Calcitonin Levels in Normal Individuals With New Highly Sensitive Chemiluminescent Enzyme Immunoassay

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Human serum calcitonin concentration in normal individuals was measured with a new assay based on the chemiluminescent enzyme immunoassay (CLEIA) method. The CLEIA assay was highly sensitive and was able to determine a calcitonin concentration of 0.04 pg/ml as sensitivity limit at a condition of 0+3SD. With this CLEIA assay, the mean value of calcitonin in males and females was 2.26 and 1.33 pg/ml, respectively, highlighting a significant difference between genders.

The mean value and range of human serum calcitonin in this assay were approximately 1/10 those reported previously in com-

petitive radioimmunoassay (RIA) methods. Since RIAs for calcitonin showed much variability at a low concentration range due to the competitive format, they seemed to lack the necessary sensitivity to cover the normal range and appeared only useful for hyper-calcitonin phenomenon in diseases such as medullary thyroid carcinoma. The CLEIA for calcitonin provided a lower detection limit than normal range, and it can therefore be assumed that it could be applied for the measurement of hypo-calcitonin phenomena typically found in some disorders such as osteoporosis. *J. Clin. Lab. Anal.* 12:218–222, 1998. © 1998 Wiley-Liss, Inc.

Key words: human calcitonin; human serum; enzyme immunoassay; chemiluminescence; age

INTRODUCTION

Calcitonin (CT), a 32-amino acid peptide hormone, is a useful serum marker for the diagnosis and monitoring of medullary thyroid carcinoma (MTC)(1). Radioimmunoassays (RIAs) for calcitonin using polyclonal antibody and ^{125}I -labeled calcitonin have so far been used for that purpose. Patients exhibiting MTC have been defined by elevated basal levels or significant increases in CT basal levels after injection of pentagastrin. However, RIAs have been hampered by several problems, including lower sensitivity for normal range and immunochemical heterogeneity of circulating calcitonin, thereby leading to variability in serum CT levels between laboratories.

Aiming to improve this method, Body et al. developed extraction methods for concentrating monomeric CT in biological samples (2,3). However, extraction methods were not deemed suitable for routine purposes because of the tedious steps involved and the large amounts of sample required.

Motte et al. reported on monoclonal antipeptide antibodies based on the immunoradiometric assay (IMRA) for the measurement of mature calcitonin form in serum (4). Although the sensitivity of the IMRA CT assay was improved to 10 pg/ml level, it remained nonetheless insufficient to cover the CT values of normal individuals (5).

CT is known to bind to osteoclasts through the CT receptor to inhibit osteoclastic bone resorption. Since lower levels of CT could result in the decrease of bone matrix and in os-

teoporosis, it seemed desirable to measure the change or the decrease in basal CT levels according to age with a highly sensitive assay for CT.

Isomura et al. recently described a chemiluminescent enzyme immunoassay (CLEIA) for CT using three monoclonal antibodies that achieved a very high sensitivity (6,7). The current report describes the basic characteristics of this highly sensitive assay and the relationship between normal CT levels and age for males and females.

MATERIALS AND METHODS

Chemiluminescent Enzyme Immunoassay (CLEIA) for Calcitonin

Three kinds of monoclonal antibodies—CT2, CT8, and OCT1—specific for human calcitonin were obtained from KRI International Corp. (Kyoto, Japan). The specificity of these monoclonal antibodies was previously analyzed with the following results: CT2 for the center portion, CT8 for the C-terminal portion, and OCT1 for the N-terminal portion of human calcitonin (7).

The solid phase of the assay was prepared by coating the carboxylated ferrite particles (Nippon Paint Corp. Ltd., Tokyo, Japan) with capture antibody CT2 as follows: 15 mg of

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carboxylated ferrite particles were incubated with 0.6 mg of CT2 monoclonal antibody in 50mM phosphate buffer pH 4.5 for 1 hr at room temperature. After washing with 50mM MES buffer pH 5.5, the carboxylated ferrite particles were incubated with 1.5 mg of 1-ethyl-3-(dimethyl aminopropyl) carbodiimide (Nakarai Chemical, Osaka, Japan) for 30 min at room temperature and then washed with 0.9% NaCl and preserved at 4°C before use.

The antibody-enzyme conjugate for the assay was prepared as follows: Fab' fragments of CT8 and OCT1 monoclonal antibodies were prepared by pepsin digestion and 2-mercaptoethanolamine reduction, respectively, and were then mixed with 1:1 molar ratio for the coupling step. The alkaline phosphatase (ALP, EC 3.1.3.1., Oriental Yeast, Tokyo, Japan) was incubated with 1-(γ -maleimidobutyryloxy) succinimide in 0.1 M Phosphate buffer pH 7.0 for 1 hr at room temperature, and then fractionated with a PD-10 gel filtration column. The fractionated ALP was coupled by incubation with a mixture of CT8/OCT1 Fab' for 3 hours at 20°C. The reaction mixture was applied to a Superdex G-200TM gel filtration column (Pharmacia, Sweden) for fractionation. The antibodies-enzyme conjugate was pooled from those fractions with a molecular weight of around 190,000.

The CLEIA used in this study was developed according to the delayed one-step enzyme immunometric assay: an equal volume of assay standard or sample and antibody-enzyme conjugate were incubated for 10 min at 37°C, following which 100 μ l of the reaction mixture were added to 250 μ l of 0.015% CT2-coated particle suspension in 50mM Tris-Cl buffer pH 7.2. After 10 min incubation at 37°C, the particles were washed and supplemented with 200 μ l of 0.2 mg/ml AMPPDTM (adamantane methoxy phosphoryloxy phenyl dioxetane, Tropix Inc., Bedford, MA) solution pH 9.5 containing 0.4 mg/ml BDMQ(poly[vinylbenzyl-(benzyl)dimethylammonium chloride]) (Tropix Inc.) as enzyme substrate. After a 5-min

incubation at 37°C, the chemiluminescent counts of the reaction mixture were performed for 2 seconds with a photon-counting instrument. These assay procedures were performed automatically by Lumipulse-1200TM instrument system (Fujirebio, Tokyo, Japan).

Controls and Samples

Synthetic human calcitonin (Peptide Institute Inc., Osaka, Japan) was diluted to 0.1 mg/ml aliquot as stock solution and stored at -80°C until use. The assay standard was generated from each freshly thawed aliquot of the stock solution by dilution with 50mM Tris-Cl buffer pH 7.2 containing 0.15 M NaCl and 0.5% BSA.

Serum samples from 95 healthy women aged 20–69 years and 90 healthy men aged 20–85 years were used for the evaluation of the normal range of calcitonin by CLEIA method. The series of three healthy women serum samples, which were bled at 5:00, 8:00, 12:00, 18:00, and 23:00 on a given day, were used for the evaluation of the variability of calcitonin value during one day.

RESULTS

Analytical Performance of CLEIA for Calcitonin

All assays for human calcitonin were performed with the Lumipulse-12TM (fully automated immunoassay instrument) following the manufacturer's instructions. A typical calibration curve is shown in Fig. 1. Since the precision of the assay standard was quite good (with a CV range of 1.4–3.5%, as shown in Table 1), the detection limit of the assay, which was calculated from the mean counts plus 3 SD of the zero standard, was 0.04pg/ml. Assay standard range was 0.1pg/ml to 500pg/ml with a linear response to the increase in human calcitonin concentration. There was no cross-reactivity to hu-

Fig. 1. Calibration curve of calcitonin assay.

TABLE 1. Precision of Calcitonin Assay

Calcitonin (pg/ml)	Sub blank	0	0.1	0.39	1.56	6.25	25	100	500
Counts	363	557	745	1300	3423	11807	48295	174484	775930
	347	601	752	1269	3238	12207	45436	174308	782602
	349	589	742	1259	3381	12186	46540	177104	838366
	341	591	720	1279	3411	11344	46974	177182	804081
	367	571							
	338	593							
	357	605							
	365	550							
Average counts	353	582	740	1277	3363	11886	46811	175020	800245
SD	11	20	14	18	85	405	1182	2774	28109
CV(%)	3.2	3.5	1.9	1.4	2.5	3.4	2.5	1.6	3.5

man calcitonin gene-related peptide and very weak cross-reactivity to rat calcitonin (less than 0.6%). The increase in concentration of bilirubin, hemolytic hemoglobin, and hematocytromicron did not have any significant influence on the assay within the range tested (data not shown).

Stability of Calcitonin in Serum

The stability of calcitonin in serum was analyzed with samples which were spiked by Calcitonin to 500 pg/ml with buffer or Calcitonin-depleted sera, then incubated in 4, 25, and 37°C for 0–8 hours and later measured by CLEIA method for CT level. The results are shown in Fig. 2. In buffer, there was no significant decrease in CT level at 4–37°C over 8 hours, whereas in serum, CT levels showed a decrease at higher temperatures at 2–8 hours incubation. The incubation of CT-spiked serum at 37°C for 8 hours resulted in almost 2/3 of relative calcitonin activity. Therefore, in order to eliminate variations in CT values during sample handling, all of the samples were treated at 4°C and kept frozen. There was no significant decrease in CT level for up to five times of freezing/thawing of serum (data not shown).

Fig. 2. Stability of calcitonin in serum sample.

Basal Levels

The distribution of calcitonin values in the group analyzed this time is shown in Fig. 3. Different distribution shapes can be observed between the male and the female group.

The calcitonin values (mean±SD pg/ml) of men and women grouped according to their age are shown in Fig. 4. The calcitonin values of all men ($2.26\text{pg/ml} \pm 1.07$) was significantly higher than those of all women ($1.33\text{pg/ml} \pm 0.94$). The lowest value of individuals in this basal study was 0.22 pg/ml, which was within the assay standard range of CLEIA.

Variability of Calcitonin Value Within a Day

The changes in the calcitonin values of three women bled at specific times during one day were analyzed (Fig. 5). While there were no significant changes in calcitonin level in the daytime (5:00–18:00), an increase was observed at night (18:00–5:00) in all three women. Although the value increase differed among the 3 individuals, the ratio of increased to nonincreased level seemed to range within 2.5 times.

DISCUSSION

This CLEIA assay achieved a highly sensitive detection of human calcitonin in serum within 30 min. The detection limit of the assay was 0.04pg/ml, calculated from the mean counts plus 3 SD of a zero standard. This sensitivity was approximately 100–1000 times higher than with the previously reported RIA or IRMA. The assay's higher sensitivity is the result of increased signals due to the sandwich-type enzyme immunoassay format using chemiluminescent enzyme substrate AMPPD and good assay precision of instrument-based measurement. Also, the combined use of 3 kinds of monoclonal antibodies specific to the N-terminal, central, and C-terminal portion of calcitonin had much influence on the achievement of a highly sensitive detection (7).

Using CLEIA assay, the mean values of basal human serum calcitonin in males and females were 2.26 and 1.33 pg/ml, respectively. These differed significantly from the nor-

Fig. 3. Distribution of calcitonin concentration.

mal values previously reported. Several investigators have suggested a 25–50 pg/ml range as the normal level of calcitonin with unextracted RIA and a 4–8 pg/ml as the normal level of monomeric calcitonin with extracted RIA. Also, the problems inherent to RIAs include immunochemical heterogeneity of circulating calcitonin and the presence of interfering substances of large molecular weight due to the polyclonal antibody preparation. Hence, a suitable calcitonin assay capable of detecting normal range values does not appear to be available yet. Although Guilloteau et al. (5) showed a level of less than 10 pg/ml of basal calcitonin measured by their IRMA using monoclonal antibodies, they failed to cover the basal value due to sensitivity limitations. Monoclonal antibody-

based IMRA reported by Motte et al. (4) and Guilloteau et al. showed a calcitonin value (approximately 1/10) that is much lower than that of polyclonal antibody-based RIA for patients' and normal serum. However, the calcitonin value in CLEIA is similar to those of monoclonal antibody-based IMRA, and it can therefore be assumed that the sandwich-type immunoassay based on monoclonal antibodies could help resolve the problems associated with polyclonal RIA in terms of specificity and accuracy of calcitonin measurement. As Isomura et al. (7) reported, and as described in this article, the combined use of multiple monoclonal antibodies specific for C-, N, and the center portion of calcitonin helped enhance sensitivity in the CLEIA system. In this system, the basal level of human calcitonin could be covered as the sensitivity of assay.

As shown in Fig. 4, the basal levels in men of each age group were significantly higher than those in women, a find-

Fig. 4. Calcitonin levels in normal women and men according to age group. Mean calcitonin levels (pg/ml) in normal women (closed circles) and men (open circles) of various age groups were plotted. Vertical lines represent the standard deviation (SD) for each mean value. Women: N=20 (Age 20–29); 20 (30–39); 20 (40–49); 20 (50–59); 15 (>60). Men: N=20 (Age 20–29); 20 (30–39); 20 (40–49); 19 (50–59); 11 (>60).

Fig. 5. Variability of calcitonin value within one day.

ing that is in agreement with most of the previously published studies (8), although the levels were fairly different. The calcitonin level tended to decrease in the group of men aged 60 or over and slightly also in that of women of the same age. These findings may suggest the physiological role of calcitonin in bone turnover in old age.

Calcitonin is known as the only hormone to inhibit the osteoclastic bone resorption through binding to the calcitonin receptor on the cell surface. With the increasing number of publications focusing on the mechanism of bone turnover and modulation of calcium or other ions, it might be safe to assume that the physiological role of calcitonin in bone turnover mechanism shall be understood soon. The measurement of calcitonin values much lower than basal level may be of great interest in the study of the hypothetical role of calcitonin deficiency in the development of osteoporosis. It might be worthwhile to measure the subjects at risk before and after the development of osteoporosis with highly sensitive calcitonin assay in physiological conditions. The CLEIA described in this study appears as a suitable tool for this purpose since it was able to measure lower levels than basal range of calcitonin. Further studies using CLEIA will be needed to confirm the clinical importance of hypo-calcitonin level in particular disorders related to bone turnover or other criteria.

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