Homologous Radioimmunoassay for Human Carboxyl-terminal Parathyroid Hormone and its Clinical Evaluation

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Abstract

An homologous radioimmunoassay for human carboxyl-terminal parathyroid hormone (PTH) was developed using the antiserum raised against synthetic human PTH (46-84) and its clinical evaluation was attempted. Antisera were produced in guinea pigs by immunization with synthetic human PTH (46-84) conjugated with BSA. One of the antisera was used at a final concentration of 1:10,000 in the assay, and synthetic human PTH (53-84) for a standard and ^{125}I -Tyr⁴⁵ human PTH (46-84) for a tracer were used. A standard curve was obtained at concentrations from 0.001 to 10 ng per tube of human PTH (53-84). The displacement curves for human PTH (51-84), human PTH (46-84), Tyr⁴⁵ human PTH (46-84) and bovine PTH (1-84) were almost identical with the standard curve made by sythetic human PTH (53-84). This antiserum, however, did not recognize the human PTH (1-34) fragment at all. Using this assay system, circulating immunoreactive PTH was measured in normal subjects and patients with parathyroid disorders. Normal PTH values in serum ranged from 0.47 ng/ml to an undetectable level (undetectable in two out of 32 normal subjects) and serum PTH values in patients with primary or secondary hyperparathyroidism or idiopathic hypoparathyroidism were almost completely discriminated from normal values. These results suggest that this homologous radioimmunoassay is a carboxyl-terminal specific assay for human PTH and can be quite useful in clinical practice.

Most of the radioimmunoassays for parathyroid hormone (PTH) in man, with very few exception (DiBella *et al.*, 1978), have been performed by using antisera raised against bovine or porcine PTH (1-84) which recognize mainly the carboxyl-terminal region of the molecule. As shown in some reports, the radioimmunoassay for carboxyl-terminal PTH is more useful in the clinical evaluation of the parathyroid function than that for amino-terminal PTH (Arnaud *et al.*, 1974; Fischer *et al.*, 1974;

Tomita *et al.*, 1980).

In the carboxyl-terminal PTH (53-84), the human molecule differs from the bovine at three positions and the porcine at four positions (Keutmann *et al.*, 1978). These molecular differences might cause nonidentical immunoreactivity of the carboxyl-terminal regions in human and bovine or porcine. Therefore, the homologous radioimmunoassay for PTH using antisera raised against the carboxyl-terminal region of human PTH might be more useful in clinical studies than the heterologous assay using antibovine PTH antisera.

In the present paper, it is demonstrated that the homologous radioimmunoassay for PTH using anti-human carboxyl-terminal PTH antisera was developed and using this assay, circulating PTH was determined and compared with PTH measured by heterologous assay using anti-bovine PTH antisera.

Materials and Methods

Synthetic human carboxyl-terminal PTH and its analogues. Synthetic human carboxyl-terminal PTH (46-84), Cys⁴⁵ human PTH (46-84), Tyr⁴⁵ human PTH (46-84) and human PTH (51-84), as shown in Fig. 1, were made by Toyo Jozo Research Laboratories, Shizuoka, Japan and human PTH (53-84) was synthesized by Peptide Institute, Osaka, Japan. Synthetic Cys⁴⁵ human PTH (46-84) was used for making bovine serum albumin (BSA)-conjugated-human PTH (46-84) for immunization, Tyr⁴⁵ human PTH (46-84) for ¹²⁵I-labeling, human PTH (53-84) for a standard and human PTH (51-84), human PTH (46-84) and Tyr⁴⁵ human PTH (46-84) were used for determination of cross-reactivities to human PTH (53-84).

Production of antisera to human PTH (46-84). An antiserum was produced in a guinea pig by immunization with 100 µg or 300 µg of BSA-conjugated-human PTH (46-84), emulsified with complete Freund adjuvant at 2 week intervals and obtained after several months. The conjugation procedure for BSA and human PTH was performed using *n*-succinimidyl-3-(2-pyridyl-dithio) propionate (SPDP) (Phar-

macia Co., Uppsala, Sweden) as a coupling reagent, BSA (Sigma Chemical Co., St. Louis, U.S.A.) and Cys⁴⁵ human PTH (46-84), according to the manual of SPDP (Pharmacia Co., Uppsala, Sweden).

The homologous radioimmunoassay for human carboxyl-terminal PTH using the anti-human PTH (46-84) antiserum. Radioiodination of synthetic Tyr⁴⁵ human PTH (46-84) was performed by a modification of the Hunter and Greenwood chloramin-T procedure (Hunter and Greenwood, 1962). ¹²⁵I-Tyr⁴⁵ human PTH (46-84) had the specific activity of 150-200 mCi per mg Tyr⁴⁵ human PTH (46-84). Assays were done under nonequilibrium conditions at 4°C. Assay procedures were as follows: 0.1 ml of synthetic human PTH (53-84) standard or serum sample, 0.1 ml of anti-human PTH (46-84) antiserum (final dilution, 10,000-fold) and 0.2 ml of pH 7.5 phosphate buffer were incubated at 4°C for 2 days, followed by incubation with 0.1 ml of ¹²⁵I-Tyr⁴⁵ human PTH (46-84), which had 5,000-10,000 cpm, at 4°C for two additional days. Separation of bound and free hormone was done by a second antibody. After centrifugation and separation of precipitates (bound hormone), bound ¹²⁵I-PTH was counted with an automatic scintillation counter. Assay results were calculated by previously reported procedures (Konagaya *et al.*, 1979) and assay values for serum samples were read from the standard curve obtained by the same assay.

Heterologous radioimmunoassay for human carboxyl-terminal PTH using the anti-bovine PTH (1-84) antiserum. Serum carboxyl-terminal PTH (-C PTH) was also measured with the radioimmunoassay kit, Eiken Kit (Eiken Chemical Co., Tokyo, Japan) using anti-bovine PTH (1-84) antiserum raised in a guinea pig which recognizes the carboxyl-terminal region of PTH. Assay procedures were previously published (Konagaya *et al.*, 1979). The detection limit of C-PTH in serum measured with this kit was approximately 0.1 ng per ml.

Serum sample. Sera for determination of the basal PTH level were collected between 7:00 and 10:00 AM after an overnight fast from 32 normal subjects, 9 patients with primary hyperparathyroidism which was proved subsequently at surgery, 24 patients with chronic renal failure on hemodialysis and 6 patients with idiopathic hypoparathyroidism.

Fig. 1. The sequence of human, bovine and porcine PTH (46-84). Human PTH (46-84) differs from bovine or porcine PTH at five positions.

Results

Human PTH (46-84) antisera. Antisera were produced in guinea pigs and 5 different antisera to human PTH (46-84) were quantitated on the basis of their capacity to bind ¹²⁵I-Tyr⁴⁵human PTH (46-84). As shown

Fig. 2. Comparison of the titration of antisera raised against human PTH (46-84). These antisera have 20 to 50% specific binding at the final dilution of 1 : 10,000 in the absence of PTH (B₀).

in Fig. 2, the titration of these antisera was tested by dilution of antisera yielding 20–50% specific binding in the absence of PTH (B₀). All antisera were good enough for the assay and then the antiserum #14 was natively used in the assay at the final dilution of 1 : 10,000.

Standard curve. Fig. 3 shows a standard curve made by using synthetic human PTH (53–84) as a standard, the antiserum #14 (the final dilution of 1 : 10,000) and ¹²⁵I-Tyr⁴⁵human PTH (46–84) as a tracer. A fairly good displacement curve was obtained at the concentratinos from 0.001 to 10 ng per tube of synthetic human PTH (53–84).

The dilution curves for serum and a parathyroid adenoma homogenate from a

patient with primary hyperparathyroidism. As shown in Fig. 4, the dilution curves for serum and a parathyroid adenoma homogenate were superimposable with a standard curve for synthetic human PTH (53–84).

Immunologic characterization of the antiserum. The cross-reactivities between synthetic human PTH (53–84) and the other synthetic carboxyl-terminal fragments, synthetic human amino-terminal PTH (1–34) or bovine PTH (1–84) were examined. As shown in Fig. 5, the displacement curve for synthetic human PTH (51–84) was almost identical with the standard curve made by using synthetic human PTH (53–84) on the molar basis, and also the displacement curves for synthetic human PTH (46–84), Tyr⁴⁵human PTH-(46–84) and bovine PTH (1–84) were approximately equal to the standard curve. On the other hand, no cross-reactivity was observed between human PTH (53–84) and human PTH (1–34). These findings indicated that this antiserum has specific affinity in the carboxyl-terminal region of PTH but not in the amino-terminal region.

As shown in Table 1, the intraassay coefficients of variation (CV's) in measurement of serum PTH in a normal subject

Table 1. Intraassay and interassay variability in the homologous radioimmunoassay for human carboxyl-terminal PTH.

No.	Intraassay variability Serum PTH (ng/ml)			Interassay variability Serum PTH (ng/ml)		
	A	B	C	A	B	C
1	0.36	2.9	1.2	0.56	2.8	1.2
2	0.42	3.1	1.1	0.68	2.4	1.0
3	0.34	3.0	1.1	0.64	2.2	0.96
4	0.38	2.9	1.2	0.74	2.9	1.3
5	0.34	3.3	0.98	0.60	2.7	1.1
6	0.35	3.2	1.2			
7	0.43	3.4	1.0			
8	0.35	2.8	1.1			
9	0.41	3.5	1.2			
10	0.40	3.0	0.96			
mean	0.38	3.1	1.1	0.64	2.6	1.1
s.d.	0.035	0.23	0.097	0.070	0.29	0.14
CV %	9.1	7.5	8.7	10.8	11.2	12.6

Fig. 3. Standard curve. This was made by using synthetic human PTH (53-84) as a standard, the antiserum #14 (final dilution, 1:10,000) and ^{125}I -Tyr 45 human PTH (46-84) as a tracer.

Fig. 4. Dilution curve of serum and parathyroid homogenate. The dilution curves for serum and a parathyroid adenoma homogenate from a patient with primary hyperparathyroidism are parallel to the standard curve.

Fig. 5. Immunologic characterization of the antisera. The displacement curves for human PTH (51-84), human PTH (46-84), Tyr⁴⁵ human PTH (46-84) and bovine PTH (1-84) are almost identical with the standard curve for human PTH (53-84).

Fig. 6. Immunoreactive carboxyl-terminal PTH levels in sera of normal subjects and patients with parathyroid disorders.

and primary hyperparathyroid patients were 9.1, 7.5 and 8.7%, respectively, and the interassay CV's were 10.8, 11.2 and 12.6, respectively. The detection limit was defined as the quantity of added hormone required to decrease the B/B₀ ratio by 10%. These ranged from 0.08 to 0.15 ng/ml (mean $\pm$ s.e., 0.11 ± 0.01 ng/ml; $n=8$). In analytical recovery studies, recovery of PTH ranged from 93.9 to 118.2% (mean, 104.5%) (Table 2).

Serum parathyroid hormone in normal subjects and patients with parathyroid dis-

orders. Fig. 6 summarizes serum PTH levels measured by this homologous radioimmunoassay in normal subjects and patients with parathyroid disorders. Serum PTH levels in normal subjects ranged from 0.47 ng/ml to undetectable (undetectable in two out of 32 normal subjects) and the mean $\pm$ s.e. was 0.23 ± 0.01 ; the mean $\pm$ 2s.d. range was 0.39–0.07 ng/ml. Serum PTH was undetectable in all 6 patients with idiopathic hypoparathyroidism. In 9 patients with surgically proved primary hyperparathyroidism, serum PTH levels ranged from 4.17 to 0.45 ng/ml which were significantly elevated from normal values ($P < 0.005$) and were not overlapped with normal values (mean $\pm$ 2s.d.). Twenty-four patients with secondary hyperparathyroidism due to chronic renal failure had serum PTH levels ranging from 2.80 to 0.31 ng/ml which were also significantly elevated from normal values ($P < 0.005$) and only one of 24 patients (4%) had a value within the normal range.

Table 2. Analytical recovery of human PTH (53–84) added to hypoparathyroid serum.

Added (ng/ml)	Measured (ng/ml)	Recovery %
1.0	1.1	110.0
3.3	3.1	93.9
10.0	9.6	96.0
33.0	39.0	118.2

Fig. 7. Correlation between serum carboxyl-terminal PTH levels measured by the homologous and heterologous assay. They are significantly correlated ($P < 0.001$).

Serum PTH values measured in two assays by using the anti-human PTH (46–84) antiserum (the homologous assay) and the anti-bovine PTH (1–84) antiserum (the heterologous assay) simultaneously under the same condition were in a highly significant positive correlation ($r=0.93$, $P<0.001$) (Fig. 7).

Discussion

Since Berson *et al.* (1963) developed the first radioimmunoassay for PTH, to date many investigators have reported a number of radioimmunoassays for human PTH but most of the radioimmunoassays which have been used in many laboratories are heterologous assays using antisera raised against bovine or porcine PTH (1–84). It is recognized that the human C-PTH (53–84) is different from both bovine and porcine PTH (53–84) at three or four positions for each. Therefore, it is quite natural that the homologous carboxyl-terminal specific radioimmunoassay than the heterologous assay might be more desirable in measuring human PTH.

It was first demonstrated by Berson and Yalow (1968) that radioimmunoassay for PTH in human serum is rather difficult because of the immunochemical heterogeneity of circulating PTH. Subsequently, Arnaud *et al.* (1970) and Sherwood *et al.* (1970) reported that PTH extracted from parathyroid glands and circulating PTH are immunologically different, and Canterbury and Reiss (1972) reported the existence of multiple immunoreactive molecular forms of circulating PTH. However, it is well recognized that the carboxyl-terminal fragments are predominantly detected because of their slower turnover than that of the intact whole molecule or the amino-terminal fragments. In fact, circulating PTH measured by the amino-terminal specific assay in patients with primary or secondary

hyperparathyroidism often overlapped with normal values (Tomita *et al.*, 1980). For this reason, the radioimmunoassay for PTH using antiserum which recognizes mainly the carboxyl-terminal region of PTH may be more useful in clinical practice than the amino-terminal assay detecting the biologically active portion of PTH (Arnaud *et al.*, 1974; Fischer *et al.*, 1974; Tomita *et al.*, 1980).

As mentioned above, most of the radioimmunoassays for human carboxyl-terminal PTH are heterologous assays, and we tried to develop an homologous radioimmunoassay for it. It was demonstrated that our antisera produced in guinea pigs recognize specifically the carboxyl-terminal region of human PTH but not the amino-terminal region of PTH. The dilution curves of serum and a parathyroid adenoma homogenate from a patient with primary hyperparathyroidism were parallel to the synthetic human PTH (53–84) standard. Therefore, PTH levels measured in the assay system could be expressed as concentrations of human PTH (53–84).

Using this assay, we measured serum PTH levels in normal subjects and patients with parathyroid disorders and observed almost complete discrimination between concentrations of PTH in normal subjects and patients with primary or secondary hyperparathyroidism. This assay system also showed a good discrimination between normal subjects and patients with hypoparathyroidism which had undetectable values in serum, while the Eiken Kit which employed an heterologous radioimmunoassay system for carboxyl-terminal PTH using anti-bovine PTH (1–84) antiserum showed an overlap between these two groups (Konagaya *et al.*, 1979; Tomita *et al.*, 1980). These results indicate that the homologous carboxyl-terminal radioimmunoassay for human PTH we developed is quite useful in the diagnosis of parathyroid diseases.

It is still not clear whether the values

measured by this homologous carboxyl-terminal assay contain some small carboxyl-terminal fragments in serum. As shown in Fig. 7, the values measured by this assay were not in accord with the values measured by the heterologous assay, although they revealed a highly significant positive correlation. This may result from the different standards used in the assays or otherwise different antisera recognizing the fragments of the carboxyl-terminal molecule.

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