Evidence for Normal Vitamin D Receptor Messenger Ribonucleic Acid and Genotype in Absorptive Hypercalciuria*

JOSEPH E. ZERWEKH, MARK R. HUGHES†, BERENICE Y. REED, NEIL A. BRESLAU, HOWARD J. HELLER, MARTHA LEMKE, IGOR NASONKIN†, AND CHARLES Y. C. PAK

Center for Mineral Metabolism and Clinical Research, University of Texas, Southwestern Medical School, Dallas, Texas 75235-8885

ABSTRACT

Absorptive hypercalciuria (a stone-forming condition) is characterized by gut hyperabsorption of calcium, hypercalciuria, and reduced bone density. Inasmuch as these features implicate enhanced calcitriol action in gut and bone, we analyzed the vitamin D receptor (VDR) gene to ascertain whether an abnormality of this gene marks patients with intestinal hyperabsorption of calcium. We have compared the frequency of a restriction fragment length polymorphism (Ram I) associated with different alleles of the VDR gene in a group of 33 well characterized absorptive hypercalciuric patients and a group of 36 normal race- and age-matched control subjects. There was no difference between the distribution of the VDR alleles in the patient population when compared with the normal population. The coding region of VDR messenger RNA was also normal, as determined by both DNA sequence analysis and chemical mismatch cleavage analysis of copy DNA from 11 index absorptive hypercalciuric patients. On the basis of these results, we propose that the enhanced intestinal calcium absorption invariably seen in absorptive hypercalciuria and attendant symptoms of this disorder are not attributable to mutations of the VDR and are not linked to a common VDR genotype. (J Clin Endocrinol Metab 80: 2960–2965, 1995)

We have recently undertaken another approach to assess whether deranged 1,25(OH)₂D action may be a pathogenetic mechanism in AH. This approach was undertaken on the basis of several observations. First, AH is known to be inherited in an autosomal dominant fashion (9–11), clearly implicating a genetic defect. Second, Li et al. (12) reported increased numbers of vitamin D receptor (VDR) in the intestine of a normocalcemic, normal calcitriolemic rat model of AH. Because the disease is passed on to successive generations, it again supports a genetic process, and more specifically a possible defect in the molecular biology of the VDR. Third, we previously reported increased VDR numbers in activated lymphocytes from some patients with AH who had normal circulating 1,25(OH)₂D levels (13). Finally, in a comparison of 62 patients with AH and 31 nonhypercalciuric stone-forming patients, the lumbar bone density was significantly lower (~10%) in the hypercalciuric group, with 74% of the patients displaying values below the mean (14). The mechanism for such bone loss in AH is unknown. If the 1,25(OH)₂D concentration is increased, this bone loss could be the consequence of enhanced calcitriol-mediated bone resorption. It could also result from an inhibition of collagen synthesis and bone formation (15). In the face of a normal serum 1,25(OH)₂D concentration, the bone loss could be the result of increased sensitivity of the skeleton to 1,25(OH)₂D. Several recent reports have described frequent polymorphisms of the VDR and have shown that common allelic variation in the VDR locus can be used as a predictor of bone turnover and bone density (16, 17). In addition, these various VDR genotypes are associated with differing levels of transcriptional expression (17). On the basis of the above obser-
vations, we chose to examine the possibility that an abnormality of the VDR gene is associated with enhanced vitamin D action in the gut in patients with AH.

Materials and Methods

Materials

All restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture medium was purchased from Gibco BRL (Grand Island, NY). AmpliTAQ DNA polymerase was purchased from Perkin Elmer (Branchburg, NJ). Two normal human fibroblast cell lines were obtained from American Type Culture Collection (Rockville, MD), and one normal fibroblast line was kindly provided by Dr. J. Griffin (University of Texas Southwestern Medical Center, Dallas, TX).

Subjects

Sixty-nine subjects, comprising 33 patients with characteristics of AH (1–3) and 36 normal volunteers, participated in this study. The patient group consisted of 29 men and 4 premenopausal women. One of the patients was African-American and the others were Caucasian. The control population was Caucasian except for one African-American participant. All normal volunteers were healthy and had no history of kidney stone disease. None of the 21 normal volunteers screened for urinary Ca had hypercalcuria. Patients were recruited from those currently participating in ongoing protocols. All patients underwent an initial 4-day inpatient evaluation while on a constant metabolic diet containing 10 mmol Ca, 26 mmol phosphorus, and 100 mmol sodium per day in the general clinical research center. Venous blood samples obtained on days 2–4 of the diet were evaluated by multichannel analysis. On days 1 and 4, fasting venous blood was obtained for PTH and 1,25(OH)2D. The fractional intestinal Ca absorption (\(\alpha\)) was measured from the fecal recovery of orally administered \(^{47}\)Ca mixed in 2.5 mmol Ca (liquid synthetic diet) given on day 1. Fasting urinary Ca was obtained on days 1 and 4, and the calcicuric response to a 1-g oral Ca load was determined on day 4. Urine was collected in three 24-h pools on days 2–4 for Ca and creatinine (Cr). Lumbar bone density (L2-L4) was obtained on day 4 by dual energy x-ray absorptiometry by quantitative digital radiography bone densitometer (Hologic, Waltham, MA). The results are expressed as the absolute bone mineral density (g/cm²) as well as the z-scores. Twenty-seven patients were classified as AH type I (24-h urinary Ca more than 5 mmol on the metabolic diet mentioned above, urinary Ca excretion after a 1-g oral Ca load more than 0.56 mmol/mmol Cr, \(\alpha > 60\%), normocalcemia, and a normal or suppressed serum PTH (Table 1). Six patients had AH type II, which is believed to be a milder form with 24-h urine Ca less than 5 mmol on a restricted diet. Seventeen patients had fasting hypercalcuria (Ca > 0.32 mmol/mmol Cr). This may represent increased bone resorption in some patients. All patients had normal liver and kidney function (Cr clearance > 70 ml/min). No subject complained of dyspnea or chronic cough. One participant had a remote history of steroid-treated symptomatic sarcoidosis. However, she had normal 1,25(OH)2D and angiotensin converting enzyme levels, and a glucocorticoid challenge (50 mg prednisone per day for 7 days) did not affect urinary Ca or Ca absorption. Within 2 weeks of inpatient admission, no patient took antacids, nonsteroidal antiinflammatory drugs, Ca, phosphorous, vitamin supplements, diuretics, anticonvulsants, or glucocorticoids. Each subject gave informed consent, and the protocol was approved by the institutional review board. Skin biopsies were obtained from 59 of the subjects, and fibroblasts were cultured. Blood samples were obtained from the remaining subjects, and DNA was extracted from whole blood cells.

Blood chemistry

Serum Ca and phosphorous were determined by a multichannel screen. Intact immunoreactive PTH was determined in 26 patients by immunoradiometric assay with a kit from Nichols Institute (San Juan Capistrano, CA). Midmolecule PTH was determined on 7 of the earlier patient samples by RIA (Endocrine Metabolic Center, Oakland, CA). Because of limitations in sample volume and the age of these older specimens, we were unable to perform the intact immunoreactive PTH assay in these 7 patients. 1,25(OH)2D was determined as previously described (18, 19). Fasting serum Ca and phosphorous were measured in the normal subjects.

Fractional intestinal Ca absorption (\(\alpha\))

Fractional intestinal Ca absorption was determined after a synthetic test meal containing 2.5 mmol Ca and 1 mcg \(^{47}\)Ca, which was given in a fasting state. \(\alpha\) was calculated from the recovery of fecal radioactivity divided by the administered dose yielded \(\alpha\). Normal subjects did not undergo this test.

Cell culture

Skin biopsies were removed by a 3-mm punch. The resulting explants were washed three times in sterile culture medium (Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum, and 2 mmol/L glutamine, 100 U/mL penicillin, 100 mcg/mL streptomycin, and 0.25 mcg/mL fungizone) and dissected into six fragments using sterile technique. Three of the fragments were placed in each of two 60-mm culture dishes, and 3 ml standard culture medium was added. Fibroblast outgrowth was evident after 10–14 days. After 21–28 days in culture, the fibroblasts were harvested by a brief treatment with trypsin-EDTA (0.25% trypsin containing 0.02% EDTA wt/vol). A frozen stock of first passage cultures was established for each cell line. The fibroblasts were tested in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum and 10% dimethyl sulfoxide and maintained under liquid nitrogen until required.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal range</th>
<th>Patient values</th>
<th>Control values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Ca mmol/L</td>
<td>2.12–2.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.32 ± 0.35</td>
<td>2.32 ± 0.08</td>
</tr>
<tr>
<td>Phosphorous mmol/L</td>
<td>0.80–1.50</td>
<td>1.08 ± 0.14</td>
<td>1.13 ± 0.19</td>
</tr>
<tr>
<td>iPTH ng/L</td>
<td>10–65</td>
<td>32 ± 18</td>
<td></td>
</tr>
<tr>
<td>mPTH ng/L</td>
<td>100–400</td>
<td>195 ± 76</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)2D pmol/L (n = 27)</td>
<td>48–125</td>
<td>125 ± 34</td>
<td></td>
</tr>
<tr>
<td>Urine Ca, mmol/day</td>
<td>&lt;5.0</td>
<td>6.90 ± 2.09</td>
<td>3.80 ± 1.20</td>
</tr>
<tr>
<td>Ca/Cr, (fast) mmol/mmol</td>
<td>≤0.32</td>
<td>0.26 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Ca/Cr, (load) mmol/mmol</td>
<td>≤0.66</td>
<td>0.62 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>Ca(OH), % absorption</td>
<td>40–60</td>
<td>74 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Control values were determined in 36 age and sex-matched normal subjects.

<sup>b</sup> All values presented as mean ± SD.
Lymphocyte isolation and immortalization

Lymphocytes were isolated from whole blood by separation on a gradient of Ficoll Paque (Pharmacia, Piscataway, NJ). Whole blood was diluted with an equal volume of RPMI 1640 medium (Gibco, Grand Island, NY) layered over a cushion of 20 ml Ficoll, and centrifuged at 400 \* g for 30 min. The buffy coat layer was collected and washed twice with RPMI 1640 medium. The isolated lymphocytes were immortalized by infection with Epstein Barr virus (20). Cells were cultured in RPMI 1640 with 10% fetal calf serum. All patient lymphocytes were maintained as a frozen stock by freezing cell aliquots in 10% dimethyl sulfoxide and 20% fetal bovine serum in RPMI 1640.

DNA analysis

DNA extraction. DNA was extracted from either cultured fibroblasts (2-5 \* 10^6 cells) or 1 mL whole blood using a Qiagen micro DNA preparation kit (Qiagen, Chatsworth, CA).

Restriction fragment length polymorphism (RFLP) analysis

Aliquots of genomic DNA were amplified by polymerase chain reaction (PCR) on a 480 Perkin Elmer thermal cycler with primers directed at the intron sequence located between exons 8 and 9 of the VDR gene (primers 76 and R1), as depicted in Fig. 1. Primer 76 represented the forward primer starting at the 5'-end of exon 8. R1 was the reverse primer and was prepared according to the sequence as given by Morrison et al. (17). All primers were obtained from the Baylor College of Medicine (Houston, TX). DNA was digested with BsmI, and the products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Digestion of the PCR product with BsmI gave two fragments of approximately 400-405 base pairs (bp) and 170-175 bp. No ApaI site was found in this PCR product. Genomic DNA from skin fibroblasts was used for all patients and 29 normal subject analyses. For the remaining 7 normal subjects, genomic DNA from immortalized lymphocytes was used.

cDNA sequencing. Samples of messenger RNA (mRNA) from 11 unrelated stone-forming AH type I patients, with a strong family history of kidney stone disease, were chosen for sequence analysis. Total RNA was isolated from cultured skin fibroblasts by using a modification of the guanidium isothiocyanate method. Chronosomal DNA was sheared by passage through a 20-gauge needle. The sample was rapidly centrifuged on a cushion of CsCl in a TL100 Beckman ultracentrifuge at 150,000 \* g for 30 min. The total RNA was then harvested from the bottom of the tube. The RNA was subjected to reverse transcription using a primer (1137) complementary to the 3'-untranslated region of the human VDR receptor (Fig. 2). Standard reverse transcriptase reactions included avian myeloblastosis virus reverse transcriptase and 2 \* 10^-6 M total RNA. One tenth of this reaction was submitted to PCR using two oligonucleotide primers, one complementary to the 5'-untranslated region (1138) and a second complementary to the 3'-untranslated region (44). This latter nucleotide was positioned internally to the initial primer used in reverse transcriptase reaction. In effect, this produced a heminested reaction with significantly improved specificity and sensitivity for low level VDR transcripts. The full length VDR product was subcloned using a TA-vector, and the product was subjected to chemical mismatch cleavage analysis (see below). Additionally, a separate PCR reaction was performed that produced two halves of reasonable length for automated DNA sequencing using the primer pairs indicated in Fig. 2. The sequences of the aforementioned primers, as well as a more detailed description of the cDNA synthesis and PCR amplification, were published previously (21). The PCR product was then sequenced using the primer pairs indicated in Fig. 2. The sequences of the aforementioned primers, as well as a more detailed description of the cDNA synthesis and PCR amplification, were published previously (21). The PCR product was then sequenced using the primer pairs indicated in Fig. 2. The sequences of the aforementioned primers, as well as a more detailed description of the cDNA synthesis and PCR amplification, were published previously (21).

Chemical mismatch cleavage analysis. Initially wild-type DNA was amplified with one of the oligonucleotide primers containing a fluorescent 5'-tagged terminus. A second PCR was performed on each of the patient DNA samples. Aliquots of the wild-type and patient DNA were mixed, heated to 94 C, and cooled to form heteroduplexes and homoduplexes. The duplexes were then chemically treated with either osmium tetroxide or hydroxylamine (22). The double-stranded DNA treated in this manner can then be cleaved opposite mismatched bases with piperidine, and the fragments can be separated by electrophoresis.

Statistical methods

The relative association of RFLP markers and patient biochemical parameters was assessed by analysis of variance and Fisher's protected least significance value for multiple comparisons. All statistical analyses were performed by using Statview 4.0 (Abacus Concepts, Berkeley, CA.). The two-sample t test was used to assess the significance of differences in the relative frequencies of the VDR genotype between normal subjects and AH patients.

Results

Samples of copy DNA (cDNA) from 11 unrelated patients with AH and a strong family history of kidney stone disease were chosen for sequence analysis. The VDR cDNA samples from these same patients were subjected to chemical mismatch cleavage analysis as described above. This analysis was performed concurrently with the DNA sequencing. Both of these methods showed conclusively that there are no point mutations, insertions, or deletions in the coding region of the VDR from patients with absorptive hypercalciuria.

All 33 patient and 36 control genomic DNA samples were then tested for common RFLPs in the VDR by using the
restriction enzyme Bsm I. Initially, we used the primers published by Morrison et al. (17) for amplification of the polymorphic Bsm I and Apa I sites located in the region of the VDR gene from exon 7 to the 3'-untranslated region. Analysis of the hVDR cDNA sequence generated with these primers showed that the 3'-end of the forward primer is on the 3'-end of exon 7. The sequence of the reverse primer as published (17) was not found in the hVDR cDNA, implying an intronic location (17). The PCR product obtained with this pair of primers was approximately 750–800 bp in length. Because primers for the hVDR exons 7 and 8 were identified previously (21), it became a simple matter to more precisely locate the polymorphic Bsm I site (as being located within the intron between exons 8 and 9). Primer 76 (5'-end of exon 8) and reverse primer R1 gave a PCR product of 570–580 bp containing a polymorphic Bsm I site, which generated two fragments of 400–405 bp and 170–175 bp (Fig. 1). This prime pair was used for all subsequent RFLP analyses. No Apa I site was found in this region.

By using the convention of Morrison et al. (16), the RFLP was coded as Bb (Bsm I): the upper case letter signifies absence of the site, and the lower case signifies presence of the site. The results obtained by PCR were confirmed by separate repeated amplification and restriction endonuclease cleavage of all samples. The genotype of 33 nonrelated patients and 36 normal subjects was determined, and the frequency of the Bsm I RFLP (located between exons 8 and 9) is shown in Table 2. There was no significant difference noted for the distribution of the various alleles between the control and AH patient populations as determined by two-sample t test (Table 2).

Table 3 summarizes the biochemical presentations for the 33 AH patients divided by VDR haplotype. Although patients with the bb haplotype tended to have higher 1,25(OH)₂D levels, urinary Ca, and intestinal Ca absorption but lower vertebral bone mineral density, the differences between the three haplotypes were not significant when analyzed by analysis of variance.

Discussion

A number of previous investigations have examined the potential pathogenetic role of 1,25(OH)₂D in the intestinal hyperabsorption of Ca characteristic of AH. To date, these studies have yielded mixed results and have suggested that AH may be a heterogeneous disorder. In the present investigation, we have examined the VDR for a possible genetic abnormality.

We initially performed DNA sequence analysis of the entire VDR coding region in 11 unrelated patients with well-established AH. To date, all previous reports of single amino acid substitution in the VDR have resulted in reduced VDR ligand binding or affinity for DNA binding sites (23). However, if remains conceivable that a single mutation could result in enhanced ligand binding or DNA affinity, ultimately leading to enhanced ligand transcriptional activation, and in the case of AH, increased intestinal Ca absorption. Despite extensive DNA sequence analysis involving 8 separate reactions for each patient, no mutations, insertions, or deletions in the coding region of the cDNA were detected. To verify this observation, chemical mismatch cleavage analysis was performed on these samples. Although single-strand conformational polymorphism analysis has been reported to be a useful mutation-scanning method (24), in our hands this technology is only able to detect 40% of the already known mutations of the VDR. Consequently, we chose chemical mismatch cleavage analysis because it detects 100% of mismatched bases in a strand of DNA. This method again confirmed the lack of any mutations in the DNA sequence encoding the VDR from these 11 selected patients with AH. This finding indicates that there is no structural abnormality in the VDR in these patients with AH. Although a VDR of normal size has been observed in an animal model of AIH as demonstrated by the Western blotting technique (12), no sequence analysis of the VDR has been performed in those animals.

We undertook an examination of the VDR alleles to ascertain whether allelic variation in the VDR locus might discriminate patients with AH from the normal population. Previous investigators have disclosed that common allelic variation in the VDR locus can be used to predict bone turnover (16) and lumbar spine density (17). Such polymorphisms in the VDR gene may be of physiological significance in regulating the VDR gene transcriptional activity and mRNA stability. Indeed, one study has demonstrated substantially greater luciferase reporter gene activity from constructs containing the 3'-nontranslated region of the VDR gene from the BB genotype vs. the bb genotype (17). Because the active form of vitamin D is a principal regulator of bone and Ca homeostasis, it seemed conceivable that patients with AH might demonstrate a particular VDR allele frequency different from that found in the nonafflicted control population and contributory to increased vitamin D action in the gut and possibly in the skeleton. Additional support for such an examination is provided by observations of reduced spinal bone mineral density in a group of patients with AH (14) and more recently in a large group of male idiopathic Ca stone-forming patients (25). To date, no VDR genotyping has been reported in any of these patients. However, in our small number of stone-forming patients, we were unable to document a VDR polymorphism unique to this group or of greater frequency than that observed for the control population. With the use of the restriction enzyme Bsm I, no unique genotype was observed for our AH patients. In addition, no significant differences were found between VDR genotype and serum or urine biochemical parameters. We did observe a general trend for the highest values of al to be associated with the bb genotype in AH patients. However,
TABLE 3. Biochemical presentation and lumbar bone density values vs. VDR genotype in 33 AH patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>VDR genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BB (n = 9)</td>
</tr>
<tr>
<td><strong>Laboratory</strong></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>Ca mmol/L</td>
<td>2.32 ± 0.07*</td>
</tr>
<tr>
<td>Phosphorus mmol/L</td>
<td>1.16 ± 0.14</td>
</tr>
<tr>
<td>iPTH ng/L</td>
<td>22 ± 18</td>
</tr>
<tr>
<td>1,25(OH)₂D pmol/L (n = 27)</td>
<td>115 ± 41</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
</tr>
<tr>
<td>Ca, mmol/day</td>
<td>0.3 ± 1.0</td>
</tr>
<tr>
<td>Ca/Cr, (fast) mmol/mmol Cr</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>Ca/Cr, (load) mmol/mmol Cr</td>
<td>0.60 ± 0.17</td>
</tr>
<tr>
<td>Ca, % absorption</td>
<td>69.7 ± 7.5</td>
</tr>
<tr>
<td><strong>Bone density</strong></td>
<td></td>
</tr>
<tr>
<td>L2-L4 (n = 30) (g/cm²)</td>
<td>0.967 ± 0.143</td>
</tr>
<tr>
<td>Z-score</td>
<td>-1.16 ± 1.60</td>
</tr>
</tbody>
</table>

iPTH, immunoreactive PTH; Ca/Cr, calcium/creatinine.

* All values presented as mean ± SD.

the lack of a determinations in the normal subjects precluded any speculation regarding the relevance of this observation.

The observed frequency of the Bb and bb genotype in our normal population is not consistent with previous reports of the BB genotype being the most prevalent (17, 26-28). This difference may simply reflect the small number of control subjects examined in the present study. Alternatively, it may represent a true difference attributable to differences in the racial and ethnic makeup of the control groups (29). However, our control and patient populations were matched with respect to race and ethnic background in the present study, thus lessening any concern over genotypic variability.

We observed for this small number of stone-forming patients that the lowest vertebral bone mineral density in patients was associated with the bb VDR genotype. This is in contrast to an initial report (17), which suggested that the Bb genotype is associated with the highest spinal bone mineral density but more consistent with recent preliminary reports that failed to observe such an association between VDR genotype and bone mineral density (26-28). Thus, the relevance of VDR genotype and the susceptibility to reduced vertebral bone mineral density must await additional confirmation.

Although the present findings have not disclosed an alteration in the VDR mRNA, they do not discount a potential increase in intestinal VDR number or action. In the genetic hypercalciuric rat model of AH, Li et al. (12) observed normal enterocyte VDR mRNA synthesis by nuclear run-on assay but reduced VDR mRNA levels. These findings suggest that mechanisms other than VDR gene expression may be operative. These could include increased VDR mRNA translational efficiency or prolongation of the VDR mRNA or protein half-life. Alterations in the long 3'-nontranslated region of the VDR mRNA may be present in the AH patients, which might play a role in VDR stability. Our examination of the Bsm I restriction site did not include the full 3'-nontranslated region of the mRNA.

Finally, the lack of a unique VDR genotype associated with the AH patients does not eliminate from consideration an alteration in VDR gene expression. We determined VDR polymorphisms in well documented cases of AH, but all patients were unrelated. Ideally, such a study should be undertaken in large kindreds wherein the AH phenotype demonstrates an inherited pattern. RFLP analyses in such kindreds might demonstrate a VDR genotype common to all afflicted individuals that is distinct from that of unaffected relatives. Although the presence of a common VDR genotype in AH would not provide evidence for a VDR defect per se, it would provide a marker for the disease that could aid in the identification of a possible disease locus.

References