Conflicting actions of parathyroid hormone-related protein and serum calcium as regulators of 25-hydroxyvitamin D$_3$-1α-hydroxylase expression in a nude rat model of humoral hypercalcemia of malignancy

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Abstract

In patients with humoral hypercalcemia of malignancy (HHM), serum levels of 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) are generally low, although the pathophysiology of the impaired vitamin D metabolism is not fully understood. In the present study, we have investigated vitamin D metabolism in our newly developed rat model of HHM in which a human infantile fibrosarcoma producing parathyroid hormone-related protein (PTHrP), named OMC-1, was inoculated s.c. into athymic nude rats. In OMC-1-bearing rats, the serum concentration of 1,25(OH)$_2$D was markedly reduced when the animals exhibited severe hypercalcemia (Ca$^{2+}$ ≥ 15 mg/dl), while it was rather elevated in those with mild hypercalcemia. To further examine whether serum Ca levels affect 1,25(OH)$_2$D concentration, we administered bisphosphonate YM529 to OMC-1-bearing rats when they exhibited severe hypercalcemia. The restoration of the serum Ca level by administration of YM529 was accompanied by a marked increase in the 1,25(OH)$_2$D level, suggesting that the serum Ca level itself plays an important role in the regulation of the 1,25(OH)$_2$D level in these rats. On the other hand, when the OMC-1-bearing rats were treated with a neutralizing antibody against PTHrP, serum 1,25(OH)$_2$D levels remained low despite the reduction in serum Ca levels. Expression of 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) in kidney was decreased in OMC-1-bearing rats with severe hypercalcemia, and markedly enhanced after treatment with bisphosphonate. This enhancement in 1α-hydroxylase expression was not observed after treatment with the antibody against PTHrP. These results suggest that PTHrP was responsible for the enhanced expression of 1α-hydroxylase in YM529-treated rats, and that hypercalcemia played a role in reducing the serum 1,25(OH)$_2$D level in OMC-1-bearing rats by suppressing the PTHrP-induced expression of the 1α-hydroxylase gene.

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Introduction

1,25-dihydroxyvitamin D(1,25(OH)$_2$D) is the active form of vitamin D, and its synthesis from 25-hydroxyvitamin D (25 OHD) is catalyzed by the mitochondrial cytochrome P450 enzyme, 25 OHD-1α-hydroxylase (1α-hydroxylase) (Henry 1997). Since 1,25(OH)$_2$D plays a critical role in maintaining serum calcium (Ca) homeostasis, the expression and the activity of 1α-hydroxylase are tightly regulated. The recent cloning of cDNA for 1α-hydroxylase has revealed that some factors, including parathyroid hormone (PTH) and calcitonin, enhance the expression of the gene, while 1,25(OH)$_2$D itself suppresses the expression in a vitamin D receptor (VDR)-dependent manner (Fu et al. 1997, Shinki et al. 1997, St-Arnaud et al. 1997, Takeyama et al. 1997). It is also reported that the promoter region of the gene possesses several cAMP-responsive elements and responds to PTH via the elements, confirming that the PTH signal plays the major role in regulation of the transcription of the 1α-hydroxylase gene (Kong et al. 1999, Murayama et al. 1999).

Synthesis of 1,25(OH)$_2$D is also modulated by serum Ca and phosphate levels (Hughes et al. 1975). Serum Ca levels regulate 1α-hydroxylase expression mainly through
the modulation of PTH secretion by the parathyroid glands (Akizawa & Fukagawa 1999); hypocalcemia stimulates, while hypercalcemia suppresses, PTH secretion leading to the modification of 1α-hydroxylase expression. However, a line of previous studies has suggested that Ca concentration itself also directly regulates the expression of 1α-hydroxylase in kidney (Fukase et al. 1982, Bland et al. 1999). For example, the recent study by Bland et al. (1999) supports a direct effect of Ca on the 1α-hydroxylase activity in a human proximal tubule cell line, although the mechanism of the effect, such as whether Ca-responsive elements are present in the 1α-hydroxylase gene promoter, remains to be established.

Cancers sometimes cause impaired Ca and vitamin D metabolism. Humoral hypercalcemia of malignancy (HHM), one of the most common paraneoplastic syndromes, is associated with an elevated serum Ca level, a low serum 1,25(OH)₂D concentration and overproduction of PTH-related protein (PTHrP) by tumors (Pierce 1993, Goltzman & Henderson 1997). In contrast, the circulating 1,25(OH)₂D and Ca concentrations are elevated in a significant number of patients with primary hyperparathyroidism. Since PTHrP shares high homology with PTH in the amino acid sequence of the N-terminus (Suva et al. 1987, Mangin et al. 1988), and exerts its function through a common effector system, the reasons for this discrepancy are unclear. Because administration of synthetic peptides for PTHrP(1–34) or PTHrP(1–36) increases the plasma 1,25(OH)₂D concentration as does the administration of PTH(1–34) (Fraher et al. 1992, Henry et al. 1997), factors other than overproduction of PTHrP have been postulated as the cause of low 1,25(OH)₂D levels. However, such factors have not been identified yet, partly due to the lack of appropriate animal models; animals bearing human tumors producing PTHrP generally do not exhibit a reduction in serum 1,25(OH)₂D levels (Ikeda et al. 1988, Ikeda & Ogata 1995).

We have previously reported a rare infantile case of HHM caused by PTHrP-producing infantile fibrosarcoma (Michigami et al. 1996). The tumor tissue obtained from the patient, and named OMC-1, was transplantable to athymic nude rats and resulted in hypercalcemia in host animals due to PTHrP overproduction, suggesting that OMC-1-bearing rats can be utilized as an animal model for HHM. When these rats exhibited severe hypercalcemia, they also showed a marked reduction in the serum 1,25(OH)₂D concentration. The aim of this study was to clarify the mechanisms underlying the impaired vitamin D metabolism in HHM using OMC-1-bearing rats. To address this issue, we investigated serum concentrations of Ca and vitamin D metabolites, and the expression of 1α-hydroxylase in these rats. The effects of therapy with bisphosphonate and anti-PTHrP neutralizing antibody on vitamin D metabolism were also examined.

Materials and Methods

Tumor line

OMC-1 tumor is a PTHrP-producing infantile fibrosarcoma originally obtained from an 8-month-old boy who manifested severe hypercalcemia. Detailed information about the patient was given previously (Michigami et al. 1996). The serum level of 1,25(OH)₂D of the patient at diagnosis of HHM was 10.6 pg/ml (normal range 33–118 pg/ml). The tumor tissue was transplantable to nude mice and nude rats by s.c. inoculation, and caused the elevation of circulating PTHrP and hypercalcemia in tumor-bearing animals, both of which were restored by resection of the inoculated tumor (Michigami et al. 1996).

OMC-1 tumor has been maintained in vivo for more than 5 years by serial s.c. transplantation into nude mice (BALB/c A Jcl-nu, nu/nu; Clea Japan, Tokyo, Japan), and still retains the capability of overproducing PTHrP.

Protocols of the animal experiments using tumor-bearing nude rats

Animal protocols were approved by the Institutional Animal Care and Use Committee at Osaka Medical Center and Research Institute for Maternal and Child Health. Seven-week-old male nude rats of a homozygous mutant strain (F344/N Jcl-nu, nu/nu) were purchased from Clea Japan and freely fed standard rodent chow and water. For experiments described below, fresh tissue of OMC-1 tumor was aseptically resected and minced into 3 mm³ fragments, and then the tumor pieces were s.c. implanted in the back of the nude rats.

Using OMC-1-bearing rats, we first studied the time-course of change in serum levels of PTHrP, Ca, phosphate and creatinine (Cr), and body weight of the host animals after the tumor inoculation (experiment 1).

In the second set of experiments (experiment 2), we focused on the vitamin D metabolism. OMC-1-bearing rats were killed to take blood samples and organs at various stages (5–7 weeks after tumor inoculation). Blood samples were subjected to the measurement of Ca, phosphate, 25 OH D, 1,25(OH)₂D and 24,25(OH)₂D as described below. Organs were kept frozen until RNA extraction. Blood and organs were also obtained from age-matched control nude rats.

Effects of bisphosphonate in OMC-1-bearing rats

We also examined the effect of the serum Ca level on vitamin D metabolism by administration of bisphosphonate. As bisphosphonate, we utilized [1-hydroxy-2-(imidazol-1,2-a]pyridin-3-yl)ethylidene]-bisphosphonic acid monohydrate (YM529, also called YH529; kindly provided by Yamanouchi Pharmaceuticals, Tokyo, Japan), because it is potent and effective in its inhibition of
osteoclastic bone resorption (Yoshida et al. 1998). For the experiments using YM529 (experiment 3), blood samples were taken from the abdominal vein at a point when OMC-1-bearing rats exhibited severe hypercalcemia, and then 0·1 mg/kg body weight of YM529 was injected i.v. Four days later, the animals were killed to take blood and organs for analyses.

Effects of neutralizing antibody against PTHrP on vitamin D metabolism in OMC-1-bearing rats

A mouse monoclonal antibody against PTHrP was kindly provided by Chugai Pharmaceuticals (Tokyo, Japan). It was previously shown that this antibody binds to PTHrP(1–34) and inhibits PTHrP function both in vivo and in vitro (Sato et al. 1993). In the current experiments, the antibody against PTHrP (3 mg/kg body weight) was injected i.v. into OMC-1-bearing rats with severe hypercalcemia after blood samples were taken. Four days later the animals were killed for further analysis (experiment 4).

Biochemical analysis

The plasma PTHrP concentration was determined using an IRMA kit (Mitsubishi Kagaku, Tokyo, Japan). Serum 1α,25(OH)₂D was measured by radioreceptor assay, and 25 OHD and 24,25(OH)₂D by a competitive protein binding assay following separation by HPLC. Serum levels of Ca, phosphate and Cr were evaluated with an auto-binding assay following separation by HPLC. The plasma PTHrP concentration was determined using an IRMA kit (Mitsubishi Kagaku, Tokyo, Japan). Serum 1α,25(OH)₂D was measured by radioreceptor assay, and 25 OHD and 24,25(OH)₂D by a competitive protein binding assay following separation by HPLC. Serum levels of Ca, phosphate and Cr were evaluated with an auto-binding assay following separation by HPLC.

RT-PCR

Total RNA was extracted from kidneys and livers obtained from OMC-1-bearing rats and control rats by a guanidinium–thiocyanate–phenol method. After treatment with DNase I (Takara Syuzo, Kyoto, Japan), the RNA samples were subjected to RT-PCR analysis. Total RNA (2–5 µg) was reverse transcribed using random hexamers and SuperScript II reverse transcriptase ( Gibco BRL, Grand Island, NY, USA) according to the manufacturer’s instructions. PCR was performed using the following sets of specific primers: rat 1α-hydroxylase, sense 5′–TTTGGACGTGGCGGAGAG–3′, antisense 5′–TGCGTGGAGTGCGCATAG–3′; rat 25-hydroxylase, sense 5′–AGAAGCAGGGCCGAGATCCC–3′, antisense 5′–AAGTGCAGGTAGCCAGATACC–3′; rat 24-hydroxylase, sense 5′–TTGGCTGAATTCTAGAAGGC–3′, antisense 5′–TTGGGTAGACGCTATTC ACC–3′; VDR, sense 5′–GGGCTATGTTCCGCTCA TGCCCATCTG–3′, antisense 5′–GGGTAGCTCTGAGCCTAG–3′, β-actin, sense 5′–CTGGGGCCGGGACAACA–3′, antisense 5′–CTGG GGCAGTACGAGCT–3′. Amplified sequences were excised by NdeI/BamHI digestion, was then labeled with [α-³²P]dCTP (NEN Life Science, Boston, MA, USA) using a Ready-to-Go labeling kit (Amerham Pharmacia, Arlington Heights, IL, USA). To prepare the probe for hybridization, a ~850 bp fragment of rat 25-hydroxylase cDNA (+143/ +1002) was amplified by PCR and cloned into pT7 Blue vector (Novagen, Madison, WI, USA). The fragment, excised by NdeI/BamHI digestion, was then labeled with [α-³²P]dCTP (NEN Life Science, Boston, MA, USA) using a Ready-to-Go labeling kit (Amerham Pharmacia, Arlington Heights, IL, USA). To prepare the probe for hybridization, a ~850 bp fragment of rat 25-hydroxylase cDNA (+143/ +1002) was amplified by PCR and cloned into pT7 Blue vector (Novagen, Madison, WI, USA). The fragment, excised by NdeI/BamHI digestion, was then labeled with [α-³²P]dCTP (NEN Life Science, Boston, MA, USA) using a Ready-to-Go labeling kit (Amerham Pharmacia, Arlington Heights, IL, USA). To prepare the probe for hybridization, a ~850 bp fragment of rat 25-hydroxylase cDNA (+143/ +1002) was amplified by PCR and cloned into pT7 Blue vector (Novagen, Madison, WI, USA). The fragment, excised by NdeI/BamHI digestion, was then labeled with [α-³²P]dCTP (NEN Life Science, Boston, MA, USA) using a Ready-to-Go labeling kit (Amerham Pharmacia, Arlington Heights, IL, USA). To prepare the probe for hybridization, a ~850 bp fragment of rat 25-hydroxylase cDNA (+143/ +1002) was amplified by PCR and cloned into pT7 Blue vector (Novagen, Madison, WI, USA). The fragment, excised by NdeI/BamHI digestion, was then labeled with [α-³²P]dCTP (NEN Life Science, Boston, MA, USA) using a Ready-to-Go labeling kit (Amerham Pharmacia, Arlington Heights, IL, USA).

Statistical analysis

The data are shown as means ± s.e.m., and significant differences were assessed by ANOVA analysis, unless otherwise indicated, using StatView 5·0 (SAS Institute, Cary, NC, USA). The regression curve was also analyzed using StatView 5·0.

Results

Changes of circulating PTHrP concentration, serum calcium level and body weight in OMC-1-bearing rats

Figure 1 describes the time-course of the circulating PTHrP concentration, serum Ca level and body weight in OMC-1-bearing rats (n=7) and age-matched non-tumor-bearing controls (n=6) examined in experiment 1. At
4 weeks after tumor inoculation, there was no significant difference between the two groups, in either Ca level or PTHrP concentration (Fig. 1A and B). However, 6 weeks after tumor inoculation, the serum levels of PTHrP in OMC-1-bearing rats were elevated to 11 ± 0.6 pmol/l, while those in control animals were 1.1 ± 0.6 pmol/l. Seven weeks after inoculation, the PTHrP levels of OMC-1-bearing animals further increased and reached 38.5 ± 3.6 pmol/l. Simultaneously with the rise in the circulating PTHrP concentration, the serum Ca level began to elevate in OMC-1-bearing rats. Associated with the elevation of serum Ca and plasma PTHrP levels, OMC-1-bearing rats lost body weight and became cachectic 6 weeks after the tumor inoculation (Fig. 1C).

Serum phosphate levels in OMC-1-bearing rats and control rats at 6 and 7 weeks after the tumor inoculation were 5.8 ± 0.6 vs 6.8 ± 0.2 mg/dl and 8.2 ± 0.6 vs 7.1 ± 0.1 mg/dl (OMC-1-bearing rats vs control rats) respectively. Although we could not find a significant difference in serum phosphate levels between the two groups at either time point, three out of six tumor-bearing rats manifested hypophosphatemia (<5.0 mg/dl) at 6 weeks. The other three tumor-bearing rats, which showed normal serum phosphate levels at this time point, exhibited increased serum Cr levels at this time point, suggesting that the normophosphatemia in these rats is due to renal insufficiency caused by hypercalcemia.

Serum Cr levels at 6 and 7 weeks were 0.8 ± 0.1 vs 0.4 ± 0.2 mg/dl and 1.3 ± 0.0 vs 0.4 ± 0.0 mg/dl (OMC-1-bearing rats vs control rats) respectively.

Serum concentrations of vitamin D metabolites in OMC-1-bearing rats

In experiment 2, blood samples were obtained from OMC-1-bearing rats at various stage (5–8 weeks after tumor inoculation) or control rats, and were subjected to the measurement of Ca, phosphate and vitamin D metabolites (Table 1). The samples from OMC-1-bearing rats were divided into two groups based on serum Ca levels; normocalcemia to mild hypercalcemia (<15 mg/dl) and severe hypercalcemia (≥15 mg/dl). Serum Ca levels of control animals, OMC-1-bearing rats with mild hypercalcemia and those with severe hypercalcemia were 10.2 ± 0.1, 11.3 ± 0.6 and 17.8 ± 0.7 mg/dl respectively (Table 1). The correlation between serum levels of Ca and 1,25(OH)2D is demonstrated in Fig. 2. It was significant ($[1,25(OH)₂D]=21·0 \times [Ca] − 151·3$; $r^²=0.778$, $P<0.001$).

![Figure 1](image-url)  
*Figure 1* Time-course of circulating PTHrP concentration (A), serum Ca level (B) and body weight (C) in OMC-1-bearing rats (●, n=7) and age-matched non-tumor-bearing control rats (○, n=6). Data are expressed as means ± s.e.m. *P<0.05, **P<0.001 (OMC-1-bearing rats vs control rats).

| Table 1 Serum concentrations of vitamin D metabolites in OMC-1-bearing and control rats. |
| Blood was taken and serum concentrations of vitamin D metabolites determined (experiment 2). OMC-1-bearing rats are divided into two groups based on the serum Ca level. Data are means ± s.e.m. (n=6 in each group) |
| **OMC-1-bearing rats** |
| **Ca** (mg/dl) | **25(OH)D** (ng/ml) | **1,25(OH)₂D** (pg/ml) | **24,25(OH)₂D** (ng/ml) |
| Mild | Severe | Control |
| Ca (mg/dl) | 11.3 ± 0.6 | 17.8 ± 0.7a | 10.2 ± 0.1 |
| 25(OH)D (ng/ml) | 19.2 ± 1.6a | 8.8 ± 1.2b | 31.4 ± 5.9 |
| 1,25(OH)₂D (pg/ml) | 85.1 ± 13.9d | 29.1 ± 5.9e | 62.5 ± 7.4 |
| 24,25(OH)₂D (ng/ml) | 11.8 ± 1.5a | 6.0 ± 0.5b | 14.6 ± 1.3 |

| a,b,cSignificantly different from the values in control rats (a, P<0.0001; b, P<0.001; c, P<0.05). |
| dSignificantly different from the values in the severe hypercalcemia group (d, P<0.005; e, P<0.01). |
Figure 2 (A) Correlation between serum Ca level and 1,25(OH)2D concentration in control rats and OMC-1-bearing rats with mild hypercalcemia. ○, non-tumor-bearing controls; ●, OMC-1-bearing rats with mild hypercalcemia (Ca<15 mg/dl). The linear regression curve between serum Ca and 1,25(OH)2D levels for the animals of both groups had a high coefficient (1,25(OH)2D=21.0×[Ca]-151.3; r²=0.778, P<0.005). (B) Serum levels of 1,25(OH)2D corresponding to serum Ca in OMC-1-bearing rats with severe hypercalcemia and those treated with YM529. ■, OMC-1-bearing rats with severe hypercalcemia (Ca≥15 mg/dl); □, OMC-1-bearing rats treated with YM529 as described in Materials and Methods. The broken line is the linear regression curve determined between serum Ca and 1,25(OH)2D levels in the animals including the control rats and OMC-1-bearing rats with mild hypercalcemia, which is shown in panel (A). (C) Serum levels of 1,25(OH)2D corresponding to serum Ca in OMC-1-bearing rats with severe hypercalcemia and those treated with the neutralizing antibody against PTHrP. ■, OMC-1-bearing rats with severe hypercalcemia (Ca≥15 mg/dl); △, OMC-1-bearing rats treated with the antibody against PTHrP as described in Materials and Methods. The broken line is the linear regression curve determined between serum Ca and 1,25(OH)2D levels in the animals including the control rats and OMC-1-bearing rats with mild hypercalcemia, which is shown in panel (A).

Table 2 Effects of bisphosphonate YM529 on biochemical parameters in OMC-1-bearing rats. YM529 (0.1 mg/kg, i.v.) was administered to OMC-1-bearing rats with severe hypercalcemia. Blood was taken before and 4 days after and biochemical analysis performed. The data are means ± S.E.M. (n=4).

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<th>Parameter</th>
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<th>After</th>
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<td>Ca (mg/dl)</td>
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<td>Phosphate (mg/dl)</td>
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<td>25OHD (ng/ml)</td>
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<td>1,25(OH)2D (pg/ml)</td>
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<td>154.5±18.0</td>
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<tr>
<td>24,25(OH)2D (ng/ml)</td>
<td>N.A.</td>
<td>6.2±1.9</td>
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*Significantly different from the value before treatment (paired t-test; P<0.05). N.A., not analyzed.

\[ P<0.005 \] in the non-tumor-bearing control group and normocalcemia to mild hypercalcemia group of OMC-1-bearing rats (Fig. 2A). The serum levels of Ca and 1,25(OH)2D were not correlated in the severe hypercalcemia group, and the levels of 1,25(OH)2D were apparently suppressed compared with those in the control group and normocalcemia to mild hypercalcemia group of OMC-1-bearing rats (Fig. 2B).

Effects of bisphosphonate YM529 on serum 1,25(OH)2D levels in OMC-1-bearing rats

To further examine whether an increased level of serum Ca was responsible for the impaired vitamin D metabolism, we administered bisphosphonate YM529 to OMC-1-bearing rats (experiment 3). Administration of YM529 reduced serum Ca levels in OMC-1-bearing rats from 16.7±0.8 to 10.7±0.5 mg/dl in 4 days (n=4, P<0.02, paired t-test). The serum 1,25(OH)2D levels after treatment with YM529 were markedly elevated (154.5±18.0 pg/ml, Table 2) compared with those in untreated OMC-1-bearing rats with severe hypercalcemia (P<0.0001), and higher even than those in untreated OMC-1-bearing rats with mild hypercalcemia (P<0.05, Fig. 2B).

Enhanced expression of 1α-hydroxylase in YM529-treated OMC-1-bearing rats

The expression of 1α-hydroxylase mRNA in kidney of OMC-1-bearing rats was examined by RT-PCR (Fig. 3). In kidney from control rats, very faint signals were detected on PCR for 1α-hydroxylase expression after 40 thermal cycles. Kidney obtained from OMC-1-bearing rats with mild hypercalcemia still retained detectable levels of 1α-hydroxylase. In contrast, kidney from OMC-1-bearing rats with severe hypercalcemia (≥15 mg/dl) exhibited a reduced expression of the gene. In kidney from OMC-1-bearing rats which were treated with YM529, expression of 1α-hydroxylase was markedly increased (Fig. 3). As to the expression of VDR, it was detected in all of the kidney samples, and was rather increased in OMC-1-bearing rats compared with non-tumor-bearing control.
The expression of 24-hydroxylase was not detected in any of the samples by 40 thermal cycles (data not shown).

**Effects of neutralizing antibody against PTHrP on vitamin D metabolism in OMC-1-bearing rats**

As described above, restoration of the serum Ca level with bisphosphonate YM529 resulted in the enhanced expression of 1α-hydroxylase, which was associated with a marked increase in serum 1,25(OH)₂D concentration. We therefore hypothesized that the large amount of PTHrP produced by the tumor刺激ated the expression of 1α-hydroxylase when the serum Ca level was restored, although it failed in the stimulation of the gene under conditions of severe hypercalcemia. To test the responsibility of PTHrP for the enhanced expression of 1α-hydroxylase, we examined the effects of neutralizing antibody against PTHrP in OMC-1-bearing rats (experiment 4). i.v. administration of 3 mg/kg body weight of the antibody caused a reduction in serum Ca level from 14.9 ± 1.2 to 12.4 ± 1.3 mg/dl in 4 days (P < 0.01, paired t-test, Table 3). Serum levels of 1,25(OH)₂D after treatment with antibody remained low (Table 3, Fig 2C). Expression of 1α-hydroxylase in kidney of the OMC-1-bearing rats treated with the antibody was shown to be suppressed in RT-PCR analysis (data not shown).

**Decreased levels of serum 25 OHD in severe hypercalcemic rats bearing OMC-1 tumor**

Serum levels of 25 OHD and 24,25(OH)₂D were also examined (Table 1). The levels of 24,25(OH)₂D were decreased in OMC-1-bearing rats compared with control rats. Interestingly, the serum levels of 25 OHD were significantly reduced in the severe hypercalcemia group compared with the control rats and normocalcemia to mild hypercalcemia group of OMC-1-bearing rats (P < 0.005).

**Expression of 25-hydroxylase**

Despite the decreased level of serum 25 OHD, RT-PCR analysis showed that the expression of 25-hydroxylase in liver was not suppressed in OMC-1-bearing rats (data not shown). Therefore, the expression of 25-hydroxylase was further investigated by Northern blot analysis (Fig. 4). Densitometric analysis showed that the relative expression of 25-hydroxylase to β-actin was 2-fold higher in OMC-1-bearing rats compared with that in non-tumor-bearing controls.

**Table 3** Effects of neutralizing antibody against PTHrP on biochemical parameters in OMC-1-bearing rats. Antibody (3.0 mg/kg, i.v.) was administered to OMC-1-bearing rats with severe hypercalcemia. Blood was taken before and 4 days after and biochemical analysis performed. Data are means ± S.E.M. (n = 4).

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<th>After</th>
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<td>14.9 ± 1.2</td>
<td>12.4 ± 1.3a</td>
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<td>Phosphate (mg/dl)</td>
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<td>6.1 ± 0.1b</td>
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<td>25OHD (ng/ml)</td>
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<td>21.0 ± 2.6</td>
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<td>24,25(OH)₂D (ng/ml)</td>
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<td>6.0 ± 0.2</td>
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a,b Significantly different from the value before treatment (paired t-test; a, P < 0.01; b, P < 0.05). NA, not analyzed.
levels of 1,25(OH)₂D are not suppressed in most of these models, although hypercalcemia occurs associated with elevated levels of PTHrP (Ikeda & Ogata 1995). This finding led us to hypothesize that serum Ca plays a major role in suppressing the level of serum 1,25(OH)₂D, which has been suggested to occur in some animal models of HHM (Ikeda et al. 1988, Nagai et al. 1998). However, serum levels of 1,25(OH)₂D are not suppressed in most of these models, although hypercalcemia occurs associated with elevated levels of PTHrP (Ikeda & Ogata 1995). This situation has made it difficult to investigate the mechanisms of impaired vitamin D metabolism in HHM. In contrast, the OMC-1-bearing rats described here manifested a marked reduction in serum 1,25(OH)₂D levels, mirroring the situation in HHM patients to some extent.

In OMC-1-bearing rats, serum levels of 1,25(OH)₂D were suppressed only during severe hypercalcemia. This finding led us to hypothesize that serum Ca plays a major role in suppressing the level of serum 1,25(OH)₂D in OMC-1-bearing rats, probably via the regulation of 1α-hydroxylase expression. Supporting the hypothesis, administration of bisphosphonate YM529 to OMC-1-bearing rats increased serum levels of 1,25(OH)₂D in response to the recovery of serum Ca levels. Further examination showed that expression of 1α-hydroxylase in kidney was markedly elevated in YM529-treated OMC-1-bearing rats to a level higher than in non-tumor-bearing control rats. There was no significant difference in serum phosphate levels between before and after treatment with YM529. The results strongly suggested that the recovery of serum Ca by bisphosphonate caused the stimulation of 1α-hydroxylase expression by PTHrP or some other factor(s). Schilling et al. (1993) reported that administration of bisphosphonate to HHM patients normalized serum Ca levels and at the same time restored 1,25(OH)₂D levels, although they found no correlation between 1,25(OH)₂D and PTHrP levels. We assumed that the large amount of PTHrP produced by the tumor was responsible for the enhanced expression of 1α-hydroxylase in YM529-treated OMC-1-bearing rats, and took advantage of a neutralizing antibody against PTHrP to investigate its contribution to the expression of the gene.

It has been reported that the anti-PTHrP utilized here specifically binds to PTHrP(1–34) and inhibits PTHrP function both in vivo and in vitro (Sato et al. 1993). Although the antibody had been shown to reduce serum Ca levels in some animal models of HHM (Sato et al. 1993), there had been no report about its effects on impaired vitamin D metabolism. In OMC-1-bearing rats, i.v. administration of the antibody to PTHrP (3 mg/kg) resulted in a decrease in the serum Ca level, although the effect in this dose was not as strong as that of YM529. As to the serum level of 1,25(OH)₂D, it remained low despite the reduction in the serum Ca level after the administration of the antibody, which was contrary to the case with YM529. The results supported that PTHrP produced by the tumor itself stimulated the expression of 1α-hydroxylase in OMC-1-bearing rats. It has been reported that treatment of HHM patients with bisphosphonates may cause an increase in serum PTH level in some patients, even though they remain hypercalcemic (Fraser et al. 1991, Fukumoto et al. 1994). However, it was unlikely that PTH was secreted in OMC-1-bearing rats which remained hypercalcemic, because the suppressed serum 1,25(OH)₂D level remained after the treatment with the antibody which specifically binds PTHrP without any effects on PTH action. In addition, serum 1,25(OH)₂D levels in the antibody-treated group were even lower than those in untreated OMC-1-bearing rats with similar serum Ca levels (normocalcemia to mild hypercalcemia).

OMC-1-bearing rats exhibited renal insufficiency at a late time point, which was suggested by the elevated serum Cr level. We can not exclude the possibility that the renal insufficiency also was involved in the impaired 1α-hydroxylase expression. However, we assume that hypercalcemia also plays an important role in the reduction of the expression of the gene in these rats, because in kidney the expression of the 1α-hydroxylase gene was still able to be enhanced in response to the change in serum Ca level caused by treatment with YM529.

A reduction in the serum level of 25 OHD was another notable feature in OMC-1-bearing rats. However, the mRNA level of 25-hydroxylase in liver was slightly increased in OMC-1-bearing rats compared with control animals. At the moment, we have two possible explanations for the reduction in serum 25 OHD level: one is malnutrition associated with cachexia, and the other is urinary leak of vitamin D binding protein/25 OHD.

Discussion

In the present study, we investigated the roles of serum Ca and PTHrP in impaired vitamin D metabolism in HHM using an animal model where PTHrP-producing infantile fibrosarcoma (OMC-1) was inoculated into nude rats. To date, a number of animal models have been developed which mimic the pathological state of patients with HHM (Ikeda et al. 1988, Nagai et al. 1998). However, serum levels of 1,25(OH)₂D are not suppressed in most of these models, although hypercalcemia occurs associated with elevated levels of PTHrP (Ikeda & Ogata 1995). This situation has made it difficult to investigate the mechanisms of impaired vitamin D metabolism in HHM. In contrast, the OMC-1-bearing rats described here manifested a marked reduction in serum 1,25(OH)₂D levels, mirroring the situation in HHM patients to some extent.

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complex. Megalin, the endocytic receptor which is responsible for renal uptake of 25 OHD (Nykaer et al. 1999), might be impaired in kidney of OMC-1-bearing rats.

In conclusion, using OMC-1-bearing rats as an animal model for HHM, we demonstrated that the expression level of the 1α-hydroxylase gene was regulated by a balance between stimulation by PTH/PTHrP signal and suppression by hypercalcemia.

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