MULTIPLE IMMUNOREACTIVE FORMS OF CALCITONIN IN HUMAN PLASMA

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SUMMARY. The nature of calcitonin in the plasma of nine patients with medullary carcinoma of the thyroid was evaluated by gel filtration on a column of Bio-Gel P-10 and radioimmunoassay of the fractions obtained. Four distinct peaks of immunoreactive calcitonin were found in each plasma; three were of higher molecular weight than monomer human calcitonin.

Calcitonin is a peptide hormone synthesized and secreted by the parafollicular cells of the thyroid in mammals. Although calcitonin appears to function in the regulation of extracellular calcium concentration in certain mammalian species, its function in man is uncertain. Efforts to define the physiological role of calcitonin in man have been hampered by difficulty in measuring the hormone in the circulation of normal subjects. At present, there is disagreement as to whether calcitonin can even be detected in unextracted normal plasma (1).

Berson and Yalow (2) first recognized that immunochemical heterogeneity of circulating peptide hormones might lead to differences in the absolute values of hormone detected by radioimmunoassays which use antisera directed against different regions of the peptide sequence. Since this phenomenon might explain the disparate results obtained by various laboratories in the measurement of calcitonin we decided to examine some of the properties of calcitonin in the plasma of patients with medullary carcinoma of the thyroid, a calcitonin-secreting tumor arising from the thyroid parafollicular cells.

MATERIALS AND METHODS. Plasma was obtained from nine patients with proven medullary carcinoma of the thyroid and stored at -20°C

until assay. Immunoreactive calcitonin in plasma and in column effluents was measured by radioimmunoassay utilizing a method previously reported (3) but with a different antiserum obtained after immunization of a rabbit with synthetic human calcitonin. The assay standard was the Medical Research Council human calcitonin standard (lot 70/234).

Plasma (2 ml) from each patient and 120,000 cpm of ^{125}I-labelled synthetic monomer human calcitonin were co-chromatographed in 0.02M phosphate, 0.12MNaCl, 0.01M disodium EDTA, 0.001% merthiolate and 0.2% crystalline egg albumin on a 0.9 x 60 cm column of Bio-Gel P-10 (Bio-Rad, Richmond, Calif.). 0.5 ml fractions were collected and radioactivity in each was measured prior to assay. In addition, plasma obtained from two patients with Paget's disease of bone after intravenous injections of synthetic monomer human calcitonin and synthetic monomer human calcitonin incubated for two hours at 25°C in normal human plasma were evaluated.

The column was calibrated with ^{125}I-labelled bovine parathyroid hormone (MW9500) and the dimer form of human calcitonin (MW6838), a peptide formed by disulfide linkages at the 1 and 7 cysteine residues of monomer human calcitonin (MW3419) (4). The elution position of the calcitonin dimer was also evaluated after incubation of the peptide in 10% mercaptoethanol at 25°C for two hours.

RESULTS AND DISCUSSION. Figure 1 illustrates an elution pattern of calcitonin immunoreactivity which is representative of that found in the plasma from each of the nine patients studied. In all plasma samples immunoreactive calcitonin eluted from the column in four distinct peaks (Table 1). Calcitonin obtained from each of the peaks was assayed in multiple dilutions and gave dose-response curves that were parallel to that of the standard human

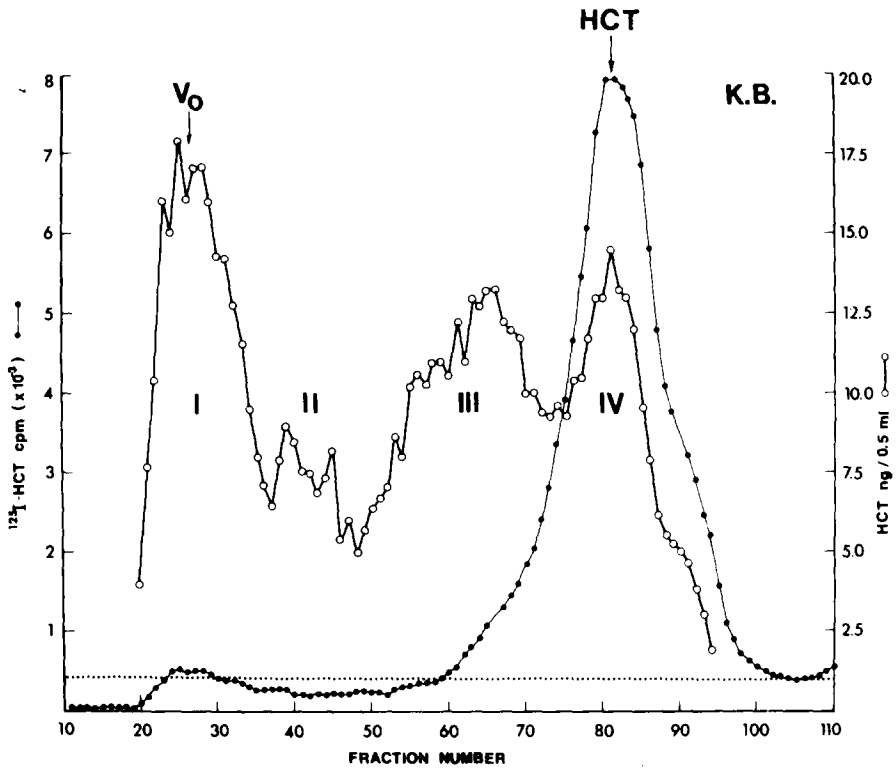

Figure 1. Gel filtration and radioimmunoassay of calcitonin in the plasma of a patient with medullary carcinoma of the thyroid. Arrows mark the void volume (Vo) of the column and the elution position of synthetic monomer human calcitonin (HCT). The detection limits of the assay are indicated by dotted lines.

calcitonin. Only 9.7 to 44% of the total immunoreactivity coincided with the elution of both ^{125}I-labelled and unlabelled monomer human calcitonin (peak IV). The remaining and major fraction of the calcitonin eluted in three peaks, all earlier than monomer calcitonin. When immunoreactive material in the fractions comprising the peaks was individually pooled and rechromatographed, greater than 90% of the immunoreactivity was recovered in the original elution volumes. Thus, the material in each peak eluted as a discrete molecular species. The immunoreactivity in the first peak (I) eluted in the void volume of the column (MW12,000 or greater), that in the second peak (II) appeared close to the elution vol-

Table I Concentration of immunoreactive calcitonin (HCT) in plasma of patients with medullary thyroid carcinoma and fractionation of calcitonin immunoreactivity by gel filtration

Patient	Plasma HCT (ng/ml)	Fractional distribution of immunoreactive calcitonin (%)* Peak I	Peak II	Peak III	Peak IV
1. K.B.	369.0	29.2	10.8	36.8	23.2
2. S.B.	325.6	22.1	15.6	32.1	30.2
3. R.F.	57.6	18.7	25.4	26.9	29
4. W.B.	36.4	20.3	6.4	30.3	43
5. S.M.	26.2	10.3	26	37.8	25.9
6. R.G.	25.8	14.5	36.8	27	21.7
7. W.R.	22.3	10.7	19.7	25.6	44
8. B.H.	20.3	18.6	22.2	29.4	29.8
9. L.M.	7.8	22.4	31.7	36.3	9.7

*Percentage of total immunoreactivity in aliquot of plasma eluted from Bio-gel column in fractions comprising peaks as represented in figure 1.

ume of bovine parathyroid hormone and the position of the third peak (III) was near to that of the dimer form of human calcitonin. The human calcitonin dimer has been found previously only in extracts of medullary carcinoma of the thyroid (4).

The possibility that the three peaks of immunoreactive calcitonin which elute earlier than monomer calcitonin (peak IV) consist of various aggregates of calcitonin appears unlikely since elution patterns similar to that shown in figure 1 were obtained when the plasma samples were chromatographed after incubation with the denaturing agent 6M guanidine. It is also unlikely that the peaks are an artefact of column chromatography since monomer human calcitonin incubated in normal human plasma eluted in a single peak coincident with peak IV of the patients' plasma samples. In addition, analyses of plasma obtained from the two patients with Paget's disease who received injections of synthetic monomer human calcitonin revealed that 99% of the hormone eluted in the position of the calcitonin monomer (peak IV), the remainder in peak I.

The observation that the immunoreactivity in peak III in the patients' plasma eluted from the column in a volume similar to that of dimer human calcitonin suggested that this immunoreactive peak might contain the calcitonin dimer. Since the amount of material which could be obtained from plasma was insufficient for direct chemical studies, the elution position of the material in peak III was re-examined after exposure to mercaptoethanol. After incubation of dimer human calcitonin in 10% mercaptoethanol, the elution of immunoreactive calcitonin shifted from the elution position of peak III to that of peak IV due to reduction of the disulfide bonds. An identical incubation of the immunoreactive calcitonin in peak III isolated by gel filtration of plasma from

two patients did not alter the elution position, indicating that the calcitonin in peak III was not dimer calcitonin.

The immunoreactive calcitonin eluting in the void volume (peak I) may be, at least in part, associated with plasma proteins since previous gel filtration and radioimmunoassay studies of calcitonin infused into dogs have indicated that a small fraction of the hormone binds to several plasma proteins (5). This probably explains the small amount of peak I calcitonin immunoreactivity found in the plasma of the two patients with Paget's disease after injection of calcitonin.

Preliminary data obtained after gel filtration of medullary thyroid carcinoma extracts and tissue culture media confirmed the existence of multiple forms of immunoreactive calcitonin. It seems reasonable to conclude that the early-eluting forms of calcitonin may be secreted directly by the thyroid carcinoma and not arise by alterations in the hormone that occur in the blood after secretion of monomer calcitonin.

Our results indicate that calcitonin circulates in a heterogeneous state as has been found for insulin (6), parathyroid hormone (7), gastrin (8), ACTH (9), and growth hormone (10). At present we do not understand the physiological significance of these apparently larger forms of calcitonin. The data, however, are compatible with the possibility that they may be precursor forms of the hormone or, perhaps, other abnormal hormonal molecules produced and secreted uniquely by the neoplastic tissue.

Although we have not yet tested the reactivity of the larger forms of human calcitonin with more than one antiserum to calcitonin, this demonstration of extensive heterogeneity of the hormone in blood (if present in normal subjects) may explain the current disagreement in assay results between different laborator-

ies each using a different antiserum. The various antisera could have widely different relative sensitivities for the detection of the various calcitonin forms, a situation encountered recently in radioimmunoassays of a higher molecular weight biosynthetic precursor of parathyroid hormone (11).

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