The Wilms' Tumor Gene Product (WT1) Modulates the Response to 1,25-Dihydroxyvitamin D₃ by Induction of the Vitamin D Receptor*

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The Wilms’ tumor gene (wt1) encodes a transcription factor involved in urogenital development, in particular in renal differentiation, and in hematopoietic differentiation. Differentiation of a number of solid tumor and leukemic cells lines can be mediated by 1,25-dihydroxyvitamin D₃. This is predominantly mediated by the nuclear receptor for 1,25-dihydroxyvitamin D₃, the vitamin D receptor (VDR). In initial experiments addressing a possible link between WT1 and VDR, we observed a correlated expression of WT1 and VDR mRNA in samples from renal tissues. HT29 colon carcinoma cells, stably transfected to express WT1, exhibited elevated endogenous VDR levels compared with control cells transfected with a control construct. Elevated VDR expression was found in wt1-transfected human embryonic kidney 293 cells, as well. In transient cotransfection experiments, we observed an activation of a vdr promoter reporter by WT1 through a WT1 recognition element, indicating transcriptional regulation of the vdr gene expression by WT1. The responsive sequence element was specifically bound by wild-type, but not by mutated WT1, in electrophoretic mobility shift assays. HT29 colon carcinoma cells, which respond to 1,25-dihydroxyvitamin D₃ with slow induction of growth arrest, were investigated for the influence of WT1 on 1,25-dihydroxyvitamin D₃-mediated growth suppression. Although HT29 cells transfected with a control construct responded moderately to 1,25-dihydroxyvitamin D₃, the response of HT29 cells expressing WT1 was strikingly enhanced. Stimulation with dihydroxyvitamin D₃ caused an up to 3-fold reduction in the growth rate of different HT29 clones expressing WT1 as compared with control cells lacking WT1 expression. Thus, induction of VDR by WT1 leads to an enhanced response to 1,25-dihydroxyvitamin D₃. We conclude that the vitamin D receptor gene is a target for transcriptional activation by WT1, suggesting a possible physiological role of this regulatory pathway.

In addition to its function as a regulator of calcium homeostasis, 1,25-dihydroxyvitamin D₃ has been demonstrated to be a potent inducer of differentiation in leukemic cells lines and cells derived from solid tumors (reviewed in Refs. 1 and 2). Physiologically, 1,25-dihydroxyvitamin D₃ directly binds to its cellular receptor, the vitamin D receptor (VDR),† resulting in transcription factor activity of the VDR. The VDR molecule dimerizes with the retinoic X receptor and activates target genes via interaction with vitamin D response elements in vitamin D-responsive promoters (1, 2). Additionally, vitamin D response element-independent functions of 1,25-dihydroxyvitamin D₃, referred to as “nongenomic signaling,” have been described, as well (3).

Our own previous experiments addressing the effects of 1,25-dihydroxyvitamin D₃ in leukemic cells pointed to an influence of Wilms’ tumor gene (wt1) expression on the induction of differentiation by 1,25-dihydroxyvitamin D₃ (4). The Wilms’ tumor gene (wt1) was isolated by deletion analysis and positional cloning in Wilms’ tumor and WAGR (Wilms’ tumor, aniridia, urogenital malformation, mental retardation) patients, leading to its definition as a tumor suppressor gene (5). The gene product contains a C-terminal zinc finger motif and a proline-glutamine-rich domain at the N terminus, similar to the early growth response-1 transcription factor (6). WT1 is expressed as four alternative isoforms containing or lacking a 17-amino acid stretch (termed 17AA) encoded by an alternatively spliced exon 5 and an insertion of three amino acids between zinc fingers 3 and 4, encoded by alternative splice II, termed KTS (7).

Expression of WT1 has been demonstrated in the kidney, the genital ridge, fetal gonads, spleen, and mesothelium (5, 8, 9). The absence of kidneys and gonads in wt1 knockout mice indicates the crucial role of wt1 for urogenital development (9). During hematopoietic differentiation, the Wilms’ tumor gene is transiently expressed, as well (10, 11). In contrast to its proposed role as a tumor suppressor, WT1 is expressed in the majority of acute leukemias (12), suggesting that the anti-oncogenic function of WT1 is dependent on the cellular context.

In initial experiments, we probed a multiple-tissue cDNA blot with probes for WT1 and VDR, observing a correlated expression pattern of VDR and WT1 mRNA. This led us to investigate a possible regulation of the expression of the VDR by WT1. In this work, we demonstrate that WT1 activates the VDR promoter in vitro. We show that that engineered expression of WT1 by HT29 colon carcinoma cells, which respond moderately to 1,25-dihydroxyvitamin D₃ (13), causes enhanced VDR levels and an enhanced response to 1,25-dihydroxyvitamin D₃. Induction of the VDR by WT1 is confirmed in human

* This work was supported by Deutsche Krebshilfe Grant 10-1290-Be 3 (to U. M. and L. B.) and Grant 10-1286-En 1 (to C. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: VDR, vitamin D receptor; HEK, human embryonic kidney; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium salts; kb, kilobase; WRE, WT1 recognition element.
embryonic kidney (HEK) 293 cells from renal origin, suggesting that the VDR gene represents a downstream target of WT1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—HEK293 and HT29 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfection, 1 × 10⁶ cells were seeded in a 10-cm dish, and after 24 h, they were transfected with 20 μg of expression plasmid using DOTAP transfection reagent (Roche Diagnostics, Mannheim, Germany). To generate stable transfectants, cells were selected for stable vector insertion with 500 μg/ml G418 (Life Technologies, Inc.). Clones derived from single cells were generated by limiting dilution and were analyzed for WT1 protein expression by Western blotting. 1,25-Dihydroxyvitamin D₃ was prepared as a 1000× stock solution (10 μM) in ethanol.

**RNAase Protection Assay**—Antisense probes for VDR and WT1 mRNA were generated by polymerase chain reaction amplification of the appropriate cDNA sequences (VDR 5′, CCCAGCTTCTCAGTCG; VDR 3′, TGACGGCGGTACTTGATGT; WT1 5′, ACACGCGCTACTCCCG; and WT1 3′, TGTCTCAGTCTCAGATGC; MWG Biotech, Ebersberg, Germany). The VDR amplicon (224 base pairs) was cloned in antisense orientation in pCR 3.1 (Invitrogen). The wt1 amplicon was digested with BamHI, and the resulting 154-base pair fragment was cloned into pCDNA 3 (Invitrogen) after digestion with BamHI/EcoRV. GAPDH and L32 probes (Pharmingen, San Diego, CA) were included for normalization. A multiple probe for cell cycle genes including p21 was also purchased from the manufacturer. In vitro transcription and ribonuclease protection assay was performed with a kit (Pharmingen, San Diego, CA) according to the manufacturer’s instructions analyzing 20 μg of total RNA isolated with Trizol reagent (Life Technologies, Inc.). Protected RNA-RNA hybrids were separated on a 6% sequencing gel and subsequently exposed to Biomax x-ray film (Eastman Kodak, Co., Rochester, NY) or to a PhosphorImager system (Molecular Dynamics).

**Western Blotting**—Protein lysate was dissolved in Laemmli buffer (500 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol, pH 6.8), heated to 95 °C, and subjected to a 10% polyacrylamide gel electrophoresis. After blotting to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and blocking with skim milk (Fluka, Steinheim, Germany), the membrane was incubated with anti-WT1 C19, anti-WT1 180, or anti-VDR C-20 (all from Santa Cruz Biotechnologies) antibodies diluted 1:1000. Detection was performed by incubation with a horseradish peroxidase-conjugated anti-rabbit antibody (dilution 1:5000), and the ECL system (both from Amersham Pharmacia Biotech). For further analysis, blots were stripped with 0.2 M glycine, pH 2.5, at 56 °C for 30 min.

**Transient Transfection Experiments**—Different murine vitamin D receptor promoter fragments were subcloned in to a pGL2 basic vector upstream of the firefly luciferase reporter gene as described elsewhere (14, 15). A mutant lacking the WT1 recognition element (WRE)-related sequence was generated by digestion of the 0.5-kb construct with FseII and SmaI, followed by treatment with T4 polymerase and religation. The expression constructs used were WT1+17AA-KTS, WT1+17AA+KTS (wt1B and wt1D, kindly provided by F. J. Rauscher, III, Wistar Institute, Philadelphia, PA), and WTAR, encoding a truncated protein (16). The wt1 coding region was subcloned into pCDNA3 (Invitrogen). NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfection, 0.5 × 10⁵ cells were seeded in a 10-cm dish and were transfected with 5 μg of expression plasmid, together with 1 μg of reporter construct using DOTAP transfection reagent (Roche Diagnostics, Mannheim, Germany) after 24 h. Transfection efficiency was determined by cotransfection of 1 μg of a renilla luciferase plasmid (pRLTK; Promega, Heidelberg, Germany) unprotected probe. Note that comparably low signal intensity of WT1 mRNA is due to the low activity of the probe in this experiment. 

**Fig. 1.** A, expression of WT1 and VDR mRNA by wt1-transfected HT29 clones and control cells. Probes protecting the mRNA coding for the L32 and GAPDH housekeeping gene were included. Control cells transfected with the empty vector and two clones with the highest expression of wt1 as assayed by previous analyses were tested in duplicate. Lane 1, undigested probes; lanes 2 and 3, HT29-neo; lanes 4 and 5, HT29-WT1A3; lanes 6 and 7, HT29-WT1A5. Lane 7 shows yeast tRNA put in the hybridization to control complete cleavage of the
WT1 Induces Endogenous VDR Expression in HT29 Colon Carcinoma and in Human Embryonic Kidney 293 Cells—To investigate the effect of WT1 on the endogenous expression of VDR, HT29 cells expressing moderate endogenous levels of VDR were stably transfected with expression plasmids encoding the -17AA-KTS isoform of WT1. Expression of WT1 mRNA was detected in different selected HT29 clones by RNase protection assay and Northern blotting (data not shown), and the protein expression was detected by Western blotting. A significant induction of VDR mRNA was observed in different, independently generated HT29 cell clones expressing WT1, compared with HT29 cells transfected with the empty vector (Fig. 1A). In accordance with the induction of VDR mRNA, we observed elevated VDR protein levels in WT1-expressing HT29 cells (Fig. 1B). Detection of elevated levels of VDR in different HT29 clones expressing WT1 suggested that this was not an effect of clonal selection. In control experiments, mRNA expression of the dimerization partner of VDR, the retinoic X receptor-α, was not elevated in clones expressing WT1 (data not shown).

To compare the extent of VDR up-regulation with a previously characterized WT1 target gene, the induction of p21 (18) was investigated in HT29 cells expressing WT1 and control cells. p21 mRNA was induced significantly, although to a somewhat lesser extent than VDR mRNA, in WT1-expressing HT29 cells (Fig. 1C).

To investigate induction of VDR by the Wilms’ tumor gene product in renal cells, human embryonic kidney 293 cells were transfected with a WT1 expression construct, and induction of VDR was assayed by immunoblotting. VDR protein was strongly elevated in HEK293 cells overexpressing WT1, suggesting that WT1 up-regulates VDR expression in the context of renal cells (Fig. 2).

WT1 Directly Activates the VDR Promoter—To address the question whether WT1 regulates the VDR expression at the transcriptional level, transient cotransfection experiments were performed. A 0.8-kb fragment of the mouse vdr gene promoter fused to a sequence coding for the firefly luciferase was cotransfected with -KTS and +KTS isoforms of WT1 and a mutant (WTAR) lacking the third zinc finger (16). The VDR promoter fragment was induced 4-fold by the -KTS-isofrom but not by the +KTS isoform of WT1. In contrast, cotransfection of the mutant WTAR slightly repressed the VDR promoter construct (Fig. 3A). The response to WT1 appeared to be in a dose-dependent manner (Fig. 3B). To narrow the sequence element responsive for WT1, the promoter construct was shortened from distal of the transcriptional start site (see Fig. 4). A 0.5-kb promoter fragment responded, as well, to WT1. A 0.2-kb fragment, located proximal to the transcription start site, was active.
induced to the same extent, suggesting that the WT1-responsive element was located in this fragment of the VDR promoter. This fragment contains four GC-rich clusters, with putative Sp1 binding sites (15). The most distal GC element, 5′-TGTGGGCG-3′, is very similar to the sequence of the recently defined WRE high affinity binding sequence, 5′-CGTGGGA(T/A)GG-3′ (19). A deletion construct lacking this site exhibited no response to WT1 anymore (Fig. 4). In contrast, mutation of a WT1/early growth response-1 site, which is present in the murine but not in the human VDR promoter, only slightly reduced the response to WT1 (data not shown). The WT1-responsive site is well conserved between the human and the mouse vitamin D receptor promoter. The element was then formally tested for its ability to bind the WT1 protein by electrophoretic mobility shift assays. The sequence element was specifically bound by wild-type WT1-KTS but not by the mutant WTAR. Mutation of a the binding sequence abrogated binding to WT1-KTS (Fig. 5). These observations suggest that WT1 activates the vdr gene expression at the transcriptional level through a WRE-related sequence element.

**HT29 Cells Expressing WT1 Exhibit Increased Response to 1,25-Dihydroxyvitamin D₃—** Because HT29 colon carcinoma cells have been described to be responsive to 1,25-dihydroxyvitamin D₃ (13, 20), we used these cells as a model for the influence of WT1 on the response to 1,25-dihydroxyvitamin D₃. Different HT29 clones expressing high levels of WT1 and HT29 control cells transfected with an empty construct were stimulated with 10 nM 1,25-dihydroxyvitamin D₃ for 7 days. Consistent with previous reports (13), HT29 cells transfected with the control vector displayed low growth cessation after treatment with 1,25-dihydroxyvitamin D₃. In contrast, HT29 cells expressing WT1 exhibited a marked stronger response to 1,25-dihydroxyvitamin D₃; after 7 days, the cell count in WT1-expressing HT29 cells was 30% compared with control vector-transfected HT29 cells after induction with 1,25-dihydroxyvitamin D₃ (Fig. 6A). At this time, HT29 control cells showed flat morphology and tight adhesion to the surface of the culture dish, whereas WT1-expressing HT29 cells was ~30% compared with control vector-transfected HT29 cells after induction with 1,25-dihydroxyvitamin D₃ (Fig. 6A). The striking growth inhibition in WT1-expressing cells reflects the enhanced VDR levels in WT1-expressing HT29 clones and suggests a major change in the molecular response to 1,25-dihydroxyvitamin D₃ dependent on WT1 expression.

**DISCUSSION**

An influence of WT1 expression on the response to 1,25-dihydroxyvitamin D₃ in hematopoietic cells was demonstrated.
by the inhibition of granulocytic differentiation mediated by 1,25-dihydroxyvitamin D₃ because of expression of WT1 (21). In initial experiments, we analyzed the expression of WT1 and the receptor gene for 1,25-dihydroxyvitamin D₃, VDR, by investigating the expression pattern of both genes using an array spotted with cDNA from different normal and tumor tissues. We detected WT1 expression predominantly in cDNA spots derived from normal adult kidney tissue. The VDR expression pattern resembled the pattern of WT1, raising the possibility of a regulatory link between WT1 and VDR expression in normal renal tissues (data not shown). Recent investigations identified Sp1 as being responsible for baseline expression of the murine vitamin D receptor (15). However, induction of the VDR by Sp1 cannot fully explain the specific pattern of VDR expression in different tissues like kidney or promyelocytes.

A number of putative WT1 target genes, with promoters being regulated in transient cotransfection experiments by WT1, could not be proved to be true targets (22). For this reason, besides investigating the regulation of a vdr promoter reporter constructs by WT1, we focused our investigations on the regulation of the endogenous vdr gene expression by transfecting two different cell lines with a wt1 expression construct and subsequent selecting for WT1 protein expression. Consistent with an induction of the vdr promoter reporter construct by the −KTS isofoms in the transient transfection assays, the endogenous VDR expression in HT29 and HEK293 cells expressing WT1 was significantly elevated.

In HT29 clones expressing WT1, the induction of VDR mRNA expression was comparable with the induction of the expression of p21 mRNA. The p21 cell cycle inhibitor gene has previously been shown to be induced by WT1 (18, 19). After generating single clones transfected with the WT1 constructs, we obtained comparably few HT29 clones stably expressing detectable WT1 protein. This suggests a selection against high levels of WT1, possibly because of induction of p21 or other growth-suppressing WT1 target genes. In addition, we observed a slight growth reduction of HT29 cells expressing WT1 compared with control cells during cell culture (Fig. 6A).

In our hands, HT29 colon carcinoma cells, but not HEK293 cells, were responsive to 1,25-dihydroxyvitamin D₃. After induction with 1,25-dihydroxyvitamin D₃, HT29 cells have been shown to exhibit elevated alkaline phosphatase activity as a marker of differentiation, which is accompanied by growth arrest and followed by a slow induction of apoptosis (20). In our experiments with HT29 clones expressing WT1, this response was markedly enhanced demonstrating that WT1 expression results in a different phenotype regarding the response to 1,25-dihydroxyvitamin D₃.

WT1 interferes with gene expression at the transcriptional and the postranscriptional levels (23), the latter being mediated predominantly by the +KTS isoforms (24). For this reason, regulation of the VDR expression by WT1 at the transcriptional level was proved by transient cotransfection assays with different murine VDR promoter reporter constructs. This led to the identification of a sequence element in the mouse VDR promoter being responsible for induction of the VDR expression by WT1. This sequence element is well conserved in the human VDR promoter (25). It is highly similar to the WRE in the amphiregulin promoter (19). This element has recently been shown to be bound by WT1-KTS with much higher affinity than the WT1/early growth response-1 target site defined earlier (26). Consistently, WT1 recognition elements in the human and murine vitamin D receptor promoters were specifically bound by WT1 in mobility shift assays.

Our data showing that WT1 induces the cellular receptor for 1,25-dihydroxyvitamin D₃ provide a new molecular mechanism by which WT1 may be involved in cellular differentiation. To date, 1,25-dihydroxyvitamin D₃ is known to induce differentiation of a number of leukemic and solid tumor cell lines, but a
physiological role of 1,25-dihydroxyvitamin D₃ in cellular differentiation is poorly understood (1, 2). The data presented here raise the possibility that the response to 1,25-dihydroxyvitamin D₃ is modulated by the Wilms' tumor gene product (WT1), which is predominantly expressed in renal and immature hematopoietic cells. In hematopoietic cells, however, we and others have found that WT1 antagonizes the induction of 1,25-dihydroxyvitamin D₃ (4, 21). The discrepancy of the data presented here could be explained by a cell-type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1. Cell-type-specific regulation of type-specific suppression or activation, respectively, of the VDR gene expression by WT1.
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J. Biol. Chem. 2001, 276:3727-3732.
doi: 10.1074/jbc.M005292200 originally published online October 24, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005292200

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