Immuonoextracted calcitonin in milk and plasma from totally thyroidec-tomized women.
Evidence of monomeric calcitonin in plasma during pregnancy and lactation

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Abstract. The level of immunoreactive calcitonin in the first produced breast milk was in totally thyroidec-tomized (TX) women 713 ± 307 pg-eq/ml (mean ± sd, N = 7) and in control women 772 ± 329 pg-eq/ml (N = 33), i.e. about 45 times higher than in plasma (see below). On gel chromatography of immunoextracted milk from TX women, immunoreactive calcitonin appeared as high molecular weight forms in the same pattern as in milk from healthy women when the same antiserum (1) was used for immunoextraction and radioimmunoassay (RIA). In another series of experiments, a new antiserum (2) raised in rabbits was used for measurement of immunoreactive calcitonin after immunoextraction with 1. Plasma levels of immunoreactive calcitonin in the TX women during pregnancy were 16 ± 6 pg-eq/ml (N = 6) and during lactation 14 ± 7 pg-eq/ml (N = 5). Immunoreactive calcitonin was undetectable (< 8 pg/ml) in plasma from those TX women in whom lactation had stopped (N = 5). Immunoextraction and gel chromatography of plasma collected during pregnancy and lactation from the TX women showed that the immunoreactive calcitonin present eluted in the region of monomeric calcitonin with both antiserum 1 and 2. In conclusion, high concentrations of high molecular weight forms of immunoreactive calcitonin have been demonstrated in milk from TX patients, most probably devoid of any calcitonin-producing thyroid C-cells. This points to a local production site in the mammary gland. Relatively high levels of immunoreactive calcitonin in plasma during pregnancy and lactation in TX women also indicate extrathyroidal production.

Immunoreactive calcitonin (iCT) in high concentrations is present in human milk in forms larger than monomeric calcitonin (CT) (Bucht et al. 1983). In degradation experiments, these large forms were resistant to reduction, denaturation and treatment with acid in unextracted milk (Bucht et al. 1983). In its largest forms immuno-extracted iCT from milk contains immunoreac-tive PDN-21 (terminology according to Tatemoto & Muff 1981), a small peptide located on the carboxy-terminal of the proforms of CT (Craig et al. 1982). Therefore, the high molecular weight forms of iCT present in milk in high concentra-tions might constitute precursors, and this, in turn, would point to a local production of the hormone in the mammary gland.

To further elucidate the place of production, we measured iCT in milk from totally thyroidec-tomized (TX) women, i.e., in subjects where the major production site of CT had been removed.

iCT exists in low concentrations in plasma from normal subjects (Body & Heath 1983), mainly in monomeric, dimeric and larger forms (Tobler et al. 1983; Bucht et al. 1985). In plasma from TX individuals, iCT is present in even lower concen-
trations (Body & Heath 1983), which makes it difficult to study its molecular forms. In this report, we measured iCT in plasma from pregnant and lactating TX women both with direct RIA and after immunoextraction and gel filtration.

Material and Methods

Subjects

Milk was obtained during the first week after delivery from 6 totally thyroidectomized women (one delivered milk after two child births) and from 33 healthy women. Furthermore, from one TX and one healthy mother, milk was collected after delivery for an extended period of 2.5 and 2 months, respectively. Plasma was collected from 6 of the TX women during the last trimester of pregnancy, from 5 during early lactation, and from 5 after cessation of lactation. The milk and plasma samples were immediately frozen.

All 6 patients had been treated for thyroid carcinoma with total thyroidectomy and 2 of them also with 131I for extinction. After treatment, thyroid function was nil or almost nil as evaluated by 131I-uptake in 5 patients. One of the 2 patients who got an extinction dose of 131I after total thyroidectomy for follicular cancer, was not checked by 131I-uptake afterwards. All patients were euthyroid under thyroxine substitution. Because of post-operative hypoparathyroidism, replacement was given with dihydrotachysterol or calcium to 2 patients.

For patients with medullary thyroid carcinoma (MTC), calcitonin production by the tumour cells must be excluded. Three such patients participated in a screening protocol for early diagnosis and treatment of hereditary MTC (multiple endocrine neoplasia type 2). At operation these patients had tumours of 3–15 mm in diameter and no metastases. During a follow-up time of 12, 10 and 6 years, respectively, there were no measurable plasma iCT levels in the annual examination even after stimulation with pentagastrin (Peptavlon®, ICI, 0.6 μg/kg iv). As there was no remaining tumour detectable, any rise in plasma CT during pregnancy and lactation must come from another source than the MTC.

This study was approved by the Committee of Ethics of the Karolinska Hospital.

Radioimmunoassay of iCT

iCT in milk and plasma was determined with a RIA earlier described in detail (Bucht et al. 1985). Antiserum 1 was raised in rabbits and was directed against the 17–32 sequence of human calcitonin (hCT). Synthetic hCT (Peninsula Lab Inc, San Carlos, CA, USA) was used as standard and for radiiodination. The detection limit of the assay was 8 pg/ml.

In addition, in this study a new antiserum (2) raised in rabbits against synthetic hCT (cibacalcin®) coupled to bovine serum albumin (BSA) was used. The equilibrium constant was $0.9 \times 10^{10}$ l/mol and the CT binding capacity of the antiserum in a dilution of 1:8000 was 1.2 $\times 10^{10}$ mol/l, as defined by Scatchard plot analysis of results of an equilibrium assay. The antigenic region specificity was investigated with six different fragments of the human CT molecule (Fig. 1). The 11–32 fragment was needed for complete competition with [131I]CT, but a considerable binding also occurred to the 17–32 and 24–32 fragments. The detection limit with this antiserum was 60 pg/ml. There was no significant cross-reactivity with salmon CT (Miacalc®), TSH, insulin, PDN-21 or PTH.

Immunoextraction

iCT was extracted from milk from 3 of the 6 TX mothers and one of the control women with CT antiserum (1) coupled to a CNBr activated Sepharose 4B column as previously described for plasma (Bucht et al. 1985). Plasma from one of the TX mothers during pregnancy, and a pool of plasma collected from 3 of the mothers during pregnancy and lactation were immuno-extracted in the same way. The recovery was 80% with synthetic hCT.

Gel chromatography

After immunoextraction, iCT from milk and plasma was gel chromatographed on Sephadex G-75 (115 x 1 cm). Immunoextracted milk iCT from the control woman mentioned above was treated with 8 mol/l urea for 24 h at 4°C and in that solution applied to a Sephadex G-75 (80 x 2 cm) column. The columns were equilibrated and developed with 0.1 mol/l ammonium-acetate, pH 6.8 with 0.01% BSA. The fractions were lyophilized and reconstituted with assay buffer: 0.05 M phosphate buffer pH 7.4, with 0.02% BSA, 0.02% sodium azide, and one ampoule of Trasylol® per l. iCT was determined in the fractions with antiserum 1 in all chromatograms. iCT was also measured with antiserum 2 in one of the chromatograms of extracted milk from a TX mother and in the chromatogram with the pooled extracted plasma.

Results

iCT in milk

At dilutions of the milk of 1:4 or more, the iCT concentrations were in TX women 713 ± 307 pg-eq/ml (mean ± SD, N = 7, median value 736 pg-eq/ml, range 236–1216) and in control women 772 ± 329 pg-eq/ml (N = 33, median value 676 pg-eq/ml, range 212–1244) during the first 5
Fig. 1.
Cross-reactivity of fragments of human synthetic calcitonin with antiserum 2 in the radioimmunoassay. 
B = number of CPM in the presence of standard or unknown hormone; B₀ = number of CPM in the absence of unlabelled hormone.

Fig. 2.
Levels of immunoreactive calcitonin in human milk from a normal mother during 2 months (○) and a TX mother during 2½ months (●).
Fig. 3.
Gel chromatography on a Sephadex G-75 column (115 x 1 cm) after immunoextraction of A: the first produced milk from a TX mother. In the fractions, iCT is determined with antiserum 1 (—•—•) and antiserum 2 (---0). B: Milk delivered after 4 weeks. iCT is determined with antiserum A. Markers: dextran blue (V₀), ovalbumin (mol wt: 43 000) (I), cytochrome C (mol wt: 12 400) (II), [¹²⁵I]CT (III), and sodium chloride (Vₛ). The broken lines indicate the detection limits of the different assays.

Fig. 4.
Gel chromatography on Sephadex G-75 (80 x 2 cm) of immunoextracted milk iCT treated with 8 mol/l Urea. iCT was determined in the fractions with antiserum 1. For markers and broken line, see Fig. 3.

iCT in plasma
During the last trimester of pregnancy, plasma from the TX women contained 16 ± 6 pg-eq/ml (mean ± SD, N = 6, median value 13 pg-eq/ml,
Gel chromatography was obtained after cessation of lactation. The plasma after extraction contained 0.7 pg-eq/ml, which was a too low level for gel chromatography.

Discussion

iCT was present in milk from totally thyroidectomized (TX) woman in similar concentrations as in milk from healthy women. In both groups, the levels were highest in colostrum to decline during the first two weeks after delivery. This agrees with our earlier findings with a commercial RIA kit (INC, Stillwater, MN, USA) (Arver et al. 1984). However, the total output of milk iCT is probably increasing with lactation time, since the milk volume is multiplied about 40 times from day two after parturition to day 14 (Casey et al. 1985). We did not have the possibility of calculating the milk volumes.

Gel chromatography of immunoextracted CT from TX women disclosed a chromatographic pattern of iCT similar to that in healthy women. 

Unextracted iCT in milk is resistant to denaturing and reducing agents (Bucht et al. 1983). In this study, where iCT was immunoextracted, about 40% was converted with 8 M urea to monomeric CT. However, 60% still eluted with a M, of about 40000. The carboxy-terminal flanking peptide of the calcitonin precursor is present in immunoextracted CT in the fractions that contain iCT with an M, of 40000. This indicates that milk iCT contains precursor forms. Preprocalcitonin has a molecular weight of 14500 (Moullec et al. 1984). Further degradation experiments of milk iCT have to be done before forms larger than preprocalcitonin can be postulated.

The same antiserum (1) was used for both immunoextraction and RIA in the previous studies. Here, a new antiserum was raised in order to support the idea that CT is present in human milk. This antiserum (2) was directed against the midportion and the carboxy-terminal of CT. It measured the same iCT forms as the antiserum (1) used for immunoextraction, even if lower levels were obtained. Antiserum 2 needed the fragment 11–32 for maximal binding, whereas 17–32 was sufficient for 1. Difficulties in conformational re-arrangements with the longer fragment, resulting in a less good fit to the antiserum, may explain the lower levels with 2.

![Fig. 5. Gel chromatography on Sephadex G-75 (115 × 1 cm) of pooled (83 ml) immunoextracted plasma from 3 TX mothers during pregnancy and lactation. iCT was determined in the fractions with antiserum 1 (●—●) and 2 (○—○). For markers and broken line, see Fig. 3.](image)
In the present study, TX women seemed to have higher levels of iCT in blood during pregnancy and lactation than under normal conditions. This was previously demonstrated for healthy women (Stevenson et al. 1979; Woloszczuk et al. 1981). Our findings indicate that iCT produced during pregnancy and lactation is not derived from the thyroid. The placenta has been suggested as an extra source of iCT during pregnancy (Galán et al. 1984). However, this cannot be the case during lactation. Since the mammary gland seems to produce iCT in high concentrations during lactation, this could be a source of iCT in blood. Immunoextraction and gel chromatography of iCT from plasma in the TX women during pregnancy and lactation disclosed the presence of iCT eluting in the region of monomeric CT. These results were verified with the new antiserum (2). The demonstration that monomer-like CT is produced during pregnancy and lactation in TX women is also a novel finding. However, this result is not surprising since the presence of the biologically active form of CT may be of importance to protect the skeleton of the mother against excessive demineralization during pregnancy and lactation (Stevenson et al. 1979). The possible presence of monomeric CT in healthy women during pregnancy and lactation remains to be demonstrated.

iCT is known to exist in low concentrations in TX individuals (Silva et al. 1978; Body & Heath 1983). The source of this iCT is not known, but extrathyroidal iCT has been demonstrated to be produced in lung carcinoma (Edbrooke et al. 1985; Zajac et al. 1985). iCT is also present in seminal plasma in large forms (Bucht et al. 1986a), which contain the carboxy-terminal flanking peptide of the CT precursor.

In summary, similar high concentrations of high molecular weight forms of iCT were demonstrated in milk from TX women and healthy women. This confirms our earlier suggestion of locally produced CT. Monomer-like CT found in plasma from TX women during pregnancy and lactation also has to be of extrathyroidal origin.

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