Radioimmunoassay for Human Calcitonin Employing Synthetic Calcitonin M: Its Clinical Application

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Synopsis

A sensitive and reliable radioimmunoassay for human calcitonin was described and applied to preliminary clinical studies.

¹²⁵I-labelled synthetic human calcitonin M was purified by gel filtration with Sephadex G-25 and G-100. A nonequilibrium incubation system was applied at the final volume of incubation mixture of 500 μ l, in which pooled plasma from normal subjects or hormone free serum was used as carrier protein at 20% incubation mixture. Dextran T 40 coated charcoal, resuspended in 1% bovine serum albumin buffer, was used for the separation of free from bound tracer. This showed the least nonspecific adsorption of tracer to charcoal. The assay was sensitive to 0.1 ng per milliliter of plasma. Recovery of synthetic human calcitonin added to plasma was found to be 101% (S.D., ± 8). Diluted plasma from a patient with medullary thyroid carcinoma showed a dilution curve parallel to standards. Basal calcitonin levels were less than 0.3 ng/ml in normal subjects. Relatively high values were found in patients with chronic renal failure and in patients with malignant tumors. Extremely elevated values were found in patients with medullary thyroid carcinoma. Provocative calcium infusion tests were also performed.

Calcitonin, a potent hypocalcemic, and hypophosphatemic peptide hormone, was first discovered by Copp *et al.* (1962) and later by Hirsch *et al.* (1963). Since then, extensive physiological and pharmacological studies of the hormone have been performed and the recent developments in the isolation, purification and synthesis of the hormone have led to the development of a sensitive and specific immunoassay for human calcitonin.

The present report describes the sensitive and reliable radioimmunoassay for human calcitonin and its application to preliminary clinical studies.

Materials and Methods

A synthetic preparation of human calcitonin, kindly supplied by Ciba Pharmaceuticals, was used for both radioiodination and as the standard. Antiserum to human calcitonin, also supplied by Ciba Pharmaceuticals, which was developed in a goat by repeated subcutaneous injection of a conjugate of synthetic human calcitonin M (Ciba) and complete Freund's adjuvant.

Radioiodination of human calcitonin M was accomplished by the method of Hunter and Greenwood (1962) to a specific radioactivity of 150–300 μ Ci/ μ g. After mixing 2 μ g of calcitonin, 2 mCi of ¹²⁵I and 10 μ l of 0.25 M phosphate buffer (pH 7.5), 35 μ g of chloramin T in 10 μ l of 0.05 M phosphate buffer (pH 7.5) was added to the mixture in a 2 ml conical flask. After a 10-sec. reaction time, 48 μ g of sodium metabisulfite in 10 μ l of 0.05 M phosphate buffer was added to the mixture as a reductant.

Excess ^{125}I and damaged or fragmented ^{125}I -calcitonin were removed from the reaction mixture by passage through a Sephadex G-25 column (1×10 cm) and Sephadex G-100 column (1×20 cm). The eluent was 0.05 M phosphate buffer at pH 7.5 containing 0.01% merthiolate, 0.01% $\text{Na}_2\text{-EDTA}$ and 1% bovine serum albumin.

Immunoassay method:

A nonequilibrium system was used routinely in flint glass test tubes (75×10 mm). Incubation mixture for the assay contained the following: 200 μl of antibody at the appropriate dilution (usually at a final dilution of 1:25,000), 100 μl of multiple aliquots of standard or sample, and either 100 μl of charcoal treated hormone free serum or 100 μl of incubation buffer consisting of 0.05 M phosphate buffer (pH 7.5), 0.01% merthiolate, 0.01% $\text{Na}_2\text{-EDTA}$ and 1% bovine serum albumin. The incubation mixtures were kept standing for 3 days at 4°C followed by a 24 hr-incubation after adding 5,000–10,000 cpm of ^{125}I -calcitonin to the mixtures. The final incubation mixture was 500 μl .

Labelled calcitonin damaged during incubation, which spuriously fractionated as antibody bound hormone, was estimated separately by incubating the tracer and the test solution without added antibody.

Charcoal dextran adsorption technique was applied for the separation of the bound radioactivity and the free radioactivity (Herbert *et al.*, 1965). Charcoal-dextran solution, composed of equal volume of charcoal suspension prepared by adding 5 g of Norit A charcoal to 100 ml of 0.1 M barbital buffer at pH 7.4, and a dextran solution prepared by adding 0.5 g of Dextran T 40 (Pharmacia Fine Chemicals) to 100 ml of barbital buffer, was stored overnight at 4°C . The suspension then was centrifuged at $1,000 \times g$ for 10 min. and supernate was decanted carefully and discarded. The dextran-coated charcoal residue was resuspended in 200 ml of fresh buffer containing 1% bovine serum albumin and mixed magnetically at 4°C for 2 hrs. and kept refrigerated. While being stirred, 500 μl of suspension was added to each incubation mixture.

The contents of the incubation tubes were mixed vigorously by a Vortex mixer for a few seconds and centrifuged at $2,000 \times g$ for 15 minutes. Then supernatant was decanted and the radioactivity in both the supernatant solution and precipitate was counted in an automatic gamma counter. All works were carried out at 4°C or on ice.

Carrier protein studies:

Since the carrier protein does influence the specific binding of tracer to the antibody in immunoassay system, the possible inhibition effects were studied in some carrier proteins as follows; plasma from total-thyroidectomized patients, pooled plasma from

normal subjects, normal plasma treated with dextran-coated charcoal, 6% bovine serum albumin buffer solution and 1% bovine serum albumin buffer solution. One hundred microliter of each carrier protein solution was incubated with 100 μl of buffer and with or without antibody for 3 days at 4°C followed by second incubation with ^{125}I -calcitonin.

Dextran coated charcoal solution:

In separation of free calcitonin from bound calcitonin, different kinds of dextran charcoal suspensions were used to study the nonspecific adsorption of calcitonin to the charcoal. Norit A charcoal coated either with dextran T 40 or dextran T 70 was suspended in either 1% or 6% bovine serum albumin solution. These four kinds of suspensions were tested for the separation of the free from bound hormone.

Recovery study:

A standard preparation of calcitonin M was diluted to the concentration of 10.0 ng/ml, 1.0 ng/ml and 0.5 ng/ml, and each 100 μl aliquots added to four different plasma from normal subjects were assayed. The assayed values were compared for the recovery of the material added.

Dilution of plasma:

For measurement of plasma calcitonin levels, dilution curves of these plasma must be parallel to the standard curve. A plasma sample from a patient with medullary thyroid carcinoma was diluted to 1/10 and 1/100 with normal pooled plasma to study the effect of dilution with pooled plasma.

Clinical study:

Basal plasma concentration of calcitonin was measured in 21 normal subjects, 10 cases with medullary thyroid carcinoma, 45 cases with chronic renal failure, 8 cases with thyroid diseases including Graves' disease and Hashimoto's thyroiditis, 8 cases with parathyroid diseases including 7 cases with primary hyperparathyroidism and one case with postoperative hypoparathyroidism, 15 cases with malignant tumors including 10 cases with breast cancer, 3 with malignant lymphoma and 2 with lung cancer, and 7 cases with diseases including 2 cases with multiple myeloma, 1 with osteomyelitis, 1 with hyperostosis, 1 with osteosarcoma and 2 with giant cell tumor.

Infusions of calcium gluconate (15 mg of calcium/kg of body weight) were given over a four-hour period as follows: in 2 cases with medullary thyroid carcinoma, 6 members of a single kindred of a patient with Sipple's syndrome, cases with chronic renal failure, 5 cases with Graves' disease, and one case with renal stone disease. Venous blood was obtained at 0, 1, 2, 3 and 4 hr. during the calcium

infusion. Plasma (obtained with heparin) was separated by centrifugation within one hour and stored at -20°C until assayed. Both plasma and serum could be used in the immunoassay, and no difference was noted between them.

Results

Antibody dilution:

In the presence of excess antiserum, 85–90% of the labelled hormone was bound to the antibody (Fig. 1). At a final dilution of 1/40,000, 50% of the labelled hormone was bound to the antibody. Consequently, antiserum was used at a final dilution of 1:25,000 in our system. The antigen-antibody complex was dissociated almost completely by acidification to pH 2 immediately before the addition of dextran coated charcoal.

Sensitivity studies:

Figure 2 illustrates a representative curve for the measurement of standard amounts of calcitonin M in an assay buffer. The assay was sensitive to about 10 pg added to the test tube of which assayed value was significantly different from the mean of zero point of the standard curve.

Carrier protein and dextran charcoal suspension:

Figure 3 illustrates the effect of various carrier proteins in the incubation mixture on the binding of ^{125}I -calcitonin to the antibody and also the influence of different kinds of dextran charcoal suspensions on the nonspecific adsorption of the tracer to charcoal. There was essentially no difference in the nonspecific inhibition between athyrotic plasma and normal pooled plasma, while a lesser amount of labelled hormone bound to the antibody was observed when dextran charcoal-treated plasma, 6% or 1% bovine serum albumin solution was used.

Theoretically, plasma obtained from thyroidectomized patients is the most de-

Fig. 1. Antibody dilution curve. Ordinate represents ratio of the bound fraction to the free fraction of ^{125}I -calcitonin, abscissa represents increasing dilution of antibody expressed as final concentration in assay mixtures.

Fig. 2. A representative standard curve. Ordinate represents B/F ratio of the tracer, abscissa represents increasing amount of standard calcitonin added to the test tube and shaded area represents the range of two S.D. of zero point of standard curve.

sirable as a carrier for a calcitonin assay, however, routinely we used either pooled plasma from normal subjects or dextran charcoal-treated plasma at 20% concentration in incubation mixture, because these nonspecific inhibitions of the immune reaction were similar to that obtained with plasma from athyrotic patients. As for the

separation, nonspecific adsorption of antibody bound tracer to charcoal was shown to be greater with dextran T 70 coated charcoal than that with dextran T 40 in the assay without antibody. And also, dextran-charcoal suspension in 1% bovine serum albumin showed a lesser nonspecific adsorption than that in 6% bovine serum albumin.

Consequently, for the separation of free from bound tracer, dextran T 40 coated charcoal suspended in 1% bovine serum albumin buffer solution was used in our system.

Fig. 3. Effect of various carrier proteins in the incubation mixture, such as plasma obtained from thyroidectomized patients (THX), normal pooled plasma (Normal), normal pooled plasma treated with dextran coated charcoal (DCC), 6% bovine serum albumin (6% BSA) and 1% bovine serum albumin (1% BSA). After each incubation, phase separation was performed either with dextran T40 (T40) or dextran T70 (T70) coated charcoal suspended either in 1% or 6% bovine serum albumin buffer. Ordinate represents B/F ratio of the tracer in the assays with excess amount of antibody (Maximal Binding) and in the assays without antibody (Non-specific Binding).

4. Recovery of standard calcitonin diluted with plasma:

Fig. 4 shows recovery of calcitonin added to the 4 different normal plasmas at concentration of 0.5 ng/ml, 1.0 ng/ml, and 10.0 ng/ml, giving the mean value and standard deviation of 0.52 ± 0.01 ng/ml, 0.96 ± 0.09 ng/ml and 10.48 ± 1.09 ng/ml respectively. This result shows the excellent recovery of synthetic calcitonin upon dilution with different normal plasma.

Dilution curve:

An important condition in the radio-immunoassay is that the concentration of

Fig. 4. Recovery of standard calcitonin added to the four different normal plasmas (○, ●, △ and □) at concentration of 0.5 ng/ml, 1.0 ng/ml and 10.0 ng/ml.

calcitonin in plasma decreases linearly with dilution of the sample.

As shown in Fig. 5, plasma from a patient with medullary thyroid carcinoma gave a parallel inhibition curve upon dilution showing 4.2 ng/ml in 1:10 dilution

and 0.42 ng/ml in 1:100 dilution. The results show that with this antiserum, there is no detectable difference in immunochemical behavior between endogenous hormone in plasma and the synthetic calcitonin M standard.

Fig. 5. Dilution of plasma from medullary thyroid carcinoma. A plasma sample from a patient with medullary thyroid carcinoma (⊙) was diluted to 1/10 and to 1/100 with normal pooled plasma.

Clinical study:

Basal fasting calcitonin concentrations in plasma distributed less than 0.3 ng/ml in all 21 normal subjects; from a normal range to 7 ng/ml in 45 cases with chronic renal failure; up to 1.6 ng/ml in 15 cases with malignant tumors; up to 1.8 ng/ml in 9 cases with thyroid disease including Graves' disease and Hashimoto's thyroiditis; up to 0.5 ng/ml in 7 cases with bone disease including 2 cases with multiple myeloma, 1 with osteomyelitis, 1 with hyperostosis, 1 with osteosarcoma and 2 with giant cell tumor; up to 0.4 ng/ml in 7 cases with primary hyperparathyroidism (Fig. 6).

In response to calcium infusion, plasma

Fig. 6. Basal plasma calcitonin in normal subjects and in various diseases.

calcitonin markedly increased in both cases with medullary thyroid carcinoma and also one member of a family with medullary thyroid carcinoma, in whom medullary thyroid carcinoma was highly suspected. No marked changes were seen in the following cases; 2 cases with renal failure, and one with treated Graves' disease which showed increased basal plasma calcitonin levels; 5 other members of the family of medullary thyroid carcinoma and 4 cases with Graves' disease (Fig. 7).

Discussion

Although bioassays for calcitonin are applicable to the measurement of the human calcitonin in plasma, these are costly, laborious, less sensitive and relatively non-specific. A radioimmunoassay for human calcitonin was first developed by Clark *et al.* (1969) and subsequent assays were reported by Dietrich and Rittel (1970), Tashjian *et al.* (1970) and Deftos (1971 a). The sensitivity of these assays were reported as ranging from 0.02 to 0.1 ng per milliliter of unknown plasma or serum. Two of these assays were not sensitive enough to measure the normal blood levels of the hormone.

The present study showed the detection limit of the assay to be about 0.1 ng per milliliter of plasma which is almost identical with previous studies. This study also demonstrated that reproducibility and the recovery of synthetic calcitonin diluted with human plasma were sufficient to measure circulating calcitonin and that no difference was detected in the assay between endogenous hormone and synthetic calcitonin upon dilution of plasma from a patient with medullary thyroid carcinoma.

Although calcitonin can be readily detected in the plasma of patients with medullary thyroid carcinoma, the levels in normal plasma are too low to be measured directly by bioassays. Estimates by bioassays have ranged from 0.2 to 1.7 ng per milliliter (Sturtridge and Kumar, 1968; Gudmundsson *et al.*, 1969), but these are not accurate due to artifacts induced by concentration procedure (Bell *et al.*, 1970). The results of the present study suggest that in the most normal adults basal concentration of calcitonin is not greater than 0.3 ng per milliliter which is equivalent to the previous reports by Tashjian *et al.* (1970), Melvin *et al.* (1970), Sizemore *et al.* (1974) and Silva *et al.* (1974 a).

Fig. 7. Changes in plasma calcitonin concentration in response to calcium infusion over four hours.

The sensitivity of this assay should make the method useful for both physiological and clinical studies. High plasma calcitonin levels have been reported in patients with Zollinger-Ellison syndrome (Sizemore *et al.*, 1974) in patients with medullary thyroid carcinoma, in some cases with hypercalcemia and in some cases with bronchogenic carcinoma (Silva *et al.*, 1974 b). The present study showed relatively high basal calcitonin levels in some cases with chronic renal failure, malignant tumors and skeletal diseases. However, no significant correlation was seen between basal calcitonin levels and serum calcium or phosphorus levels. At present the exact implication of these findings are unknown, but they can provide an important clue in clarifying the derangement of calcium metabolism. The value of radioimmunoassay for calcitonin in the diagnosis of medullary thyroid carcinoma, a neoplasma of calcitonin-secreting cell (C-cells), is well established (Melvin *et al.*, 1972; Deftos *et al.*, 1971 b). Most patients with this tumor have diagnostically an increased concentration of this hormone. However, some patients with this tumor reported to have a relatively low concentration of calcitonin and calcium infusion tests are necessary to establish the diagnosis (Jackson *et al.*, 1973; Deftos, 1974; Parthemore *et al.*, 1974). The present study showed that 2 patients with medullary thyroid carcinoma had markedly elevated basal calcitonin levels and both of them showed striking increase in calcitonin levels after the provocative calcium infusion.

Because of the known familial nature of medullary thyroid carcinoma, a provocative calcium infusion test is of great value in the early detection of clinically occult cases with medullary thyroid carcinoma. Jackson *et al.* (1973) reported positive calcium infusion tests for 22 individuals in 76 members of 3 kindreds with known familial medullary thyroid carcinoma. Seventeen of the 22 cases with positive calcium in-

fusion tests had undergone surgery and bilateral medullary thyroid carcinoma was confirmed in 16 of these cases and C cell hyperplasia which presumably preceded the neoplasma was present in the one remaining case. They emphasized the usefulness of the provocative calcium infusion procedures in detecting the tumors at the earliest possible stage.

Radioimmunoassay for calcitonin also provides a useful method in the long-term postoperative management of patients with medullary thyroid carcinoma. Goltzman *et al.* (1974) demonstrated the usefulness of this assay in localization of recurrent or residual tumor and in the planning and evaluation of proper treatment. Fig. 8 illustrates a serial determination of basal

Fig. 8. Basal calcitonin levels in a patient with medullary thyroid carcinoma before and after total thyroidectomy.

calcitonin levels in a patient with medullary thyroid carcinoma before and after total thyroidectomy. This 48 year-old female underwent total thyroidectomy and excision of bilateral pheochromocytoma. Basal calcitonin after the surgery dropped to 10 ng per milliliter, however this was much greater than the normal levels and residual tumor tissue was suspected. A gradual rise in basal calcitonin levels was demonstrated in her postoperative course, suggesting recurrence of the disease. Another neck mass was noted 10 months after surgery and a second surgery is now under consideration.

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