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Production and characterisation of immunoreactive calcitonin gene-related peptide (CGRP) from a CGRP receptor-positive cloned osteosarcoma cell line (UMR 106.01)

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A subclone of an osteoblast-like osteosarcoma cell line (UMR 106.01) has recently been shown to possess specific binding sites for calcitonin gene-related peptide (CGRP) linked to adenylate cyclase. The present study provides the first demonstration for the production of immunoreactive CGRP from CGRP-receptor positive osteosarcoma cells. Mean immunoreactive CGRP levels were 15 pmol/g and 1 pmol/l for acid extracts of cells and cell-exposed media respectively. On gel filtration and high performance liquid chromatography, a major proportion of immunoreactive CGRP was found to co-elute with synthetic rat $CGRP_{(1-37)}$. Only negligible quantities of calcitonin were detected in cell extracts or cell-exposed supernatant. The production of authentic CGRP from a CGRP-receptor positive tumour suggests that the peptide may have autocrine effects on its producer cell.

The calcitonin/calcitonin gene-related peptide (CGRP) gene complex encodes a small family of peptides: calcitonin, CGRP and katacalcin. Calcitonin gene-related peptide (CGRP) is extensively distributed in neural, vascular and endocrine tissues. There is a significant regional overlap between the distribution of CGRP and the CGRP receptor (for review 1). However, it remains uncertain whether the peptide and its receptor co-exist on single normal cells. Cancer cells, such as bronchogenic carcinoma (BEN) cells are known to express both calcitonin and its receptor (2, 3); CGRP behaves as a weak calcitonin agonist on calcitonin receptors (4). However, CGRP binds specifically to membrane preparations and stimulates cAMP formation independently of calcitonin in a subclone of rat osteogenic sarcoma cells, UMR 106.01 (5). With a view that CGRP may influence tumour cell behaviour, we have attempted to investigate the production of the peptide from CGRP receptor-positive UMR cells.

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MATERIALS AND METHODS

Cell culture: Rat osteoblast-like osteosarcoma (UMR 106.01) cells were incubated at a high density in RPMI-1640 (1X) (90% v/v; Flow Laboratories, UK, Ltd) with foetal calf serum (10%, v/v; Gibco, UK), streptomycin, penicillin, glutamine and sodium bicarbonate. The cells were subcultured at confluence by washing in EDTA, followed by treatment with trypsin (0.025%, w/v; Sigma) (2 minutes), addition of complete medium before centrifugation (x 200 g) and resuspension in complete medium. The cells were maintained in plastic tissue culture flasks (Sterilin) at 37°C, in an atmosphere of CO_2 and were harvested in their log growth phase (5).

Peptide extraction: Peptides were extracted as previously described (6). An acid extraction medium (7) was used to acidify supernatant (50 µl medium per ml) and homogenise cells (10 ml medium per g wet weight of cells). After incubation (1 hour 4°C) and centrifugation, samples were passed through prewetted Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, USA) and the cartridges washed with aqueous TFA (0.5% v/v). Finally, the adsorbed peptide was eluted in aqueous methanol (80% v/v) containing TFA (0.5% v/v). The eluate was vacuum dried and reconstituted in 750 µl phosphate buffer (0.05 mol/l, pH 7.4) with aprotinin proteinase inhibitor (Trasylol, Bayer, Newbury, Berks., 40 KIU/ml). CGRP values were corrected for percentage recovery of synthetic rCGRP (Peninsula Laboratories Europe Ltd., St. Helens, Merseyside) added to medium and control tissue. The mean recovery and the coefficient of variation were >75% and <10% respectively.

Chromatography: For gel filtration chromatography, acid extracts were loaded onto a Sephadex G50 superfine column (80 x 1.5 cm) and eluted with ammonium acetate (0.2 mol/l) containing bovine serum albumin (Sigma, Dorset; 0.1% w/v) and sodium chloride (0.2 mol/l), at a flow rate of 10ml/hour. The column was calibrated using blue dextran (BDH), $Na^{125}I$ (Amersham) non-radioactive synthetic rCGRP (2.5 nmol) (molecular weight 3876) and ^{125}I-iodotyrosyl^{o}rCGRP (molecular weight 1186; 50 µl of 12,000 cpm in 1ml distilled water) in separate runs. Fractions were collected every 30 minutes, freeze dried and their CGRP content measured by radioimmunoassays described below. For high performance liquid chromatography (HPLC), supernatants of cell extracts were reconstituted in 250 µl acetonitrile : water : TFA (10 : 89.9 : 0.1 by volume), loaded onto a Spherisorb 5µ-ODS column (25 x 0.46 cm) and eluted using a gradient of 10 to 90% acetonitrile in aqueous TFA (0.1% v/v) at a flow rate of 0.8 ml/minute.

Radioimmunoassays: Two highly sensitive radioimmunoasays (detection limit 300 atomoles CGRP per tube) were used to measure immunoreactive CGRP (i-CGRP) levels in column fractions and acid extracts. An assay using antiserum CG/39 was highly specific for the carboxy-terminus of the CGRP molecule (8), while another assay using an antibody (CC2/1) recognised the intact peptide (9). Both antisera were used in a final dilution of 1 : 300,000 (incubation volume 300 µl) in a disequilibrium assay requiring a long preincubation step: HPLC-purified ^{125}I-labelled iodotyrosyl^{o}rCGRP$_{(28-37)}$ was added after the sample had been incubated with the antibody and 100 µl bovine serum albumin (0.25% w/v; Sigma, Poole, Dorset) for 3 days, at 4°C (7). Following another 2-day incubation (4°C), the antibody-bound label was separated using cellulose-coated sheep anti-rabbit antibody (IRE, Fleurus, Belgium) and the radioactivity was counted on a Nuclear Enterprises NE 1000 gamma counter. Rat CGRP (Peninsula) was used as standard. The inter- and intra-assay variations were <10% for a pool of 12 fmol/tube synthetic rCGRP. Immunoreactive calcitonin (i-CT) was measured by a method previously described (9).

RESULTS

Immunoreactive CGRP levels: Immunoreactive CGRP was consistently demonstrable in acid extracts of cell pellets and cell-exposed medium of UMR 106.01 cells. Serial dilutions of cell extracts and culture supernatant resulted in tracer displacement curves that were not parallel to that of rat CGRP (Figure 1). Table 1 shows mean i-CGRP and i-CT values of the cells and and cell-exposed medium respectively. The mean levels have been corrected for the recovery of the respective intact peptides from the extraction process (recovery, mean ± sem; leu^{16}-hCT, 79 ± 2%; rCGRP, 72 ± 4.2%). It is also evident that values obtained using the C-terminal specific, CG/39-based radioimmunoassay were approximately 4-fold higher than those obtained using the CC/2-based assay. The production of i-CGRP from UMR 106.01 cells maintained in 2 ml culture medium was found to increase with the duration of the culture period at least upto 5 days (Figure 2).

Chromatography: Radioimmunoassay, using antiserum CC2/1 of gel filtration fractions of cell extracts revealed a prominent immunoreactive peak (fraction numbers 32 to 42, Kav 0.42) that co-eluted with rCGRP and an additional small peak having a Kav of 0.32 (approximately 12% of total immunoreactivity recovered from the column). When fractions were measured using the C-terminal-specific CG39-based radioimmunoassay, another major peak of smaller molecular weight (Kav 0.75) was evident constituting approximately 38% of the total immunoreactivity (Figure 3). HPLC showed that the major component of CGRP-immunoreactivity of UMR 106.01 cells was indistinguishable from the synthetic rat peptide (retention time 53-62 minutes). There was an additional peak that eluted earlier indicating the presence of less hydrophobic cross-reacting species (Figure 4).

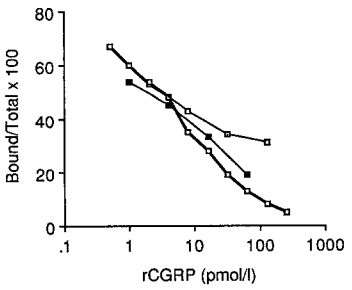

Figure 1
Displacement of ^{125}I-iodotyrosyl^{0}rCGRP$_{(28-37)}$ from antibody CC2/1 by synthetic rat calcitonin gene-related peptide (rCGRP) (open squares) and acid extracts of cell pellets (dotted squares) and cell-exposed medium (closed squares) of rat osteoblast-like osteogenic sarcoma (UMR106.01) cells.

TABLE 1

Peptide	Antibody	Cell content (pmol/g)	Medium content (pmol/l)
i-CGRP	CC2/1	36 ± 7.5	3.0 ± 0.7
	CG/39	110 ± 12.6	17 ± 3.8
i-CT	OC/31	8.7 ± 1.9	< 10

Immunoreactive calcitonin gene-related peptide (i-CGRP) and calcitonin (i-CT) concentrations in acid-methanol extracts of cells (Cell; pmol/g weight ± SEM; n = 4) and cell exposed supernatant (Medium; pmol/l ± SEM; n = 5) of rat osteogenic sarcoma (UMR 106.01) cells, using antisera CC2/1, CG/39 (for i-CGRP) and OC/31 (for i-CT).

DISCUSSION

The present study demonstrates the production of authentic CGRP from CGRP receptor-positive clonal rat osteoblast-like osteogenic sarcoma cells (UMR 106.01). Chromatography of cell and supernatant extracts revealed that a large proportion of CGRP-immunoreactivity co-eluted with synthetic monomeric rat CGRP. This is consistent with, though not proof of, of structural identity. In addition, a substantial proportion of the immunoreactive material consisted of cross-reacting species of lower molecular weight and lesser hydrophobicity. Previous studies have

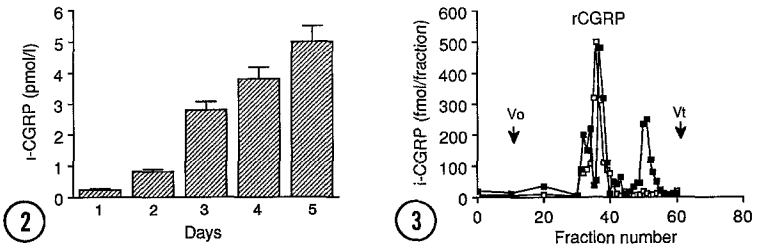

Figure 2
Production of immunoreactive calcitonin gene-related peptide (i-CGRP) by 0.8 x 10^6 rat osteogenic osteosarcoma (UMR 106.01) cells for periods of 1 to 5 days in 2 ml culture medium.

Figure 3
Gel filtration profile of an acid extract of rat a osteogenic sarcoma (UMR 106.01) cell line using a Sephadex G50 superfine column. Fractions (5 ml collected every 30 minutes) were dried down and assayed for immunoreactive calcitonin gene-related peptide (i-CGRP; fmol/fraction) using antibodies CC2/1 (open squares) and CG/39 (closed squares). V_0 - void volume and V_t - total volume.

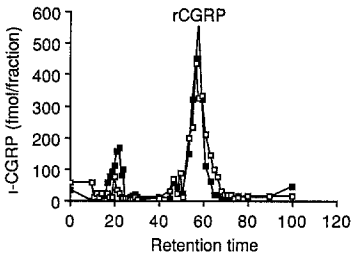

Figure 4
High performance liquid chromatograms of an acid extract of rat osteogenic sarcoma (UMR 106.01) cells using a 5μ-ODS column and an acetonitrile : water gradient. Fractions (0.8 ml) were collected every 1.2 minutes and assayed for immunoreactive calcitonin gene-related peptide (i-CGRP; fmol/fraction) using antibodies CC2/1 (open squares) and CG/39 (closed squares).

demonstrated the production of the calcitonin gene peptides in other non-thyroid tumour cells, namely lung (BEN) and breast (T47D and MCF7) cancer cells and leukaeima (HL60) cells in culture, as well as in a number of cancers *in vivo* (10-13). Whereas, ectopic calcitonin production is largely associated with the production of high molecular weight species (10-12) with small amounts of monomer, we demonstrate that UMR 106.01 cells produce significantly lower amounts of 'big' CGRP. These results are consistent with our recent observations with BEN and HL60 cells (14), and though unexpected, this might suggest that tumour cells can discriminate between the two very similar precursors for calcitonin and CGRP (1, 14). The significance of the ectopic production of calcitonin, a calcium-regulating hormone, or CGRP, a potent vasodilator from tumour cells is unclear. Nevertheless, such peptide-producing tumour cell lines will continue to be used for studies on the regulation of gene expression. Interestingly enough, negligible amounts of calcitonin are produced from UMR 106.01 cells. This may either point towards the sole expression of the non-calcitonin-producing β-CGRP gene (15) or else reflect the complete switching to a low calcitonin/high CGRP producing state (16).

The demonstration of CGRP production from CGRP-receptor positive UMR cells is indeed a novel observation. Michaelangeli and colleagues have recently demonstrated that CGRP acts independently of calcitonin on a specific binding site (5). Thus, it is possible that the secreted peptide may act on its own membrane receptor to modify growth or function. A number of peptides including calcitonin, bombesin, substance P and somatostatin have been found to effect the growth of tumour cells (17), and it would be interesting to determine any effects of CGRP on the growth of CGRP receptor-positive cancer cells. Martin and co-workers have also elegantly demonstrated the presence of calcitonin receptors on a variety of lung and breast cancer cells, reviving the possibility that such tumours might trap circulating peptides *in vivo* and progressively release them. This systemic effect might be of vital importance when circulating calcitonin is elevated (18). It is also known that tumours that do not normally express peptide

receptors may do so when exposed to elevated peptide levels (18). Thus, at some stage, it would be interesting to investigate whether the production of CGRP and the synthesis of its receptor is regulated by structurally-related regions of the genome.

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