Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D₃

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(Received 7 June 1999; returned for revision 21 July 1999; finally revised 30 July 1999; accepted 7 October 1999)

Summary

OBJECTIVE In the vitamin D receptor (VDR) gene a BsmI restriction fragment length polymorphism (RFLP) in intron 8 and a translational start-site polymorphism, identified as a FokI RFLP, have been described. Crucial for a proper interpretation of these polymorphisms in association studies is the knowledge whether they have direct consequences for 1,25-(OH)₂D₃ action at cellular level. The present study was designed to assess functional significance of the FokI and BsmI VDR gene polymorphisms in peripheral blood mononuclear cells (PBMC) with a natural occurring VDR genotype for cell growth inhibition by 1,25-(OH)₂D₃.

DESIGN PBMC of women were isolated, VDR genotyped and in vitro inhibition by 1,25-(OH)₂D₃ of Phytohemagglutinin (PHA)-stimulated growth of PBMC was examined in relation to VDR genotype.

RESULTS PHA-stimulated growth and maximal growth inhibition were independent of VDR genotype. However, the FF genotype had a significant lower ED₅₀ than the Ff genotype corresponding to an allele dose effect of 0.32 nM per f allele copy (P = 0.0036). For BsmI genotypes no differences in ED₅₀ were observed.

CONCLUSION The present study demonstrates for the first time in cells with a natural VDR genotype a direct functional consequence of the VDR gene translational start-site polymorphism for the action of 1,25-(OH)₂D₃. Especially under conditions of vitamin D insufficiency these findings might have clinical implications.

Over the last years the genetic basis of osteoporosis has been intensively studied. In relation to this, several polymorphisms in the vitamin D receptor (VDR) gene have been identified. Firstly, the BsmI restriction fragment length polymorphism (RFLP) is located in intron 8 at the 3’ end of the VDR gene. Several studies have shown an association between this VDR polymorphism and bone mineral density (Morrison et al., 1994; Fleet et al., 1995; Spector et al., 1995; Uitterlinden et al., 1996), but other studies have not found such relationship (Hustmeyer et al., 1994; Garnero et al., 1995; Kröger et al., 1995). Putting all these data together, there seems to be a weak association with bone mass (Cooper & Umbach, 1996). Also associations with other phenotypes, like primary hyperparathyroidism, prostate cancer, and radiographic osteoarthritis have been described (Carling et al., 1995; Taylor et al., 1996; Inglés et al., 1997; Uitterlinden et al., 1997).

Recently, a polymorphism of the translational start-site has been identified in the VDR gene (Saijo et al., 1991; Sturzenbecker et al., 1994; Miyamoto et al., 1996; Arai et al., 1997). This polymorphism is characterized by the presence of either two ATG start codons separated by 6 nucleotides or due to a T to C substitution in the 5’ ATG site only the presence of the most 3’ ATG codon. The presence of the 5’ ATG-site results in recognition sequence for the FokI restriction enzyme (Gross et al., 1996). For this VDR polymorphism also both the presence and the lack of association with bone mineral density has been described (Gross et al., 1996; Arai et al., 1997; Harris et al., 1997; Eccleshall et al., 1998). In contrast to the BsmI polymorphism, the FokI polymorphism has distinct structural consequences for the VDR. The absence of the FokI restriction site, indicated as F, predicts a VDR protein of 424 amino acids, whereas the presence of the FokI site results in a VDR of 427 amino acids.

Crucial for proper interpretation of genetic association studies is the demonstration of functional consequences of these polymorphisms. For the BsmI polymorphism some in vitro studies have been performed showing potential association with
parameters related to bone turnover and bone mineral density (Howard et al., 1995; Kralj et al., 1995; Matsuyama et al., 1995; Graafmans et al., 1997; Kiel et al., 1997). However, these in vivo studies are complex and do not provide direct insights into the consequences for 1,25-(OH)\(_2\)D\(_3\) action at cellular level. A few in vitro studies have been performed to address functionality of VDR gene polymorphisms. In these studies cells were transfected with either one of the VDR genotypes (Morrison et al., 1994; Arai et al., 1997). A potential disadvantage of transfecting cells with VDR genotypes is the absence of control over subtle differences in expression of the gene of interest. Especially, these differences may be very important for the differential phenotypic effect of natural occurring gene polymorphisms. The present study was designed to investigate the functional consequences of the BsmI and FokI RFLP of the VDR gene for the action of 1,25-(OH)\(_2\)D\(_3\) in cells, peripheral blood mononuclear cells (PBMC) expressing the natural VDR genotypes. PBMC form a readily accessible target of vitamin D which is used as a model for studying genotype-dependent 1,25-(OH)\(_2\)D\(_3\) effects.

Materials and methods

Subjects

Fasting blood samples from 72 healthy postmenopausal women, aged 59–75 years (mean age ± SD, 65.7 ± 4.3 years) were taken. Women were randomly selected from independently living subjects of the Rotterdam Study (Hofman et al., 1991) whereby those using hormonal replacement therapy, cytostatics, vitamin D, thyroid hormone or known to have diabetes were excluded. Four women were excluded from analysis as no growth dose–response curves were present because no genotype data were available. Written informed consent was obtained from each participant. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam.

Cell culture

PBMC were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation (Böyum, 1968). The cells were suspended in phenol red-free RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO) supplemented with 10% charcoal-treated FCS, 2 mm glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Breda, The Netherlands) and 24 mm sodium bicarbonate. Immediately after isolation the cells were used for the proliferation analysis. For this purpose cells (4.4 x 10\(^6\) cells per ml) were cultured for 96 h in 24-wells plates (300 μl/well) in the presence of 3 μg/ml phytohemagglutinin (PHA) and 10\(^{-10}\) M to 10\(^{-7}\) M 1,25-(OH)\(_2\)D\(_3\) (kindly donated by Dr L. Binderup of LEO Pharmaceutical Products, Ballerup, Denmark). At the end of the incubation, DNA content of adherent and nonadherent cells was measured according to the ethidium bromide method of Karsten & Wollenberger (1977). For each individual two PBMC cultures were performed each consisting of two DNA measurements.

Due to ethical reasons it was not possible to perform both PBMC growth analyses and VDR measurements in one and the same individual. For the PBMC growth study 3–4 tubes of blood were needed (8–10 ml). In addition to this 3–4 other tubes had to be taken for clinical analyses. This would mean that over 10 tubes of blood from volunteers were needed in order to have at least the possibility of measuring VDR. For measurement of VDR in monocytes only we have calculated that even 21 tubes would be necessary.

Genotyping procedure

The BsmI RFLP at the 3’ end of the VDR gene was assessed by a direct haplotyping PCR procedure as previously described (Uitterlinden et al., 1996). The FokI RFLP was analysed by PCR with the primers described previously (Gross et al., 1996). The amplification protocol consisted of 28 cycles of 94, 60 and 72°C for 1 minute each. Ten microliters of the PCR products were digested with 10U FokI and 1.2 μl of a 10x buffer (containing 150 μM Tris- HCl, pH 7.5, 250 mM NaCl and 35 mM MgCl\(_2\)) by incubating for 30 minutes at 37°C. Digestion products were analysed on a 3% NuSieve agarose gel run in 0.5X TBE. Capital letters denote absence and lowercase letters the presence of the site for the restriction enzyme BsmI (B/B) or FokI (F/f).

Statistical analysis

For each individual a best fitted growth curve and maximal inhibition and ED\(_{50}\) values were calculated and analysed using GraphPad Prism software (GraphPad Software, Inc., San Diego, USA). Next, per genotype the mean maximal inhibition and ED\(_{50}\) (± SE) were calculated. Analysis of variance (ANOVA) was performed and for pairwise comparison student’s t-test was used. The relation between allele dose and ED\(_{50}\) was quantified by linear regression analysis.

Results

As shown in Table 1 incubation of PBMC with PHA resulted in an almost 2-3 times stimulation of growth compared to control cells. PHA-stimulated cell growth was strongly dose-dependently inhibited by 1,25-(OH)\(_2\)D\(_3\) (Fig. 1a) with a maximal inhibition of about 75% at 10\(^{-7}\)–10\(^{-5}\) M (Table 1). The half-maximal
inhibition was achieved at 1.05 nm 1,25-(OH)_2 D_3 (Fig. 1b). Neither PHA-stimulated cell growth nor maximal inhibition by 1,25-(OH)_2 D_3 appeared to be VDR genotype dependent. However, the ED_{50} showed differences between FokI genotypes in a gene-dose dependent manner (Fig. 1a,b). The PMBCs with the FF-genotype had the lowest ED_{50} value (0.82 ± 0.072) Ff heterozygotes had an intermediate ED_{50} value (1.24 ± 0.15), while ff homozygotes had the highest ED_{50} value (1.39 ± 0.3). Linear regression analysis demonstrated an allele dose effect of 0.32 nm per allele copy (P = 0.0036). No significant differences were observed when the 3 end of the VDR was genotyped by the direct haplotyping procedure (Uitterlinden et al., 1996), which may be due to too low numbers per genotype group to allow analysis (data not shown). Also when the analysis was restricted to the single BsmI polymorphism no with differences in ED_{50} (Fig. 1b) and maximal inhibition were observed (Table 1). The Apal and TaqI polymorphisms were also not associated with differences in maximal inhibition by 1,25-(OH)_2 D_3 and ED_{50} (data not shown).

Discussion

The current study demonstrates direct functional consequences of natural occurring VDR gene polymorphisms for the cellular action of 1,25-(OH)_2 D_3 in PBMC of healthy postmenopausal women. In most previous studies the functional consequences of VDR genotypes at the cellular level were only studied in cells transfected with different VDR alleles (Morrison et al., 1994; Arai et al., 1997; Gross et al., 1998a). Our study demonstrates that the half maximal concentration for 1,25-(OH)_2 D_3 inhibition of PHA-stimulated growth is significantly different between PBMC characterized by different alleles of the VDR translational start-site while the maximal inhibition is similar for all genotypes. The ED_{50} for cells homozygous for the 424 amino acids long VDR, i.e. the FF genotype, is lower than the ED_{50} for cells containing the 427 amino acids long VDR, i.e. the Ff and ff genotypes. A significant allele dose effect was observed which means that PBMC containing both forms of the VDR, the heterozygotes, have an intermediate ED_{50}. Arai et al. (1997) have also shown a more potent effect by the short VDR using HeLa cells transfected with a VDR FokI genotype and a 24-hydroxylase VDRE-reporter construct. They tested only one concentration of 1,25-(OH)_2 D_3 and it is not clear whether this reflects the maximal concentration or approaches the half-maximal effective dose for the response analysed by Arai et al. (1997). Also others showed a more active 424 amino acids VDR (Jurutka et al., 1998; Remus et al., 1998) while one other study did not observe differences between VDR FokI genotypes (Gross et al., 1998a). Together, our data support the hypothesis that the 424 amino acids-long VDR is more efficient in exerting 1,25-(OH)_2 D_3 effects than the 427 amino acids-long VDR. Although we were not able to measure VDR levels in the same PMBC of the individuals used in the growth study (see Materials and Methods), it is unlikely that the differences in effect are due to increased VDR expression by the FF genotypes (Arai et al., 1997). Arai et al. (1997), even reported that the FF genotype appears to have a somewhat (± 20%) reduced VDR expression. Maybe the numbers of ff are too low.
to show significance, but the somewhat higher maximal inhibition by the ff genotypes may reflect an increased VDR level to compensate for the lower affinity.

In view of the localization of the polymorphism in the N-terminal A/B region of the VDR it is unlikely that it will have an effect on 1,25-(OH)2D3 binding to the ligand binding domain in C-terminal E/F region. It is tempting to speculate that the difference in amino terminal sequence between the VDR genotypes directly affects binding of the VDR to its target genes. In this respect it is noteworthy that the VDR has a very short A/B region and therefore the sequence variation is located close to the DNA binding domain (C-region). Interesting in this respect are the observations by Jurutka et al. (1998) that the 424 amino acids VDR interacts more efficiently with the transcription cofactor TFII B and possesses elevated transcriptional activity compared to the 427 amino acids VDR.

The VDR FokI genotype effect on ED50 and not on maximal inhibition has potential clinical significance. In this way it is conceivable that dependent on the vitamin D status a VDR genotype effect is present or absent. So, especially under conditions of vitamin D insufficiency, for instance in elderly subjects, the biological consequences may become apparent, while at sufficient 1,25-(OH)2D3 levels the genotype consequences will be absent.

In contrast to the FokI RFLP, no relationship was observed between the VDR BsmI RFLP and 1,25-(OH)2D3 action which is in line with earlier findings (Gross et al., 1998b). An explanation for the dissociation with some in vivo data showing an association between BsmI VDR genotypes and response to 1,25-(OH)2D3 (Howard et al., 1995; Krall et al., 1995; Matsuyama et al., 1995; Graaffmans et al., 1997; Eccleshall et al., 1998) is unknown and yet purely speculative. Considering the localization of the BsmI polymorphism it is unlikely that it will have a direct structural effect on the VDR. The BsmI RFLP can be in linkage with polymorphisms in the 3′ UTR (Morrison et al., 1994; Ingles et al., 1997) and in this way be related to stability of VDR mRNA and VDR expression (Nesic et al., 1993). Maybe the present experimental system is not sensitive sufficient to observe effects of potential differences in VDR mRNA stability between BsmI genotypes. Some data showed that the BsmI RFLP does not affect the abundance of the VDR mRNA (Mocharla et al., 1997), while other studies indicated a difference in abundance of VDR mRNA between TaqI genotypes without an effect on VDR mRNA stability (Verbeek et al., 1997). Unfortunately as mentioned above, it was not possible to relate our present observations directly to VDR levels in PBMCs of the individuals in this study. Although the 3′ polymorphisms are not in linkage with the 5′ start site polymorphism analysis of combinations VDR genotypes may reveal further diversity in VDR activity (Remus et al., 1998). Preliminary analysis of the various combinations of FokI and BsmI genotypes in the current study didn’t provide additional information over the FokI analysis alone. It must, however, be noted that the number of individuals per genotype combination group is low and limits statistical analysis.

In conclusion, the current study provides evidence for direct functional consequences of the vitamin D receptor translational start-site polymorphism for the cellular action of 1,25-(OH)2D3 and provides insights into the vitamin D receptor genotype–phenotype association studies. The relationship with 1,25-(OH)2D3 concentrations is interesting and potentially important under conditions related to low 1,25-(OH)2D3 levels such as vitamin D deficiency and renal impairment. More generally, the present data demonstrate that differences in responsiveness between individuals may occur on the basis of naturally occurring variants of a single gene. Finally, it should be noted that in the present study one specific response of one specific target cell of 1,25-(OH)2D3 has been examined. For a full 1,25-(OH)2D3 response interaction of the vitamin D receptor with cofactors (e.g. transcription factor II B, see above) is essential and these cofactors may vary between responses and cell types. Therefore, additional analyses of other responses and cell types are needed to further assess the generality of the concept that the 424 amino acid vitamin D receptor is the more active form.

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**Table 1** Growth stimulation by PHA and maximal growth inhibition by 1,25-(OH)2D3 of PBMC according to VDR genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (%)</th>
<th>PHA-stimulated growth§</th>
<th>Maximal 1,25-(OH)2D3 effect</th>
</tr>
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<tbody>
<tr>
<td>All†</td>
<td>2·25±0·07</td>
<td>72·8±1·46</td>
<td></td>
</tr>
<tr>
<td>FF</td>
<td>34 (50·0)</td>
<td>2·18±0·10</td>
<td>71·0±2·15</td>
</tr>
<tr>
<td>Ff</td>
<td>25 (36·8)</td>
<td>2·28±0·12</td>
<td>71·6±2·18</td>
</tr>
<tr>
<td>ff</td>
<td>9 (13·2)</td>
<td>2·31±0·19</td>
<td>79·5±3·73</td>
</tr>
<tr>
<td>BB</td>
<td>10 (15·2)</td>
<td>2·31±0·14</td>
<td>72·4±4·55</td>
</tr>
<tr>
<td>Bb</td>
<td>35 (53·0)</td>
<td>2·21±0·11</td>
<td>73·7±5·91</td>
</tr>
<tr>
<td>bb</td>
<td>21 (34·9)</td>
<td>2·30±0·13</td>
<td>71·5±2·33</td>
</tr>
</tbody>
</table>

* n = the number of subjects. § PHA growth is expressed as fold stimulation over cell growth in the absence of PHA. † All = Effect independent of genotypes. Data are presented as means ± SEM.
References


