Immunoreactive Calcitonin Production by a Human Promyelocytic Leukemia Cell Line HL60

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Using a sensitive radioimmunoassay, we detected human immunoreactive calcitonin in cell extracts and in cell-exposed media of the HL60 cell line derived originally from a patient with acute promyelocytic leukemia. The cell extract was chromatographed on a reverse-phase high-pressure liquid chromatography system. Radioimmunoassay of the fractions showed that the immunoreactive calcitonin was heterogeneous but had peaks correspond-

CALCITONIN, a 32 amino acid peptide hormone, is normally secreted by the parafollicular cells (C cells) of the thyroid. Its therapeutic role in the management of Paget's disease is well known; although its physiologic role is not yet clearly defined, it may prevent excessive resorption of calcium from bone during periods of calcium stress such as pregnancy and lactation. Increased circulating levels of immunoreactive human calcitonin (i-hCT) are found in patients with medullary carcinoma of the thyroid and also in a wide variety of nonthyroid neoplasia. We have found raised plasma levels of i-hCT in the majority of patients with acute and chronic myeloid leukemia. The hormone may be derived from the leukemic cells themselves, since i-hCT can be detected in the supernatant media of short-term cultures of peripheral blood leukocytes from patients with acute and chronic myeloid leukemia and in extracts of the cells. In order to obtain further evidence of ectopic i-hCT production by leukemic cells, we studied the HL60 cell line established by Collins et al. from the peripheral blood of a woman with acute promyelocytic leukemia.

MATERIALS AND METHODS

Cell Culture Conditions

The HL60 cells provided by Dr. M. F. Greaves (Imperial Cancer Research Fund, London) were grown in tissue culture flasks (Sterilin, U.K.) in RPMI 1640 medium with 10% horse serum and penicillin (50 µg/ml) and streptomycin (50 µg/ml), and harvested in their logarithmic growth phase. The culture media were removed, centrifuged, and the supernatants were assayed for i-hCT.

RIVA and SCOTT cell lines provided by Mr. K. H. Th'ng (MRC Leukaemia Unit) and normal fibroblasts and normal buffy coat cells in short-term culture were used as controls.

Extraction Procedure

HL60 and RIVA cells stored in liquid nitrogen were homogenized using a Braun microdisemembranator and extracted at 4°C in a mixture of trifluoroacetic acid (TFA) 15% w/v, formic acid 5% w/v, sodium chloride 1% w/v, and hydrochloric acid 1 M. The extract was adsorbed to a 1 ml bed volume porasil octadecasilyl (ODS) silica column pre-equilibrated with 1% TFA in distilled water and eluted with a mixture of 80% methanol and 1% TFA. The extract was vacuum-dried and reconstituted in phosphate buffer before assay for i-hCT. Calcitonin monomer is partly converted to its sulphoxide by this extraction procedure.

High-Pressure Liquid Chromatography

The porasil ODS extract of 2g cells was chromatographed in a reverse-phase high-pressure liquid chromatography (HPLC) system using a 10 x 0.46 cm Spherisorb 10 µ ODS column, eluted with an aqueous gradient of 40% methanol-1% TFA, rising to 90% methanol-1% TFA. Bovine insulin (250 µg), supplied by Dr. Rittel of Ciba-Geigy, was used as an internal marker, since it elutes near to human calcitonin and does not cross-react with the human calcitonin antiserum when tested at concentrations up to 250 µg/tube. The HPLC fractions were dried in a vacuum oven and redissolved in 250 µl phosphate buffer containing 0.05% w/v human serum albumin. Two 100-µl aliquots of each redissolved fraction were assayed for i-hCT.

Gel Filtration

The porasil ODS extract of 1.5g cells was chromatographed on a 83 x 1.6 cm Sephadex G-50 (superfine) column, eluted with 10% (v/v) propan-2-ol/0.1 M formic acid in water. The column was calibrated with bovine serum albumin, trypsin inhibitor, cytochrome-C, insulin, 125I-hCT, glucagon, and sodium chloride. Column fractions were vacuum dried and reconstituted in 250 µl phosphate buffer containing 0.5% w/v human serum albumin. Two 100-µl aliquots of each redissolved fraction were assayed for i-hCT.

Calcitonin Radioimmunoassay

Calcitonin was measured using a modification of the radioimmunoassay for calcitonin previously described. The synthetic human calcitonin for labeling was supplied by Ciba-Geigy, and the standard preparation of human calcitonin was obtained from the National Institute of Biological Standards and Control, London, (70/324).
Antisera 827/4 (Burroughs Wellcome) was incubated overnight with samples at a final dilution of 1:60,000. This antisera is directed against the central and C-terminal parts of the calcitonin molecule. Single or double amino acid substitutions at the antigenic determinants of the hormone produce either a loss or inhibition of binding by the antisera or a change in shape of the displacement curves. Cross-reactivity of the antisera with any other peptide hormone has not been detected.

RESULTS

I-hCT was consistently found in the supernatant media of cultures of HL60 cells, but not in media from RIVA, SCOTT, or normal cells or RIVA cell extracts. The quantity of I-hCT in the supernatant media after culturing cells for 5 days related to the number of cells originally plated (Fig. 1). The production of I-hCT by 0.75 x 10^6 cells maintained in 1 ml of culture medium increased with the duration of the culture period at least up to 7 days (Fig. 2). The number of cells almost doubled every 24 hr. Serial dilution of the HL60 cell extract resulted in a tracer displacement curve of shape similar to that given by extracts of other I-hCT-producing tumors, but not parallel to the displacement curve of synthetic human calcitonin monomer (Fig. 3).

Immunooassay of the reverse-phase HPLC fractions of the cell extract revealed four peaks (A, B, C, D—Fig. 4). Peaks C and D were similar in position to those obtained with calcitonin monomer sulphoxide and calcitonin monomer, respectively, but serial dilutions were not parallel to the standard curve for synthetic I-hCT. However, only three I-hCT peaks (X, Y, Z—Fig. 5) were observed in the Sephadex G-50 fractions, none of which coeluted with 125I-hCT.

DISCUSSION

The concept that leukemic cells may produce ectopic hormones is not widely accepted. There is one report of a patient with acute myeloid leukaemia in whom hypercalcemia was thought to be due to ectopic parathyroid hormone production and another report, so far unconfirmed, that leukemic leukocytes can synthesize vasoactive intestinal polypeptide. We have shown that elevated plasma levels of I-hCT are present in patients with acute and chronic myeloid leukemias. This has been confirmed by Pflüger and colleagues, who have also reported a case of ectopic ACTH syndrome in a patient with acute myeloid leukemia. In this article we report that extracts of the HL60 cell line derived from the blood of a patient with acute promyelocytic leukemia also contain I-hCT, as do supernatant media harvested from HL60 cells in culture. These findings support the concept that myeloid leukemic cells in general can produce I-hCT.

The absence of complete parallelism between a dilution curve of the HL60 cell extract and that of synthetic human calcitonin monomer (Fig. 3) suggests that the two are not identical. Reverse-phase HPLC of HL60 extracts revealed two hydrophilic I-hCT peaks (A, B) in addition to the two peaks (C, D) that resembled synthetic hCT and its sulphoxide. Sephadex G-50 gel filtration, however, showed the presence of two I-hCT components of apparently higher molecular weight than 125I-hCT (X, Y), and one that was smaller (Z). There are a number of possible explanations for the heterogeneity of the I-hCT extracted from HL60 cells. It is known for example that mRNA isolated from human medullary carcinoma of the thyroid directs the synthesis of several high molecular weight forms of calcitonin that are subsequently cleaved by
proteolytic enzymes to the mature form. Cells that are not the major physiologic source of calcitonin may not possess all the cleavage enzymes present in thyroid C cells. It is therefore possible that some of the i-hCT extracted from HL60 cells represents a precursor molecule. The immunologic characteristics of circulating i-hCT were identical to synthetic hCT in some, but not all, patients, suggesting that in the latter, incorrect processing of the CT precursor molecule had occurred. Medium conditioned by a human epidermoid bronchial carcinoma cell line also contains i-hCT, which on serial dilution gives a tracer displacement curve that is not parallel to synthetic human calcitonin monomer.  

Virtually all of the i-hCT secreted by this cell line has a higher molecular weight than calcitonin monomer, and this may be due to absence of specific processing enzymes at some point in the secretory pathway. Alternatively, Baylin et al. have recently shown that some of the calcitonin obtained from medullary thyroid or oat cell carcinomas may be glycosylated, and this is another possible explanation for the heterogeneity of i-hCT extracted from these tumors. It is unlikely, however, that any of the i-hCT detected in the HL60 cells would be glycosylated in view of the acidic conditions of the extraction procedure that was used. Since we have yet to determine the molecular weights

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**Fig. 3.** Cross-reaction of an ODS-silica extract of HL60 cells in a radioimmunoassay for human calcitonin. (O-O) Human calcitonin standard; (•-•) HL60 cell extract.

**Fig. 4.** HPLC of the HL60 cell extract showing four peaks of i-hCT (A, B, C, D). Peaks C and D correspond to the positions of calcitonin sulphoxide and calcitonin monomer, respectively.
of the various i-hCT fractions obtained from HL60 cells, it also remains possible that some may be fragments of calcitonin monomer.

Various theories, such as gene derepression or tissue dedifferentiation, have been proposed to account for ectopic hormone production by nonendocrine tumors. However, the finding of so-called ectopic hormones in normal nonendocrine tissues may mean that many ectopic hormones are normal cell products elaborated in particular by immature differentiating cells. In a possible analogy, membrane antigens, such as the common-ALL antigen thought at one time to be specific for leukemia, have since been identified on the surface of normal hemopoietic stem cells. In carcinomas, this explanation for ectopic hormone production may be difficult to demonstrate in part because the pathways by which tumor stem cells produce mature progeny are poorly understood. The finding of ectopic hormone production in a leukemic cell line is therefore of importance, in that the steps of normal myeloid differentiation are well documented. In particular, the fact that HL60 cells can be induced to differentiate into mature granulocytes will permit the relationship between cell differentiation and i-hCT production to be studied in more detail.

REFERENCES


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