benzylpenicillin potassium, and 0.1 mg/ml of kanamycin sulphate with 94% air and 6% CO\(_2\) at 37°C. The outgrowth of cells from the surface of the trabecular bone occurred within seven days, and reached confluence by 21 to 28 days. The adherent cells were dissociated with 0.25% tripsin (Difco Laboratories, Detroit, Michigan) in calcium- and magnesium-free PBS and were plated at the density required for the experimental producers. The cells were used for the studies from passages 3 to 5.

Restriction fragment length polymorphism (RFLP) analysis. The genomic DNA was extracted from osteoblast-like cells using standard methods. The VDRG was amplified by using the polymerase chain reaction (PCR). Detection of the BsmI site was achieved by amplifying a region spanning the site, with one primer originating in exon 7 (primer 1: 5’-CAACCAAAGACTCAAGTACGGGCTCGGT-3’) and the other in intron 8 (primer 2: 5’-AACCGGAAAGGTCAAGGG-3’) producing an 825 base pair (bp) fragment. Detection of ApaI and TaqI sites facilitated using a single amplification with one primer in intron 8 (primer 3: 5’-CAGAGCATGGAGGAGGGAAG-3’) and the other in exon 9 (primer 4: 5’-GCAACTCCTCATGGCTAGGCTACA-3’) producing the
740 bp fragment. Each sample was subjected to 30 amplification cycles in fast capillary PCR using an FTS-1 thermocycler (Corbett Research, Sydney, Australia). The RFLPs were analysed using restriction endonuclease BsmI, ApaI, and TaqI. We expressed the presence of the polymorphic restriction end nuclease site as b, a and t and its absence as B, A and T.

**Assay of cell proliferation.** We measured the proliferation of osteoblast-like cells by determining the content of DNA. Cells were plated at an initial density of 2 x 10^4 cells/cm² in 24-well plates and were cultured in DMEM supplemented with 10% FCS for 24 hours. At that time, the medium was removed and replaced with DMEM containing 0.025% bovine serum albumin (Gibco BRL, Grand Island, New York). The cells were precultured for 12 hours and then cultured for 48 hours with varying concentrations of 1,25(OH)₂D₃. They were dissolved by Triton X-100 and the content of DNA was measured on the cell extracts by the method of Kapuscinski and Skoczylas. Briefly, the solution of DNA in 0.01M NaCl was mixed with 4',6-diamidino-2-phenylindole 2HCl (DAPI) in water and buffered with HEPEs, at a final concentration of 0.005M, to pH7. These samples were then measured immediately after mixing using lengths of 362 and 454 nm.

**Assay of osteocalcin synthesis.** We determined the production of osteocalcin in the presence and absence of 10⁻⁸M 1,25(OH)₂D₃. The cells were precultured and cultured as described above for determination of the content of DNA. After incubation, the supernatant was collected and kept frozen at -20°C until assay and then tested by sandwich enzyme immunoassay (ELISA) using an osteocalcin kit (Takara, Otsu, Japan). We expressed the presence of the polymorphic restriction end nuclease site as b, a and t and its absence as B, A and T.

**RNA isolation and Northern blot analysis of osteocalcin and VDRG.** Total RNA was extracted from cell cultures using the total RNA isolation reagent (Gibco BRI). For electrophoresis, 20 μg of total RNA was loaded on 1% agarose gel containing 0.5 μg/ml of ethidium bromide. The RNA was capillary blotted on to nylon membranes (GeneScreen DUPONT, Boston, Massachusetts) in tenfold saline sodium citrate (SSC) and cross-linked with ultraviolet light. The human cDNA probes of osteocalcin and vitamin D receptor were generously provided by Dr Koshihara (Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). The blots were prehybridised in a solution containing 50% formamide, 0.9M NaCl, 0.1M NaPO₄ (pH7.4), 0.1% sodium dodecyl sulphate (SDS), 10 mg/ml of herring sperm DNA and five fold Denhardt’s solution at 42°C for four hours and then hybridised overnight at 42°C in the same solution containing the 32-labelled probes. The membranes were exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, New York) and an imaging plate and analysed using a Bio Imaging Analysis BAS 2000 (Fujix, Tokyo, Japan).

**Statistical analysis.** We performed analysis of variance (ANOVA) using the Statview statistical package (Abacus Concepts Inc, Berkeley, California). The significance of differences was determined by the F-test.

**Results**

The patients were divided into four groups according to VDRG genotype as follows: bbAaTT in eight, bbaaTT in six, BbAaTt in three, and BbAATt in one. The last was therefore excluded from analysis. We analysed the three major groups for VDR polymorphism and found no statistically significant differences in age or clinical data among the genotypes with BsmI, ApaI, and TaqI RFLPs (Table I).

Administration of 1,25(OH)₂D₃ did not affect the proliferation of the cells for 48 hours. In the bbaaTT group the concentration of DNA showed an increase of 13% compared with the control group without administration of 1,25(OH)₂D₃, in the bbAaTT group a decrease of 14.3% and in the BbAaTt group, an increase of 1.7% (Fig. 1). There was no significant difference between the effects of 1,25(OH)₂D₃ according to the genotype.

Basal production of osteocalcin by osteoblast-like cells was undetectable in the supernatant in the absence of 1,25(OH)₂D₃ but in its presence the cells produced osteocalcin in a dose-dependent manner. The concentrations of osteocalcin were corrected for the content of DNA. The geometrical mean of osteocalcin was 5.5, 16.7 and 13.8 ng/ml for the BB, AA and TT genotypes, respectively. One-way ANOVA of treated measurements showed that the mean differences between the three genotypes were significant (F = 5.24, p < 0.05). The
BbAaTt mean was equal to 32% of the bbAaTT mean (95% confidence interval (CI) 16 to 69) and 40% of the bbaaTT mean (95% CI 18 to 86) (Fig. 2). The concentration of 1,25(OH)₂D₃ required to produce a maximal effect was 10⁻⁸ M (data not shown).

We also examined the effect of differences in VDR genotype on osteocalcin and VDR mRNA expression. Figure 3a shows the pattern in each group. The Northern blot probed with osteocalcin and VDR revealed main transcripts of approximately 0.6 kb and 4.5 kb, respectively. Osteocalcin mRNA was not expressed unless 1,25(OH)₂D₃ was added to the culture. Figure 3b shows that treatment of the cells with 1,25(OH)₂D₃ led to an increase of osteocalcin mRNA of six to eight times compared with the control group, but there were no statistically significant differences in the levels of mRNA of the three genotypes. VDR mRNA was expressed without treatment with 1,25(OH)₂D₃, but the expression of VDR mRNA did not increase significantly after its addition. There were no statistically significant differences in the levels of VDR mRNA according to VDR genotype (Fig. 3c).

Discussion

Gene RFLP analysis is a useful method for finding individual differences in the base sequences in genes. Morrison et al. first reported that VDR gene polymorphism affects the maximum BMD, but there has been much debate as to its effect on bone metabolism. One of the reasons for this is that many factors are involved in bone metabolism and it is important to investigate directly the response to 1,25(OH)₂D₃ in osteoblasts involved in bone metabolism in each genotype.

Osteocalcin is the most abundant non-collagenous protein of the bone extracellular matrix and is synthesised only by osteoblasts. During bone remodelling it is a determinant of bone formation and is a key protein in regulating BMD. Therefore the varying amounts of osteocalcin stimulated by 1,25(OH)₂D₃ may affect the regulation of BMD. In our study the greatest production of osteocalcin was in the osteoblast-like cells of the bbAaTT genotype.

It is also known that in osteoporosis the effect of treatment with vitamin D varies among patients. Matsuyama et al. reported that the VDR genotype affects the response to 1α-hydroxy vitamin D. In particular, the more common genotypes (bbaaTT and bbAaTT) are associated with a positive response to treatment with 1α-hydroxy vitamin D. By contrast, BbAaTt, the most common type in Caucasians, has the poorest response. Our study showed the same tendency regarding the production of osteocalcin in osteoblast-like cells.

Northern blot analysis, however, for osteocalcin and VDR mRNA did not show a statistically significant difference among the VDR genotypes. Thus the question remains as to which process VDR polymorphism affects during the production of osteocalcin in osteoblast-like cells.

In this instance, vitamin D has an important role in regulating the production of osteocalcin. When vitamin D binds to the VDR, it recognises the vitamin-D-responsive elements (VDRE) in the gene. VDRE is located in the 5' region of the osteocalcin gene so that the transcription and translation processes for osteocalcin are stimulated by vitamin D. This discrepancy may be caused by a difference in the translation or stability of the message. Verbeek et al. and Mocharla et al. used the reverse-transcriptase polymerase chain reaction assay to measure the abundance of the mRNAs from two different VDR genotypes in normal lymphocytes, and in leukaemia and cell lines of prostate carcinoma. They observed no difference in the half-life of these two polymorphic transcripts. Gross et al. detected no significant differences in VDR abundance, binding affinity, and combined with these findings.
levels of VDR mRNA or responsiveness to 1,25(OH)₂D₃ in cultured skin fibroblasts of BB and bb genotypes, but Carling et al.²⁴ reported lower levels of the VDR message in parathyroid adenomas of patients with the BA/t genotype. These differing results suggest that the response to 1,25(OH)₂D₃ varies among the cell strains. Although our study does not measure the stability and translation of the message, the discrepancy between the protein and mRNA suggests that VDR polymorphism may alter this process.

Our study has several limitations. The number of each genotype was small. The patients who contributed the isolated osteoblast-like cells did not have osteoporosis. There may have been problems with the primary culture.

It is the first report, however, about the effect of 1,25(OH)₂D₃ on osteoblast-like cells of primary culture according to VDR polymorphism. We detected a difference in the amount of osteocalcin produced in the cell in the VDR genotypes. This suggests that the responsiveness to 1,25(OH)₂D₃ on bone metabolism varies according to the VDR polymorphism.

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References


