1,25-Dihydroxyvitamin D₃ Receptors in Human Carcinomas: A Pilot Study

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ABSTRACT

1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] receptor concentration was measured by an accurate immunoradiometric assay in primary tumors from 10 patients with colorectal carcinoma and 11 patients with non-small cell carcinoma of the lung. Measurements were also performed on a nontumorous sample of the same origin as the tumor from each patient. All of the tumors contained receptor with a mean concentration of 123.4 fmol/mg protein for colorectal and 75.1 fmol/mg protein for lung carcinoma. Compared to normal tissue from the same patient, 100% of the lung tumors and 70% of the colorectal tumors had significantly higher levels of the 1,25-dihydroxyvitamin D₃ receptor. A correlation was found between well-differentiated colorectal tumors with or few metastases and high levels of 1,25-dihydroxyvitamin D₃ receptor. Receptor concentration was also assayed in metastatic lesions of malignant melanoma from 7 patients. 1,25-Dihydroxyvitamin D₃ receptor was present in 85% of the metastases at a mean level of 26 fmol/mg protein. For these patients an inverse correlation was found between receptor level and age.

The results obtained in this pilot study suggest that an alteration in 1,25-dihydroxyvitamin D₃ receptor regulation may occur in vivo when a cell undergoes malignant transformation.

INTRODUCTION

The active form of vitamin D, 1,25-(OH)₂D₃, is believed to act through an intracellular receptor to modulate transcription of specific genes in a manner analogous to that of other steroid hormones (1, 2). In recent years, the presence of a specific VDR has been demonstrated in a number of cell lines and tissues of both normal and malignant origin (3, 4). Both in vitro and in vivo evidences suggest that 1,25-(OH)₂D₃ plays a role in cell replication and differentiation (5). There is evidence that VDR is essential for these responses to 1,25-(OH)₂D₃ and that the magnitude of the response appears to be correlated to receptor number (6, 7). In vitro, the hormone has been shown to inhibit the replication of cell lines derived from cancers of the breast and malignant melanomas (8), as well as promoting the human colon carcinoma cell line HT-29 to differentiate into functional enterocytes (9). Several in vivo studies also indicate a hormone dependency of VDR-positive tumors and tissues. Experiments in immunosuppressed mice showed that injection of 1,25-(OH)₂D₃ suppresses the growth of human colon cancer xenografts and a metabolite of vitamin D was found to prolong the survival time of mice with Lewis lung carcinoma (10, 11).

Futhermore, an epidemiological study found dietary vitamin D and calcium intake to be inversely correlated with the risk of colorectal cancer (12). In a study of primary carcinoma of the breast, VDR-positive tumors were found to be associated with longer disease-free survival than those with receptor-negative tumors, thus implying a prognostic value of VDR measurements (13).

In this study we measured VDR in surgically obtained tumors and adjacent normal tissue from the same patient using an IRMA (14) and by ligand-binding assay (15). We chose to study tumors from colon and lung and malignant melanoma of the skin, since functional 1,25-(OH)₂D₃ receptors have been identified in tumor cell lines derived from these tissues (5), combined with a relatively high incidence in the population (16).

The aim of the study was to investigate whether 1,25-(OH)₂D₃ receptor number is altered as a result of malignant transformation and whether VDR measurements could be used as a prognostic marker to furnish a basis for endocrine therapy.

MATERIALS AND METHODS

Vitamin D Compounds. Radioactive 1,25-(OH)₂D₃ (160 Ci/mmol; 1 Ci = 37 GBq) was produced by DuPont/NEN and prepared as described previously (17). Nonradioactive 1,25-(OH)₂D₃ was a gift from Hoffmann-La Roche (Nutley, NJ).

Buffers. Buffers used were: PBS (1.5 mM KH₂PO₄-8.1 mM Na₂HPO₄, pH 8.0-137 mM NaCl-2.7 mM KCl; PBS-0.5% (v/v) Triton X-100; 50 mM Tris-HCl, pH 7.4-1.5 mM EDTA; TED; TED-150 mM NaCl; TEDK300; TEDK300-0.5% (v/v) bovine serum albumin-0.02% NaN₃; TEDK300-5 mM disopropyl fluorophosphate.

Sample Preparation. Tissues were surgically excised, trimmed free of muscularis, and immediately taken to the Pathology Department (University of Wisconsin-Madison) where samples of control and tumor tissues were obtained. Specimens were frozen in liquid nitrogen or on dry ice and stored at -70°C until use. All steps in tumor extract preparation were carried out at 0-4°C unless otherwise noted. Frozen tissue was cut into small pieces with a razor blade, washed twice with 15 ml TED-50 mM NaCl and once with 15 ml TED, followed by homogenization in 2 volumes (v/v) TEDK300-5 mM disopropyl fluorophosphate, using 6-8 strokes with a glass-Teflon homogenizer. Homogenate was centrifuged for 1 h at 170,000 x g in a Beckman L5-50 ultracentrifuge and the supernatant was frozen in liquid nitrogen and stored at -70°C.

Clinical Staging of Tumors. Colorectal cancers were staged according to a modified Dukes' classification of carcinomas (18). Lung cancers were staged I-III based on a tumor-nodes-metastasis classification (19).

Immunoradiometric Assay. The immunoradiometric assay was carried out as described previously (14). Briefly, tumor sample was mixed with a biotinylated monoclonal antibody (VD2F12) and a second, 125I-labeled monoclonal antibody (IVG8C11) directed against a different and nonadjacent epitope (20). After incubation overnight at +4°C, avidin-Pharose was added. The mixture was kept on ice for 1 h with mixing every 20 min. The Sepharose was precipitated by centrifugation at 800 x g for 5 min and the pellet was washed 3 times with PBS-0.5% (v/v) Triton and then counted in a Packard Multi-Priacs Auto-Gamma counter (Packard Instruments, Downers Grove, IL). Non-specific binding (i.e., incubation mixture without receptor) was subtracted from all sample values. Receptor concentration in the sample was determined from a standard curve using purified receptor.

Assay of Specific [³H]-1,25-(OH)₂D₃-binding Capacity. Samples were diluted in 3 volumes (v/v) TEDK300 and 0.5 ml was labeled with 1 nM
1,25-(OH)2D3, overnight at +4°C. Parallel samples were incubated in the presence of 100 nm 1,25-(OH)2D3 to assess nonspecific binding. Receptor-bound hormone was separated from unbound hormone by a modified (21) hydroxyapatite assay (15, 22). Bound radioactivity was counted in a Packard Pria 400 CL/D liquid scintillation counter (Packard Instruments).

Protein Assay. Protein concentration in tumor preparations was measured by Bio-Rad Microassay using bovine serum albumin as a standard.

RESULTS

Colorectal Cancer. Ten primary colorectal adenocarcinomas were analyzed. Normal colorectal mucosa from the same patients served as an "internal control." All patients were male ranging in age between 48 and 77 years.

Using the IRMA we detected VDR in all normal and tumor samples. Normal mucosa contained between 26 and 175 fmol receptor/mg protein with a mean of 90 fmol/mg protein, while tumor preparations had a mean of 123.4 fmol VDR/mg protein ranging from 32 to 200 fmol (Table 1). There was a statistically significant higher VDR content in the tumors than in the normal colorectal mucosa (P < 0.05) using a paired t test. For two patients there was no difference and in one case the normal tissue had a higher VDR content than the corresponding tumor.

When the ligand binding assay was used to assess VDR levels, 3 normal samples and 1 tumor sample had unmeasurable quantities of receptor (<3 fmol/mg protein). Values ranged from 0 to 39 for normal and from 0 to 101 for tumor extracts. (The means were 13 and 36 fmol/mg protein, respectively.) This method revealed higher VDR levels in the tumors compared to normal mucosa in 7 of 9 patients. One sample set showed no difference and one had more receptor in normal mucosa. When comparing the VDR levels of the normal group to those of the tumor group using the Wilcoxon signed-ranks test, a difference was indicated both by the IRMA and by the ligand binding assay (P < 0.05).

To assess the change in receptor number between normal and tumor tissue, we calculated the ratio of VDR in tumor sample over VDR in control sample for each patient. The mean ratio was 1.87 for tumors classified as Dukes' stage B and 1.18 for tumors classified as Dukes' stage C (not shown). A significant difference (P < 0.05) between ratios of the two groups was observed using the Mann-Whitney U test, where a higher ratio was correlated to a more differentiated tumor with few or no metastases. No significant correlation could be found between age and VDR levels in either normal mucosa or tumors (data not shown). However, the small number of samples available for this study is a major limitation to any conclusions that can be drawn with regard to correlations between VDR levels, age, or tumor stage.

Lung Cancer. Primary carcinomas and normal lung parenchyma from 11 patients were analyzed. Nine of the tumors were adenocarcinomas and two were squamous cell carcinomas. One of the patients was female and the age range was 47–82 years.

When analyses were done by IRMA, all tumor samples and 10 of 11 normal samples were found to contain VDR. Normal lung had between 0 and 21 fmol VDR/mg protein with a mean of 11.0, while the tumors contained 16 to 173 fmol/mg protein with a mean of 75.1 (Table 2). By using the ligand-binding assay, only 1 of 11 normal samples showed specific 1,25-(OH)2D3 binding activity, 9 of 11 had no binding activity (<3 fmol/mg protein), and in one case the limited amount of

### Table 1 Determination of VDR levels in colorectal carcinomas and normal colorectal mucosa by IRMA and ligand binding assay

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Tumor stage (Duke's)</th>
<th>IRMA (fmol/mg)</th>
<th>Ligand binding (fmol/mg)</th>
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</thead>
<tbody>
<tr>
<td>5065</td>
<td>72</td>
<td>C2</td>
<td>90 ± 3.3</td>
<td>35.5</td>
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<tr>
<td>5115</td>
<td>67</td>
<td>B2</td>
<td>194 ± 10.4</td>
<td>—</td>
</tr>
<tr>
<td>5143</td>
<td>75</td>
<td>B1</td>
<td>146 ± 6.1</td>
<td>47.3</td>
</tr>
<tr>
<td>5156</td>
<td>63</td>
<td>C2</td>
<td>32 ± 2.5</td>
<td>0</td>
</tr>
<tr>
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<td>65</td>
<td>C1</td>
<td>120 ± 5.7</td>
<td>69.4</td>
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<tr>
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<td>67</td>
<td>B2</td>
<td>56 ± 2.1</td>
<td>10.2</td>
</tr>
<tr>
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<td>C2</td>
<td>106 ± 1.4</td>
<td>17.9</td>
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<tr>
<td>5197</td>
<td>73</td>
<td>B2</td>
<td>200 ± 12.0</td>
<td>17.5</td>
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<td>48</td>
<td>C2</td>
<td>108 ± 7.8</td>
<td>28.4</td>
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<td>5255</td>
<td>71</td>
<td>B2</td>
<td>182 ± 0.7</td>
<td>101.8</td>
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</table>

* Significantly higher VDR levels in the tumor compared to control tissue using the paired t test (P < 0.05).

### Table 2 Determination of VDR levels in lung carcinomas and normal lung parenchyma by IRMA and ligand binding assay

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Tumor stage</th>
<th>IRMA (fmol/mg)</th>
<th>Ligand binding (fmol/mg)</th>
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<tr>
<td>5131</td>
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<td>I</td>
<td>111 ± 10.5</td>
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<td>I</td>
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<td>I</td>
<td>173 ± 1.0</td>
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<td>81</td>
<td>II</td>
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<td>I</td>
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<td>I</td>
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<td>54</td>
<td>II</td>
<td>63 ± 0.7</td>
<td>11.8</td>
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</tbody>
</table>

* Significantly higher VDR levels in the tumor compared to control tissue (P < 0.05 using the paired t test).

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material did not allow quantitation of VDR by the ligand-binding assay. The mean VDR value in this group was 1.7 fmol receptor/mg. In the tumor group, 9 of 11 samples contained VDR between 3.9 and 65.6 fmol/mg protein with a mean of 10.9 fmol/mg protein. Two samples were not analyzed with this method due to insufficient material.

By comparing the VDR levels of the tumor group with those of the normal group using the Wilcoxon signed-ranks test, there was a significantly higher level of VDR in the tumors as measured both by the IRMA and by the ligand-binding assay ($P < 0.05$). For all samples analyzed, the tumor contained significantly more VDR than its corresponding control sample ($P < 0.05$ with paired $t$ test) as measured by the IRMA.

By calculating the ratio between receptor number in tumor sample over receptor number in control sample for each patient, we compared lung tumors of different stages with respect to alteration in VDR levels. However, due to a large interindividual variation and a low number of samples, no significant difference could be observed with the Mann-Whitney $U$ test.

In lung tumors no correlation between VDR levels and age could be found using linear regression analysis (data not shown).

Malignant Melanoma. Due to difficulties in locating the primary tumor, only metastatic lesions of malignant melanoma were obtained from 7 patients between 31 and 80 years of age. No internal controls were available for these tumors.

By IRMA, 6 of 7 tumors were found to contain measurable VDR levels. The mean was 26 fmol VDR/mg protein and the range was between 0 and 95 fmol/mg protein (Table 3). A reverse correlation could be demonstrated between VDR levels and age indicating higher VDR levels in tumors from younger subjects ($r = -0.8762; P < 0.01$) (Fig. 1).

By ligand binding assay 4 of 5 metastases contained VDR with a mean of 8.8 fmol/mg and a range of 0–23 fmol/mg. Two samples could not be analyzed because of the limited amount of material. The relationship between tumor stage and VDR levels was not investigated since the primary tumors were not available.

**DISCUSSION**

It is now widely accepted that 1,25-(OH)$_2$D$_3$, apart from its well-known role in calcium homeostasis, is involved in the regulation of growth and differentiation of normal and malignant cells. It has been established that this effect is dependent on the presence of a specific receptor for 1,25-(OH)$_2$D$_3$.

In our study we used an immunoradiometric assay to measure 1,25-(OH)$_2$D$_3$ receptor in colorectal and lung carcinomas as well as in malignant melanomas. Radioligand binding and qualitative techniques for VDR determinations do not take into consideration occupied receptor, inactive receptor, or receptor that has been denatured by manipulation, thereby underestimating the actual receptor concentration. With the IRMA we were able to detect VDR in 100% of the tumors investigated even in the absence of ligand binding activity. It should be noted, however, that only functional receptor binds ligand. A majority of individual colorectal and lung cancers contained significantly higher levels of VDR compared to corresponding normal tissue. This difference was also observed when comparing VDR levels in the two tumor groups to VDR levels of the control groups. These results suggest that the VDR receptor in lung and colorectal tumors may be regulated differentially as compared to control tissue. The mechanism behind this difference in VDR expression is not understood but could possibly be used clinically as a marker for malignant transformation of colon and lung tissue. In addition, we observed that high tumor/control sample ratios for VDR may be associated with a more favorable tumor stage in colorectal cancers (not shown).

This is in good agreement with previously published data reporting a prodifferentiation and antiproliferative effect of 1,25-(OH)$_2$D$_3$ both in vitro and in vivo (5).

For other steroid hormone receptors such as the glucocorticoid receptor, a decrease in receptor levels has been reported with age (23). Our observation that VDR levels in malignant melanoma. Receptor content was determined by IRMA. Values are expressed as the mean of three or more determinations ± SD (bars) ($r = -0.8762; P < 0.01$).

![Fig. 1. Correlation between VDR levels and age in metastatic malignant melanoma. Receptor content was determined by IRMA. Values are expressed as the mean of three or more determinations ± SD (bars) ($r = -0.8762; P < 0.01$).](image)

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For other steroid hormone receptors such as the glucocorticoid receptor, a decrease in receptor levels has been reported with age (23). Our observation that VDR levels in malignant melanomas seem to decrease in specimens from older subjects could be a similar age-related decline in VDR number. The possibility that receptor levels may correlate to a more or less favorable prognosis in malignant melanomas could not be investigated at this time. However, data on clinical course and overall survival of the patients will become available and can be compared to VDR number of their tumors.

Carcinogenesis has been described as a multistep process that involves activation of oncogenes in combination with loss or inactivation of tumor suppressor genes (23, 24). The VDR belongs to the "superfamily" of ligand-dependent transcriptional factors with demonstrated high homology to oncogenes (22, 26). Based on accumulated data, it seems likely that the effect of 1,25-(OH)$_2$D$_3$ on differentiation and proliferation of tumors may be related to the expression of specific oncogenes thereby suppressing or promoting tumor growth. The close relationship to oncogenes is exemplified by the finding that...
1,25-(OH)₂D₃ decreases the expression of the c-myc oncogene (4), presumably through a receptor-mediated mechanism. If 1,25-(OH)₂D₃ via its receptor can inhibit tumor growth, VDR determination could become a valuable marker for prognosis and therapeutic regimen in lung and colorectal carcinomas. Based on receptor determinations, endocrine treatment has already proved useful in estrogen- and progesterone receptor-positive breast cancer (27). It should be noted, however, that there are data suggesting that a certain level of receptor is necessary to induce a cellular response to a specific hormone (28).

Our results indicate that an alteration in VDR regulation takes place when a normal cell undergoes malignant transformation. In light of our observations, we believe that VDR determinations could become an important tool in diagnosing, predicting prognosis, and treating patients with lung and colorectal carcinomas and possibly other malignancies.

REFERENCES


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