Identification and Measurement of Calcitonin Precursors in Serum of Patients with Malignant Diseases

Pascale P. Ghillani, Philippe Motté, Frédéric Troalen, Annick Jullienne, Paule Gardet, Thierry Le Chevalier, Philippe Rougier, Martin Schlumberger, Claude Bohuon, and Dominique Bellet

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ABSTRACT

Previous studies have suggested that molecular species larger than the mature calcitonin (CT) are produced by tumors of different origin. In order to study these species, we developed a monoclonal immunoradiometric assay for calcitonin precursors (CT-pr). This assay was based on both monoclonal antibody Kc01 directed to the 1-11 region of katacalcin and monoclonal antibody CT-08 directed to the 11-17 portion of CT. The sensitivity of this monoclonal immunoradiometric assay for CT-pr was <100 pg/ml. Only one of 131 healthy subjects had CT-pr serum levels >100 pg/ml; this value was therefore selected as the standard serum value in healthy individuals. CT-pr was present in the serum of seven of ten patients with metastatic disease and in that of 21 of 52 patients (40%) with benign liver disease but was undetectable in sera of patients with other benign diseases. The serum CT-pr level was correlated with that of mature CT in patients with medullary carcinoma of the thyroid. In contrast, the serum CT-pr level was frequently elevated in the absence of a detectable CT level in patients with various malignant tumors and, particularly, in those with either tumors of the neuroendocrine system (60%) or hepatocellular carcinomas (62%). CT-pr was detected in tumor extracts from a patient with a hepatocellular carcinoma. Moreover, hybridization experiments with total RNA extracted from this tumor demonstrated the presence of RNAs hybridizing with complementary DNA encoding for common region, calcitonin, and katacalcin sequences. These results show that CT precursors are excreted by numerous cancers and might well be useful biological markers for the follow-up of productive tumors.

INTRODUCTION

The hypocalcemic hormone CT is a 32-amino-acid-long polypeptide in its mature form which derives from the post-translational processing of a larger precursor in thyroid C-cells (1). The complete sequence of this precursor, designated preprocalcitonin, has been deduced on the basis of the nucleotide sequence of cloned cDNAs encoding the peptide (2). Within the preprocalcitonin, CT is linked to the KC sequence and preceded by an 84-amino-acid-long N-terminal region that terminates in a Lys–Arg cleavage signal. The biosynthesis of the hormone involves consecutive proteolytic events affecting preprocalcitonin: the signal peptide is removed as preprocalcitonin enters the endoplasmic reticulum to produce procalcitonin and the N-terminal region is then cleaved. The resulting 57-amino-acid-long polypeptide is composed of the CT sequence separated from the KC sequence (21 residues) by the Gly–Lys–Lys–Arg cleavage amiation site (3, 4). Finally, calcitonin is released by proteolysis, and the proline C-terminal residue is simultaneously amidated (5).

Neoplastic C-cells secrete large amounts of calcitonin in serum, and CT is used as a tumor-associated marker of MCT. Several groups have reported the heterogeneity of circulating forms of calcitonin and the presence of immunoreactive molecular species of higher molecular weight than that of monomeric CT, but the precise nature of these forms has not been clearly determined (6). However, immunoprecipitable calcitonin-specific precursor molecules have been demonstrated following cell-free translation from either rat and human tissues or cell lines (7–9). These data suggested that the heterogeneity of immunoreactive forms of serum CT as well as the ectopic secretion of calcitonin described with radioimmunoassays based on polyclonal antibodies might be due to the presence of circulating CT-pr.

This study was aimed at both identifying the presence and determining the level of CT precursors in serum from healthy individuals and patients with either benign or malignant diseases. We used a library of mAbs directed against distinct epitopes of the calcitonin or katacalcin molecule to develop two m-IRMAs for the specific identification and quantification of mature calcitonin or biosynthetic CT precursors. We report here the simultaneous presence of both CT and CT-pr in sera of patients with MCT. In contrast, the presence of CT-pr, usually in the absence of a detectable CT level, was found in sera of patients with malignant tumors of other origin.

MATERIALS AND METHODS

Subjects. We studied serum samples collected from a total of 876 individuals. The first time, 131 healthy individuals aged 20 to 60 yr and 30 pregnant women were evaluated as well as 101 patients with various benign diseases and 27 patients with MCT. The latter were divided into three different groups. A first group included untreated patients whose diagnosis of MCT was later confirmed (n = 5). A second group was composed of patients with no evidence of relapse and considered to be in complete remission (n = 19). This group was divided into two subgroups depending upon whether patients had (N+; n = 10) or did not have (N−; n = 9) lymph node involvement. Patients with recurrence of the disease (local neck recurrence or distant metastases; n = 7) were included in the third group. Patients whose sera were tested both before and after treatment or recurrence could be classified into two different groups. Moreover, pentagastrin stimulation tests were carried out in five patients with MCT and one healthy subject. After informed consent from each individual was obtained, the stimulation of CT secretion was performed by i.v. injection of pentagastrin (0.5 μg/kg; Peptavlon; I.C.I. Pharma, Cergy, France) and serum samples were collected before and 2 and 5 min after infusion.

Furthermore, serum samples from 587 patients with non-MCT malignant tumors of various origin were also studied. The presence of liver metastases was assessed by echographic examination. Finally, serial studies were carried out on samples collected from 3 subjects with SCLC.

After drawing, blood samples were rapidly separated by centrifugation for 15 min at 1500 x g, and sera were rapidly stored frozen until analysis. After thawing, samples were heat inactivated (30 min at 56°C) to prevent enzymatic degradation of calcitonin in the serum (10) and

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: CT, calcitonin; m-IRMA, monoclonal immunoradiometric assay; CT-pr, calcitonin precursor; mAb, monoclonal antibody; MCT, medullary carcinoma of the thyroid; cDNA, complementary DNA; KC, katacalcin; SCLC, small cell lung carcinoma; NSE, neuron-specific enolase.
assayed using different m-IRMAs. We have previously determined that heating the samples had no effect on immunoreactivity of either calcitonin or its precursors (data not shown).

Production and Characterization of Monoclonal Antibodies to Calcitonin or to Katacalcin. The production and characterization of monoclonal antibodies directed to either mature CT or KC have already been described (11, 12). Briefly, these antibodies were obtained after fusion of splenocytes from mice immunized with either CT or KC, both conjugated to tetanus toxoid. Two monoclonal anti-CT antibodies designated CT07 and CT08 were directed against distinct and separate epitopes. \((a)\) mAb CT07 (IgG2, \(K_{\text{ass}} = 0.9 \times 10^{10} \text{M}^{-1}\)) recognized an epitope located in the 26–32 region of mature CT and did not bind to procalcitonin (11). It was previously shown that CT07 bound to the 26–32 sequence bearing the C-terminal carboxamide group present on mature CT and did not bind to a similar sequence bearing a C-terminal carboxyl group. \((b)\) mAb CT08 (IgG1, \(K_{\text{ass}} = 3.0 \times 10^{9} \text{M}^{-1}\)) recognized an epitope present in the 11–17 portion of CT and which was also expressed on peptides mimicking the sequence of procalcitonin. A monoclonal anti-katacalcin antibody identified as KC01 has also been characterized (12). mAb KC01 (IgG1, \(K_{\text{ass}} = 1.5 \times 10^{9} \text{M}^{-1}\)) recognized an epitope localized to the 1–11 region of katacalcin and also bound its epitope within the precursor molecule. The antibody binding sites of these three antibodies are presented in Fig. 1.

Development of Monoclonal Immunoradiometric Assays for Either Mature Calcitonin or Calcitonin Precursors. Monoclonal antibodies CT07, CT08, and KC01 were utilized to construct two different monoclonal immunoradiometric assays developed in a “one-step” (simultaneous) format. One assay based on CT07 and CT08 antibodies (m-IRMA CT07-CT08) was used for the specific measurement of mature CT, while the other, based on KC01 and CT08 antibodies (m-IRMA KC01-CT08) was constructed for the specific quantification of CT-pr (12). Briefly, either CT07 or KC01 served as “capture antibody” and was linked to a solid phase support by incubating polystyrene beads (Polystyrene Plastic Balls, Chicago, IL) overnight at room temperature, the beads were washed with distilled water, and fractions were collected every 5 min. The unbound fractions were then incubated with the gel coupled with mAb CT07. This latter gel was also stirred 18 h at 4°C before packing into a column and collection of nonadsorbed material, as previously described. Bound material was eluted from both columns with 2.5% acetic acid, and fractions were collected every 5 min. After neutralization, 200 \(\mu\)l of all fractions diluted 1:1 in normal human serum were assayed by m-IRMA CT07-CT08 and m-IRMA KC01-CT08, respectively; the limit of detection of both assays was determined as previously described (12).

Preparation of Tissue Extract. Liver tumor and normal liver tissues were placed in liquid nitrogen immediately after resection and stored at \(-80^\circ\text{C}\) until time of extraction. The tissues were pulverized while still frozen and then homogenized with Polytron in phosphate-buffered saline containing 0.1% Aprotinin. After centrifugation (40,000 \(\times\) g for 30 min at 4°C), the supernatant was collected and assayed by both m-IRMA CT07-CT08 and m-IRMA CT-pr.

Affinity Chromatographies. Monoclonal antibody CT07 or KC01 (ascitic fluid) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. Then, both gels were equilibrated with a NaH$_2$PO$_4$ 0.05 M starting buffer (pH 8.5). Serum sample was incubated with the gel coupled with mAb KC01. After a rotating agitation for 18 h at 4°C, the gel was packed into a column; nonadsorbed material was eluted by extensive washing with distilled water, and fractions were collected every 5 min. The unbound fractions were then incubated with the gel coupled with mAb CT07. This latter gel was also stirred 18 h at 4°C before packing into a column and collection of nonadsorbed material, as previously described. Bound material was eluted from both columns with 2.5% acetic acid, and fractions were collected every 5 min. After neutralization, 200 \(\mu\)l of all fractions diluted 1:1 in normal human serum were assayed by m-IRMA CT07-CT08 and m-IRMA KC01-CT08. Fig. 2 shows the sequence of affinity chromatographies followed by specific assays of bound and unbound fractions.

Neuron-specific Enolase Measurement. NSE serum levels were determined using a commercial radioimmunoassay (Pharmacia, Uppsala, Sweden). This assay was performed according to the manufacturer’s instructions. The normal range of NSE serum levels observed by this technique is <12.5 ng/ml.

Extraction of Total RNA and Dot-Blot Hybridization. Total RNA was prepared by a modification of the method of Chomczynski and Sacchi (14), in which tissue was homogenized in guanidinium thiocyanate and then extracted with phenol and chloroform-isoamyl alcohol mixture, before isopropanol precipitations. Purification of RNA was achieved by LiCl precipitation. RNA was quantified by measurement of absorbance at 260 nm. Purified total RNA denatured in formaldehyde for 15 min at 60°C was spotted onto GeneScreen (New England Nuclear, Boston, MA). The paper was air dried and then baked at 80°C for 2 h. The paper was then prehybridized in a buffer containing 50% formamide for 3 h, after which the nick-translated radiolabeled probe was added for 18 h at 42°C. In this experiment, we have used a cDNA encoding for common region, calcitonin and katacalcin sequences. After incubation, the paper was twice washed in a buffer containing 0.15 M NaCl, 0.015 M sodium citrate, and 0.1% sodium dodecyl sulfate at 55°C for 30 min each. The filter was then autoradiographed.

RESULTS

All serum samples were tested in two different assays. In one, we measured CT levels with the CT07-CT08 m-IRMA, which
is specific for mature calcitonin and displays a limit of detection of 10 pg/ml. In the other, we measured CT-pr levels with KC01-CT08 m-IRMA, which is specific for biosynthetic CT precursors and has a limit of detection of 100 pg/ml.

In an attempt to estimate the normal range of serum CT-pr levels, we measured CT-pr values in serum samples collected from healthy individuals. We found that all but one subject had undetectable CT-pr serum levels (Fig. 3). The normal range was therefore defined as <100 pg/ml. We also studied CT-pr levels in sera of patients with various benign diseases. Seven of 10 patients with advanced renal failure and 40% (21 of 52) of those with benign liver disease had elevated CT-pr serum values, while CT-pr was undetectable in sera of other patients (Fig. 3). It was noteworthy that 2 of 10 patients with renal failure had simultaneous elevation of both CT and CT-pr values. In contrast, CT was undetectable in sera of those with benign liver disease. Indeed, we had previously found an elevation of serum CT in 33% of patients with renal failure, but none in those with benign liver disease (15). Thus, the elevation of CT-pr in sera of patients with benign liver disease appears to be independent of secretion of mature calcitonin.

Identification and Measurement of CT-pr in Patients with MCT. We measured both CT and CT-pr serum levels in MCT patients either untreated or with a residual tumor burden. These patients had CT levels in the range of 12 to 220,000 pg/ml. In those sera, CT-pr serum levels were consistently detectable and ranged from 112 pg/ml to 6.8 μg/ml. As shown by a regression curve, CT and CT-pr levels were correlated (r = 0.97), and it was estimated from this curve that CT-pr values were 11 times as high as CT values (Fig. 4). MCT patients in complete clinical remission, with no residual tumor burden and a serum CT level <10 pg/ml, had undetectable CT-pr levels, as did patients who underwent total thyroidectomy. Taken together, these results strongly suggested that neoplastic C-cells secrete calcitonin precursors as well as mature calcitonin.

Furthermore, we investigated the effect of pentagastrin on CT and CT-pr secretion. For this purpose, sera from one healthy subject and from 5 MCT patients were evaluated before and 2 and 5 min after injection of pentagastrin. The healthy individual had undetectable CT and CT-pr levels during the stimulation test. In contrast, the five MCT patients had an elevated basal serum CT level (range, 19 to 1,180 pg/ml) and displayed a positive response to pentagastrin stimulation. The peak CT values ranged from 207 to 35,700 pg/ml and the peak/basal ratios were higher than 2 (mean ± SEM = 14.4 ± 7.4). Interestingly, the peak CT-pr values ranged from 189 to 91,800 pg/ml, but the peak/basal ratios of CT-pr were consistently lower than those of calcitonin (mean ± SEM 3.6 ± 1.7) (Table 1).

Using sequential affinity chromatography with either mAb KC01 or mAb CT07 linked to a CNBr-activated Sepharose column, we separated biosynthetic CT precursors from CT in serum of a given MCT patient whose initial serum levels of CT and CT-pr, determined by specific m-IRMAs, were 10,130 pg/ml and 3,000 ng/ml, respectively. The serum was first laid onto the KC01 affinity column. After elution of unbound and then bound material, m-IRMAs performed on fractions demonstrated that the unbound material showed CT immunoreactivity (Fig. 5, A and B), while the bound material collected after Fraction 10 displayed the peak of CT-pr immunoreactivity (Fig. 5, C and D). CT immunoreactive fractions (A) were then laid onto the CT07 affinity column. Assays performed on both unbound and bound fractions after elution demonstrated both the absence of significant CT-pr immunoreactivity in those fractions and the presence of a CT immunoreactive peak in the bound fractions collected after elution (Fig. 5, E to H).

Measurement of CT-pr in Various Non-MCT Malignant Tumors. After identification and measurement of biosynthetic calcitonin precursors in sera of patients with MCT, we extended the clinical study to non-MCT malignant tumors. First, we performed assays in sera of patients with tumors deriving from the neuroendocrine system, as does MCT. For this purpose, and in line with the Amine Precursor Uptake and Decarboxylation concept (16, 17), we tested sera from patients with either insulinoma, neuroblastoma, carcinoid tumors, malignant pheochromocytoma, small cell lung carcinoma, or nonspecific apudoma. These tumors are known for their neural crest origin and their potentiality for multiple peptide secretion (18). In particular, such malignancies have been described as producing neuropeptides (19, 20). We found that 7% of such patients had simultaneous elevation of CT and CT-pr levels, whereas 53% of patients had a detectable CT-pr level in the absence of a detectable CT value (Fig. 6). As 60% of patients with SCLC had elevated CT-pr serum levels, while only 6% had simultaneous elevation of the CT level, we studied whether or not the CT-pr level was increased in lung cancers of other histological

![Fig. 3. CT-pr serum levels in healthy subjects, pregnant women, and patients with benign diseases. □, 10 cases; ●, ○, 1 case; ○, patient with detectable CT. Numbers in parentheses, serum level of CT. The percentage represents the incidence of detectable CT-pr serum level in each group of patients.](image-url)
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Fig. 4. CT and CT-pr serum levels in patients with medullary carcinoma of the thyroid.

Table 1  Effect of pentagastrin stimulation on CT and CT-pr secretion

<table>
<thead>
<tr>
<th>Patients</th>
<th>CT (pg/ml) at the following times (min)</th>
<th>CT-pr (pg/ml) at the following times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>&lt;10&lt;sup&gt;*&lt;/sup&gt; &lt;10 &lt;10 &lt;100 &lt;100 &lt;100</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>19 207 143 100 123 189</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>30 504 433 181 451 272</td>
<td></td>
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<tr>
<td>D</td>
<td>90 1,690 973 1,100 5,600 3,800</td>
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<tr>
<td>E</td>
<td>486 1,775 2,251 1,910 3,400 4,300</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1,180 35,700 11,600 12,000 68,000 91,000</td>
<td></td>
</tr>
</tbody>
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* Serum levels measured before and 2 and 5 min after pentagastrin injection.

Fig. 5. Pattern of CT and CT-pr immunoreactivities in serum samples from a patient with MCT, as revealed by specific assays for either CT or CT-pr after a sequence of affinity chromatographies. A to H refer to the fractions shown in Fig. 2.

Fig. 6. CT-pr serum levels in patients with tumors of the neuroendocrine system. 10 cases; O, +, 1 case; O, patient with detectable CT level (value indicated in parentheses); +, patient with liver metastases. The percentage represents the incidence of detectable CT-pr serum level in each group of patients.

Fig. 7. CT-pr serum levels in patients with lung tumors. 10 cases; O, +, 1 case; O, patient with detectable CT level (value indicated in parentheses); +, patient with liver metastases. The percentage represents the incidence of detectable CT-pr serum level in each group of patients.

Fig. 8. Pattern of CT and CT-pr immunoreactivities in serum samples from a patient with MCT, as revealed by specific assays for either CT or CT-pr after a sequence of affinity chromatographies. A to H refer to the fractions shown in Fig. 2.

Types, namely, squamous cell carcinoma, large cell carcinoma, adenocarcinoma, and undifferentiated carcinoma. Results presented in Fig. 7 show that the percentage of those patients with an elevated CT-pr level ranged from 17.5% (squamous cell carcinoma) to 53% (large cell carcinoma) and that only 2% of patients with non-SCLC lung tumors had simultaneous elevation of the serum CT level. We also determined CT-pr serum levels in patients with various malignancies (Fig. 8). While CT-pr was undetectable in differentiated carcinoma of the thyroid, the percentage of positivity in the sera of other patients varied...
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widely, ranging from 7% (breast tumor) to 62% (hepatocellular carcinoma) depending on the cell type and stage of the tumor. Moreover, it was noteworthy that only 2 of these 298 patients had detectable levels of CT. Finally, we tested samples of patients with MCT and non-MCT malignant tumors at multiple dilutions to determine whether reactivity in the m-IRMA KC01-CT08 was comparable (parallel) to that of standard. Fig. 9 shows that the dose-response curve of the standard (PTN47) was parallel to those displayed by various serum samples of patients with MCT and non-MCT tumors taken as representative.

Measurement of CT and CT-pr Gene Transcript in a Hepatocellular Carcinoma. To confirm the production of CT-pr by a non-MCT tumor, we studied the presence of CT-pr at the tumor level. We prepared tumor extracts from both a normal liver tissue and a liver carcinoma of a patient displaying a serum CT-pr level of 11,000 pg/ml. Using the m-IRMA KC01-CT08, we did not find any significant binding in the extract of normal liver tissue, while we found a level of 28.2 pg/mg of tissue in the extract of liver carcinoma. Moreover, we performed hybridization experiments on the same tumor to detect CT-related RNAs. As a positive control in this experiment, we spotted an equivalent amount (25 μg) of total RNA isolated from a human MCT. As a negative control, we spotted 25 μg of yeast RNA. We saw more hybridization of the radiolabeled CT probe to tumor than to normal liver RNAs (data not shown).

Serial Studies in Patients with Small Cell Lung Carcinoma. As patients with SCLC were those with the highest incidence of detectable CT-pr levels among patients with lung cancers (60%), we performed serial studies of CT-pr levels to evaluate the usefulness of CT precursors for the follow-up of these tumors. Simultaneously, we determined the serum levels of both CT and NSE, since the latter parameter has been described as a useful marker for follow-up of patients with SCLC (21). These studies were carried out in 3 patients during the course of their disease. In one patient, CT-pr levels remained consistently elevated, whereas both CT and NSE levels were undetectable during the clinical disease-free period. In fact, a slight decrease in CT-pr levels during the first 2 mo of treatment was followed by a regular increase in levels up until evidence of liver metastases by echographic examination. Similar results were observed in the other two patients, in whom elevation of CT-pr levels preceded clinical or echographic evidence of recurring disease by 3 to 10 mo. However, in one of these patients, CT levels remained undetectable, while NSE and CT-pr levels increased simultaneously; in the other patient, both NSE and CT remained undetectable, while CT-pr levels increased.

DISCUSSION

Polyclonal antibodies directed to peptide hormones such as somatostatin, vasoactive intestinal polypeptide, and calcitonin appear to recognize heterogeneous immunoreactive molecules produced by various malignant tumors (22). For example, 59% of tumor tissues collected at random contain immunoreactive CT (18). Among these immunoreactive molecules are molecular species of higher molecular weight than those of native hormones. Previous studies suggested that such species might well be precursor forms of these hormones (9). Hitherto, radioimmunoassays based on polyclonal antibodies were not capable of determining the precise nature of these forms. Recently, it was demonstrated that m-IRMA s enable the distinction of closely related gene products as well as the direct measurement of hormone precursors (12, 23). In fact, m-IRMA CT07-CT08 is an assay specific for mature CT, since antibody CT07 has the unique property of recognizing the 26–32 portion bearing a carboxamide function on its C-terminal amino acid residue as found on mature CT. The specificity of this assay was confirmed by the absence of reactivity of both CT-pr and KC in the m-IRMA KC01-CT08. In contrast, the construction of m-IRMA KC01-CT08 based on one mAb directed to KC and the other one to CT enables the specific recognition of CT-pr. The specificity of this assay was confirmed by the lack of reactivity of both CT and KC in the m-IRMA KC01-CT08. We used such
m-IRMAs to measure CT precursors separately from mature CT. Our results confirm the heterogeneity of immunoreactive CT in serum and demonstrate the presence of circulating CT precursors in patients with either benign or malignant diseases. Elevated values of up to 50,000 pg/ml were detected in 40% and 70% of patients with benign liver disease and advanced renal failure, respectively, while all except one healthy subject had an undetectable serum CT-pr level (<100 pg/ml). The presence of CT-pr in the blood of patients with renal failure might be due to delayed clearance of CT precursors; such a mechanism had already been suggested by the finding of mature CT in patients with advanced renal failure (15, 24). In contrast, the isolated presence of CT-pr in 40% of patients with benign liver diseases such as hepatitis or cirrhosis, although CT is consistently undetectable in such patients, might be due to an ectopic production of precursors by hepatic cells as well as to a defect in the hepatic metabolism of these molecules.

Based on sera from patients with MCT, we established that neoplastic C-cells released incompletely processed CT precursors in addition to mature CT. It was noteworthy that, in those patients, CT-pr levels were correlated with CT levels, with the former value being approximately 11 times higher than the latter. This observation does not favor the hypothesis of alterations in specific mechanisms of CT precursor processing in those neoplastic C-cells. Such alterations would lead to marked variations in the ratios of precursors to mature hormones, as observed in other tumor tissues containing prohormones and hormones, for example, gastrinomas producing progastrin and gastrin (25). Moreover, it was striking that, after pentagastrin injection, the peak/basal ratio of CT-pr was lower than that of CT, suggesting that the reserve of precursor forms was smaller than that of mature CT in secretory granules of neoplastic C-cells. Previous studies performed by gel filtration separation followed by m-IRMA KC01-CT08 on sera of patients with MCT demonstrated the presence of immunoreactive products with molecular weights of 14,000 and 8,000. Molecular weights of these products were consistent with the hypothesis that the KC01-CT08 combination binds to procalcitonin and to intermediates of biosynthesis which include the CT sequence linked to the KC sequence (12).

In contrast to MCT tumors which produce both precursors and mature hormones, non-MCT tumors, in their large majority, appeared to secrete CT precursors in the absence of mature CT. It was noteworthy that the dose-response curves observed with multiple dilutions of serum of patients with MCT and non-MCT tumors were parallel to that of standards. This observation strongly suggests that m-IRMA KC01-CT08 detects molecular species in sera comparable to the standard. Rises in serum CT-pr level in non-MCT patients were consistent with previously reported ectopic secretion of immunoreactive CT (18, 26). This production of immunoreactive hormone was found with radioimmunoassays which measured any species reacting with a given polyclonal anti-CT antibody. Thus, it is likely that a large number of tumors previously described as producing "immunoreactive CT" do, in fact, produce CT precursors. In order to confirm the production of CT-pr by non-MCT tumors, we performed experiments with a liver carcinoma from a patient displaying a detectable serum CT-pr level in the absence of a detectable CT level. The finding of immunoreactivity on the tumor extract with the m-IRMA KC01-CT08 strongly suggests that the material measured in serum of this patient was produced by its tumor. Moreover, we found CT-related RNAs in this tumor. This observation shows that the tumor was transcribing the calcitonin gene. All together, these latter results are consistent with the production and the excretion of CT precursors detected in serum by the tumor of this patient.

Other studies suggested that many cancers are associated with ectopic hormone elaboration, but only a portion elaborate biologically active hormones and produce clinically recognizable syndromes (27). Our data demonstrate that, at least in the case of the calcitonin hormone, many cancers are associated with ectopic hormone precursor elaboration. The absence of both a detectable circulating CT level and a clinically recognizable ectopic CT syndrome might be due to the lack of enzymes capable of metabolizing CT precursors to bioactive CT. Alternatively, the absence of complete processing of CT precursors in cancer patients might result from structural differences between CT-pr present in their blood and precursors elaborated in normal thyroid C-cells; modification in the biochemical structure would prevent the proteolytic cleavage of precursor molecules. Such alterations have been shown in the small cell lung carcinoma cell line DMS53, which biosynthesizes CT precursors. In these cells, glycosylation of CT precursors is not a feature of posttranslational processing, and the lack of glycosylation seen in DMS53 cells might explain why apparently identical intracellular and extracellular precursors are detectable and perhaps why mature forms are only rarely present (9).

Among the cancer patients with detectable CT-pr levels in their blood, the highest incidence (62%) was found in patients with hepatocellular carcinomas. Interestingly, 80% of patients with hepatocellular carcinomas, as well as 10 to 40% of patients with benign liver diseases, produced α-fetoprotein (28). Thus, patients with either benign or malignant liver diseases have the capacity to produce molecules as different as oncofetal protein or hormone precursors. A variety of substances, including several peptide hormones such as calcitonin, were found to be present in serum from patients with lung cancers, particularly SCLC (29, 30). Biosynthesis of high-molecular-weight calcitonin by human small cell carcinoma cells in tissue cultures was reported by several groups (9, 31, 32). We tested patients with lung cancers of different histopathological types for the presence of CT and/or CT-pr in their blood and found the highest incidence (60%) of detectable CT-pr levels in patients with SCLC. Surprisingly, 53% of patients with large cell carcinomas also displayed an elevation in CT-pr serum levels. It is noteworthy that these latter lung tumors do not belong to the neuroendocrine system as do SCLC, MCT, and other tumors derived from Amine Precursor Uptake and Decarboxylation cells, a significant percentage of which produce CT-pr. However, the histological typing of lung cancers has often been found to be difficult, and the histology of such cancers as well as their capacity to produce CT-pr might change after therapy, mutation, or new oncogene expression (33). Alternatively, the production of CT-pr by numerous lung tumors of various types might be explained by the existence of a common stem cell for all cell types; the existence of such stem cells has been suggested (34), and tumors deriving from these cells might have the common capacity to produce CT-pr.

Since 60% of patients with SCLC had detectable CT-pr levels, we performed serial measurements of CT precursors to evaluate the usefulness of CT-pr determination for the follow-up of patients with SCLC. Our preliminary data showed that CT-pr levels paralleled changes in the clinical status and tumor burden of these patients. Interestingly, the rise in CT-pr levels preceded evidence of tumor recurrence by several months. None of several biological markers previously proposed as indicators of either the extent of disease or the clinical response to cytotoxic therapy...
appeared sensitive or specific enough for general use in the management of patients with SCLC. In light of our data, the measurement of CT-pr might be useful for the follow-up of these tumors.

In conclusion, our study demonstrates that numerous patients with malignant tumors and, in particular, patients with tumors deriving from neuroendocrine cells had detectable levels of CT. Finally, the development of m-IRMAs for different hormone precursors is important for measuring molecules that might be useful as tumor-associated markers and for understanding the processing of these precursors in cancer cells.

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