Purification and partial characterization of high-molecular-weight forms of ectopic calcitonin from a human bronchial carcinoma cell line

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Studies on the high-molecular-weight immunoreactive calcitonin produced ectopically in culture by an epidermoid bronchial carcinoma cell line are reported. In cell-exposed medium, the principal component has a molecular weight of 40 000 and molecules of mol.wts. 13 000 and 10 000 also occur. Only a trace amount of material co-eluting with 3500-mol.wt. human calcitonin is detectable. None of the calcitonins show cross-reactivity with anti-corticotropin serum. The 40 000-mol.wt. immunoreactive calcitonin is readily proteolysed to the 13 000- and 10 000-mol.wt. components, but the 10 000-mol.wt. component behaves as a comparatively stable 'core' molecule. By using immunoprecipitation and high-pressure liquid chromatography (h.p.l.c.), it is possible to prepare radiochemically homogeneous 10 000-mol.wt. immunoreactive calcitonin from cells grown in the presence of individual ³⁵S- or ³H-labelled amino acids. Peptide mapping of enzymic digests of this material by h.p.l.c. shows that it contains peptides in common with synthetic human calcitonin.

Calcitonin is a 32-amino-acid polypeptide hormone that is normally secreted by the C-cells of the thyroid in mammals (Potts, 1976). Because there are insufficient amounts of calcitonin present in normal human thyroid and serum, a detailed characterization has been made only of human calcitonin derived from medullary carcinoma of the thyroid (a neoplasm of the C-cells; Neher *et al.*, 1968*a,b*). Subsequent work showed that circulating immunoreactive calcitonin in patients with medullary carcinoma of the thyroid exhibited considerable and variable molecular-weight heterogeneity (Singer & Habener, 1974; Deftos *et al.*, 1975; Sizemore *et al.*, 1975). This heterogeneity was also observed in tumour extracts (Heath & Sizemore, 1979) and in culture medium exposed to thyroid medullary carcinoma cells (Lichtenberger & Singer, 1975; Goltzman & Tischler, 1978).

Immunoreactive calcitonin is also secreted ectopically by a number of non-thyroid tumours (Coombes *et al.*, 1974) and is notably common in cases of bronchial carcinoma (Silva *et al.*, 1974; Milhaud *et al.*, 1974; Hansen & Hummer, 1979). The molecular-weight distribution of lung tumour immunoreactive calcitonin has been studied in serum

(Snider *et al.*, 1977; Becker *et al.*, 1978) and in cell culture medium (Bertagna *et al.*, 1978). Becker *et al.* (1978) found two predominant molecular-weight forms (>30 000 and ~12 000–15 000) and commented that these could be degraded to smaller molecules on incubating the serum at room temperature; Bertagna *et al.* (1978) obtained mol.wts. of 14 000 and 7000 for immunoreactive calcitonin from a small-cell bronchial carcinoma cell line.

Ellison *et al.* (1975) established a BEN cell line (an epithelial cell line from an epidermoid bronchial carcinoma), which produced immunoreactive calcitonin *in vitro*. Radioimmunoassay studies suggested that the tumour-derived calcitonin was not identical with synthetic human calcitonin and a preliminary gel-filtration study of cell-exposed culture medium confirmed the presence of high-molecular-weight forms of immunoreactive calcitonin (Coombes *et al.*, 1976). Hunt *et al.* (1977) further showed that the BEN cell line had a calcitonin-responsive adenylate cyclase and that this was probably linked to specific calcitonin receptors on the cell surface (Martin *et al.*, 1976). The discovery that mild trypsin treatment of BEN-cell-line immunoreactive calcitonin converts it into a form with similar molecular weight to human calcitonin raises a possible parallel with the 'auto stimulation' hypothesis of tumour cell growth by

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

endogenously synthesized polypeptide factors (for review, see Shields, 1978).

In the present paper, we describe work that further characterizes the immunoreactive calcitonin from the BEN cell line in terms of its molecular weight and, by a combination of immunoprecipitation, trypsin digestion and fractionation by h.p.l.c., demonstrates the incorporation of radio-labelled amino acids into peptides that can also be proteolytically derived from synthetic human calcitonin.

Materials and methods

Cell culture

Cells were routinely cultured as described by Ham *et al.* (1980). For experiments involving the incorporation of [35 S]methionine, cells were depleted of endogenous methionine and cysteine by incubation for 24 h in 12.5 ml of 'depletion' medium, prepared from a Selectamine kit (Gibco), lacking these two amino acids but containing normal concentrations of all other components. Subsequently, the cells were transferred to 12.5 ml of the same medium, supplemented with unlabelled cysteine and [35 S]methionine (0.5 mCi at a specific radioactivity of 200 Ci/mol). A similar approach was used for labelling with [3 H]methionine (sp. radioactivity 4.9 Ci/mmol), [3 H]cysteine (sp. radioactivity 1.2 Ci/mmol) and [3 H]leucine (sp. radioactivity 1.0 Ci/mmol). Labelling medium was changed daily over a period of 4 days. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Processing of medium and cells

Initial processing of cell-exposed culture medium was by the 'mini-column' h.p.l.c. system previously described (Nice *et al.*, 1979; Ham *et al.*, 1980). In the case of radiolabelling experiments, medium was collected and processed daily by mini-column chromatography during the 4-day labelling period. Finally, all four samples were removed from storage (in liquid N₂), pooled and re-chromatographed before gel filtration or analytical h.p.l.c.

To obtain intracellular immunoreactive calcitonin, cells were harvested by scraping into extraction buffer (0.1 M-HCl, 20 μ g of pepstatin/ml) at 4°C. The cell suspension was transferred to a plastic universal bottle (Sterilin, Teddington, Middx., U.K.) in an ice bath and sonicated (4A) for four 15 s periods with 1 min cooling intervals. The sonicated material was clarified by centrifugation at 48000 g for 30 min at 4°C in a Sorvall SS34 rotor. The supernatant was then fractionated by using the mini-column as described above.

Radioimmunoassay

Calcitonin was assayed as described before (Ham

et al., 1980). H.p.l.c. buffer for the preparation of standard curves was supplemented with 0.5 mg of bovine serum albumin/ml. Corticotropin was assayed as described by Rees *et al.* (1971), except that the poly(ethylene glycol) separation method of Ham *et al.* (1980) was used. Standard corticotropin and 125 I-labelled corticotropin were gifts from Dr. P. J. Lowry and Professor L. H. Rees (St. Bartholomew's Hospital) respectively.

Gel-filtration chromatography

Analytical chromatography was performed on Sephadex G-50 as described by Ham *et al.* (1980). Molecular weights, rather than Stokes' radii, were used to enable direct comparison with other reported studies. It should be appreciated, however, that if immunoreactive calcitonin has a more extended tertiary structure than the globular proteins used as markers, the molecular weights assigned here would be overestimates.

For preparative work with radiolabelled samples, a column (90 cm $\times$ 2.2 cm) of Sephadex G-75 equilibrated with 10 mM-formic acid, containing 10% (v/v) propan-2-ol, 0.1 M-NaCl and bovine serum albumin (0.5 mg/ml), was used. Sample volume was less than 2 ml and flow rate was 4.5 ml/h. Fractions (3.0 ml) were collected and 100 μ l portions were used for calcitonin radioimmunoassay. Calcitonin-containing fractions were pooled as appropriate, concentrated by mini-column chromatography and dried under a stream of N₂. Recoveries of immunoreactive calcitonin were generally 90% or better.

Immunoprecipitation

Calcitonin preparations from Sephadex G-75 chromatography were redissolved by vortex-mixing in 1 ml of buffer [0.1 M-Tris/HCl, pH 8.2, containing 0.1% (v/v) Triton X-100, 1 mM-benzamidine, 1 mM-EDTA and 1.2 mM-phenylmethanesulphonyl fluoride]. Anti-(human calcitonin) serum, which had been pretreated for 30 min with 1.2 mM-phenylmethanesulphonyl fluoride, was then added to the calcitonin solution (150 μ l/ μ g of immunoreactive calcitonin). After incubating for 48 h at 4°C, 20 vol. of goat anti-(rabbit immunoglobulin G) serum was added per volume of anti-(human calcitonin) serum and incubation was continued for a further 24 h. The precipitates were then recovered by centrifugation for 30 min at 4800 g and the pellets were washed with buffer and then water and then stored in liquid N₂.

Reduction and carboxamidomethylation

(a) Immunoprecipitates were redissolved in 1 ml of 0.1 M-Tris/HCl, pH 8.2, containing 1 mM-EDTA and 6 M-guanidine hydrochloride, preheated to 100°C, and the solution was heated for a further 3 min at 100°C. After cooling to room temperature,

dithiothreitol was added to give a final concentration of 4 mM and the solution was incubated overnight at 37°C under an N₂ atmosphere. Iodoacetamide was then added to a final concentration of 30 mM, followed by an incubation under N₂ for 1 h at room temperature in the dark. Finally, more dithiothreitol was added to a concentration of 0.725 M and the incubation was continued for 36 h at 37°C. This last treatment should reduce any methionine sulfoxide that may have formed during the purification procedure (Houghten & Li, 1979).

The reduced and carboxamidomethylated immunoreactive calcitonin was then diluted 10-fold with mini-column buffer and the pH was adjusted to 2.1 with 1 M-HCl. The carboxamidomethylated immunoreactive calcitonin was recovered free from reagents by mini-column chromatography as described (Ham *et al.*, 1980), except that fractions were collected into 20 µg of carboxamidomethylated bovine serum albumin and 20 µg of pepstatin.

(b) Human calcitonin (0.1 µmol) was treated as described in (a) except that iodo[1-¹⁴C]acetamide was added at a specific radioactivity of 13 Ci/mol. This procedure should result in labelling only the cysteine residues at positions 1 and 7.

(c) Partially purified immunoreactive calcitonin from a Sephadex G-50 column run was reduced and extensively carboxamidomethylated by the method of Ewenstein *et al.* (1978), then rechromatographed on the same column.

H.p.l.c.

This was performed as described by Nice *et al.* (1979), except that the column was packed with Nucleosil 5-C₁₈ (Macherey-Nagel, Düren, Germany). A standard marker peptide (bombesin, obtained from Bachem, Torrance, CA, U.S.A.) was added to all calcitonin samples to check the reproducibility of the chromatographic separation. Although the absolute values of retention times varied due to modifications made to the liquid chromatograph, which changed the dead volume of the system, the retention times relative to this standard peptide were invariant. Fractions were collected at 0.5 min intervals with bovine serum albumin and pepstatin as before and portions were removed for liquid-scintillation counting and calcitonin radioimmunoassay.

Digestion with trypsin and mapping of the resultant peptides

Purified immunoreactive-calcitonin fractions obtained by h.p.l.c. were dissolved in 0.5 ml of 0.1 M-Tris/HCl, pH 8.2, containing 2 mM-CaCl₂. Trypsin (5 µg; Worthington 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated grade from Cambrian Chemicals, Croydon, Surrey, U.K.) was added, giving an enzyme/protein ratio of

approx. 1:100, and digestion was allowed to proceed for 4 h at 37°C. Any insoluble material was then removed by centrifugation and the supernatant was acidified to pH 2.1 with 1 M-HCl and fractionated by h.p.l.c.

Results

Gel-filtration chromatography

The elution profile of cell-exposed medium chromatographed directly on Sephadex G-50 is shown in Fig. 1(a). There is a principal peak of mol.wt. 28 000 and a broad area of immunoreactive calcitonin in the mol.wt.-region 10 000–13 000 with no detectable 3500-mol.wt. human calcitonin. By comparison, Fig. 1(b) shows the Sephadex G-50 profile for culture medium that had been processed by mini-column chromatography before gel filtration. This had originally been envisaged simply as a useful purification and concentration step as the bulk of serum proteins is removed and the immunoreactive calcitonin is recovered in a small volume (Nice *et al.*, 1979). However, it was found that the principal peak of immunoreactive calcitonin (about 60% of the total) now eluted much nearer to the void volume ($V_e/V_0 = 1.08$, instead of 1.15) and had an apparent mol.wt. of 40 000. These differences in elution profile were highly reproducible. The 40 000-mol.wt. peak could be reduced and extensively carboxamidomethylated without altering its elution position on Sephadex G-50, ruling out the possibility of non-covalent or disulphide-linked aggregates. Better defined peaks were also obtained at mol.wts. 13 000 and 10 000 (about 40% of total immunoreactive calcitonin), as well as a very small proportion (1–2%) of material eluting in the human calcitonin position. None of these components cross-reacted with anti-corticotropin serum.

Mini-column-pretreated extracts of BEN cells gave the same pattern of peaks as in Fig. 1(b), but the relative amounts of the different immunoreactive calcitonin types were more variable, with a lower proportion of the 40 000-mol.wt. peak generally being obtained. This effect may be attributable to the action of lysosomal proteinases in the cell extract.

H.p.l.c.

The 40 000-mol.wt. components from medium and from cell extracts had identical elution times on h.p.l.c. (Figs. 2a and 2b). However, the cell-derived material appeared to be less stable and, on aging, an increasing proportion of an earlier-eluting peak of immunoreactive calcitonin was found (Fig. 2b). This peak, eluting 4 min after bombesin, was also present in the h.p.l.c. elution profile of the 10 000–13 000-mol.wt. region (Fig. 3) and was identified by re-chromatography on Sephadex G-50 as the

Fig. 1. Gel filtration chromatography of immunoreactive calcitonin from culture medium

(a) Medium (8 ml) that had been exposed to BEN cells for 24 h was applied to a column (120 cm $\times$ 2.5 cm) of Sephadex G-50 (Superfine grade), equilibrated with 50 mM-ammonium acetate, pH 5.0, containing 0.1 mg of bovine serum albumin/ml. A flow rate of 18 ml/h was used and fractions (5.2 ml) were collected; (b) 100 ml of medium was chromatographed on the mini-column h.p.l.c. system as described in the text and the resulting eluate was applied to the Sephadex G-50 column as in (a). $\blacktriangledown$ indicates the elution position of the internal marker, (Me - ^{14}C)-labelled bovine serum albumin. The numbers above the peaks refer to molecular weights ($\times 10^{-3}$).

Fig. 2. H.p.l.c. of 40 000-mol.wt. immunoreactive calcitonin from (a) cell-exposed medium and (b) an extract of BEN cells

Chromatography was performed as described in the text. A tripartite linear gradient of acetonitrile was used for elution: this consisted of a sharp rise from 0–10% between $t = 2.5$ and $t = 5.0$ min, followed by a rise to 40% at $t = 45$ min and finally a sharp rise to 60% at $t = 50$ min. Symbols: $\circ$, ^{35}S radioactivity; $\bullet$, immunoreactive calcitonin (ng/ml). The arrow marks the elution position of the marker peptide, bombesin.

10 000-mol.wt. component. Sephadex G-50 re-chromatography of the other more diffuse peak of immunoactivity in Fig. 3 gave a profile of immuno-reactive calcitonin across the whole 10 000–13 000 region.

Purification of isotopically-labelled immuno-reactive calcitonin

A protocol involving prolonged (4 days) exposure of cells to labelling medium was found necessary to obtain sufficient incorporation of isotope. This might reflect a slow turnover of endogenous intracellular immuno-reactive calcitonin. Attempts to purify the 40 000-mol.wt. component were complicated by its instability; unless it was kept at low pH and in the

Fig. 3. *H.p.l.c. of 10000–13000-mol.wt. immunoreactive calcitonin from cell-exposed medium*

Chromatography was performed as described in the legend to Fig. 2. Symbols: O, ^{35}S radioactivity (d.p.m.); ●, immunoreactive calcitonin (ng/ml). The arrow marks the elution position of the marker peptide, bombesin.

presence of pepstatin, it was rapidly degraded into fragments. Of these, the 10000-mol.wt. fragment appeared to be comparatively resistant to further proteolysis and acted as a stable 'core' molecule. Thus, immunoprecipitation of a mixture of immunoreactive calcitonin pooled from a Sephadex G-75 eluate was shown by h.p.l.c. to result primarily in the 10000-mol.wt. component (Fig. 4). The elution profile shows two regions that have a constant ratio of immunoreactive calcitonin to radioactivity. This implies the presence of two radiochemically homogeneous substances and further studies were concentrated on the more prominent of these, which corresponds to the 10000-mol.wt. component ($t = 40$ min).

Peptide mapping after digestion with trypsin

Fig. 5(a) is the peptide profile on h.p.l.c. after digestion with trypsin of [^{14}C]carboxamidomethylated human calcitonin. This shows two major peaks, a radiolabelled one (35 min) and an unlabelled immunoreactive one (33 min), presumably the (1–18)-octadecapeptide and (19–32)-tetradecapeptide respectively. The late-eluting peptide (43.5 min), with both radioactivity and immunoactivity, corresponds to residual uncleaved human calcitonin. In addition to these, there are a number of minor radioactive peptides, presumably derived from further cleavage of the (1–18)-octadecapeptide by contaminant proteinases in the trypsin preparation used. Byfield *et al.* (1972) have previously shown that the peptide

Fig. 4. *H.p.l.c. of immunoprecipitated calcitonin from cell-exposed medium*

Calcitonin-containing fractions from Sephadex G-50 chromatography were pooled, immunoprecipitated, reduced and carboxamidomethylated as described in the text. The immunoreactive calcitonin was then chromatographed as described in the legend to Fig. 1, except that the acetonitrile concentration increased linearly from 10% at $t = 5$ min to 50% at $t = 72$ min.

Fig. 5. Digestion by trypsin of human calcitonin analysed by h.p.l.c.

(a) $[^{14}\text{C}]$ Carboxamidomethylated human calcitonin and carrier bovine serum albumin were digested with trypsin at an enzyme/protein ratio of 1:100 for 4 h at 37°C . (b) As for (a), except that the enzyme/protein ratio was 1:20 and digestion was for 8 h at 37°C . Chromatography was performed as described in the legend to Fig. 4 and in the text. Symbols: $\circ$, ^{14}C radioactivity (d.p.m.); $\bullet$, immunoreactive calcitonin (ng/ml)

bond between tyrosine-12 and threonine-13 of human calcitonin is particularly susceptible to hydrolysis. This effect could be made more pronounced by prolonged incubation of the human calcitonin (1-18)-octadecapeptide with an excess of trypsin (Fig. 5b).

Figs. 6(a)-6(c) show similarly prepared peptide maps of 10000-mol.wt. immunoreactive calcitonin from BEN cell line labelled with $[^3\text{H}]$ leucine, $[^3\text{H}]$ methionine and $[^3\text{H}]$ cysteine respectively. It can be seen that these share up to six peaks in common with the peptide maps of human calcitonin, although the relative proportions are quite different and variable. Nevertheless, the close correspondence of the patterns obtained from the 10000-mol.wt. immunoreactive calcitonin with that of the minor human calcitonin-derived peptides makes it probable, though not certain, that they are the same peptides; only one peak (27.5 min in the leucine-labelled digest) does not correspond with any peptide

Fig. 6. Digestion by trypsin of 10000-mol.wt. immunoreactive calcitonin analysed by h.p.l.c.

Immunoreactive calcitonin (mol.wt. 10000) was prepared as described in the text and in Fig. 4 from cell-exposed medium supplemented with (a) $[^3\text{H}]$ leucine, (b) $[^3\text{H}]$ methionine and (c) $[^3\text{H}]$ cysteine. Digestion with trypsin and chromatography were performed as described in the legend to Fig. 5(a).

from the human calcitonin (1-18)-octadecapeptide sequence. A partial amino-acid sequence would be necessary to clarify this point (Dayhoff & Orcutt, 1979) and the present study has shown that it is possible to incorporate a level of radioactivity (approx. 2000 c.p.m. per residue) into the ectopic material sufficient to make this a feasible approach.

Discussion

Starting from tumour extracts, Lowry and his colleagues (Ratcliffe *et al.*, 1973; Lowry *et al.*, 1976) have purified two peptides, which have the same amino-acid compositions and end-groups as the (18-39)- and (2-38)-fragments of corticotropin.

Although other workers have demonstrated the incorporation *in vitro* of labelled amino acids into immunoreactive hormone from an ectopic source (see Rees, 1975, for a review), these latter studies did not provide any evidence of chemical relatedness to the hormone from the normal source.

This was made possible in our own investigations of the immunoreactive calcitonin products of a bronchial carcinoma cell line by the application, at three different stages, of recently developed h.p.l.c. methodology. The mini-column system has shown its value as an initial purification and stabilization technique; separation of the hormone from the bulk of the contaminating proteins is carried out under conditions (low pH and the presence of pepstatin) that minimize proteolytic degradation. As a result, we have been able to detect a form of ectopic calcitonin larger (mol.wt. 40000) than any previously reported.

Analytical h.p.l.c. has proved of use not only in the separation of these large molecules but as a method of analysing smaller amounts of radio-labelled peptides by peptide mapping after digestion with trypsin. It is on the similarities in the patterns of peptide fragments obtained by using this latter h.p.l.c. technique that our evidence for the chemical relatedness of the ectopic calcitonin to human calcitonin is based. It is not possible to conclude anything about the number of copies of the human calcitonin sequence in the 10000-mol.wt. molecule since the only peptide not derived from this sequence ($t = 27.5$ min in the leucine map) contains so little radioactivity that it can probably be ascribed to a contaminant such as the 13000-mol.wt. immunoreactive calcitonin.

The possibility that the high-molecular-weight ectopic calcitonin is related to the normal precursor form of human calcitonin in the thyroid must be considered (Rees, 1975). The occurrence of large molecules with a precursor-product relationship to calcitonin has been reported for ultimobranchial glands of trout (mol.wt. 9000; Roos *et al.*, 1974; Roos & Frelinger, 1978) and of chicken (mol.wt. 13000–15000; Moya *et al.*, 1975). More recently, cell-free translation of mRNA from rat and human medullary carcinoma of the thyroid and from cod ultimobranchial glands has demonstrated the existence of a 15000-mol.wt. translation product with calcitonin immunoreactivity that is cleaved to a 12000-mol.wt. immunoreactive calcitonin by microsomal preparations (Goodman *et al.*, 1979; Jacobs *et al.*, 1979). These recall the common finding of ectopic calcitonin in the molecular-weight range 10000–15000 reported by Becker *et al.* (1978) and Bertagna *et al.* (1978), as well as in the present study, and a chemical comparison of the two classes of molecule might shed light on the mechanisms of ectopic calcitonin synthesis.

A complementary question is that of whether the normally occurring prohormones are also proteolytically derived from a larger, more labile molecule similar to our 40000-mol.wt. immunoreactive calcitonin. There have been two reports of larger primary translation products: a 26000-mol.wt. component (Roos & Frelinger, 1978) and a 65000-mol.wt. component (Lips *et al.*, 1978; van der Donk *et al.*, 1978). On the basis of immunological evidence, the latter group of workers have proposed the 65000-mol.wt. molecule to be a common precursor of several polypeptide hormones. This hypothesis has yet to be confirmed but in any case we have failed to find any association of immunoreactive corticotropin with our immunoreactive calcitonin, in common with studies of the pituitary (Weber *et al.*, 1979), medullary carcinoma of the thyroid (Shibasaki *et al.*, 1979) and lung carcinoma (Bertagna *et al.*, 1978) systems.

Finally, the frequency of occurrence of immunoreactive calcitonin in cases of bronchogenic carcinoma (Hansen & Hummer, 1979) and its potential use as a marker in therapy (Silva *et al.*, 1979) lend some clinical interest to the further characterization of the 10000-mol.wt. fragment and the possibility of developing a specific assay for the ectopic hormone.

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