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Calcitonin and PDN-21 as Tumour Markers in MEN-2 Family Screening for Medullary Thyroid Carcinoma

Søren Schifter

Calcitonin is expressed in medullary thyroid carcinomas (MTC). It is processed from a large molecular weight precursor and is flanked at its C-terminal end by a 21 aminoacid peptide (PDN-21) formed in equimolar concentrations to calcitonin by enzymatic cleavage of the prohormone. This investigation compared basal measurements of calcitonin and PDN-21 and the response of the two peptides following pentagastrin stimulation in normal controls and in family members with C-cell hyperplasia or early neoplasia. The results showed that calcitonin and PDN-21 may both be used in family screening for the MEN-2 syndrome, but the unstimulated circulating concentrations of calcitonin were higher and more influenced by C-cell hypersecretion than PDN-21 ($P < 0.01$), and the increase in stimulated concentrations of calcitonin were significantly higher than for PDN-21 ($P < 0.01$). These findings may be explained by differences with respect to secretion and metabolic clearance rate for the two peptides.
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INTRODUCTION

CALCITONIN HAS been used as a tumour marker for medullary thyroid carcinoma (MTC) for two decades [1–3] and measurement of calcitonin is used in family screening for multiple endocrine neoplasia, type 2 (MEN-2) [4–7]. It is important to point out individuals with the inherited disposition for MTC at an early stage of the disease [8] and supplementary tumour markers to calcitonin may improve the possibility for early diagnosis and thereby hopefully contribute to improvement of the prognosis for these patients.

The calcitonin gene has been isolated [9, 10] and its regulatory peptides and peptide products have been identified [11, 12]. Assays for some of these peptides may be useful as supplementary tumour markers to calcitonin, thereby improving the diagnostic sensitivity and specificity in screening for MEN-2. The calcitonin gene is peculiar by giving rise to two regulatory peptides calcitonin and calcitonin gene-related peptide (CGRP) [13, 14]. The signal peptide and prosequence are identical for the two regulatory peptides but procalcitonin also gives rise to a C-terminal cleavage peptide named PDN-21 or katacalcin [15, 16] (Fig. 1).

Calcitonin is normally expressed in C-cells of the thyroid [1–3] and has a calcium lowering effect [17], while CGRP is a neuropeptide with widespread distribution in the central and peripheral nervous system [13, 18–20].

Preliminary investigations indicated that PDN-21 was an active hormone with calcium lowering effects [15, 21] but these

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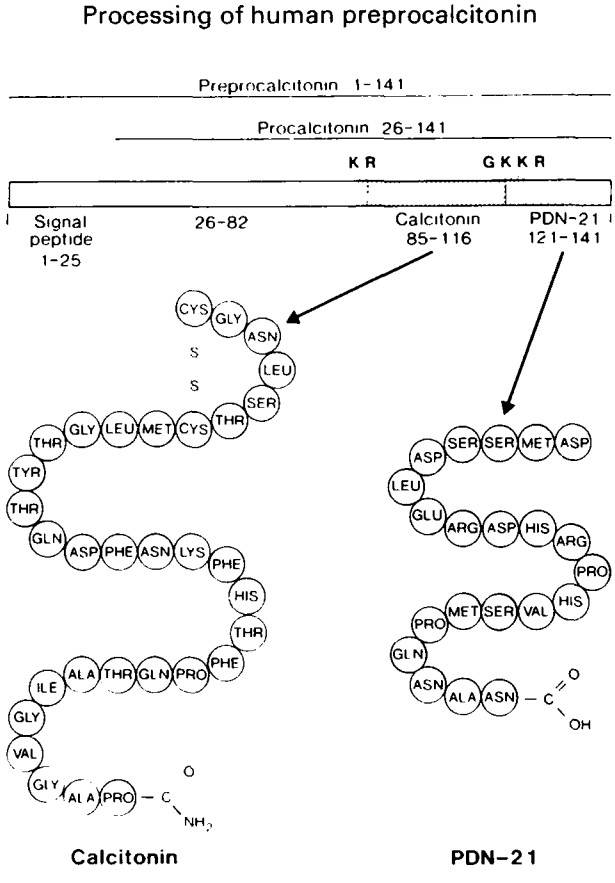

Fig. 1. Processing of human pro-calcitonin and aminoacid structure of calcitonin and PDN-21.

observations have not been confirmed [22, 23] and it is now regarded as an inactive peptide released as a byproduct to the intracellular processing of procalcitonin.

Most radioimmuno assays (RIA) for calcitonin have problems with respect to sensitivity when employed for determination of circulating concentrations in normal subjects [24–26], while assays for PDN-21 have been claimed to be more sensitive with measurable concentrations in most normal subjects [27]. This may indicate that PDN-21 is a more sensitive marker for MTC than calcitonin. The present investigation therefore compares the response to pentagastrin stimulation in normals and in MEN-2 family members with C-cell hyperplasia or early neoplasia employing a new developed highly sensitive RIA assay for calcitonin and a commercial highly sensitive RIA for PDN-21.

PATIENTS AND METHODS

Patients

Pentagastrin stimulation tests were performed in 12 normal controls, mean age 26, range 14–43 years and in 20 MEN-2 family members, mean age 30, range 10–60 years. 8 of these showed elevated calcitonin response to pentagastrin stimulation indicating C-cell hyperplasia or early neoplasia. Informed consent was obtained from all individuals according to the Helsinki Declaration II.

Procedures

Blood samples were collected in dry tubes and serum separated by centrifugation at 2000 *g* for 10 min. Samples were stored at −20°C until analysis.

Pentagastrin stimulation tests were performed by intravenous bolus injection of pentagastrin (Peptavlon, ICI) 0.5 μg/kg at $t = 0$. Basal levels were routinely determined at −3 and −1 min before injection and stimulated values at +2 and +5 min after injection of pentagastrin.

Analytical methods

Calcitonin was determined with a new highly sensitive RIA. Standards (Peninsula Laboratories, USA) were prepared in 0.1 mol/l phosphate buffer pH 7.5 containing 0.1% bovine albumin, 0.01% sodium azide and aprotinin (Trasylol, Bayer AG), 20 KIE/ml. Tracer was prepared employing the Iodo-Gen method and using the total molecule as substrate. Purification was performed on high performance liquid chromatography (HPLC). Total separation was obtained between mono- and diiodinated calcitonin. The monoiodinated form was used as tracer. The antibody employed was obtained by immunisation of rabbits with a complex of human calcitonin covalently linked with glutaraldehyde to bovine serum albumin (DAKO A/S, Denmark). The antibody is specific for the C-terminal end of the human calcitonin molecule but does not discriminate between the amidated or non-amidated peptide. It is used in a final dilution of 1:400.000. The assay is performed in a non-equilibrium system with incubation for 4 + 2 days. Sample volume is 200 μl, antibody 100 μl, and tracer 100 μl. Separation is performed with a solid phase separation system (Tachisorb, Hoechst, AG). The specificity has been tested against salmon calcitonin, which showed no interaction (<0.001%). The detection limit is <0.4 pmol/l. Intra- and interassay varations are 5% and 10%, respectively. Normal range is 4.0–13.0 pmol/l [mean (2 S.D.), $n = 70$].

PDN-21 was determined by a commercial RIA kit (IRE-MEDGENIX, Belgium). The assay is performed in a non equilibrium system with preincubation for 24 h followed by incubation with 125-I PDN-21 for 24 h. Total volume 300 μl, sample volume 100 μl, antibody volume 100 μl, tracer volume 100 μl. Separation is performed with a polyethylene glycol (PEG) facilitated second antibody system by centrifugation at 1500 *g* for 15 min following a brief incubation for 20 min. The detection limit for the assay is 2.5 pmol/l, intra- and inter-assay variation 3% and 6%, respectively.

Statistics

The statistical calculations were performed using mean values for the two determinations before stimulation and the two poststimulation values. Levels of calcitonin and PDN-21 were compared before and after stimulation in each of the three groups using Wilcoxon's test for paired comparison. This test was also used for testing increases in calcitonin and PDN-21 concentrations following pentagastrin stimulation. Calculation of correlation coefficients for CT compared with PDN-21 were performed using the Spearman test and basal concentrations for the three groups were compared using the Mann-Whitney test.

RESULTS

The concentrations of calcitonin and PDN-21 were unaffected by pentagastrin simulation in normal controls and in normal family members (Fig. 2a and b). The concentration of PDN-21 increased significantly to supranormal levels ($P < 0.01$) following pentagastrin stimulation in all family members with pathological calcitonin response ($P < 0.01$) indicating C-cell hyperplasia or early neoplasia (Fig. 2c).

Slightly elevated basal concentrations of calcitonin as well as PDN-21 were found in 3 of the 8 patients with a pathological

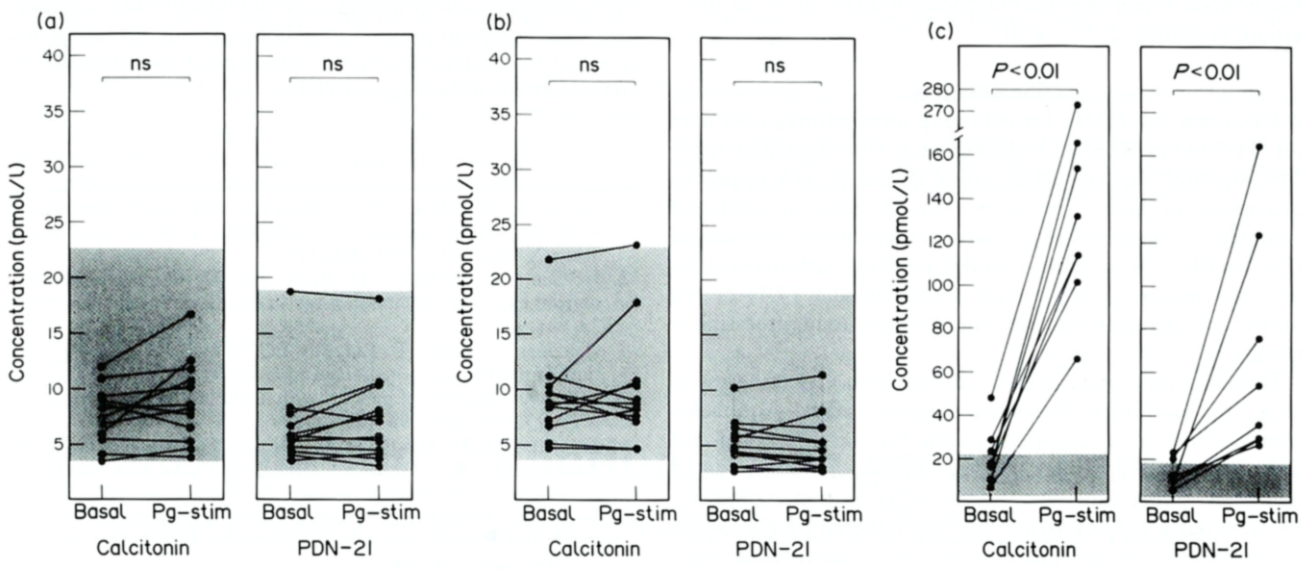

Fig. 2. (a) Basal and pentagastrin stimulated (Pg stim.) serum immunoreactivity for calcitonin and PDN-21 in 12 normal controls. (b) Basal and pentagastrin stimulated serum immunoreactivity for calcitonin and PDN-21 in 12 normal MEN-2 family members. (c) Basal and pentagastrin stimulated serum immunoreactivity for calcitonin and PDN-21 in 8 MEN-2 family members with pathological calcitonin response to stimulation. Shaded areas represent range for normal concentrations in response to pentagastrin stimulation. Pentagastrin stimulation and collection of blood samples were performed as described in the text.

pentagastrin test. The basal concentrations of calcitonin were elevated significantly in individuals with pathological pentagastrin test when compared with the groups of normal controls ($P < 0.01$) and the same was found for the basal concentration of PDN-21 in these individuals ($P < 0.01$).

The relative increase in concentrations of calcitonin and PDN-21 was compared in patients with C-cell hyperplasia or early neoplasia as demonstrated in Fig. 3, showing no significant difference while the absolute rise from basal concentrations was significantly higher for calcitonin as compared to PDN-21 ($P < 0.01$). Correlation between unstimulated concentrations of calcitonin and PDN-21 was relatively low but significant ($r = 0.46$, $P < 0.01$, slope = 0.39, intercept = 3.65, $n = 32$). The correlation increased when adding the post-stimulation values, ($r = 0.59$, $P < 0.01$, slope = 0.49, intercept = 1.53, $n = 64$). The correlation between pre- and post-stimulation values for calcitonin and PDN-21 following logarithmic transformation is shown in Fig. 4.

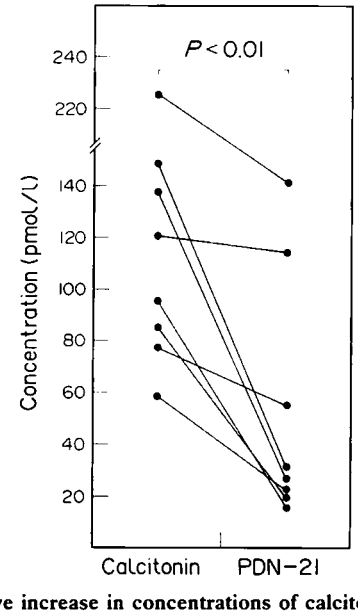

Fig. 3. Relative increase in concentrations of calcitonin and PDN-21 following Pg stim. in the patients with C-cell hyperplasia or early neoplasia. The relative increase in concentrations is not significantly different for the two peptides.

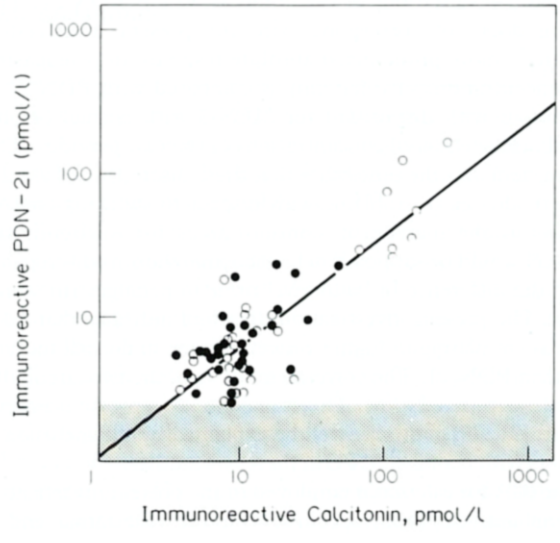

Fig. 4. Relation between concentration of calcitonin and PDN-21 for all individuals investigated including both pre- (●) and post-stimulation (○) measurements. *X* and *Y* represent log values to concentrations of calcitonin and PDN-21, respectively. The shaded area indicates concentrations below the detection limit for the PDN-21 RIA. $r = 0.59$; $P < 0.001$; $y = ax + b$; a = 0.75; b = 0.06.

The unstimulated concentrations of calcitonin were significantly higher than the concentrations of PDN-21 in all three groups investigated ($P < 0.01$).

DISCUSSION

Calcitonin and PDN-21 may both be used as tumour markers for MTC and in MEN-2 family screening with stimulation tests. This investigation demonstrates total agreement with respect to diagnostic specificity, but shows that increases in basal and stimulated concentrations of calcitonin are more pronounced than for PDN-21 in MEN-2 family members with hyperplasia or early neoplasia even though calcitonin and PDN-21 are formed in equimolar amounts when pro-calcitonin proessing is normal.

Hormone processing may be disturbed in endocrine carcinoma cells resulting in the secretion of prohormone. Antibodies employed in assays are often specific for the C-terminal end and may even discriminate between amidated and unamidated molecules. When employing an assay with C-terminal specificity for calcitonin underestimation of synthesis therefore might occur due to secretion of pro-calcitonin which is undetectable when employing a C-terminal antibody with affinity for molecules with a free C-terminal end. This is in contrast to measurement of PDN-21 as the C-terminal end of this peptide also forms the C-terminal end of the prepro- and prohormone. PDN-21 may therefore be detected with C-terminal antibodies even when present as part of the primary translation product.

This investigation demonstrates that circulating concentrations of calcitonin are significantly higher than for PDN-21 when comparing concentrations in normal individuals and individuals with the inherited disposition for MEN-2 with slightly elevated levels of calcitonin and PDN-21. Prior investigations have shown equimolar concentrations of calcitonin and PDN-21 when comparing patients with manifest medullary thyroid carcinomas [21, 27], thereby indicating the same clearance-rate from the circulation for the two peptides. The investigation by Ittner *et al.* [27] also showed comparable changes in concentrations of calcitonin and PDN-21 following pentagastrin stimulation in patients with manifest MTC and significantly elevated concentrations of calcitonin as well as PDN-21. This finding does not correspond with the present investigation showing a more pronounced absolute response to pentagastrin for concentrations of calcitonin as compared with PDN-21 in individuals with disposition for MEN-2 with normal or only slightly elevated basal concentrations of the two peptides. This finding excludes the possibility that these discrepancies are due to methodological problems with respect to standard concentrations as the increase in concentrations for calcitonin and PDN-21 would be supposed to be the same, the only discrepancy being the difference in basal level prior to pentagastrin stimulation. The present investigation therefore indicates that calcitonin is circulating in higher concentrations in normal individuals than PDN-21. The correlation between the two circulating peptides is relatively low. This is in contrast to previous investigations [21, 27], but may well be explained by the inclusion of patients with manifest MTC in these two studies.

The RIA for calcitonin employed in the present investigation uses an antibody with specificity for the C-terminal end of the molecule but independent of C-terminal amidation. The antibody does therefore not discriminate between processed CT and the prohormone, and this may be important as the prohormone processing may be disturbed in cancer cells.

In conclusion, the results show total agreement with respect to selection of individuals with pathological pentagastrin tests when comparing the two assays. Calcitonin and PDN-21 assays may therefore both be used for MEN-2 family screening and choosing the preferable assay should depend on the specificity and sensitivity of the assays available.

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Phenotypic and Functional Analysis of Tumour-infiltrating Lymphocytes from Patients with Melanoma and Other Metastatic Cancers

Claire Mathiot, Eric Robin, Alain Gey, Xiao Weng, Thierry Dorval, Pierre Pouillart, Xavier Sastre, Marc Zerbib, Jean Pierre Hamelin, Rémy Salmon, Jean Claude Durand, Maud Brandely and Wolf H. Fridman

Thirty tumour specimens, among which were 17 melanomas, were cultured with recombinant interleukin-2 (IL-2) in order to produce tumour-infiltrating lymphocytes (TIL). In the melanomas, three categories of TIL were characterised. The first, containing mostly CD3+ and CD8+ cells, lysed only autologous tumour cells; the second, containing mostly CD3+ and CD4+ cells, lysed both autologous tumour cells and allogeneic cells lines; the third, with mixed phenotype although cytotoxic for K562 targets, did not kill melanoma cells. The optimal conditions for a good development of TIL were established: we found that the lymph node or cutaneous origin of the tumour was unimportant, a 2 h enzymatic treatment was optimum and that TIL grew well in AIM V serum free medium. Therefore the easiness and the reproductibility of the TIL cultures from melanoma tumour samples allows the rapid development of therapeutic trials in metastatic melanoma.
Eur J Cancer, Vol. 28, No. 2/3, pp. 345–350, 1992.

INTRODUCTION

ADOPTIVE IMMUNOTHERAPY consists of the transfer of immunologically competent cells, such as natural killer cells (NK), T lymphocytes or monocytes, to cancer patients in order to induce an antitumour response either by a direct cytotoxic effect or by the release of immunostimulatory or cytotoxic factors. This procedure was initiated by Rosenberg *et al.* who, after demonstrating the cytotoxic activity of lymphoid cells, previously stimulated with interleukin-2 (IL-2), against fresh murine [1] and human [2] tumours, reinjected them into tumour-bearing hosts. Three different cells have since been used: lymphokine-activated killer cells (LAK), interferon-activated monocytes, and tumour-infiltrating lymphocytes (TIL).

LAK cells are derived from peripheral blood mononuclear cells of patients previously stimulated *in vivo* by IL-2. The cells are then activated *in vitro* with IL-2 for 4 days, which results in a reinforcement of their cytotoxic activity and an increase in their number. The cells are reinjected into the patients together with IL-2. LAK cells are cytotoxic against fresh autologous and allogeneic tumour cells. This approach has been shown to be effective in inducing tumour regressions both in murine models and in humans with metastatic cancers of various origins (kidney, colon, melanoma) [3]. However, the cytotoxicity is not tumour-specific and the adverse effects of the treatment are considerable, as a large number of cells need to be reinjected and as IL2 associated with the reinjection must be administered in high doses.

Monocytes also play a role in antitumour defences; the isolation and culture of circulating blood monocytes gives rise

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