Increased Serum Levels of a Parathyroid Hormone-like Protein in Malignancy-Associated Hypercalcemia

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Study Objective: To measure the serum levels of a newly described parathyroid hormone-like protein (PLP), which was isolated from malignant tumors associated with hypercalcemia, and determine whether PLP is a humoral factor in malignancy-associated hypercalcemia.

Design: A cross-sectional study of serum levels of PLP using a newly developed radioimmunoassay.

Setting: A university-affiliated Veterans Administration hospital in San Francisco, California, a University hospital in Hong Kong, and a private hospital in Danville, Pennsylvania.

Patients: Patients with hypercalcemia (calcium > 2.65 mmol/L) and a diagnosis of malignancy were studied. Control groups included normocalcemic patients with malignancy, patients with hyperparathyroidism, and normal subjects.

Measurements and Main Results: Serum immunoreactive PLP (iPLP) levels in normal subjects were less than 2.5 pmol eq/L (10 pg/mL), and 68% of subjects had undetectable levels. The serum concentration of iPLP was normal in 15 of 16 hypercalcemic patients with hyperparathyroidism. Serum iPLP was increased (> 2.5 pmol eq/L) in 36 of 65 (55%) patients with malignancy-associated hypercalcemia, with a mean value of 6.1 ± 0.9 pmol eq/L (24 pg/mL). In a subgroup of patients with solid tumors, serum iPLP was increased in 30 (71%) of 42 hypercalcemic patients, with a mean value of 6.5 ± 0.9 pmol eq/L. Serum iPLP was elevated in only 3 of 23 normocalcemic patients with cancer. In patients with solid malignancies ($n = 59$), levels of iPLP were positively correlated with the total serum calcium ($r = 0.43$, $P < 0.01$).

Conclusions: The data indicate a relation between the serum concentration of iPLP and the presence of hypercalcemia in solid malignancies. The results support a role for PLP as a humoral mediator of hypercalcemia in most patients with solid tumors. Measurement of iPLP should be useful in the differential diagnosis of hypercalcemia.

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Albright (1) first put forth the hypothesis that a humoral substance akin to parathyroid hormone is the cause of hypercalcemia complicating malignancy, and several lines of evidence can now be marshalled to support this view. In patients with solid tumors (especially squamous and renal carcinomas), as in those with primary hyperparathyroidism, hypercalcemia is associated with hypophosphatemia and with increased excretion of nephrogenous cyclic adenosine 3',5'-monophosphate (cAMP) (2, 3). In some patients with increased nephrogenous cAMP excretion, a parathyroid hormone-like substance is also detectable in plasma by bioassay (4), however, levels of parathyroid hormone itself are not increased (2). The tumor content of parathyroid hormone-like bioactivity is also correlated with the occurrence of hypercalcemia (5). These observations led to the recent identification of a human parathyroid hormone-like protein (PLP) in squamous (6-8), breast (8), and renal tumors (9-11) associated with hypercalcemia. The parathyroid hormone-like protein is a 16 000 molecular-weight protein with a predicted sequence of 139 to 172 amino acids, which is homologous with parathyroid hormone only at the amino terminus. Synthetic fragments that contain the 1-34 sequence of PLP activate parathyroid hormone receptors in kidney and bone (12, 13), increase cellular and urinary cAMP levels, and exhibit the bone-resorbing, phosphaturic, and hypocalciuric properties of parathyroid hormone (14-16). When injected or infused into rodents, amino-terminal PLP peptides are at least as potent as parathyroid hormone in causing hypercalcemia (16, 17). Thus PLP has most properties predicted of a humoral mediator of malignancy-associated hypercalcemia; it has not been shown, however, that PLP is secreted by tumors in vivo or that secretion of PLP is sufficient to cause hypercalcemia.

To determine whether PLP in fact has a humoral role in normal or in hypercalcemic states, we developed a radioimmunoassay of PLP and assayed serum from normal subjects and patients with malignancy or hyperparathyroidism. The finding of elevated serum levels of immunoreactive PLP in a high proportion of patients with solid malignancies complicated by hypercalcemia supports a significant role for PLP in the pathogenesis of hypercalcemia.

Methods

Radioimmunoassay

Synthetic PLP(1-34)amide (Merck Sharp & Dohme, West Point, Pennsylvania) (15 μg) was iodinated with chlora-

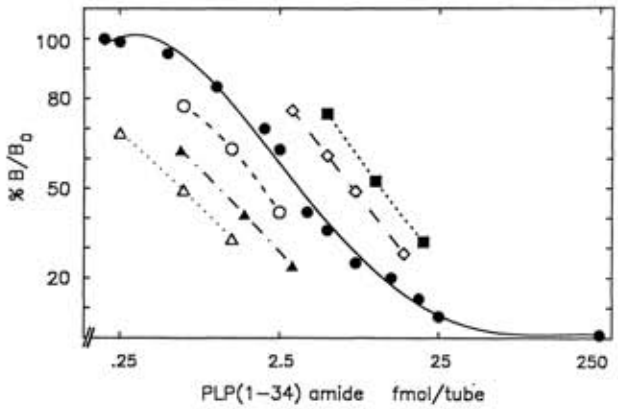

Figure 1. Radioimmunoassay of PLP. Binding of ^{125}I-PLP(1-34)amide to antiserum in the presence of PLP(1-34)amide (*closed circle*), PLP derived from tumor cells (9) added to normal serum and extracted using antibody-Sepharose (*open diamond*), immunoextract of 30 mL of normal serum (*closed squares*), or immunoextracts of serum from patients with malignancy-associated hypercalcemia (*open circles, open triangles,* and *closed triangles* for three different subjects). Multiple dilutions of a sample are plotted on a logarithmic scale.

mine T as previously described (12). Iodinated peptides were purified by chromatography over C-18 SEP-PAK cartridges (Waters Associates, Milford, Massachusetts) followed by reverse-phase HPLC on a Vydak 218TP column (The Separations Group, Hesperia, California) eluted with 0% to 80% acetonitrile in 0.1% trifluoroacetic acid. After solvent exchange using Sephadex LH-20 (12) (Pharmacia, Piscataway, New Jersey), labeled peptides were stable for at least 1 month at −80 °C in 10 mmol/L acetic acid containing 0.1% bovine serum albumin. Because direct addition of serum or plasma interfered with radioimmunoassay of PLP, the PLP was extracted from serum samples by immunoaffinity chromatography before assay. A rabbit antiserum to synthetic PLP(1-37) (Peninsula Laboratories, Belmont, California) with a titer of 1:10 000 and an affinity of 10 ng/mL was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, New Jersey). The 40% $(NH_4)_2SO_4$ precipitate from 6.3 mL of the antiserum was dialyzed against coupling buffer (0.2 mol/L $NaHCO_3$, 0.5 mol/L NaCl, pH 8.5) and coupled by incubation overnight at 4 °C with 4 g of Sepharose in a final volume of 30 mL. Unreacted CNBr groups were then blocked by mixing with 0.1 mol/L Tris-HCl, 0.5 mol/L NaCl, pH 8 for 2 hours followed by extensive washing. The resulting antibody-Sepharose complex had a binding capacity of 7.5 nmol PLP(1-34)amide/75 μL of conjugate.

Serum samples (1 to 5 mL) were stored at −80 °C before assay. For immunoextraction, samples were incubated overnight at 4 °C in capped 6-mL chromatography columns (Evergreen Scientific, Los Angeles, California) containing 75 μL of the antibody-Sepharose conjugate. Columns were then drained, the conjugate was washed with buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5), and bound PLP was eluted with 2 mL of 0.3 mol/L acetic acid. Lyophilized eluates were redissolved in 0.3 to 0.5 mL of RPMI-1640 medium. The recovery of ^{125}I-PLP(1-34)amide added to normal serum was assessed with every assay and ranged from 70% to 90%. Columns were regenerated for additional use by washing with 10 mL 1 mol/L acetic acid.

Assays contained 0.1 mL of sample or the PLP(1-34)amide standard in a total volume of 0.4 mL containing assay diluent (0.1% bovine serum albumin, 5% aprotinin [Mobay Corporation, New York, New York], 0.05 mol/L sodium barbital, 0.05 mol/L sodium acetate, pH 8.6) and a 1:400 000 dilution of a rabbit antiserum to PLP(1-34) (Peninsula Laboratories, Belmont, California). After overnight incubation at 4 °C, 4000 cpm ^{125}I-PLP(1-34)amide was added in 0.1 mL, and the incubation was continued overnight. Bound and free fractions were then separated using dextran-coated charcoal as previously described (18), except that the final albumin concentration was 0.2%. Both fractions were then assessed for ^{125}I-radioactivity. Each sample was assayed at two or more dilutions, and a control tube without antiserum was included for each dilution to correct for nonspecific binding of ^{125}I-PLP(1-34)amide according to the following formula: bound/free (B) = cpm in supernatant − [control × (cpm in supernatant + cpm in pellet)]/cpm in pellet, where "control" represents the fraction of counts in the supernatant of control tubes (19). Data are expressed as percentage B/B_0 where B_0 is the bound/free in tubes with no added sample or standard. In this assay, controls ranged from 0.05 to 0.08 in the absence of serum extracts, and from 0.05 to 0.15 in tubes containing serum extracts. The B_0 ranged from 0.3 to 0.4. Final immunoassay results are presented as pmol equivalents of PLP(1-34)amide/L, denoted as pmol eq/L.

The limit of detection, defined as the concentration producing a $B/B_0 = 80\%$, was 1.0 to 1.5 fmol PLP(1-34)amide/tube (Figure 1) giving a detection limit of 1.7 pmol eq/L (7 pg eq/mL) in 3 mL of serum. The calculated detection limit varied with the volume of sample extracted. For large-volume (30 mL) normal serum pools, the detection limit for iPLP was 0.2 pmol/L. For the purpose of data analysis, serum samples with undetectable iPLP were assigned values equal to their detection limit. The intra-assay and inter-assay coefficients of variation were 7.5% and 19%, respectively, based on the measurement in ten assays of iPLP in a sample of conditioned culture medium from human renal carcinoma cells (9). When a sample of this conditioned medium was added to normal serum to give 12 pmol eq/L PLP, 58% ± 4% of iPLP was recovered in the immunoextracts (Figure 1). On the basis of assay of this immunoextracted sample, the intra-assay coefficient of variation was 22%. The iPLP levels shown herein were not corrected for recovery.

To compare the detection of different forms of the hormone by the radioimmunoassay, recombinant PLP(1-141) (Genentech Inc., South San Francisco, California) was assayed. Recombinant PLP(1-141) was expressed in *Escherichia coli* (20); the preparation was homogeneous by criteria including amino acid content and partial amino acid sequence analysis, and had an intact carboxyl terminus by lysyl endopeptidase digestion followed by molecular ion mass spectrometry. Seventy to eighty percent of ^{125}I-PLP(1-141) (iodinated using the procedure outlined for PLP[1-34]-amide) added to normal serum was recovered in immunoextracts. The PLP(1-141) and the PLP(1-34)amide produced parallel immunodilution curves, but PLP(1-141) was fourfold less potent on a molar basis (data not shown). The radioimmunoassay did not detect human parathyroid hormone(1-34) or human parathyroid hormone(1-84) at concentrations as high as 2500 fmol/tube.

Other Assays

Intact parathyroid hormone was measured by an immunoradiometric assay (21) (Allegro PTH kit, Nichols Institute, San Juan Capistrano, California) with a normal range of 1.3 to 6.8 pmol/L. Serum creatinine, phosphate, and total calcium levels were measured by standard automated methods.

Patients

Patients with hypercalcemia were identified by review of laboratory values at the participating hospitals in San Francisco, California; Danville, Pennsylvania; and Hong Kong. Serum samples were obtained on fasting patients with confirmed hypercalcemia (serum calcium ≥ 2.65 mmol/L on at least two occasions) who had a diagnosis of malignancy. A suitable sample of serum could not be obtained from some patients and they were therefore excluded from the study. Two patients with malignancy whose hypercalcemia

was shown to be secondary to other factors (lithium therapy in one patient and vitamin D toxicity in the other) and one patient with myeloma and hypercalcemia who had an increased parathyroid hormone level were excluded from the analysis. The presence of bone metastases was ascertained by bone scan. Patients with a diagnosis of malignancy and no history of hypercalcemia (cancer controls) were drawn from the same hospital populations. Serum samples were obtained preoperatively from patients hospitalized for surgical treatment of hyperparathyroidism. Normal subjects were laboratory and hospital workers. Informed consent was obtained from study patients under protocols approved by the Human Research Committees at the participating institutions.

Statistical Analysis

Statistical analyses were done using the Student *t*-test for unpaired comparisons. The Bonferroni adjustment was made where appropriate. All data are expressed as the mean ± SE.

Results

Serum immunoreactive PLP (iPLP) in normal subjects ranged from less than 1 to 2 pmol eq/L, with 68% of normal subjects displaying undetectable levels (Figure 2). When the value of the limit of detection was assigned to samples with undetectable PLP, the mean level in normal subjects was 1.45 ± 0.13 pmol eq/L. To study further the circulating level of PLP in normal individuals, we did the following experiment. Serum obtained from normal volunteers was pooled. Pools of 30-mL serum were immunoextracted with 350 µL of antibody-Sepharose, concentrated 30- to 40-fold, and assayed in the PLP radioimmunoassay. In four such pools (totaling 12 subjects) the mean level of iPLP was 0.86 ± 0.17 pmol eq/L. The displacement of ^{125}I-PLP(1-34) binding by dilutions of these immunoextracts paralleled the standard curve (Figure 1).

We studied 16 patients with surgically proven hyperparathyroidism. The serum calcium was 2.98 ± 0.13 mmol/L and phosphate was 0.93 ± 0.10 mmol/L. In 15 patients (10 with adenomas, 3 with secondary hyperparathyroidism and chronic renal failure, and 2 with parathyroid carcinomas) serum levels of iPLP were less than 2.5 pmol eq/L. One patient with multiple endocrine neoplasia type I and parathyroid hyperplasia had a serum iPLP level of 3 pmol eq/L.

In 23 patients with cancer without a history of hypercalcemia, serum iPLP was less than 2.5 pmol/L in 20 (Figure 2). The serum levels of calcium and phosphate were 2.20 ± 0.04 mmol/L and 1.20 ± 0.03 mmol/L, respectively. The serum creatinine level was 90 ± 10 µmol/L. The distribution of tumor types in this group is given in the legend to Figure 2. Sixteen patients (70%) had metastatic disease (9 with skeletal metastases and 7 with metastases to other sites) whereas 5 patients had no evidence of metastatic disease. The metastatic status was not known in 2 patients. Elevated serum iPLP in the absence of hypercalcemia was seen in 1 patient with squamous carcinoma of the lung, 1 with melanoma, and 1 with ovarian carcinoma. All 3 patients died from their disease within 2 to 3 months of study without developing hypercalcemia.

We studied 65 patients with malignancy-associated hypercalcemia. These patients had mean serum calcium and phosphate levels of 3.20 ± 0.05 mmol/L and 1.15 ± 0.04 mmol/L, respectively, and creatinine levels of 140 ± 10 µmol/L ($P < 0.02$ compared with normocalcemic patients with cancer). Table 1 shows the tumor types in this series. Measurements of intact parathyroid hormone were available in 46 patients; levels were below the detection limit of 1.3 pmol/L in 42 patients; the remaining 4 patients had measurable but normal levels of 2.1 pmol/L or less. Seven patients had a normal parathyroid hormone level when other assays were used.

Of the 65 patients with malignancy-associated hypercalcemia, 36 (55%) had serum iPLP levels that exceeded 2.5 pmol eq/L (the upper limit of normal). Mean iPLP in this group exceeded mean iPLP in normal persons (6.22 ± 0.99 pmol eq/L compared with 1.45 ± 0.12, $P < 0.001$) and in normocalcemic patients with cancer (1.72 ± 0.30, $P < 0.001$). Increased iPLP levels were commonest in patients with

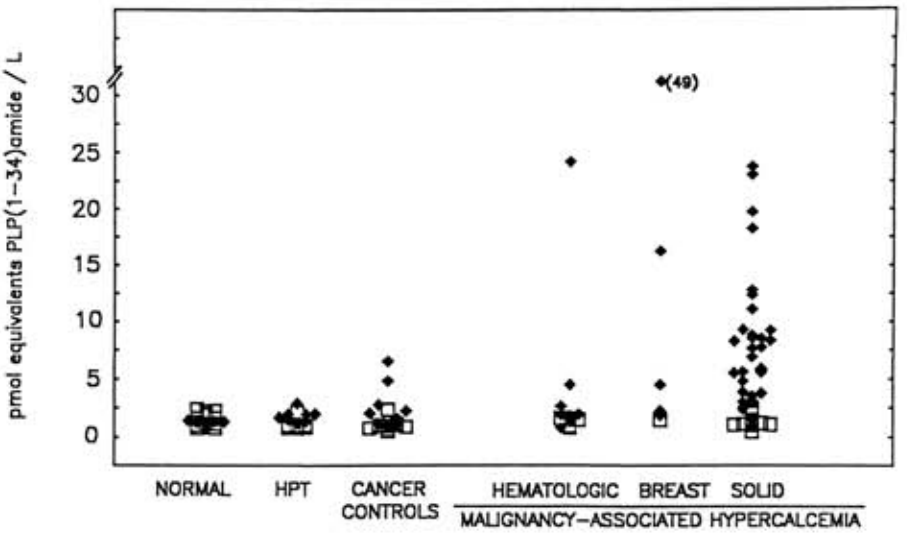

Figure 2. Serum iPLP levels in 17 normal subjects and patients with hyperparathyroidism (HPT) ($n = 16$) or malignancy ($n = 88$). Control patients with cancer ($n = 23$) are normocalcemic patients with malignancy. Malignancy-associated hypercalcemia includes patients with hematologic malignancy (myeloma, lymphoma, and leukemia, [$n = 15$]), breast cancer ($n = 6$), and solid tumors other than breast cancer ($n = 44$). Samples with an undetectable level of iPLP are indicated by open squares and plotted at the iPLP value corresponding to the calculated detection limit. Control patients with cancer included 9 with squamous carcinomas, 6 with adenocarcinomas, 3 with lymphomas, 1 with myeloma, and 5 with other malignancies. HPT = hyperparathyroidism.

Table 1. *Levels of PLP and Tumor Types in 65 Patients with Malignancy-Associated Hypercalcemia*

Tumor Type	Patients	PLP Level	
		> 2.5	≤ 2.5
	n(%)	*pmol eq/L*	
Squamous	20(31)		
Lung		4	1
Head and neck		8	1
Other		5	1
Adenocarcinoma	19(29)		
Lung		4	3
Kidney		3	2
Other		4	3
Transitional cell	3(5)	1	2
Anaplastic	2(3)	1	1
Breast carcinoma	6(9)	3	3
Hematologic malignancy	14(22)		
Multiple myeloma		3	6
Lymphoma		0	4
Leukemia		0	1
Small cell lung carcinoma	1(2)	0	1
Total	65(100)	36*	29†

* Comprised 55% of total.
† Comprised 45% of total.

solid tumors. Of the 42 hypercalcemic patients with solid tumors other than breast or prostate carcinoma, 30 (71%) had elevated iPLP levels (mean 6.61 ± 0.91 pmol eq/L), including 17 of 20 with squamous carcinoma and 3 of 5 with renal carcinoma.

Among the 30 patients with solid tumors and elevated iPLP levels, 17 were negative and 9 were positive for bone metastasis. This information was unavailable in 4 patients. Of 12 patients with solid tumors and no increase in iPLP, 7 had evidence of bone metastases, whereas 3 of 12 had no osseous metastases (1 patient each with squamous and large cell carcinoma of the lung and 1 patient with a renal cell carcinoma); metastatic status was unknown in 2. Thus 17 of 20 (85%) patients with solid tumors who met the classical definition of humoral hypercalcemia (hypercalcemia in the absence of bone metastases) had elevated iPLP levels.

Of the 29 hypercalcemic patients without elevated iPLP levels, 17 (59%) had tumor types previously associated with local osteolytic hypercalcemia (prostate or breast carcinoma or a hematologic malignancy). However, 6 of 23 patients with prostate, breast, or hematologic malignancy (26%) had skeletal involvement and elevated serum iPLP levels (3 with breast carcinoma and 3 with myeloma). All 23 patients had evidence of malignant disease in bone.

In all patients with malignancy, serum iPLP was positively correlated with serum calcium ($r = 0.34$, $P < 0.01$) and was negatively correlated with serum phosphate ($r = -0.30$, $P < 0.01$). In the subgroup of patients with solid tumors, iPLP was correlated with serum calcium ($r = 0.43$, $P < 0.01$) and was negatively correlated with serum phosphate ($r = -0.26$, $P < 0.05$). There was no significant correlation between serum levels of iPLP and creatinine in patients with malignancy-associated hypercalcemia.

Discussion

The existence of a humoral parathyroid hormone-like substance in malignancy-associated hypercalcemia was previously suggested by the finding of increased nephrogenous cAMP excretion (2, 3) and in a few patients by the detection of parathyroid hormone-like activity by cytochemical bioassay of serum (4). Our results establish that PLP is a humoral factor that is most prevalent in patients with solid tumors–the category previously associated with expression of PLP (6-11)–and with increased nephrogenous cAMP (2, 3). The finding that serum iPLP is increased in 71% of hypercalcemic patients with solid tumors can be compared with previous results that 85% of patients in this category had elevated nephrogenous cAMP (22). Several lines of evidence suggest that humoral secretion of PLP may be the primary etiologic basis of hypercalcemia in many patients with solid tumors. In our study, 17 of 30 patients with solid tumors and increased iPLP levels had humoral hypercalcemia according to the classical definition of hypercalcemia without evidence of bone metastases. The serum levels of iPLP found in these hypercalcemic patients are comparable to the levels of intact, biologically active parathyroid hormone in primary hyperparathyroidism (21, 23). Such concentrations of PLP may be sufficient to produce hypercalcemia, given the hypercalcemic potencies in vivo of PLP and parathyroid hormone (16). We found a significant correlation between serum iPLP and serum calcium in patients with solid tumors. The correlation was relatively weak, probably reflecting influences of fluid intake, renal function, nutritional status, or immobilization on the serum calcium. Blockade of the action of PLP by infusion of neutralizing antibodies has recently been shown to reverse humoral hypercalcemia in nude mice bearing human tumors (24), suggesting that secretion of PLP is the primary cause of hypercalcemia in this model.

Increased serum iPLP was also seen in 26% of patients with breast carcinoma, lymphoma, or multiple myeloma. Nearly all hypercalcemic patients with these tumor types have lytic bone lesions, suggesting that local osteolytic factors may predominate in the etiology of hypercalcemia. However, previous studies (22) involving these tumors have shown high nephrogenous cAMP levels in 39% of hypercalcemic patients. In fact, one of the tumors from which PLP was first purified was a breast carcinoma (8). Although it is not possible to distinguish the contributions of local and systemic factors to hypercalcemia, elevated levels of nephrogenous cAMP and iPLP are consistent with the interpretation that in some patients humoral hypercalcemia may co-exist with extensive bone metastases.

Each tumor group had hypercalcemic patients with normal serum levels of iPLP. Several possibilities could account for this apparent heterogeneity. Some patients had bone metastases, which could cause hypercalcemia by local osteolysis, a mechanism that may involve the local production of any of several bone-re-

sorbing factors. (Local elaboration of PLP itself by tumor deposits in bone is one mechanism that could cause osteolytic hypercalcemia without humoral secretion.) It is also possible that more sensitive assays would permit further discrimination between normal and increased iPLP levels. Further, iPLP determinations in the present radioimmunoassay may underestimate the serum concentration of circulating PLP in some patients. Thus, we found that intact PLP (recombinant PLP[1-141]) is approximately fourfold less immunoreactive than PLP(1-34)amide standard. The circulating forms of PLP in malignancy may be heterogeneous, and it is possible that the predominant form is one that, like intact PLP, would be underestimated by the radioimmunoassay. Finally, it is likely that hypercalcemic factors (some of which may be humoral) other than PLP are etiologically implicated in some patients with malignancy. Available evidence (25) favors a role for lymphokines such as tumor necrosis factor-β, 1,25 dihydroxyvitamin D_3, or prostaglandins in some patient groups. Determining the forms of PLP in serum, correlating serum iPLP and nephrogenous cAMP levels, and further understanding of the roles of other factors will aid in distinguishing among these possibilities.

The normal levels of iPLP in patients with parathyroid adenomas demonstrate that increases in serum iPLP levels in malignancy are not the consequence of hypercalcemia. Previous studies (26) have indicated that the PLP gene is commonly expressed in parathyroid adenomas, but elevated circulating iPLP levels in such patients are evidently rare. It should be noted, however, that we did not assess the expression of PLP in parathyroid adenomas in our patients.

The specificity of increased iPLP for malignancy-associated hypercalcemia suggests that the radioimmunoassay of PLP will be useful in the differential diagnosis of hypercalcemia, where the intercurrence of malignancy and hyperparathyroidism poses a relatively common dilemma. The occurrence of increased serum PLP levels in patients with hypercalcemia and nonsolid malignancies also suggests that measurement of iPLP could be of value in the future for selecting patients for specific therapeutic interventions aimed at reducing excessive PLP or blocking its effects.

The serum from some normal subjects also had detectable levels of PLP. This observation lends support to the possibility that PLP could have a systemic role in normal physiology; PLP is expressed in normal skin and dermal keratinocytes (26, 27) and in lactating mammary tissue (28). The normal source, controlling factors, and physiologic targets for circulating PLP, however, are all unknown. The ability to measure iPLP should be of use in unraveling the physiologic roles of this new hormone.

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