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Demonstration of Immunoreactive Calcitonin in Sera and Tissues of Lung Cancer Patients*

W. LUSTER,† C. GROPP,†‡ H. SOSTMANN,† H. KALBFLEISCH§ and K. HAVEMANN†
†*Department of Hematology and Oncology and* §*Department of Pathology, University of Marburg/Lahn, Federal Republic of Germany*

Abstract—*In sera of* 194 *patients with lung carcinoma, calcitonin was estimated by radioimmunoassay with an antibody against human calcitonin. Increased levels of calcitonin-immunoreactive protein were found in* 57% *of the patients with small cell carcinoma, in* 10% *of the patients with squamous carcinoma and in only* 2 *patients with large cell carcinoma. In patients with small cell carcinoma, serial determinations of calcitonin were accomplished during therapy. Significantly decreased calcitonin levels were found in patients who responded to therapy with cytostatics and X-ray. Increased calcitonin levels were measured from* 1 *to* 2 months *before clinical symptoms of a relapse were detectable. Investigations on the biochemical nature of this calcitonin-immunoreactive protein were made on both serum of lung cancer patients and tumor tissue. Evidence for the production of calcitonin-immunoreactive protein directly by the tumor was given by immune histology and by determinations of calcitonin in tumor tissue. Three protein fractions, which were immunoreactive with anti-human calcitonin, with molecular weights of about* 100,000, 48,000 *and* 20,000, *were separated by gel filtration. The two higher molecular weight fractions were degraded to molecular weights of about* 17,000 *and about* 3400—*the molecular weight of physiological calcitonin—by incubation with sodium dodecyl sulphate under reducing conditions. These results led to the conclusion that it may be possible to characterize a tumor-specific calcitonin precursor molecule; in addition to its use in monitoring therapy it may be useful in the differential diagnosis of small cell carcinoma.*

INTRODUCTION

THE ASSOCIATION of hormone excess by a tumor of non-endocrine origin has been documented for a wide range of peptide hormones [1–4]. Some recent reports have suggested that the peptide calcitonin can be detected as an 'ectopic hormone' in body fluids, especially in lung cancer patients. The first question raised was whether the tumor itself synthesized the hormone or if the tumor caused increased release of the hormone from its physiological source. Silva *et al.* [1] succeeded in performing an arteriovenous calcitonin gradient across the tumor bed. These results gave evidence that the high serum calcitonin level in lung cancer patients is tumor-derived. Moreover, Silva *et al.* [1] reported that production of calcitonin may be specific for small cell lung carcinoma.

For some ectopic hormones the existence of high molecular weight 'prohormones' have been described [5, 6]. Biosynthesis of a high molecular weight calcitonin by small carcinoma cells in culture was reported by Bertagna *et al.* [7]. In the present paper we report on a long-term study of calcitonin-immunoreactive protein in sera of patients with small cell lung carcinoma before, during and after therapy. Calcitonin biosynthesis by the tumor directly is evidenced by immunohistological methods. Moreover, the molecular nature of the calcitonin-immunoreactive protein in serum of tumor patients and fresh tumor tissue is examined.

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*Supported by the Deutsche Forschungsgemeinschaft.
‡Address requests for reprints to: C. Gropp, M.D., Medizinische Universitätsklinik, Mannkopffstraße 1, D-3550 Marburg/Lahn 1, Federal Republic of Germany.

MATERIAL AND METHODS

Patients

Serum samples from 194 patients with histologically proven lung cancer (according to the WHO classification) and from 54 control persons were assayed for calcitonin. Assays were performed before and in serial determinations during chemotherapy and radiotherapy. Prior to therapy the extent of the disease was evaluated by means of chest and bone roentgenograms, bronchoscopy and/or bronchomediastinoscopy, and liver and bone scans.

Tumor tissue pieces were obtained from 2 patients by autopsy not longer than 3 hr after death.

Assay for calcitonin

Calcitonin was determined by means of two commercial radioimmunoassays (Immuno Nuclear Corporation, Stillwater, MN and Byk Mallinckrodt, Dietzenbach, Federal Republic of Germany), as described previously [8].

Extraction of calcitonin from tissue material

Tumor tissue pieces were homogenized in 3 vols of distilled water or in distilled water containing the following proteinase inhibitors: 0.03 mg/ml phenylmethylsulfonyl fluoride, 0.03 mg/ml *p*-mercuric benzoate and 0.01 M EDTA, and centrifuged for 20 min at 20,000 *g*.

Gel filtration

Three-milliliter samples of serum or tissue extracts were subjected to gel filtration on a 1.5 × 90-cm column of AcA 54 (LKB Stockholm, Sweden), equilibrated with 80 mM KH_2PO_4, 0.4 M EDTA and 0.1% NaN_3, pH 7.4, or with the same buffer containing proteinase inhibitors. Protein was eluted in the presence of the above buffers under a flow rate of 8 ml/hr. Four-milliliter fractions were collected, lyophilized and resuspended in 400 µl of distilled water in order to estimate calcitonin. The column was calibrated with blue dextran (Pharmacia, Uppsala, Sweden), aldolase (Boehringer, Mannheim, Federal Republic of Germany), bovine serum albumin, ovalbumin, chymotrypsinogen A (Pharmacia, Uppsala, Sweden), myoglobin whale, cytochrome *c*, cyanocobalamin, bromophenol-blue (Serva, Heidelberg, Federal Republic of Germany) and [^{125}I]-calcitonin (Immunonuclear, Stillwater, MN).

Affinity chromatography

Whole serum, lyophilized pools of immunoreactive calcitonin obtained by gel filtration or synthetic human calcitonin were applied at 22°C to 1-ml columns of concanavalin A Sepharose (Pharmacia, Uppsala, Sweden) which were equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl and 0.02% NaN_3. The columns were washed with a 10-fold volume of the same buffer. For elution of specifically bound protein 1 ml of buffer containing 0.2 M methyl D-mannoside [9] was applied to the column. Exactly the same procedure was performed with protein A Sepharose CL-4B. Specifically bound protein was eluted with 0.1 M glycine–HCl, pH 3.0 [10].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to Weber and Osborn [11]. Fifty-microliter aliquots of lyophylized fractions obtained by gel filtration were run on 10% polyacrylamide gels. Electrophoresis was also carried out in parallel, in the absence and in the presence of 10 mM dithiothreitol. Gels were cut into 1-mm slices and each slice was shaken for 16 hr at 4°C in 0.2 ml of radioimmunoassay buffer prior to the calcitonin assay [12].

Immunofluorescence

Unfixed 6 to 8-µm thick frozen normal lung and tumor sections were air-dried for 20 min and then exposed to rabbit antiserum which had been diluted 1:50 to calcitonin. Normal rabbit IgG, as well as phosphate-buffered saline, pH 7.2, served as controls and showed no reaction on all specimen [13]. Antigen–antibody complexes were visualized with FITC-conjugated goat anti-rabbit immunoglobulin (Behringwerke AG, Marburg, Federal Republic of Germany). Readings were performed with a Zeiss Standard microscope with the filter combination BP 455, BP 490, FT 510, LP 520.

RESULTS

Calcitonin levels in serum of lung cancer patients during progression of disease

Calcitonin was determined by radioimmunoassay in sera of 135 patients with small cell lung carcinoma, in 40 patients with squamous lung carcinoma, in 19 patients with large cell carcinoma and in 54 sera from healthy persons. Levels of 30–120 pg calcitonin/ml were found in the sera of the control persons. In our study patients with calcitonin levels of more than 180 pg/ml were defined as patients with increased calcitonin. Of 135 patients with small cell carcinoma 76 (56%) showed elevated calcitonin levels. Five patients with squamous carcinoma and only 2 patients with large carcinoma showed slightly higher levels than normal (Fig. 1).

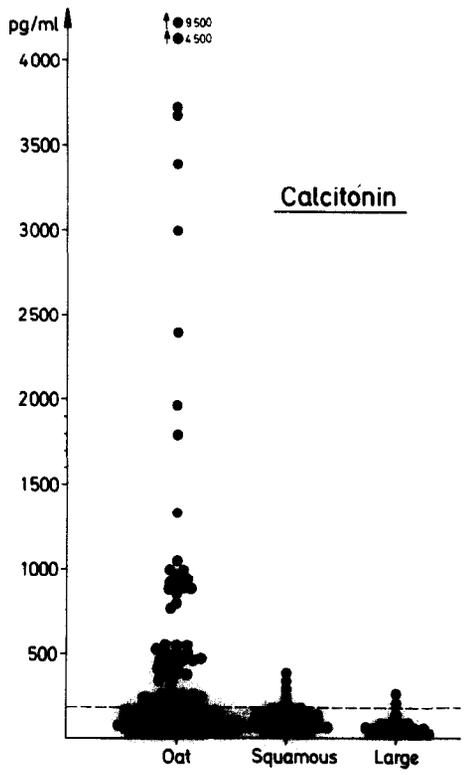

Fig. 1. Calcitonin values in patients with lung cancer at diagnosis.

Nevertheless, only one of all the examined patients exhibited a reduced calcium level. Patients with large cell and squamous carcinoma were no longer examined in this study.

Serial determinations of calcitonin were performed in patients with small cell carcinoma before and during X-ray therapy and chemotherapy. Patients who responded to therapy with tumor decrease showed a corresponding decrease of serum calcitonin in all cases. In patients who achieved a complete remission a decrease of calcitonin even down to normal values was measured (Fig. 2). Patients who did not respond to therapy as judged by clinical parameters did not show any significant change in their serum calcitonin levels (Fig. 2).

In patients who responded to therapy with a total tumor degradation and with a resumption of a normal serum calcitonin level the therapy was discontinued; however, calcitonin determinations were continued routinely. Subsequent increase of serum calcitonin in these patients indicated tumor progression 4–8 weeks earlier than was evidenced by clinical parameters.

Localisation of calcitonin immunoreactive protein in the tumor

In this step the aim was to confirm the opinion that immunoreactive calcitonin is produced directly by the tumor. In tumor cells intracellular and extracellular deposits reactive with rabbit anti-human calcitonin were demonstrated by immunofluorescence (Fig. 3); sections of normal lung tissue from the same patient reacted completely negatively to anti-calcitonin.

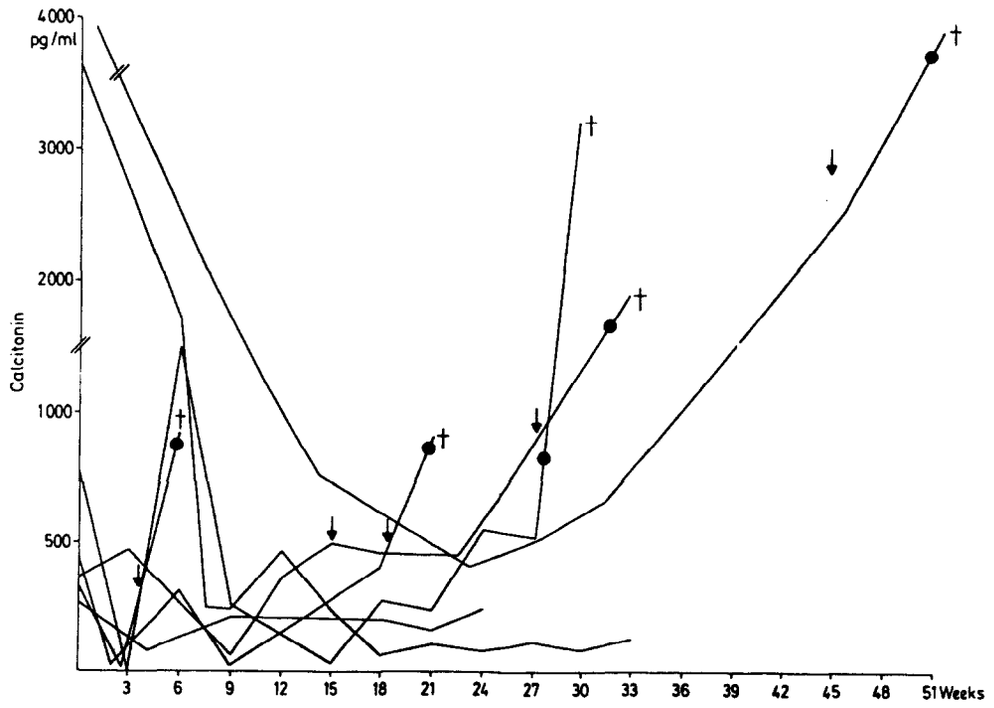

Fig. 2. Serial calcitonin determinations in lung cancer patients before and during therapy (↓ = clinical tumor progression, ● = end of therapy).

Studies on the tumor-derived calcitonin-immunoreactive molecule

The presence of three immunoreactive forms of calcitonin, with molecular weights of 100,000, 48,000 and 20,000, all larger than physiological calcitonin, were shown in sera of tumor patients by gel filtration of their total serum on AcA 54 (Fig. 4). To corroborate these results the same experiments were repeated on extracts of tumor tissue which were obtained by autopsy; here three high molecular weight calcitonin-like fractions could also be separated (Fig. 4). Quantitative comparison between gel filtration peaks from tumor tissue and from serum showed an increase of the lowest molecular weight calcitonin in serum accompanied by a decrease of the two higher molecular peaks (Fig. 4). Comparison of column profiles in the absence and in the presence of proteinase inhibitors did not show any difference. A protein with the exact molecular weight of physiological calcitonin was undetectable in both tissue and serum.

Interaction of physiological calcitonin with plasma proteins was excluded by incubation of calcitonin-free plasma with synthetic human calcitonin. Calcitonin was eluted from the AcA 54 column in one peak in the position of physiological calcitonin.

When the calcitonin level in sera decreased during therapy all serum calcitonin fractions decreased without any change in the ratio of the higher to lower molecular weight fractions. Gel filtration of sera from patients who were establishing a new tumor progression resulted in the same elution pattern of calcitonin-immunoreactive fractions than that obtained before therapy (Fig. 5). Whole sera, calcitonin fractions from AcA 54 columns as well as synthetic human calcitonin were subjected to affinity chromatography on protein A Sepharose. Calcitonin was not bound to the protein A. This result, together with the elution pattern from AcA 54 columns, gives evidence that high molecular weight calcitonin is not an aggregate of normal calcitonin with various immunoglobulin fractions. The same fractions were analyzed by affinity chromatography using concanavalin A Sepharose to detect glycoprotein content. No immunoreactive calcitonin was bound to concanavalin A. To differentiate between the various fractions they were incubated in the presence of sodium dodecyl sulphate and then separated by electrophoresis on 10% polyacrylamide gels. The two highest molecular weight serum fractions were no longer detectable after this treatment. Only two calcitonin-immunoreactive bands with molecular weights of 17,000 and 3400—similar to physiological calcitonin—appeared on the gels (Fig. 6). When the two higher molecular weight calcitonin fractions obtained by gel filtration of tumor extracts were subjected to electrophoresis in the presence of sodium dodecyl sulphate the same two low molecular weight bands were found. Electrophoresis of all of these calcitonin fractions under reducing conditions did not result in any significant change of their protein pattern.

DISCUSSION

Production of calcitonin by tumors was first described for C cell carcinoma of the thyroid gland by Riniker *et al.* [14]. This same phenomenon was demonstrated in patients with lung cancer by Silva *et al.* [1] some years later. These results have been confirmed by several authors [4, 7].

Hansen and Hummer [15] described an ectopic synthesis of calcitonin in 66% of 73 patients with small cell carcinoma, which corresponds to our results where immunoreactive serum calcitonin was found in 56% of 135 patients. Similar to the results of Milhaud *et al.* [16] and Hansen and Hummer [15], only a few patients with squamous or large cell carcinoma showed slightly increased serum calcitonin levels. High serum calcitonin seem to be typical of patients with small cell carcinoma. These findings are reinforced by the work of Roos *et al.* [17], who also gave evidence that their determinations of elevated calcitonin levels in sera of patients with epidermoid or anaplastic lung cancer may be artifactual.

Our results of the serial calcitonin determinations during and after therapy lead to the conclusion that serum calcitonin level may correlate with tumor biosynthetic activity. This would therefore suggest the usefulness of calcitonin measurements. Indeed, elevated values could indicate that tumor cells are still present, although this may not be apparent in light of the clinical data, and hence treatment should be continued. Serum calcitonin measurements may also be an indicator for the sensitivity of the tumor to one sort of therapy and continual high values may indicate that a change is necessary. Hansen *et al.* [18] found similar data for a smaller number of patients. In patients with normal pretreatment levels of calcitonin elevated levels were observed during therapy which were perhaps induced by the cytotoxicity of the drugs used. Hence in our hospital, only patients who had high serum calcitonin before treatment had their calcitonin values monitored during therapy.

As well as being a tumor marker, elevated calcitonin measurements may aid the diagnosis of small carcinoma. However, elevated levels may occur in other diseased states such as C cell carcinoma of the thyroid gland, phaeochromacytoma, renal

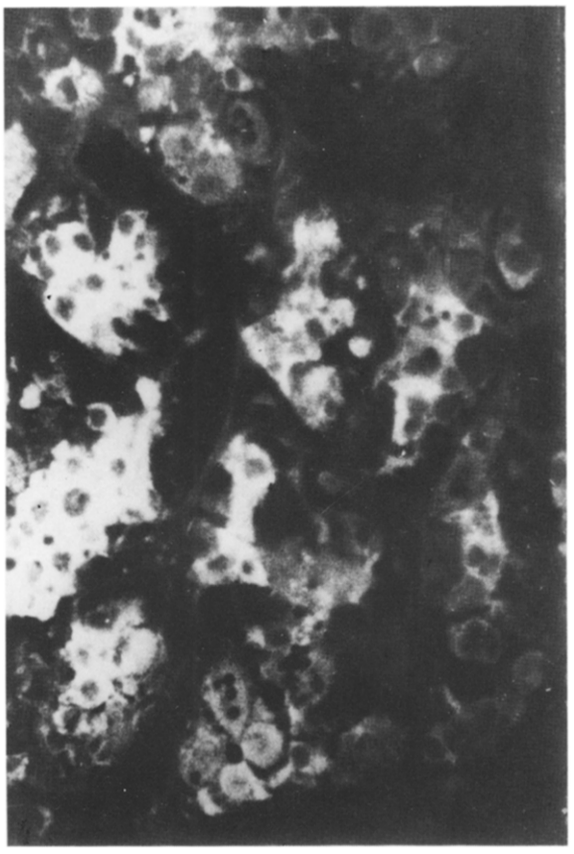

Fig. 3. Immunofluorescence staining of a tissue section from small cell carcinoma with an antiserum against normal calcitonin.

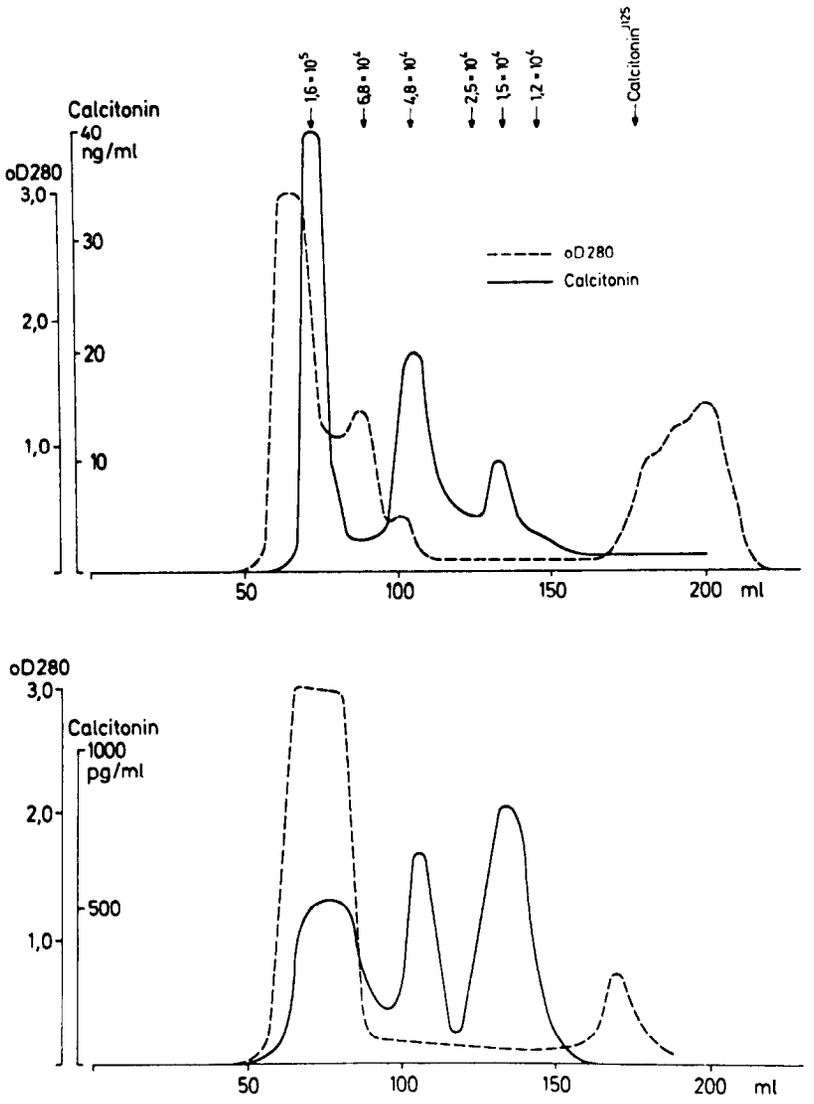

Fig. 4. AcA 54 column chromatography of one patient's serum (part below) and the tumor extract of the same patient (upper part). Fractions were assayed for calcitonin.

insufficiency and hypercalcaemia, although these raised levels are only greatly marked in C cell thyroid carcinoma and small cell lung carcinoma [19–21].

In lung tumor patients with high calcitonin levels normal serum calcium levels were measured, which give evidence that tumor calcitonin may be biologically inactive. Biochemical examination of the nature of the tumor calcitonin, presented in this paper, shows that the immunoreactive materials are indeed different from the physiological 1–32 calcitonin. Three molecules with a higher molecular weight than normal calcitonin were separated [22–24]. Non-specific aggregation of normal calcitonin with immunoglobulin fractions was excluded by affinity chromatography on protein A Sepharose. The existence of carbohydrate side-chains with α-D-mannosyl or sterically related residues in the calcitonin-immunoreactive molecules was excluded by affinity chromatography with concanavalin A. These results correspond to those of Goltzman and Tischler [12], but seem to contrast with those of Jacobs *et al.* [25]. This latter group were able to show a glycosylated translation product structurally related to calcitonin by immunoprecipitation with antiserum against synthetic calcitonin in a heterologous medullary thyroid mRNA cell-free system. However, the situation in small cell lung tumors *in vivo* may be quite different.

Incubation of the two highest molecular weight calcitonin fractions under reducing and non-reducing conditions in the presence of sodium dodecyl sulphate results in immunoreactive calcitonin with a molecular weight of 17,000 and 3500 molecular weight of normal calcitonin. Our results suggest that the two highest molecular weight calcitonin-immunoreactive fractions contain a 17,000-dalton protein which is relatively stable. The 3500-dalton calcitonin also formed on

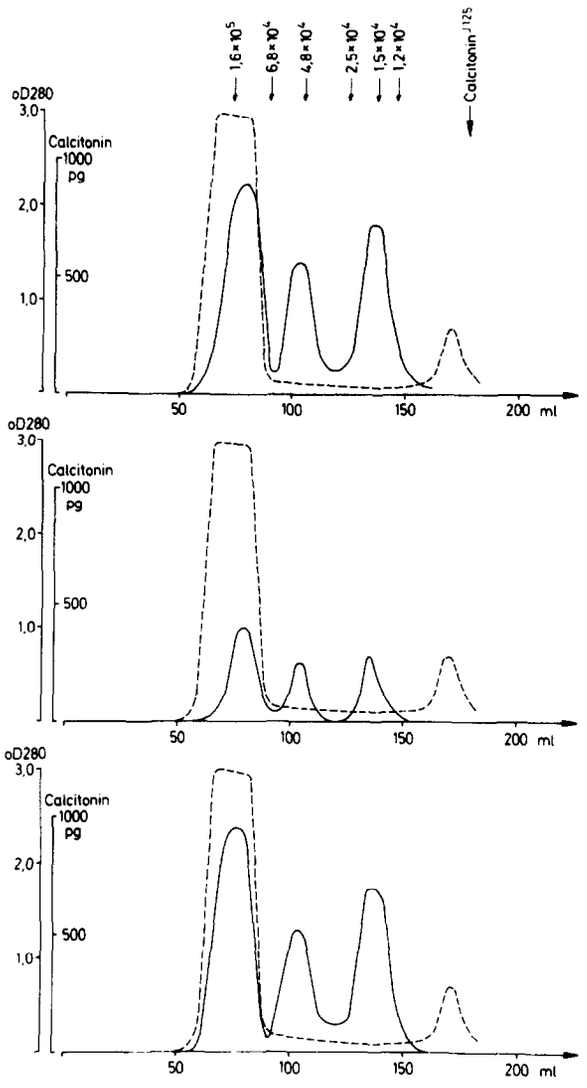

Fig. 5. AcA 54 column chromatography of one patient's serum before therapy (above), during therapy (middle) and at the time of tumor progression (below). Fractions were assayed for calcitonin. This result is representative for a number of six patients' sera.

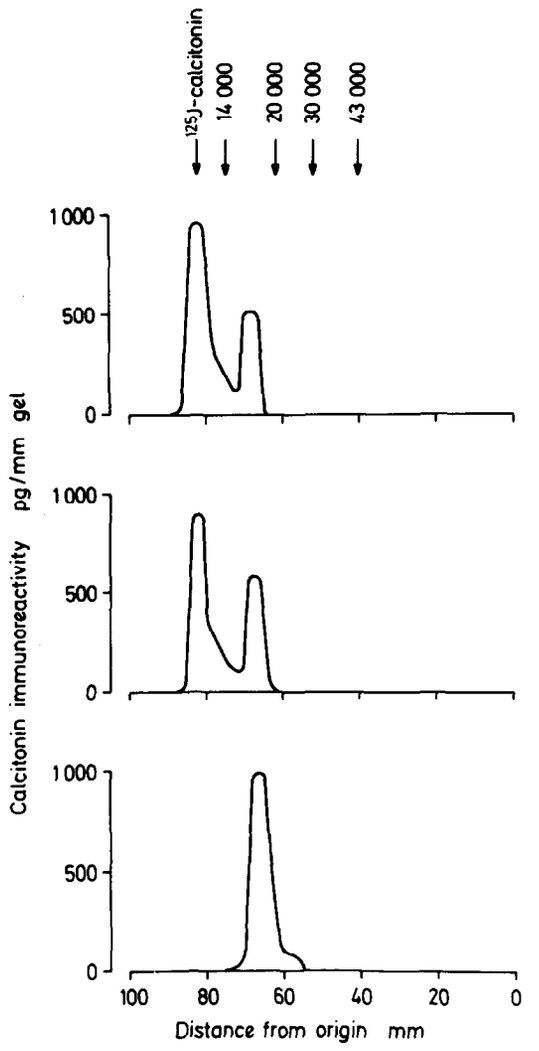

Fig. 6. SDS polyacrylamide gel electrophoresis of the three calcitonin fractions obtained by gel filtration from highest to lowest molecular weight (see Fig. 4). Gels were cut into 1-mm pieces and calcitonin was estimated. These findings are representative of 6 sera and 2 tumor extracts.

SDS gel electrophoresis is unlikely to be derived from the 17,000-dalton product and would therefore suggest the existence of more than one form of high molecular weight calcitonin.

Our results are supported by those of Jacobs *et al.* [25] and Desplan *et al.* [26], who show a calcitonin-immunoreactive translation product in the molecular range of 15,000–17,000. Moreover, they are able to isolate a 55,000-dalton calcitonin from medullary thyroid carcinoma tissue. Desplan *et al.* [26] suggest that it might be a product of the post-translational modifications of the precursor, perhaps involved in the formation of storage granules. Roos *et al.* [17] have shown calcitonin of molecular weights 16,000 and 8000 are synthesized by small cell lung tumors, the former being in good agreement with our 17,000 material.

In summary, the protein with the molecular weight of 17,000 seems to be a prohormone synthesized by small cell lung tumors. Development of a specific immunoassay against this calcitonin-immunoreactive tumor protein would be advantageous in the differential diagnosis of small cell lung carcinoma. At the moment measurements of this material could be used for defining remission and also for the early detection of a relapse [27, 28].

REFERENCES

1. Silva OL, Becker KL, Primack A, Doppman I, Snider RH. Ectopic section of calcitonin by oat-cell carcinoma. *N Engl J Med* 1974, **290**, 1122–1124.

2. HORAI T, NISHIHARA H, TATEISHI R, MATSUDA M, HATTORI S. Oat-cell carcinoma of the lung simultaneously producing ACTH and serotonin. *J Clin Endocrinol Metab* 1973, **37**, 212–219.
3. BERSON SA, YALOW RS. Parathyroid hormone in plasma in adenomatous hyperparathyroidism, uremia and bronchogenic carcinoma. *Science* 1966, **153**, 907–909.
4. ELLISON ML, WOODHOUSE D, HILLYARD C *et al.* Immunoreactive calcitonin production by human lung carcinoma cells in culture. *Br J Cancer* 1975, **32**, 373–379.
5. MAINS RE, EIPPER BA, LING N. Common precursor to corticotropins and endorphins. *Proc Natl Acad Sci USA* 1977, **74**, 3024–3022.
6. LIPS CJM, VAN DER SLUYS VEER J, VAN DER DONK JA, VAN DER DAM RH, HACKENG WHL. Common precursor molecule as origin for the ectopic-hormone-producing-tumor syndrome. *Lancet* 1978, **i**, 16–18.
7. BERTAGNA XY, NICHOLSON WE, PETTENGILL OS, SORENSON GD, MOUNT CD, ORTH DN. Ectopic production of high molecular weight calcitonin and corticotropin by human cell carcinoma cells in tissue culture. *J Clin Endocrinol Metab* 1978, **47**, 1390–1393.
8. GROPP C, HAVEMANN K, SCHEUER A. Ectopic hormones in lung cancer patients at diagnosis and during therapy. *Cancer* 1980, **46**, 347–354.
9. GOLDSTEIN IJ, HOLLERMAN CE, SMITH EE. Protein–carbohydrate interaction. II. Inhibition studies on the interaction of concavalin A with polysaccharides. *Biochemistry* 1965, **4**, 876–883.
10. HJELM H, HJELM K, SJÖQUIST J. Protein A from *Staphylococcus aureus*, its isolation by affinity chromatography and its use as immunosorbent for isolation of immunoglobulins. *FEBS Lett* 1972, **28**, 73–76.
11. WEBER K, OSBORN M. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* 1969, **244**, 4406–4412.
12. GOLTZMAN D, TISCHLER AS. Characterization of calcitonin released by medullary thyroid carcinoma in tissue culture. *J Clin Invest* 1978, **61**, 449–458.
13. GOLDSTEIN G, SLYZYS IS, CHASE MW. Studies on fluorescence with fluorescein-coupled sheep anti-rabbit globulin. *J Exp Med* 1961, **114**, 89–94.
14. RINIKER B, NEHER R, MAIER R *et al.* Menschliches Calcitonin. I. Isolierung und Charakterisierung. *Helv Clin Acta* 1968, **51**, 1738–1742.
15. HANSEN M, HUMMER M. Ectopic hormone production in small cell carcinoma. In: MUGGIA F, ROZENCWEIG M, eds. *Lung Cancer Progress in Therapeutic Research*. New York, Raven Press, 1979, 199–207.
16. MILHAUD G, CALMETTE C, TABOULET J, JULIENNE A, MOUKHTAR MS. Hypersecretion of calcitonin in neoplastic conditions. *Lancet* 1974, **i**, 462–463.
17. ROOS BA, LINDALL AW, BAYLIN SM *et al.* Plasma immunoreactive calcitonin in lung cancer. *J Clin Endocrinol Metab* 1980, **50**, 659–666.
18. HANSEN M, HUMMER M, HUMMER L. ACTH, ADH and calcitonin concentrations as markers of response and relapse in small cell carcinoma of the lung. *Cancer* 1980, **46**, 2062–2067.
19. MELVIN KEW, TASHJIAN AH, JR, MILLER HH. Studies in familial medullary thyroid carcinoma. *Recent Prog Horm Res* 1972, **28**, 399–470.
20. GOLTZMAN D, POTTS JT, JR, RIDGWAY ED, MALOOF F. Calcitonin as a tumor marker: use of the radioimmunoassay for calcitonin in the postoperative evaluation of patients with medullary thyroid carcinoma. *N Engl J Med* 1974, **290**, 1035–1039.
21. COOMBES RC, HILLYARD C, GRENNBERG PB, MACINTYRE I. Plasma immunoreactive calcitonin in patients with non-thyroid tumors. *Lancet* 1974, **i**, 1080–1083.
22. SIZEMORE GW, HEATH H. Immunochemical heterogeneity of calcitonin in plasma of patient with medullary thyroid carcinoma. *J Clin Invest* 1975, **55**, 1111–1118.
23. DEFTOS LJ, ROOS BA, BRONZENT D, PARTHEMORE JG. Immunochemical heterogeneity of calcitonin in plasma. *J Clin Endocrinol Metab* 1975, **40**, 409–412.
24. HAM J, ELLISON ML, LUMSDEN J. Tumor calcitonin, interaction with specific calcitonin receptors. *Biochem J* 1980, **190**, 545–550.
25. JACOBS JW, LUND PK, POTTS JT, JR, BELL NH, HABENER JF. Procalcitonin is a glycoprotein. *J Biol Chem* 1981, **256**, 2803–2807.
26. DESPLAN C, BENICOURT C, JULLIENNE A *et al.* Cell-free translation of mRNS coding for human and murine calcitonin. *FEBS Lett* 1980, **117**, 89–92.
27. ROOS BA, OKANO K, DEFTOS LJ. Evidence for a pro-calcitonin. *Biochem Biophys Res Commun* 1974, **60**, 1134–1140.
28. MOYA F, NIETO A, R-CANDELA JL. Calcitonin biosynthesis: evidence for a precursor. *Eur J Biochem* 1975, **55**, 407–413.