Increased Expression of 25-Hydroxyvitamin D-1 α -Hydroxylase in Dysgerminomas

A Novel Form of Humoral Hypercalcemia of Malignancy

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Humoral hypercalcemia of malignancy (HHM) is a common paraneoplastic disorder usually associated with increased synthesis of parathyroid hormone-related peptide (PTHrP). Unlike non-cancer forms of hypercalcemia, HHM does not routinely involve increased circulating levels of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). Dysgerminomas are a notable exception to this rule, previous reports having described hypercalcemia with elevated serum 1,25(OH)₂D₃. To investigate the etiology of this form of HHM we have characterized expression and activity of the enzyme that catalyzes synthesis of 1,25(OH)₂D₃, 25-hydroxyvitamin D-1 α -hydroxylase (1 α -hydroxylase), in a collection of 12 dysgerminomas. RT-PCR analyses indicated that mRNA for 1 α -hydroxylase was increased 222-fold in dysgerminomas compared to non-tumor ovarian tissue. Parallel enzyme assays in tissue homogenates showed that dysgerminomas produced fivefold higher levels of 1,25(OH)₂D₃ compared to normal ovarian tissue. Immunolocalization studies indicated that 1 α -hydroxylase was expressed by both tumor cells and by macrophages within the inflammatory cell infiltrate associated with dysgerminomas. The immunological nature of the increased 1,25(OH)₂D₃ production observed in dysgerminomas was further emphasized by correlation between expression of 1 α -hydroxylase and the endotoxin recognition factors

CD14 and toll-like receptor 4 (TLR4). These data suggest that inflammatory mechanisms associated with dysgerminomas are the underlying cause of the increased expression and activity of 1 α -hydroxylase associated with these tumors. We further postulate that this autocrine/paracrine action of 1 α -hydroxylase may lead to increased circulating levels of 1,25(OH)₂D₃ and a form of HHM which is distinct from that seen with PTHrP-secreting tumors. (*Am J Pathol* 2004, 165:807–813)

Humoral hypercalcemia of malignancy (HHM) is a paraneoplastic disorder which affects up to 30% of cancer patients and is associated with a wide variety of tumor types, including breast and lung cancer and multiple myeloma.^{1–3} The hypercalcemia that occurs with HHM is rapid and severe and usually acts as a marker of poor tumor prognosis, with low mean survival times.⁴ In the vast majority of cases, HHM occurs as a result of tumor secretion of parathyroid hormone-related peptide (PTHrP), which exerts potent effects on renal calcium handling and bone resorption similar to parathyroid hormone (PTH) itself.^{3,5,6} However, in a small proportion of cases, PTHrP does not appear to be the central cause of HHM, the disorder instead stemming from abnormal synthesis of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). For example, increased circulating levels of 1,25(OH)₂D₃ have been described in patients with Hodgkin's and non-Hodgkin's lymphomas,^{7–13} although, in some cases there may still be some involvement of PTHrP.¹⁴ Until recently the precise cellular source of the raised 1,25(OH)₂D₃ associated with tumors was unclear. Serum levels of 1,25(OH)₂D₃ are normally dependent on the enzyme 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α -hydroxylase) which is abundantly expressed in the kidney. At this site 1 α -hydroxylase is tightly regulated by phosphate, calcium, and 1,25(OH)₂D₃ itself^{15,16} and, consequently, the kidney seems an unlikely source of dysregulated 1,25(OH)₂D₃ synthesis. In-

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deed, in severe cases of PTHrP-driven HHM serum $1,25(\text{OH})_2\text{D}_3$ may be suppressed, possibly as a direct response to increased calcium concentrations.¹⁷

An alternative proposal is that the increased circulating levels of $1,25(\text{OH})_2\text{D}_3$ associated with lymphomas are due to abnormal expression of 1α -hydroxylase at extra-renal sites. In a recent case study of a patient with hypercalcemia and raised serum $1,25(\text{OH})_2\text{D}_3$ due to a B-cell lymphoma, we used immunohistochemistry to demonstrate the presence of 1α -hydroxylase in macrophages associated with the lymphoma.¹⁸ Resection of the tumor normalized serum calcium and $1,25(\text{OH})_2\text{D}_3$ levels suggesting that the tumor secreted a factor(s) which stimulated 1α -hydroxylase expression by macrophages. Thus, it would appear that certain types of tumors are able to mimic the aberrant vitamin D metabolism which has been well characterized for a wide variety of granulomatous disorders.¹⁹

In the present study, we have investigated further the pathophysiology of tumor-associated vitamin D metabolism in a cohort of patients with dysgerminomas.²⁰ Compared to other tumors, dysgerminomas have a relatively low incidence of hypercalcemia, although there have been previous reports documenting raised serum calcium and $1,25(\text{OH})_2\text{D}_3$ with dysgerminomas and seminomas.^{21–25} We have used a collection of 12 dysgerminomas to assess expression of 1α -hydroxylase, and to relate this to circulating hormone levels, macrophage markers, and other components of vitamin D metabolism and signaling. Data indicate that abnormal expression of 1α -hydroxylase in dysgerminomas may result in a form of HHM distinct from that seen in PTHrP-secreting tumors, and which is associated with increased circulating levels of $1,25(\text{OH})_2\text{D}_3$.

Materials and Methods

Tissue Samples

Tissue from the index dysgerminoma case (see Table 2) together with control ovarian tissue from non-neoplastic ovaries were collected after surgical resection for reasons unrelated to the study and with written informed consent. The protocol was approved by the Human Investigation Review Board of Lutheran Medical Center, Cleveland Clinic Health System, Cleveland, OH. All other dysgerminoma samples as well as accompanying serum specimens, where available, were generously provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. For all analyses, tissues were snap-frozen in liquid nitrogen and stored at -80°C . Tumor tissue from the index case dysgerminoma was also fixed in 10% buffered formaldehyde solution and embedded in paraffin wax before sectioning at $3\text{-}\mu\text{m}$ thickness. Donors of non-dysgerminomatous ovary tissue were genetic females and were aged 11, 25, 37, 43, 51, 52, and 83 years. Except for those aged 51 and 83 years, all other donors were menstruating at the time biopsies were taken.

RNA Extraction and Reverse Transcription

RNA was extracted from dysgerminomas and non-neoplastic ovary tissue using the GenElute Mammalian Total RNA kit as detailed by the manufacturer (Sigma, Poole, UK). RNA was eluted in Rnase-free elution solution and then treated with amplification grade deoxyribonuclease 1 (DNase 1) (Sigma) as stated by the manufacturer. The DNA-free RNA was stored at -80°C and aliquots ($1.5\text{ }\mu\text{g}$) were reverse-transcribed using AMV reverse transcriptase as described by the manufacturer (Promega, Southampton, UK).

Quantitative RT-PCR Analysis of Gene Expression

Expression of specific mRNAs was quantified using an ABI 7700 sequence detection system (PE Biosystems, Warrington, UK) as described previously.²⁶ Briefly aliquots ($25\text{ }\mu\text{L}$) of PCR reactions were set up containing TaqMan Universal PCR Master mix in a 2X solution (PE Biosystems), 3 mmol/L $\text{Mn}(\text{OAc})_2$, 200 $\mu\text{mol/L}$ dNTPs, 1.25 U AmpliTaq Gold polymerase, 1.25 U AmpErase uracil-N-glycosylase (UNG), 5 or 1.25 pmoles/ μL TaqMan probe, and 5 or 9 pmoles/ μL primers. Approximately 50 ng of cDNA were used per reaction. All reactions were multiplexed with the housekeeping gene product 18S rRNA, provided as an optimized control probe labeled with VIC (PE Biosystems), enabling data to be expressed in relation to an internal reference to allow for differences in sampling. All fluorogenic probes for genes of interest were labeled with five-carboxy fluorescein (FAM). Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacturer's guidelines, and used to determine ΔCt values (Ct of target gene – Ct of housekeeping gene). All reactions were performed in triplicate and expressed as a mean of three separate experiments $\pm$ SD (SD). Samples were amplified using primers and probes detailed in Table 1 under the following conditions: 50°C for 2 minutes; 95°C for 10 minutes; followed by 44 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Analysis of 1α -Hydroxylase Activity in Tissue Samples

Activity levels for 1α -hydroxylase in dysgerminomas and non-neoplastic ovaries were assessed by quantifying the metabolism of 25-hydroxyvitamin D_3 (25OHD_3) in homogenates from these tissues. For each assay 10 nmol/L [^3H]- 25OHD_3 (specific activity, 152 Ci/mmol; Amersham, London, UK) was added to tissue homogenates prepared from snap-frozen dysgerminomas ($n = 7$) or non-neoplastic ovaries ($n = 4$). Aliquots of homogenate used in the assays contained 0.4 mg protein, 0.2 mol/L co-factor (NADPH, Sigma), and 0.5 mmol/L protease inhibitor (PMSF, Sigma). Homogenate/substrate mixtures were incubated for 5 hours at 37°C and the reaction terminated by freezing at -20°C . Vitamin D_3 metabolites were then

Table 1. Primer and Probe Sequences for Real-Time RT-PCR Analyses

Gene	Primer probe sequence 5'–3'
1 α -OHase	Forward 5' primer TTGGCAAGCGCAGCTGTAT-3' Reverse 5' primer TGTGTTAGGATCTGGGCCAAA-3' TaqMan probe TTGCAATTCAAGCTCTGCCAGGCG-3'
VDR	Forward primer 5'-CTTCAGGCGAAGCATGAAGC-3' Reverse primer 5'-CCTTCATCATGCCGATGTCC-3' TaqMan probe 5'-AAGGCACTATTACCTGCCCTTCAA-3'
24-OHase	Forward primer 5'-CAAACCGTGAAGGCCTATC-3' Reverse primer 5'-AGTCTTCCCCTTCCAGGATCA-3' TaqMan probe 5'-ACTACCGCAAAGAAGGCTACGGGCTG-3'
p21	Forward primer 5'-GCAGACCAGCATGACAGATTTTC-3' Reverse primer 5'-GGATTAGGGCTTCTCTTGA-3' TaqMan probe 5'-CCACTCCAAACGCCGGCTGATCTT-3'
CD45	Forward primer 5'-AGTATCCCCGGACTCTTTGGA-3' Reverse primer 5'-CGGAGCCGCTGAATGTCT-3' TaqMan probe 5'-TGCTAGTGCTTTTAATACCACAGGTGTTTCATCAGT-3'
HOXA10	Forward primer 5'-AAGGATTCCCTGGGCAATTG-3' Reverse primer 5'-CCAGTGTCTGGTGCTTCGTGTA-3' TaqMan probe 5'-CGACCACTCTTTGCCGTGAGACAG-3'
CD14	Assays-on-Demand (ABI) primer and probe mix IDs Hs00169122_g1
TLR4	Assays-on-Demand (ABI) primer and probe mix Hs00152939.m1
PTHrP	Assays-on-Demand (ABI) primer and probe mix Hs00174969.m1

extracted from the reaction mixtures in 2.5 ml chloroform: methanol (4:1 vol:vol) and the conversion of [^3H]-25OHD $_3$ to [^3H]-1,25(OH) $_2\text{D}_3$ quantified by scanning thin layer chromatography (TLC) as described previously.²⁷ Results were expressed as mean fmole [^3H]-1,25(OH) $_2\text{D}_3$ produced per hour per mg of protein $\pm$ SD.

Immunohistochemistry for 1 α -Hydroxylase

Immunohistochemical analysis of 1 α -hydroxylase localization in paraffin-embedded dysgerminoma tissue sections was carried out using methods as described previously.²⁶ Briefly, sections were de-waxed and underwent antigen retrieval by processing them in 0.01 mol/L sodium citrate buffer in a pressure cooker at 103 kPa for 2 minutes. Slides were then incubated in 3% methanol-hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, washed in Tris-buffered saline (TBS, pH 7.6), and then incubated with sheep anti-mouse 1 α -hydroxylase antiserum in 10% normal swine serum in TBS at dilutions of between 1:100 and 1:300 for 45 minutes at room temperature. The mouse peptide sequence is 71% homologous to the human 1 α -hydroxylase protein and only 17% to the human 24-hydroxylase; cross-reactivity with human 1 α -hydroxylase has been described in previous studies.^{18,26,27} After a 15-minute TBS wash, donkey anti-sheep IgG peroxidase conjugate with 10% normal swine serum in TBS at 1:100 dilution was added to sections for 45 minutes. DAB was used to visualize the secondary antibody, thus localization of the protein, which precipitates as a brown-colored deposit. Mayer's hematoxylin was used to counter-stain sections, followed by dehydration through ethanol washes and xylene, and mounted in xylene-based dibutyl polystyrene (DPX).

Data Analysis

Data from quantitative RT-PCR were reported as either the fold increase of the mean $\pm$ SD or as the raw mean

ΔCt values. Statistical analysis was performed on the ΔCt data using one-way analysis of variance (analysis of variance) with Student-Newman-Keuls multiple comparison post-hoc test or Pearson correlation (Sigma-Stat3 V2.03).

Results

Case Study of Hypercalcemia Associated with a Dysgerminoma

The index case dysgerminoma patient presented with a histologically-verified dysgerminoma of the left ovary which was associated with hypercalcemia (15.9 mg/dL, normal range 8.5 to 10.5 mg/dL) (Table 2). This was shown to occur in the presence of suppressed PTH (11.3 pg/ml; normal range, 10 to 65 pg/ml) and PTHrP (0.3 pmol/L; normal range, <1.3 pmol/L) and low normal levels of 25OHD $_3$ (18 ng/ml; normal range, 9 to 52 ng/ml). However, circulating levels of 1,25(OH) $_2\text{D}_3$ were high (71 pg/ml; normal range, 15 to 62 pg/ml). Repeat PTHrP values 3 days preoperatively after intravenous pamidronate therapy for hypercalcemia were 0.4 pmol/L. PTH

Table 2. Biochemical Data for the Index Case Patient with a Dysgerminoma of the Left Ovary

Age	18 years	
Total calcium	15.9 mg/dL	(8.5–10.5 mg/dL)
Ionized calcium	1.98 mmol/L	(1.15–1.35 mmol/L)
Albumin	3.8 g/dL	(3–5 g/dL)
Phosphate	1.8 mg/dL	(2.2–4.6 mg/dL)
PTH	11.3 pg/ml	(10–65 pg/ml)
PTHrP	0.3 pmol/L	(<1.3 pmol/L)
Bun/Cr	19/1.5 mg/dL	(7–18/0.6/1.3 mg/dL)
25OHD $_3$	18 ng/ml	(9–52 ng/ml)
1,25(OH) $_2\text{D}_3$	71 pg/ml	(15–60 pg/ml)
Total calcium (1 year after resection)	9.8 mg/dL	(8.5–10.5 mg/dL)

Figure 1. Immunolocalization of 1α -hydroxylase in dysgerminoma tissue and a non-neoplastic ovary. Paraffin-embedded biopsies from the index case dysgerminoma (**A**) and a non-dysgerminomatous benign cystic ovary (**B**) were used to immunolocalize 1α -hydroxylase (**A** and **B**; magnification, $\times 200$). Brown staining for 1α -hydroxylase was observed in macrophages and tumor cells within the dysgerminoma, while non-neoplastic tissue showed only weak staining in cystal epithelial cells.

values 3 days and 1 day preoperatively were 4.0 and <4.0 pg/ml, with serum calcium values of 10.5 and 10.3 mg/dL and phosphorous values of 2.3 and 4.7 mg/dL, respectively. One day postoperatively, serum calcium was 9.3 mg/dL, phosphorous was 1.8 mg/ml, PTH was 44 pg/ml, PTHrP was 0.4 pmol/L, $1,25(\text{OH})_2\text{D}_3$ was 39 pg/ml, and 25OHD_3 was 13 ng/ml.

Immunohistochemical analysis of tissue from the index case dysgerminoma using a specific antiserum for 1α -hydroxylase showed strong expression of protein for the enzyme in both neoplastic cells and in tumor-associated macrophages (Figure 1). By contrast control, non-dysgerminomatous tissue showed only discrete staining for 1α -hydroxylase in cystal epithelial cells (Figure 1A).

Quantitative Analysis of 1α -Hydroxylase mRNA Expression in Dysgerminomas

To quantify the induction of 1α -hydroxylase in the dysgerminoma from the index case patient and to determine whether or not this effect was common to dysgerminomas in general, tissue from an additional 11 dysgerminomas and 8 non-neoplastic ovaries were used to generate mRNA for reverse transcription and quantitative real-time PCR analysis. The retrospective nature of these studies meant that a complete set of biochemical data was not available for the majority of dysgerminoma samples and non-neoplastic controls. However, serum-ionized calcium, $1,25(\text{OH})_2\text{D}_3$, and PTH levels were obtained for four dysgerminoma patients. Mean values were: ionized calcium, 2.14 ± 0.04 mmol/L (normal range, 1.15 to 1.35 mmol/L); $1,25(\text{OH})_2\text{D}_3$, 68.8 ± 14.4 pg/ml (normal range, 15 to 62 pg/ml); and PTH, 22.8 ± 10.9 pg/ml (normal range, 8 to 65 pg/ml). All of the patients showed normal serum levels of 25OHD_3 (26.7 ng/ml ± 8.1 , normal range, 9 to 52 ng/ml).

Data shown in Figure 2 confirmed the up-regulation of 1α -hydroxylase mRNA in the index case dysgerminoma detailed in Table 2 but also revealed a consistent increase in 1α -hydroxylase expression for dysgerminomas as a whole when compared to non-neoplastic ovaries. The collective mean ΔCt value for the control samples (22.09 ± 2.03 SD) was statistically different from the mean 1α -hydroxylase ΔCt for the dysgerminomas (14.29 ± 1.84), $P < 0.001$. This corresponded to a striking 222-fold increase in 1α -hydroxylase expression. Donors of non-dysgerminomatous samples (aged 11 to 83 years) included three postmenopausal subjects, but there was no correlation between age of donor and the

level of 1α -hydroxylase expression (data not shown). Further studies were carried out in parallel to assess changes in the expression of other genes associated with $1,25(\text{OH})_2\text{D}_3$ signaling and metabolism (Figure 2). Mean ΔCt values for VDR mRNA were also statistically higher in dysgerminomas compared to control ovaries ($P < 0.001$) and reflected a 41-fold increase in expression. By contrast, the catabolic enzyme 24-hydroxylase was weakly expressed by both dysgerminomas and non-neoplastic ovaries and there was no statistical difference between ΔCt values for the two sets of samples.

Increased Synthesis of $1,25(\text{OH})_2\text{D}_3$ in Dysgerminomas

The functional significance of the increased levels of 1α -hydroxylase mRNA and protein in dysgerminomas was determined by enzyme assays to assess the conversion of

n=	8	12	8	12	8	12
mean ΔCt	22.1	14.3	23.4	23.2	23.8	18.5
SD	2.0	1.8	7.7	3.1	1.9	1.8

Figure 2. Expression of genes associated with vitamin D metabolism and signaling in dysgerminomas. Data show the expression of mRNAs assessed by real-time RT-PCR. In each case mRNA expression is represented by: i) ΔCt values ($\pm$ SD) which represent the number of PCR amplification cycles (Ct) at which logarithmic PCR plots cross a calculated threshold line. ΔCt values = Ct of the target gene – Ct of the housekeeping gene; ii) fold-increase in mRNA expression relative to the mean of non-neoplastic ovarian samples expressed as an arbitrary value of 1. The target genes analyzed in this fashion were 1α -hydroxylase (1α -OHase), 24-hydroxylase (24-OHase), and vitamin D receptor (VDR). In each case, the mean fold-change in gene expression for the dysgerminoma index case is shown as a horizontal line. Data shown are the mean $\pm$ SD. ***, $P < 0.001$ compared with non-neoplastic ovarian tissue, based on ΔCt values.

Figure 3. 1α -hydroxylase activity is increased in dysgerminomas. Substrate [3 H]-25OHD₃ (10 nmol/L) was added to cell homogenates in the presence of NADPH (0.2 mol/L) as cofactor and incubated for 5 hours. Conversion of the inactive substrate to active [3 H]-1,25(OH)₂D₃ was determined by TLC and reported as mean fmole/hour/mg protein of [3 H]-25OHD₃ converted to active [3 H]-1,25(OH)₂D₃ in either dysgerminomas ($n = 7$) or non-neoplastic ovaries ($n = 4$) $\pm$ SD. All statistics were performed on raw data using analysis of variance (***, $P < 0.001$ compared with non-neoplastic ovaries).

[3 H]-25OHD₃ to [3 H]-1,25(OH)₂D₃ in tissue homogenates from the tumors and non-neoplastic ovaries. Scanning TLC analysis data shown in Figure 3 revealed a fivefold increase in 1,25(OH)₂D₃ production by dysgerminomas and this activity correlated with tumor 1α -hydroxylase mRNA expression ($P < 0.01$, data not shown). By contrast, 24-hydroxylase activity, as determined by production of [3 H]-24,25-dihydroxyvitamin D₃, was undetectable in both dysgerminomas and non-neoplastic ovaries (data not shown).

Increased Expression of 1α -Hydroxylase Expression in Dysgerminomas Is Associated with Macrophage Infiltration and Activation

To investigate the mechanism for up-regulation of 1α -hydroxylase expression in dysgerminomas we also characterized genes associated with different aspects of tumor function (Figure 4). Expression of mRNA for PTHrP was enhanced in the dysgerminomas. However, this was not apparent at the protein level as the index case dysgerminoma detailed in Table 2 presented with low circulating PTHrP despite having increased PTHrP mRNA within the dysgerminoma. Expression of the cell cycle-associated gene p21 and the homeobox gene HOXA10, both of which are potential target genes for 1,25(OH)₂D₃, showed no significant change in dysgerminomas. However, mRNA for CD45, the common leukocyte antigen, was increased 85-fold in dysgerminomas underlining the characteristic inflammatory nature of these tumors. To study this in more detail, further RT-PCR analyses were carried out for CD14 and toll-like receptor 4 (TLR4) which together form a recognition complex for endotoxins such as lipopolysaccharide (LPS). Both CD14 and TLR4 were

Figure 4. Expression of putative target genes for 1,25(OH)₂D₃ in dysgerminomas. Data show the expression of mRNAs assessed by real-time RT-PCR. In each case, mRNA expression is represented by: i) Δ Ct values ($\pm$ SD); ii) fold-increase in mRNA expression relative to the mean of non-neoplastic ovarian samples as an arbitrary value of 1. The target genes analyzed in this fashion were CD45, PTHrP, p21, and HOXA10. In each case, the mean expression level for the dysgerminoma index case is shown as a **horizontal line**. ***, $P < 0.001$ compared with normal ovarian tissue, based on Δ Ct values.

strongly up-regulated in dysgerminomas (12.7- and 22.9-fold, respectively, $P < 0.001$ for both). Their expression also correlated closely with levels of 1α -hydroxylase mRNA (see Figure 5), suggesting that leukocyte invasion and/or local inflammation are closely associated with increased synthesis of 1,25(OH)₂D₃ in dysgerminomas.

Discussion

The physiological significance of extra-renal 1α -hydroxylase activity has been recognized for almost a quarter of a century following the seminal observation that an anephric patient with sarcoidosis was able to maintain normal circulating levels of 1,25(OH)₂D₃ despite having no classical source of the hormone.²⁸ Since then, extra-renal synthesis of 1,25(OH)₂D₃ has been demonstrated for a wide range of granulomatous disorders,¹⁹ and activated macrophages have been identified as the source of dysregulated 1α -hydroxylase activity in these

Figure 5. Expression of 1α -hydroxylase (1α -OHase) in dysgerminomas and normal ovaries correlates with CD14 and toll-like receptor 4 (TLR4). Levels of mRNA for 1α -hydroxylase were compared to CD14 (A) and TLR4 (B) mRNA levels (relative to 18S rRNA) and data shown as scatter plot for mean Δ Ct values for each dysgerminoma ($n = 12$) and non-neoplastic ovary ($n = 8$). Correlation statistics (R value and statistical significance p) were performed on raw Δ Ct triplicate means using Pearson product moment correlation.

diseases.^{29,30} However, recent studies have suggested a much broader role for the enzyme. RNA, protein, and activity analyses indicate that the enzyme is expressed in a wider range of tissues than originally thought. Many of the cells that express 1α -hydroxylase are within "barrier sites" such as the skin, colon, vasculature, and placenta.^{26,31,32} This has stimulated interest in the role of 1α -hydroxylase in normal physiology, as well as its potential impact on autoimmune disease and cancer.²⁹ In *ex vivo* analyses, the enzyme has been shown to be up-regulated in colonic cancer^{33–35} and parathyroid adenomas.³⁶ By contrast, studies *in vitro* have reported decreased synthesis of $1,25(\text{OH})_2\text{D}_3$ by primary cultures of prostate tumor cells and cell lines compared to normal prostate cells.³⁷ This has raised key questions concerning the source of 1α -hydroxylase in tumors and the putative effects of $1,25(\text{OH})_2\text{D}_3$ produced by neoplastic tissues. In a recent case study of a B-cell lymphoma patient with hypercalcemia, we highlighted the similarities between this condition and granulomatous disease by showing that the increased production of $1,25(\text{OH})_2\text{D}_3$ by the lymphoma was due to tumor-associated macrophages rather than the tumor itself.¹⁸ To investigate this further, we characterized 1α -hydroxylase expression and activity in another type of tumor that has been linked to abnormal vitamin D metabolism, namely dysgerminomas.

Although previous reports have documented hypercalcemia in women with dysgerminomas,^{21–24} the contribution of $1,25(\text{OH})_2\text{D}_3$ to this problem has yet to be fully defined. Data presented here suggest that the cause of HHM in dysgerminomas is distinct from that observed for other types of cancer, with tumor-associated 1α -hydroxylase activity being the putative etiological mechanism. Nevertheless, it should be emphasized that in the absence of serum PTHrP data for all of the dysgerminoma patients we cannot rule out the contribution of this factor to aberrant calcium homeostasis. There has been one previous study which has documented increased serum PTHrP in a dysgerminoma patient with hypercalcemia.³⁸ However, although PTHrP mRNA was up-regulated in our tumors, this did not appear to have any impact on circulating levels of PTHrP in the index case. Certainly, there does not appear to be a clear link between PTHrP and 1α -hydroxylase activity. Firstly, in our previous report of a B-cell lymphoma with hypercalcemia and raised serum $1,25(\text{OH})_2\text{D}_3$, PTHrP was normal¹⁸ and secondly, in most cases of HHM due to PTHrP, serum $1,25(\text{OH})_2\text{D}_3$ levels appear to be suppressed, potentially as a result of direct regulation by raised calcium levels.¹⁷

In common with our previous B-cell lymphoma report, the increased production of $1,25(\text{OH})_2\text{D}_3$ in dysgerminomas appears to be closely linked to macrophage function, as this type of tumor is characterized by extensive infiltration by immune cells.²⁰ The vast majority of cells that make up the inflammatory infiltrate in both dysgerminomas and testicular seminomas are CD8⁺ T-cells but it is now recognized that there are significant numbers of macrophages present in these tumors as well.^{39–41} Furthermore, macrophage colony stimulating factor (M-CSF) is increased in patients with malignant germ cell tumors of the ovary, particularly in dysgerminomas.⁴² It was

therefore interesting to note that 1α -hydroxylase expression in dysgerminomas was closely correlated with two components of the innate immune system, CD14 and TLR4. Despite this, immunohistochemical analysis showed that 1α -hydroxylase was expressed by both tumor cells and macrophages associated with the tumor. The relative contribution of these cells to the increased synthesis of $1,25(\text{OH})_2\text{D}_3$ by dysgerminomas remains unclear as both CD14 and TLR4 have been detected on epithelial cells as well as macrophages.^{43,44} However, we can postulate that macrophages make a significant contribution to the levels of 1α -hydroxylase activity in dysgerminomas because of the apparent absence of the "feedback control" enzyme, 24-hydroxylase, which synthesizes inactive $1,24,25$ -trihydroxyvitamin D_3 from $1,25(\text{OH})_2\text{D}_3$.³⁴ Most VDR-expressing cells characteristically show a sensitive induction of 24-hydroxylase expression in the presence of $1,25(\text{OH})_2\text{D}_3$ but this does not appear to occur in macrophages.²⁹ The up-regulation of 1α -hydroxylase without a parallel induction of 24-hydroxylase may also provide an explanation for the relative infrequency of hypercalcemia associated with dysgerminomas.²⁰ Specifically we can postulate that, in common sarcoid patients, raised serum calcium concentrations associated with some dysgerminomas are due to unregulated localized synthesis of $1,25(\text{OH})_2\text{D}_3$ that eventually spills over into the circulation. In the absence of any feedback control this will not only be dependent on the level of 1α -hydroxylase expression, but also on the availability of substrate, namely 25OHD_3 . All of the patients studied had normal serum levels of 25OHD_3 but if these were to increase, as a consequence of increased exposure to sunlight or dietary vitamin D, then this could lead to higher levels of extra-renal $1,25(\text{OH})_2\text{D}_3$ production.

Although expression of 1α -hydroxylase has been described for a variety of different neoplasms,^{18,33–37} the impact of the enzyme on the tumor itself remains unclear. In view of the established anticancer effects of $1,25(\text{OH})_2\text{D}_3$,⁴⁵ we can speculate that localized production of the hormone may be part of an endogenous tumor-defense mechanism. In particular, data presented here suggest that, in dysgerminomas, increased local synthesis of $1,25(\text{OH})_2\text{D}_3$ may be linked to specific facets of tumor immunology. These include possible effects on natural killer cells, suppressor and regulatory lymphocytes,³¹ as well as tumor-associated macrophages.^{46,47} It seems likely that similar responses will also occur in other neoplasms, albeit to a lesser degree, and thus dysgerminomas may act as a useful model for further analysis of the role of extra-renal 1α -hydroxylase and local $1,25(\text{OH})_2\text{D}_3$ production in cancer.

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