Overproduction and Secretion of a Novel Amino-Terminal Form of Parathyroid Hormone from a Severe Type of Parathyroid Hyperplasia in Uremia

Toshio Arakawa,*† Pierre D’Amour,‡ Louise Rousseau,‡ Jean-Hugues Brossard,‡ Makoto Sakai,§ Hiroomi Kasumoto,§ Naoya Igaki,§ Takeo Goto,§ Tom Cantor,‖ and Masafumi Fukagawa*

*Division of Nephrology & Dialysis Center, Kobe University School of Medicine, Kobe, Japan; †Arakawa Renal Clinic, Takasago, Japan; ‡Centre de recherche, Centre hospitalier de l’Université de Montréal, Hôpital Saint-Luc, and Département de médecine, Université de Montréal, Montréal, Québec, Canada; §Department of Internal Medicine, Takasago Municipal Hospital, Takasago, Japan; and ‖Scantibodies Laboratory Inc., Santee, California

Measurement of bioactive parathyroid hormone (PTH) is essential for optimal management of bone abnormalities in dialysis patients. This can be accomplished by PTH measurements using third-generation PTH assays, which detect more or less of the first six amino acids of the PTH structure. Such assays do not detect non-(1-84) PTH fragments, such as human PTH (7-84), which are recognized by the second-generation PTH assays that use a detection antibody that recognizes an epitope within the 13-34 region of the PTH structure. Therefore, third-generation PTH results are expected to be lower than those that are obtained with second-generation PTH assays. Rare exceptions to this rule have been reported for patients with severe primary hyperparathyroidism or parathyroid cancer. Sera and gland extracts were analyzed from a dialysis patient with high bone turnover disease and with surprising higher PTH levels by a third-generation assay than by a second-generation assay. This finding normalized after the surgical removal of an enlarged gland with a single nodule, an advanced type of nodular hyperplasia. HPLC fractionation of sera and gland extracts revealed the overproduction and secretion of a PTH molecule with an intact amino-terminus structure distinct from (1-84) PTH. This form of PTH was readily detectable by third-generation PTH assays but was poorly reactive in second-generation PTH assays. Therefore, parathyroid glands with advanced uremic nodular hyperplasia may overproduce and secrete a novel, biologically active form of PTH with an intact 1-6 region but a presumably modified 12-18 region required for the detection in second-generation PTH assays.


Measurement of bioactive parathyroid hormone (PTH) levels still provides one of the most useful pieces of information for the optimal management of bone abnormalities in dialysis patients (1,2). For this purpose, the second generation of PTH assays has been most widely used (3,4) until it was demonstrated also to detect large carboxyl-terminal (C)-PTH fragments with a partially preserved amino terminal (N) structure, also called non- (1-84) PTH, or N-truncated PTH fragments (N-PTH) (5,6). The prototype of these fragments, (7-84) PTH, reacts in these assays because of the recognition site of amino-terminal detection antibodies in the 13-34 region (7). Furthermore, low clearance from the kidneys may result in increased serum levels of such fragments in chronic kidney disease (5,8). Therefore, a third generation of PTH assays, with greater specificity for more or less of the first six amino acids of the PTH structure, has been developed (9–15). PTH levels that are detected by this third generation of PTH assays are expected to be lower than those that are obtained by the second generation of PTH assays because they do not detect non-(1-84) PTH fragments (10). Exceptions to this rule have been reported in patients with severe primary hyperparathyroidism or with parathyroid carcinoma (16–18).

In dialysis patients, the third-generation PTH/second-generation PTH ratio has been reported to be approximately 60% (10,19–21). This ratio can be reduced in severe secondary hyperparathyroidism by therapy with intravenous vitamin D analogues (22,23) but has not been modified by calcimimetic therapy (24). In a limited number of dialysis patients with severe secondary hyperparathyroidism, the ratio can be elevated to >100% (25) for reasons that remain unclear.

To elucidate the pathogenesis of such an elevated third-generation PTH/second-generation PTH ratio that was seen in a dialysis patient, we analyzed the patient’s sera and gland extracts by HPLC fractionation and found the overproduction and secretion of an additional peak of N-PTH that was distinct from (1-84) PTH. This peak was detectable mainly by a third-
generation PTH assay and poorly reactive in a second-generation PTH assay. Our data suggest a significant role of this new PTH molecular form that was produced in this patient with advanced parathyroid disease as a result of chronic kidney disease.

Materials and Methods
The patient was a 61-yr-old woman who had been treated by maintenance hemodialysis since 1972. The cause of end-stage renal failure was chronic glomerulonephritis. In 1982, she developed severe secondary hyperparathyroidism, and 3.5 parathyroid glands were surgically removed. In 1999, despite the maintenance of intact PTH (l-PTH) levels between 200 and 300 pg/ml with serum calcium levels between 9.8 and 10.3 mg/dl (normal range for the institution 8.5 to 10.5 mg/dl) by intravenous injection of the 1,25-dihydroxyvitamin D₃ analog maxacalcitol therapy (5 to 10 μg at the end of each dialysis session, three times per week), serum alkaline phosphatase activity increased progressively up to 730 IU/L (normal range 100 to 325 IU/L). Evidence of previous bone fracture by x-ray and a marked decrease of bone mineral density also were noted.

When we measured circulating PTH levels by two different assays, the value that was detected by the third-generation PTH assay (498 pg/ml) paradoxically was more than twice that detected by the second-generation PTH assay (200 pg/ml). Although no enlargement of the residual parathyroid gland was seen, an ectopic parathyroid gland that measured 2 cm in diameter was detected in the right upper mediastinum by technetium-99m sestamibi scintigraphy, for which a surgical parathyroidectomy was performed in February 2004.

Blood Samples and Examination of Surgically Removed Gland
Blood samples were taken monthly from the patient at the start of the dialysis sessions. The collected blood was centrifuged, and the separated sera were aliquotted before storage at −80°C until further analysis.

Half of the surgically removed parathyroid glands was used for routine pathologic analysis, and the other half was frozen immediately and stored at −80°C until extraction. The removed gland was homogenized in ice-cold PBS-EDTA buffer in a glass homogenizer for PTH extraction. The supernatant was prepared by centrifugation of the homogenized tissue at 2 to 8°C, diluted at 1:1000, and used for HPLC analysis.

The study was designed according to the Helsinki Declaration and was approved by the institutional ethics committee. Informed consent was obtained from the patient before the study.

PTH Assays
Circulating PTH levels, present in serum and HPLC fractions, were measured by second- and third-generation PTH assays (Santanibodies Laboratory Inc., Santee, CA) according to the instructions provided by the manufacturer. The whole-PTH assay, also called cyclase-activating PTH assay, is a third-generation PTH assay that reacts with synthetic human PTH (hPTH) (1-84) but not with hPTH (7-84) or with non-(1-84) PTH fragments that are present in the circulation (7,9,10). It has an early epitope in region 1-6, where position 1 and, to a much lesser extent, position 2 are implicated (7,9,10). Its revealing antibody was raised against hPTH (1-84) in goats and purified by affinity chromatography against hPTH (1-6) (26). It recognizes a major peak of immunoreactivity co-eluting with hPTH (1-84), after separation of circulating PTH by HPLC, and also a minor peak corresponding to a new N-PTH. The latter normally represents <7% of whole-PTH immunoreactivity in normal individuals but up to 15% in dialysis patients (16). Third-generation PTH assays do not detect non-(1-84) PTH fragments that are detected by second-generation PTH assays (7,9,10). The total-PTH assay behaves like a typical second-generation PTH assay and reacts equally well with synthetic hPTH (1-84) and hPTH (7-84). Its epitope has been approximated to region 12-18 of the hPTH structure by saturation analysis of its revealing antibody (7). This is similar to the epitope of the original intact second-generation PTH assay developed by Nichols Institute (7). After HPLC separation of circulating PTH, it detects a peak of immunoreactivity co-eluting with hPTH (1-84) and a second peak of C-PTH fragments with a partially-preserved N-structure, also called non-(1-84) PTH, or N-truncated C-fragments. This assay reacts poorly with N-PTH that is detected by third-generation PTH assays, possibly because of a posttranslational modification in region 12-18 of this new PTH molecular form (7,16).

HPLC Analysis of Sera and Gland Extracts
Sera and gland extracts were fractionated by HPLC, as described previously (7,16,27). The gland extracts and all presurgery stocked sera (10 samples from November 5, 2002, to January 6, 2004) were applied to Sep-Pak Plus C-18 cartridges (Waters Chromatography Division of Millipore Canada, Quebec, Canada) to extract circulating PTH molecular forms. Samples were eluted from the cartridges with 3 ml of 800 ml/L acetonitrile in 1 g/L trifluoroacetic acid (TFA). Acetonitrile was evaporated from the elute with nitrogen; the residual volume was freeze-dried and reconstituted in 2 ml of 1 g/L TFA for HPLC analysis. Each 2-ml sample was loaded on a C18 µBondapak analytical column (3.9 × 300 mm; Waters Chromatography Division of Millipore Canada) and eluted with a noncontinuous linear gradient of acetonitrile (19 to 35%) in 1 g/L TFA delivered at 1 ml/min by an Agilent 1100 Series HPLC system (Wilmington, DE) over 60 min. The 1-ml fractions were evaporated, freeze-dried, and reconstituted to 1 ml with 7 g/L BSA in water. PTH was measured in each fraction by both PTH assays. Recovery of immunoreactivity during these procedures was >75% for both assays, comparing the PTH content of all HPLC fractions with the amount initially loaded on Sep-Pak Plus C18 cartridges. For ensuring that PTH degradation did not occur during the procedures, hPTH (1-84) was added to hypoparathyroidism serum and internally labeled PTH was added to cultured parathyroid cells from the patient with typical secondary hyperparathyroidism. When the serum and parathyroid cell supernatants were fractionated by HPLC, a single peak of immunoreactivity or of radioactivity, corresponding to the elution position of hPTH (1-84), was identified (28).

Results
Circulating PTH Levels before and after Surgical Parathyroidectomy
As shown in Figure 1, the values of the third-generation PTH assay were higher than those of the second-generation PTH assay before surgical parathyroidectomy. After the operation, the values became lower than those of the second-generation PTH assay, as should be the case in uremia. A similar time course was confirmed with the third-generation and second-generation assays from another company (Nichols Institute Diagnostics, San Juan Capistrano, CA). Before surgery, the second-generation PTH value was 270 pg/ml (28.6 pmol/L) and the third-generation PTH value was 648 pg/ml (68.8 pmol/L), whereas after surgery, the second-generation PTH value was 28 pg/ml (3 pmol/L) and the third-generation PTH value was 19 pg/ml (2.0 pmol/L).
The excised glands presented hyperplasia of the parathyroid cells without the normal rim; however, there was no evidence of malignancy, such as multiple mitosis, poor differentiation, or invasion of blood vessels and surrounding tissues. Therefore, the gland was diagnosed as a single nodule, the most advanced type of parathyroid hyperplasia in uremia (29,30) (data not shown).

**HPLC Analysis of Sera and Gland Extracts**

Figure 2 illustrates the sera PTH HPLC profile of our patient before surgery compared with the serum profile of a patient with typical secondary hyperparathyroidism. In both cases, three regions of interest are outlined. They correspond to hPTH (1-84), region 40-44; N-PTH, region 34-38; and non-(1-84) PTH, region 22-32. In the control patient, the third-generation PTH assay typically detected a major hPTH (1-84) peak, a small N-PTH peak, and minute amounts of immunoreactivity in the non-(1-84) PTH region. In our patient, the same assay detected a minor peak of hPTH (1-84), a large peak of N-PTH, representing more than three times the amount of the former, and also other N-forms of PTH present in the non-(1-84) PTH region in large amounts. Again, in the control patient, the second-generation PTH assay typically detected a major peak of hPTH (1-84), another major peak of non-(1-84) PTH, and little N-PTH. In our patient, the same assay detected hPTH (1-84), non-(1-84) PTH fragments in larger quantity, and a much smaller amount of N-PTH than the third-generation PTH assay.

Finally, we compared the HPLC profile of parathyroid cell supernatants from a control patient with that of parathyroid gland extracts from the reported patient (Figure 3). The peak of N-PTH detected with the third-generation PTH assay in our patient was smaller in the extracts than that observed in sera and much larger than the N-PTH peak secreted by the parathyroid cells of the patient with uremic secondary hyperparathyroidism. The proportion of non-(1-84) PTH fragments that were detected by the second-generation PTH assay also was higher in the gland extracts than in sera.

**Discussion**

Third-generation PTH assays predominantly detect (1-84) PTH in the circulation (7,10,16,28). However, as revealed recently (7,16,28), they also react with a small amount of a new N-form of PTH that is distinct from (1-84) PTH after HPLC separation of circulating PTH with better performance acetonitrile gradients. Second-generation PTH assays detect both (1-84) PTH and non-(1-84) PTH fragments (5,6,8). Because non-(1-84) PTH fragments represent a much larger fraction of circulating PTH than N-PTH, second-generation PTH values are expected to be higher than third-generation PTH values (7,16,28). This is of particular relevance in patients with renal failure, in whom non-(1-84) PTH fragments may represent 50% of I-PTH (5,6,8,27), as a result of their reduced clearance in the absence of functional kidneys. Therefore, it was suggested that
in our patient (A).

The main finding was overproduction of N-PTH and region 1-2 of the PTH structure, and the latter has a 12-18 PTH; solid line) PTH assays. The former has an early epitope in generation (W-PTH; dotted line) and second-generation (T-PTH; solid line) PTH assays. The former has an early epitope in region 1-2 of the PTH structure, and the latter has a 12-18 epitope. The main finding was overproduction of N-PTH and of other amino-terminal forms of PTH that migrate in the non-(1-84) PTH region detected by third-generation PTH assay in our patient (A).

Figure 3. PTH HPLC profile of patient gland extracts (A) compared with the profile of PTH that was secreted by parathyroid cells that were isolated from the parathyroid glands of a patient with typical secondary hyperparathyroidism. Again, the PTH that was present in HPLC fractions was revealed by third-generation (W-PTH; dotted line) and second-generation (T-PTH; solid line) PTH assays. The former has an early epitope in region 1-2 of the PTH structure, and the latter has a 12-18 epitope. The main finding was overproduction of N-PTH and of other amino-terminal forms of PTH that migrate in the non-(1-84) PTH region detected by third-generation PTH assay in our patient (A).

PTH values that are detected by these new assays more accurately reflect the biologic activity of circulating PTH than values that are obtained by second-generation PTH assays.

We recently reported the case of a chronic dialysis patient in whom third-generation PTH values paradoxically were much higher than second-generation PTH values (25). In that patient, the reversed third-generation PTH/second-generation PTH ratio was normalized by surgical parathyroidectomy, indicating that the enlarged parathyroid glands or a part of these glands may have been responsible for this phenomenon. Similar reversed third-generation PTH/second-generation PTH ratios were reported previously only in three patients with severe primary hyperparathyroidism and three patients with parathyroid cancer (16–18). In these patients, a high concentration of an N-form of PTH, distinct from (1-84) PTH, as in other reported cases of primary hyperparathyroidism (16–18). Other N-forms of PTH that migrate in the region of non-(1-84) PTH fragments also were identified in large quantities in this patient. These molecular forms of PTH usually are seen in minute amounts and only in patients with the highest PTH levels (28). They represent the largest amount found in a patient to date. The amount of hPTH (1-84) and non-(1-84) PTH fragments relative to N-PTH was larger in the parathyroid cell extracts than in the peripheral blood in our patient. A possible explanation for this phenomenon could be reduced peripheral clearance of N-PTH in this patient compared with PTH (1-84) or non-(1-84) PTH fragments. Further studies will be required to elucidate the finding. The precise reason for the overexpression of N-PTH molecular forms in this and other rare patients remains unknown but may represent a possible phenomenon or expression of a different PTH molecule by different areas of monoclonal growth. Other patients with primary hyperparathyroidism (16–18) had large, poorly differentiated parathyroid tumors and severe hypercalcemia, but this was not the case in our patient with normocalcemia and well-differentiated cells forming nodular hyperplasia. A specific relationship between the overproduction of N-PTH and genetic abnormalities that are present in some parathyroid tumors and carcinoma has yet to be elucidated (31).

The processing and action of several PTH molecules, including non-(1-84) PTH fragments and C-PTH fragments, have been reviewed extensively recently (32). The major non-(1-84) PTH fragment, (7-84) PTH (28), has been demonstrated to antagonize the calcemic and bone-resorbing effects of (1-84) PTH (33–37). The clinical applications of this information to uremic patients (15,38,39) and the regulation of these fragments (40) have generated significant debate. The new N-form of PTH recognized in this study was detectable with the antibody used in the third-generation PTH assay but only poorly by the antibody used in the second-generation PTH assay. As in other cases with primary hyperparathyroidism (16–18), it indicated the structural integrity of the 1-2 region detected by the third-generation PTH assay but presumably a modified 12-18 region, corresponding to the epitope of the N-terminal antibody of the second-generation PTH assay (7). This notion is supported further by the finding that a second-generation PTH assay with a more distal 26-32 epitope reacted with the N-PTH HPLC peak as well as the third-generation PTH assay did (7). The mode of posttranslational modification of N-PTH remains unclear, although oxidation has been ruled out (16).

N-PTH has been demonstrated mainly in the sera of patients with large, poorly differentiated parathyroid tumors (16–18). In this study and in our previous case (25) of dialysis patients with reversed PTH ratio, the pathologic diagnosis of an excised
gland was a single nodule, the most advanced form of nodular hyperplasia in dialysis patients (29,30). It has been suggested that patients with nodular hyperplasia usually are beyond the control of medical therapy (41,42). In dialysis patients, the complication of sporadic primary hyperparathyroidism may be rare but remains a possibility. There was no evidence of multiple mitosis or poor differentiation suggestive of malignancy in this case. Furthermore, the absence of normal rims supports the diagnosis of hyperplasia rather than adenoma. Even by checking the monoclonality, it is still very hard to exclude a primary adenoma in our patient because each nodule that formed in parathyroid glands in uremia also has shown to be of monoclonal origin (43). Expression levels of parathyroid adenomatosis 1/cyclin D1 may be helpful as suggested by Tomi- naga et al. (44). After all, dialysis patients with either a single nodule or primary adenoma should be refractory to vitamin D therapy.

So far, much lower serum levels of N-PTH have been demonstrated in dialysis patients (16). Very high serum levels, as seen in this patient, could be a marker for the severity and controllability of hyperparathyroidism in these cases. Such a possibility needs to be examined in future clinical studies, especially in comparison with previously known markers, such as parathyroid size (42,45) and serum fibroblast growth factor 23 levels (46,47).

Another unique characteristic of this case was the high level of serum alkaline phosphatase activity, indicating high bone turnover, accompanied by progressive severe osteopenia and several episodes of fracture despite the control of second-generation PTH levels between 200 and 300 pg/ml, levels that suggested good PTH control according to Kidney Disease Outcomes Quality Initiative guidelines (48). Therefore, there is a strong probability that the new form of PTH, not detectable by second-generation PTH assay, has a significant biologic effect on bone turnover in our patient. Although high serum level of bone-specific alkaline phosphatase that was confirmed in a sample before the surgery (data not shown) further supports such a possibility, additional basic studies are required to demonstrate this possibility. It also is suggested that conventional second-generation PTH assays not only may overestimate the real activity of PTH on bone, as previously shown (49,50), but also occasionally may underestimate its activity, as in this case.

**Conclusion**

We have demonstrated that a possible new active N-form of PTH molecule was produced in and secreted from the most severe type of parathyroid hyperplasia in a dialysis patient. The clinical significance of this molecular form and the abnormal ratio of third-generation PTH/second-generation PTH need to be studied further in future investigations.

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