Increased Expression of the 25-Hydroxyvitamin D₃-1α-Hydroxylase Gene in Alveolar Macrophages of Patients with Lung Cancer

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25-Hydroxyvitamin D₃-1α-hydroxylase (1α-hydroxylase) plays a central role in calcium metabolism by synthesizing the active hormone 1α,25-dihydroxyvitamin D₃ (D₃) in the kidney. Its increased expression in the extrarenal tissues has been found in alveolar macrophages in sarcoidosis but not in any other pathological conditions. We found that 1α-hydroxylase-mRNA in alveolar macrophages measured by semiquantitative RT-PCR was 2-fold greater in patients with lung cancer than in control subjects (0.61 ± 0.20 vs. 0.34 ± 0.11, respectively; P < 0.0001). When the clinical stages of lung cancer were divided into early (stage IA–IIIA) and advanced (stage IIIB and IV), the expression of 1α-hydroxylase gene was compared among the control, early, and advanced groups, the advanced group showed the highest expression, followed by the early group, then the control group (0.34 ± 0.11, 0.52 ± 0.11, and 0.69 ± 0.23 for control, early, and advanced groups, respectively; P < 0.0001). The 1α-hydroxylase-mRNA level was well correlated with serum 1α,25-dihydroxyvitamin D₃ concentration and the 1α,25-dihydroxyvitamin D₃ to 25-hydroxyvitamin D₃ ratio, but none of the findings related to calcium metabolism among the patients with lung cancer. Increased local production of 1α,25-dihydroxyvitamin D₃ may be associated with the pathological conditions, such as immunosuppression, in lung cancer. (J Clin Endocrinol Metab 88: 5704–5709, 2003)
TABLE 1. Clinical characteristics of patients with lung cancer

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Pathology</th>
<th>Clinical stage</th>
<th>Cigarette smoking</th>
<th>Treatment</th>
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<tr>
<td>1</td>
<td>57</td>
<td>F</td>
<td>Adeno</td>
<td>IA</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>78</td>
<td>M</td>
<td>Adeno</td>
<td>IA</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>F</td>
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<td>IA</td>
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</tr>
<tr>
<td>5</td>
<td>76</td>
<td>M</td>
<td>Adeno</td>
<td>IB</td>
<td>Smoker</td>
<td>Before treatment</td>
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<tr>
<td>6</td>
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<td>M</td>
<td>Adeno</td>
<td>IIIA</td>
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<td>Before treatment</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>F</td>
<td>Adeno</td>
<td>IIIB</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>M</td>
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<td>IIIB</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>9</td>
<td>75</td>
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<td>Adeno</td>
<td>IV</td>
<td>Nonsmoker</td>
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<td>44</td>
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<td>Adeno</td>
<td>IV</td>
<td>Nonsmoker</td>
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</tr>
<tr>
<td>11</td>
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<td>F</td>
<td>Adeno</td>
<td>IV</td>
<td>Nonsmoker</td>
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</tr>
<tr>
<td>12</td>
<td>69</td>
<td>F</td>
<td>Adeno</td>
<td>IV</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>13</td>
<td>75</td>
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<td>Small</td>
<td>IIIA</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>14</td>
<td>68</td>
<td>M</td>
<td>Small</td>
<td>IIIIB</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>15</td>
<td>56</td>
<td>M</td>
<td>Small</td>
<td>IIIIB</td>
<td>Smoker</td>
<td>Chemotherapy, rad</td>
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<tr>
<td>16</td>
<td>53</td>
<td>M</td>
<td>Small</td>
<td>IIIIB</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
<tr>
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<td>70</td>
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<td>Squamous</td>
<td>IA</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>18</td>
<td>79</td>
<td>M</td>
<td>Squamous</td>
<td>IIIA</td>
<td>Nonsmoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>19</td>
<td>77</td>
<td>M</td>
<td>Squamous</td>
<td>IIIB</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>20</td>
<td>51</td>
<td>M</td>
<td>Squamous</td>
<td>IIIB</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>21</td>
<td>77</td>
<td>M</td>
<td>Squamous</td>
<td>IIIB</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
<tr>
<td>22</td>
<td>83</td>
<td>M</td>
<td>Squamous</td>
<td>IIIB</td>
<td>Smoker</td>
<td>Before treatment</td>
</tr>
</tbody>
</table>

M, Male; F, female; Adeno, adenocarcinoma; Small, small-cell carcinoma; Squamous, squamous cell carcinoma; Chemo, chemotherapy; rad, radiotherapy.

The study protocol was approved by our institutional review board for human research. Informed consent was given by each subject.

**BAL procedures, preparation of BAL cells, and purification of AMs**

BAL was performed as previously reported (18). Briefly, both the upper and lower respiratory tracts were anesthetized with topically warmed sterile 0.9% saline were instilled, and the returns were gently aspirated through the side channel of the bronchoscope (19). Bronchoalveolar cells were separated by centrifugation at 900 x g for 10 min at 4 C and washed with PBS. The total cell count was determined using a hemocytometer. The BAL cells were seeded in the culture medium at a density of 1 x 106 cells/ml. A differential cell count was performed on cytocentrifuged smears stained with Wright-Giemsa. As in the previous study (14), purified AMs were obtained by the method of removing nonadherent cells (20). The cells were plated into dishes coated with fetal calf serum and incubated at 37 C in 5% CO2 for 2 h. All adherent cells were macrophages.

**Measurement of serum markers of calcium metabolism**

Fasting blood samples were collected at the time of bronchoscopy. The serum calcium level, which was measured by automated methods, was corrected for the serum albumin level. The serum levels of PTH and PTHrP were determined by chemiluminescent immunoassay (normal range, 10–65 ng/l) and immunoradiometric assay (normal range, < 0.6 pmol/l), respectively. The serum ionized calcium level was measured using an ion-selective electrode (normal range, 1.20–1.35 mmol/l) (21). The serum levels of 1,25(OH)2-D3 (normal range, 16–65 ng/l) and 25-OH-D3 (normal range, 25–137 pmol/l) were measured by RIA and competitive protein-binding analysis (22), respectively.

**RNA isolation and RT-PCR**

Total RNA was extracted from BAL cells using the acid guanidinium thiocyanate-phenol-chloroform technique (23). Two micrograms were used for the first-standard cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD) and random hexanucleotide. After terminating the reaction by heating at 70 C for 10 min, the reaction mixture was diluted 5-fold with distilled water.

The level of human 1α-hydroxylase-mRNA was measured by PCR amplification. PCR was performed in 50 μl reaction mediums containing 5 μl of cDNA solution, 0.2 μm of forward and reverse primers, 0.2 μm of deoxynucleotide triphosphates, and 1.25 U of Pyrobest DNA polymerase (Takara Shuzo Co., Tokyo, Japan). After 5 min of initial denaturation at 96 C, amplification was performed in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) for 42 cycles with 30-sec denaturing at 96 C, 40-sec annealing at 60 C, and 1-min extension at 72 C. We designed the sequences of the primers spanning an intron of each genome, 5′-GTGTTCCACGGTGTGACCATG-3′ and 5′-GAATTCCAGGTACCACAGG-3′. When semiquantitative RT-PCR was performed, PCR amplification was carried out for 30–42 cycles for 1α-hydroxylase and for 28–38 cycles for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control for gene expression. The PCR products were separated on 1% agarose gel by electrophoresis, stained with ethidium bromide, and visualized. We confirmed the linearity of the PCR products up to 36 cycles for 1α-hydroxylase and GAPDH; the values obtained were plotted against the numbers of cycles. Each best-fit straight line was obtained and extrapolated back to zero cycle. The intercepts at the zero cycle were used to determine the relative abundance of both mRNAs, and the value for 1α-hydroxylase was normalized to GAPDH. The RTPCR product of 1α-hydroxylase was confirmed by Southern blot analysis as described previously (14).

**Statistical analysis**

All values were analyzed with StatView J 4.5-software (SAS Institute, Cary, NC). The BAL cells, serum markers of calcium metabolism, and 1α-hydroxylase mRNA level were compared among groups by ANOVA, and when ANOVA showed significant difference, Fisher’s protected least significant difference test was performed. P < 0.05 was considered significant. The correlation between relative intensity of the 1α-hydroxylase mRNA level and markers of calcium metabolism were evaluated using the Pearson’s correlation coefficient. All continuous values are expressed as means ± sd.
of 1. We combined them in a control group. The expression of 1α-hydroxylase was measured by semiquantitative RT-PCR in AMs and phagocytes and lymphocytes (Table 2). There were no significant differences in the total counts of BAL cells obtained and the percentages of macrophages and lymphocytes (Table 2).

The 1α-hydroxylase-mRNA was detected in AMs obtained from every subject. The relative intensity of its level was approximately 2-fold greater than that in the controls (0.61 ± 0.20 vs. 0.34 ± 0.11, P < 0.0001).

**Correlation of the 1α-hydroxylase-mRNA level and the findings concerning with lung cancer**

When we compared the 1α-hydroxylase-mRNA expression level among the clinical stages of lung cancer, it was difficult to make a clear conclusion because the number of the patients in each group was small, although a tendency might be seen that the enzyme expression increased with advancing clinical stage (Fig. 2A). Therefore, we divided the clinical stage into early and advanced stage, according to the clinical classification for operability (24). Early lung cancer includes stage I and II.A, whereas advanced lung cancer includes stage IIIB and IV. When the expression of 1α-hydroxylase gene was compared among control, early, and advanced groups, the advanced group had the highest mRNA levels, followed by the early group, and then the control group (Fig. 2B). No difference was observed among the pathological types of lung cancer (Fig. 2C), or between genders, smoking habits, or prior treatments (data not shown).

**Relationship between the 1α-hydroxylase-mRNA level and calcium metabolism**

The 1α-hydroxylase-mRNA level showed a significant correlation with the serum 1,25-(OH)₂ D₃ concentration (P = 0.03, Fig. 3A) and with the ratio of 1,25-(OH)₂ D₃ to 25-(OH) D₃ (P = 0.0008, Fig. 3B) among the patients with lung cancer. The correlation became much higher when analyzed in all 40 patients, including patients with lung cancer, control patients with other pulmonary diseases, and healthy volunteers (Fig. 3, A and B). As shown in Table 2, none of the findings related to calcium metabolism, such as serum calcium, ionized calcium, PTH, PTHrP, 25-(OH) D₃, and 1,25-(OH)₂ D₃, were significantly different between the lung cancer group and the controls. However, the ratio of 1,25-(OH)₂ D₃ to 25-(OH) D₃ was significantly higher in the patients with lung cancer than control subjects, suggesting that the enzyme was functional. When compared among control, early, and advanced groups, the serum 1,25-(OH)₂ D₃ level tended to be higher, although not significant, in the advanced group; and the ratio of 1,25-(OH)₂ D₃ to 25-(OH) D₃ was significantly higher in the advanced lung cancer group than other groups (Fig. 4B).

**Discussion**

In the previous study, we demonstrated the presence of 1α-hydroxylase gene expression in human AMs and observed a significant correlation between the 1α-hydroxylase gene expression and the disease activity in patients with sarcoidosis (14). In the present study, using the same semiquantitative RT-PCR technique, we found that the expression of 1α-hydroxylase mRNA in AMs was significantly enhanced in patients with lung cancer. Except for sarcoidosis, there are few reports demonstrating the enhanced expression.
of 1α-hydroxylase gene in human extrarenal tissues under pathological conditions.

A significant correlation was obtained between the 1α-hydroxylase-mRNA level in AMs of patients with lung cancer and their serum 1,25-(OH)2 D3 concentration and the 1,25-(OH)2 D3 to 25-(OH) D3 ratio. This suggests that 1α-hydroxylase in AMs in patients does actually function to catalyze the synthesis of active vitamin D3 sterol. In contrast to sarcoidosis, however, the increased expression of this enzyme did not correlate with serum total and ionized calcium concentrations. The enhancement of 1α-hydroxylase might not be adequate to induce an increase in serum calcium because the patients with sarcoidosis in our previous study showed a 6-fold increase in 1α-hydroxylase-mRNA, whereas the patients with lung cancer in the present study manifested only twice as much of the enzyme level as the controls.

It is of interest that the 1α-hydroxylase gene expression tended to be higher in advanced stages of lung cancer. When we classified the patients based on their clinical stages from IA–IV, we did not obtain a significant difference in the 1α-hydroxylase-mRNA level among the groups because of the insufficient number of patients. The patients were, therefore, divided into two groups, early and advanced; the early group included stages IA–IIIA, and the advanced group included stages IIIB and IV. The basis of this classification is that patients with non-small-cell lung cancer with clinical stages IA–IIIA are usually considered to be operable, whereas patients with stages IIIB and IV are not. The mRNA level of 1α-hydroxylase was the highest in the advanced group and lowest in the control group (Fig. 2B). In addition, the advanced group showed a significantly higher level of the 1,25-(OH)2 D3 to 25-(OH) D3 ratio and a tendency to have a higher 1,25-(OH)2 D3 concentration than the control group (Fig. 4).

Although 1α-hydroxylase is almost exclusively expressed in the kidney under normal circumstances (2–4, 25), the presence of this enzyme in some extrarenal cells, such as macrophages and keratinocytes, has been suggested under some pathological conditions by the assay of the enzyme activity (2, 4, 25). After cloning of the 1α-hydroxylase cDNA, the detection of the extrarenal expression of this enzyme at the mRNA and protein levels has become possible. We demonstrated 1α-hydroxylase-mRNA in human AMs for the first time (14). Zehnder et al. (26) synthesized a specific antibody against an antigenic region of the mouse 1α-hydroxylase amino acid sequence and examined the extrarenal expression of this enzyme in human AMs for the first time (14). Zehnder et al. (26) synthesized a specific antibody against an antigenic region of the mouse 1α-hydroxylase amino acid sequence and examined the extrarenal expression of this enzyme. Their immunohistochemical study confirmed the discrete expression of 1α-hydroxylase in several human tissues including skin (basal keratinocytes), lymph nodes (granulomata), colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex), and placenta (decidual and trophoblastic cells). Thus, the expression of 1α-hydroxylase is more widespread than previously suggested, but the function of this enzyme in these extrarenal tissues is unknown at present.

It may be intriguing to consider the functional relevance of 1α-hydroxylase enhancement in lung cancer. Augmented enzyme expression did not relate to the serum calcium level.
but did relate to the increased synthesis of circulating 1,25-(OH)2 D3, which probably reflected the increased local production induced by the enhanced 1α-hydroxylase activity in AMs. It was reported that 1,25-(OH)2 D3 plays immunomodulatory roles in a variety of immune responses. In vitro studies have revealed that 1,25-(OH)2 D3 inhibits activated T cell functions, resulting in suppression of cytokine synthesis and antibody-stimulating activities (2–5, 27). 1,25-(OH)2 D3 has also been demonstrated to inhibit natural killer cell activity at a physiological concentration (28) and suppress the proliferation and maturation of antigen presentation cells (29, 30). Moreover, administration of 1,25-(OH)2 D3 in vivo diminished the TNFα and interferon (IFN)-γ production by Th1 cells, resulting in prevention of experimental autoimmune encephalomyelitis (31). Vitamin D analogs have been effectively used to prolong transplant survival in animal models of transplantation (32, 33). These findings suggest that 1,25-(OH)2 D3 is a potent immunosuppressive agent and raise the possibility that the locally synthesized 1,25-(OH)2 D3 in AMs function for immunosuppression in lung cancer. It was reported that patients with lung cancer exhibit dysfunction of the immune system, such as the impairment of tumor-specific immune response during the initial stage of tumor growth and generalized immunodeficiency during the late stage of tumor development (34, 35). Alterations in T cell functions occur in patients with various cancers including melanoma (36), renal cell carcinoma (37), cervical cancer (38), and others (39). In addition, mice bearing slowly progressive tumors induced by carcinogen demonstrated significantly diminished functions of T cells and natural killer cells and impaired capacity to produce Th1 cytokines (40).

The mechanism of how 1α-hydroxylase is regulated in AMs of patients with lung cancer remains to be elucidated. It is known that renal 1α-hydroxylase synthesis is tightly regulated by the concentration of serum calcium, PTH, and 1,25-(OH)2 D3 (2–4, 25, 41). In contrast, extrarenal synthesis of 1α-hydroxylase by macrophages does not respond to these regulatory influences, but rather, it is sensitive to immune signals, such as IFN-γ, TNFα, and lipopolysaccharide (42–46). In patients with malignancy, overproduction of various cytokines, including IFN-γ, TNFα, IL-2, and TGF-β, have
been identified either by cancer cells or host immune cells (34, 35). Their production was most evident in patients with advanced stages of cancer. Although we have currently no data indicating augmentation of these cytokines in BAL, it is possible that they may play a role in enhanced expression of 1α-hydroxylation in AMs.

In conclusion, the present study demonstrated that patients with lung cancer have increased expression of 1α-hydroxylation in AMs, which correlates with serum 1,25-(OH)₂ D₃ concentration. It is possible that increased production of 1,25-(OH)₂ D₃ may be associated with immunosuppression in lung cancer.

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References


18. Zehnder B, Blum R, Williams MC, McNinch RW, Howie AJ, Stewart PM, Hewison M. 2001 Extrarenal expression of 25-hydroxyvitamin D₃(3)-1\α-


tamin D₃ is a positive regulator of the anti-encephalitogenic cytokines TGF-β and IL-4. J Immunol 160:5314–5319.


