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CALCITONIN GENE-RELATED PEPTIDE: AN AUTOCRINE GROWTH FACTOR WITH REGULATORY ACTIVITY IN VITRO

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We show that an autocrine system for calcitonin gene-related peptide (CGRP) exists in F9 teratocarcinoma cells. Synthesis of CGRP by F9 cells was demonstrated by measuring the peptide concentration in cells and medium and by determining specific mRNA in cells. During six days of culture, CGRP secretion did not vary significantly in the medium, while intracellular CGRP and CGRP mRNA levels increased. F9 cells contained a CGRP-sensitive adenylate cyclase system and CGRP increases the accumulation of cAMP in the culture medium. Interestingly affinity purified antibodies against CGRP specifically inhibited growth of F9 cells by 50%.CGRP therefore stimulates F9 cell growth by an autocrine process, suggesting that CGRP may be a growth factor during early embryogenesis. © 1992 Academic Press, Inc.

Embryonic development is mainly the result of temporally and locally coordinated events, i.e. cell proliferation, adhesion, migration, differentiation and cell death. Ectopic production of soluble factors, including growth factors and hormones (1), and altered responsiveness to different hormones (2), play a critical role in mediating these events.

Teratocarcinoma cells in culture offer an in vitro model system to study early biochemical events involved in embryonic development. F9 cells derived from OTT 6050 embryonal carcinoma cells, resemble the inner cell mass of the early (day 4-5) post-implantation mouse embryo (3). They are nullipotent cells with a limited capacity to differentiate spontaneously (4).

We have previously shown that salmon calcitonin (sCT) was to be a more potent stimulator of adenylate cyclase activity than human calcitonin (hCT) in F9 cells (5) and that the presence of calcitonin (CT) receptors was a marker of teratocarcinoma stem cells (6). We also observed that F9 cells secreted immunoreactive CT, suggesting that the hormone may have autocrine effects on these cells (7). The pioneering work of Rosenfeld and his colleagues (8) revealed that the CT/calcitonin gene-related peptide (CGRP) gene is a complex transcription unit. Tissue-specific alternative RNA processing results in the production of mRNA specific for CGRP, a 37-amino acid peptide expressed in neurons, and in the production of CT mRNA coding for the precursor of

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CT in thyroid C cells (9-10).CGRP is a neuropeptide with multiple biological effects on the nervous and cardiovascular system.(11). CGRP receptors are coupled to the adenylatecyclase system (12).

Transfection studies have established that splicing mechanisms leading to the production of CGRP predominate in F9 cells (9). Therefore, it was of interest , using these teratocarcinoma cells as a model to study the secretion of CGRP and its possible involvement in early embryonic development.

MATERIALS AND METHODS

The hormone sources used were as follow : human CGRP (hCGRP) from Peninsula Laboratory and chicken CGRP (cCGRP) from Interchim.

($a^{32}P$) ATP, ($a^{32}P$) dCTP, ($a^{32}P$) UTP and ^{125}I-CGRP were purchased from Amersham, and bovine serum albumin (fraction V) from Pentex .

Cell culture : F9 cells, kindly provided by Dr. Jetten (NIH), were grown on 100 mm tissue culture dishes (Costar) at 37°C in Dulbecco-Vogt modified Eagle's medium (GIBCO) containing 10% fetal calf serum (Seromed), antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin), and 4mM glutamine (Flow) in a 5% CO_2 humidified atmosphere. Cells were plated at a low cell density at time 0 (250000 cells/ dish). The growth medium was changed every 48 hrs.

CGRP radioimmunoassay (RIA): At each experimental time (1 to 6 days), 24-hour conditioned medium (10ml) was collected and stored at -20°C until assay; cells were scraped with a rubber policeman and extracted in 0.1M acetic acid, lyophylized and stored at -20°C until assay. CGRP was measured using a sensitive and specific radioimmunoassay after purification of the culture media on an AMPREP column (Amersham). Eluted CGRP was lyophylized and dissolved in 500 µl of RIA phosphate buffer. Serial dilutions (10 to 100 µl) were incubated in the presence of sheep antiserum against hCGRP (Bachem) at a final dilution of 1/250000 in 0.05 M phosphate buffer containing 0.3% albumin and 10 mM EDTA in a final volume of 400 µl for 4 days. Then 100 µl of ^{125}I-CGRP (Amersham) was added and incubation continued for 3 days. Free and bound hormone were separated by charcoal adsorption in RIA buffer in the presence of 0.25% gelatine.

mRNA extraction : Nucleic acids were extracted from F9 cells using phenol / chloroform / isoamyl alcohol (50/50/2) in the presence of 0.1M Tris-HCl and 0.01 M EDTA (13).

Total RNA was precipitated with 10 M LiCl and poly (A)-rich RNA was purified using an oligo-dT cellulose column .

Dot - blot analysis : 5 µg of poly (A) RNA, extracted from F9 cells after 2, 4, and 5 days of culture and 1, 2, 4, 8 µg of poly (A) RNA extracted from rat thyroids were denaturated with formaldehyde (14) and spotted in duplicate, on "GeneScreen" (New England Nuclear-NEN) membrane. The membrane was then baked at 80° C for 2 h.

Northern blot analysis : 10 µg of poly (A) RNA extracted from F9 cells after 6 days of culture were denaturated with glyoxal, subjected to electrophoresis on 1% agarose gel (15) and transferred to "GeneScreen" membrane. The membrane was then dried and baked for 2h at 80° C.

Hybridization with the CGRP RNA probe :

Dot-blot and northern blot membranes were prehybridized for 16-h at 50°C in 50% formamide, 50 mM sodium phosphate pH 6.5, 0.8 M NaCl, 1mM EDTA, 0.1% SDS, 2.5x Denhardt (1xDenhardt = 0.02% bovine serum albumin, ficoll, and polyvinyl-pyrrolidone), 250 µg/ml denaturated fetal calf thymus DNA, 500 µg/ml yeast RNA,and 10 µg/ml poly (A). Hybridization was carried out for 18-h at 50°C in the same buffer containing a ^{32}P-labelled specific CGRP RNA probe obtained from the pSP-CGRP plasmid (gift of Pr. Craig). The membranes were then washed 4 times for 20 minutes at 60°C with 50 mM NaCl, 20 mM sodium phosphate pH 6.5, 1 mM EDTA and 0.1% SDS.

Autoradiographies were performed using Hyperfilm MP (Amersham) for 4 days at -80°C. The autoradiogram was scanned on a densitometer and results were expressed as the mean of two values in arbitrary units.

cAMP radioimmunoassay (RIA) :

The medium was removed and the cells (1200 000 cells./ 60 mm dish) were washed in serum free medium , then incubated for one hour in 4 ml of serum-free medium containing 0.1 mM 3

isobutyl, 1-methylxanthine, an inhibitor of phosphodiesterase.CGRP was added at the indicated conxentrations and the cells incubated for a further hour. The supernatant was frozen until use.The cells were scrapped in 1 ml serum free medium with 0.1 mM 3 isobutyl, 1-methylxanthine and boiled for 2 minutes. The protein content of the cells was measured by fluorometric assay (16). cAMP concentrations in the cells and/either in the supernatants were estimated by means of a radioimmunoassay in 100ul, using a rabbit anti-cAMP antiserum (kindly donated by J. Saez, INSERM U 307). The antibody bound- cAMP was separated from free cAMP by the use of a second antibody (Read-PR-1000, CIS, France). Non-specific binding was determined by the addition of excess of cAMP. The results are expressed as pmoles cAMP per mg cell protein.

CGRP antibody : The antibody was raised in sheep against unconjugated synthetic human CGRP (Bachem) and purified (17) on an affinity column of CGRP coupled to Sepharose 4B. For proliferation experiments, the purified CGRP antibody was added to the medium at time 0 only or every two days at a final dilution of 1/4000 corresponding to a CGRP antibody concentration of $1.83\ 10^{-9}$ M. Antiserum stripped of CGRP antibody by the affinity column of CGRP was used as control .

Cell count : Cell numbers (mean ± SEM) were determined in triplicate from three dishes after detachment with a trypsin / EDTA (0.05% / 0.01%) solution. Cells were resuspended and counted in a cell counter (Coultronic).

RESULTS

Hybridization under stringent conditions was observed when the human probe specific for CGRP was applied to F9 cell mRNA dotted or transferred onto "GeneScreen " membranes (Fig.1-A insert, 1-B). In a similar way, the ratio of CGRP mRNA / total mRNA increased progressively with time in culture (Fig. 1-A). CGRP mRNA levels were higher in F9 cells than in rat thyroid. Poly (A) RNA extracted from F9 cells after 6 days of culture and analysed by Northern blotting and hybridization with the CGRP probe showed a specific band of ≈1200 bases corresponding to mature CGRP mRNA (Fig.1-B)

In RIA, displacement of ^{125}I hCGRP by F9 cell culture media and cell extract was almost identical to that with synthetic hCGRP when the specific antibody (18) was used (data not shown). As shown in Figure 1-C, immunoreactive CGRP was found in F9 cells and in their medium. CGRP concentrations are expressed as the mean of results obtained by displacement of human labelled CGRP from its specific antibody by multiple dilutions of F9-conditioned medium. F9 cells secreted CGRP(1.5 to 2 ng/10^7 cells/24 h, corresponding to a concentration of $2.5x10^{-11}$ M) and no significant change was observed during the 6 days of culture. In contrast, the amount of intracellular CGRP (0.09 ng to 0.26 ng/10^7 cells) increased progressively during the 5 days of culture.

In order to study the possible involvement of CGRP in F9 cell growth, an affinity-purified antibody against hCGRP was added to the F9 culture medium. After affinity chromatography, proteins not specifically retained by the column did not bind ^{125}I hCGRP. The full binding capacity of the antiserum was recovered in the fraction eluted from the column. After four days of culture in the presence of the antibody, F9 cell growth was inhibited by 50% (Figure 2-panel A). Further addition of this antibody every two days was necessary to maintain the inhibitory effect on F9 cell growth. The antiserum stripped of CGRP antibody by the affinity column of CGRP was used as control. Added to the F9 cell culture at the same dilution as the purified CGRP antibody, they did not inhibit growth. As shown in Fig 2-panel B, the inhibitory effect of CGRP

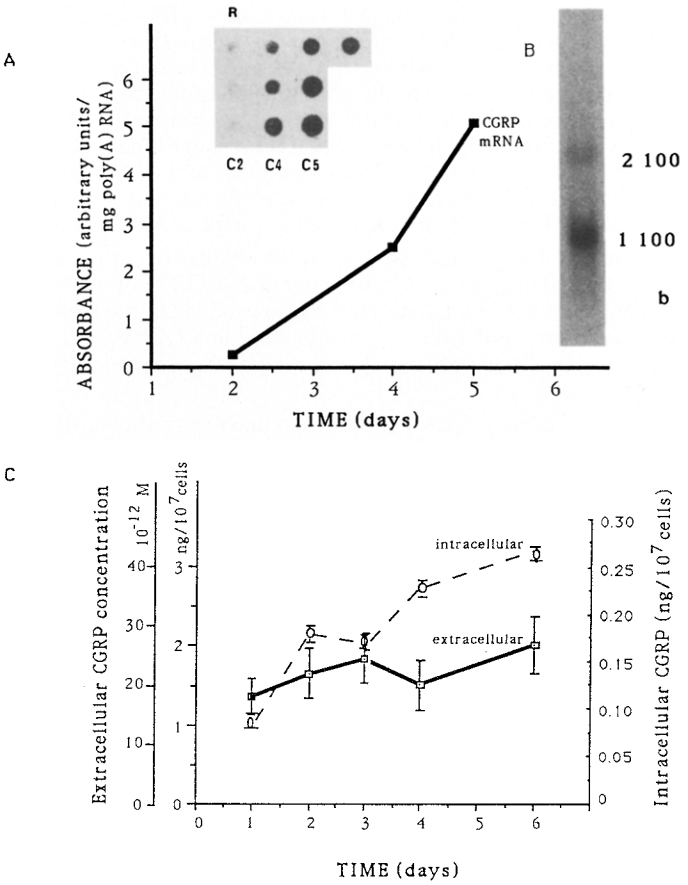

Fig. 1 : CGRP mRNA (panel A, B) and F9 cell CGRP content and secretion (panel C).
Panel A : Dot blot analysis of CGRP mRNA from F9 cells. Aliquots of poly (A) RNA extracted from F9 cells (5 µg in duplicate) after 2 (C2), 4 (C4) and 5 (C5) days of culture and aliquots (1,2,4,8 µg) of poly (A) RNA extracted from rat thyroids (R) were dotted on GeneScreen membranes. The dot-blot was hybridized with a specific ^{32}P-labelled CGRP RNA probe. Autoradiography of the dot-blot membrane (in the insert) was analysed with a densitometric scan. Results are expressed (arbitrary units / mg of poly (A) RNA) as the mean of the two results for each experimental time.
Panel B : Northern blot analysis of CGRP mRNA in F9 cells. Ten µg of poly (A) RNA extracted from F9 cells after 6 days of culture was analysed on 1% agarose gels transferred to GeneScreen membranes, hybridized with a specific ^{32}P-labelled CGRP RNA probe and autoradiographed on Hyperfilm MP (Amersham).
Panel C : Intracellular CGRP and extracellular CGRP having accumulated in the culture medium for 24 h were assayed using a specific RIA as described in Methods. For each experimental time, results are expressed as the mean of 4 dilutions of CGRP extracted from 10^7 cells with 0.1 M acetic acid, purified using a Amprep column (Amersham) and lyophylised. The results of extracellular CGRP are also expressed in molar concentrations in the medium.

antibody on F9 cell growth was dependent on the antibody titer. Increasing dilutions of the antibody leads to a progressive decrease in the inhibitory effect on growth. Furthermore, addition of exogenous hCGRP (10^{-6} M) blocked the inhibitory effect of the purified CGRP antibody (data not shown). Two days after the addition of antibody specific for CGRP, F9 cells proliferation was significantly reduced ($P< 0.001$), but the addition of the same antibody to differentiated

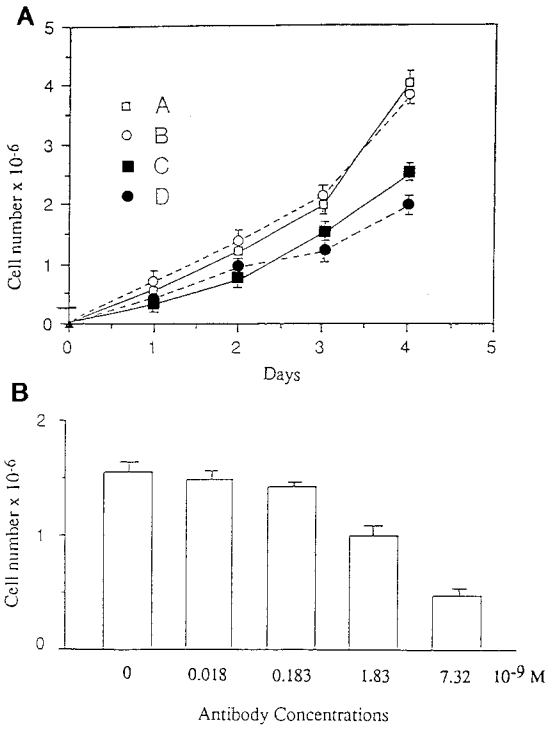

Fig.2 : Effect of an antibody against CGRP on the growth of F9 cells.
Panel A : Control F9 cells (A) (□—□) were grown in 6-well culture dishes, as described in the legend of fig 1, in 10% FCS-supplemented Dulbecco's medium, and the medium was changed every two days. At time 0, 100,000 F9 cells were plated. Affinity -purified antibody against CGRP was added at a final dilution of 1/4000 (concentration , 1.83×10^{-9} M) every two days (D) (●------●) or only at day 0 (C) (■—■). Antiserum stripped of CGRP antibody by the affinity column was used as control and added at time 0 (B) (o------o)
Panel B :Cells were cultured for two days in the presence of affinity-purified antibodies at the final dilutions indicated and counted as described elsewhere. Cell numbers are expressed as the mean ± S.E.M. of two experiments (3 dishes each).

TABLE 1: Effect of the anti-CGRP antibody on the growth of different cells

	Control	+ anti-CGRP antibodies
Undifferentiated teratocarcinoma cells (F9 cells)	$1.464\ 10^6 \pm 167\ 10^3$ cells	$1.044\ 10^6 \pm 21\ 10^3$ cells
Differentiated teratocarcinoma cells (PYS-2)	$0.498\ 10^6 \pm 7\ 10^3$ cells	$0.491\ 10^6 \pm 5\ 10^3$ cells
Human fibroblasts	$0.454\ 10^6 \pm 13\ 10^3$ cells	$0.468\ 10^6 \pm 13\ 10^3$ cells

Cells were cultured in 6 well culture dishes for two days in the presence or not of affinity purified anti-CGRP antibodies (final dilution 1/4000 corresponding to 1.83×10^{-9} M concentration) and counted as previously described. At time 0, 200,000 cells were plated. Cell number was expressed as the mean of triplicate counts of 3 dishes ± SEM.

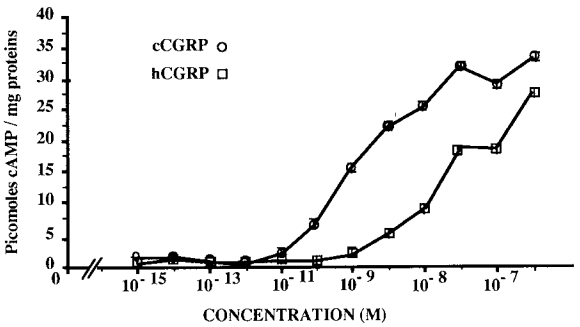

Fig.3: Effect of increasing concentrations of CGRP on extracellular cyclic AMP level.
F9 cells were incubated for 90 minutes in serum free medium in presence of the CGRP (○) cCGRP (□) hCGRP. Cyclic AMP was extracted and measured by radioimmunoassay as described in materials and methods. The results are expressed as means ± SE of triplicate determinations.

teratocarcinoma cells (parietal yolk sac cells, PYS-2) or to human fibroblasts was without effect on these non-producing CGRP cells. The inhibitory effect of the anti-CGRP antibody on cell proliferation appeared to be specific for undifferentiated cells producing CGRP (table 1).

We previously demonstrated that CGRP stimulates F9 cell adenylate cyclase activity (18) with cCGRP being a more potent activator than hCGRP. Similarly when F9 cells were exposed to cCGRP or hCGRP, a clear dose dependent increase in the extracellular medium concentrations of cAMP were observed (Figure 3) . The maximal increase of extracellular cAMP levels was observed with 5 10^{-8} M cCGRP and 10 $^{-6}$ M hCGRP.

DISCUSSION

Our results show that F9 teratocarcinoma cells synthesize and actively secrete CGRP. Furthermore, F9 cells possess specific hormonal responsiveness for CGRP and inhibition of the endogenous hormone by specific antibodies reduces cell proliferation by 50%.

We used for these studies antibodies and probes specific for human CGRP (hCGRP), as neither the sequence of mouse CGRP nor its messenger has yet been established. However in a closely related rodent, the rat, both the amino acid sequence of the protein and the nucleotide sequence of the coding exons are highly homologous to their human counterparts. In addition, hybridization of the human probe to F9 RNA was achieved in highly stringent conditions, indicating that mouse CGRP mRNA is highly homologous to the human CGRP mRNA.

In a preliminary experiment, we confirmed the predominant existence of CGRP mRNA in F9 cells reported by Rosenfeld and colleagues in transfection studies (9). The level of CGRP mRNA increased with culture time. Northern analysis of poly (A) RNA extracted from F9 cells after 6 days of culture showed that RNA was not degraded and the size of mature CGRP mRNA in F9 cells was identical to that in rat thyroid (≈1200 bases).

Our data confirmed that the putative mouse CGRP and human CGRP share antigenic determinants, as dilution curves of mouse CGRP-like material paralleled synthetic human CGRP in the radioimmunoassay. The level of CGRP in the medium was 10 times higher than that originally reported for CT in these cells (7). This is in agreement with the higher levels of CGRP mRNA in comparison to CT mRNA in F9 cells (unpublished data).

As previously shown for calcitonin (5), non-mammalian cCGRP was more effective than mammalian molecules in stimulating the adenylate cyclase activity of F9 cells (19, 20).

In numerous cell types growth factors regulate cell growth via an autocrine process (21). Addition of an antibody against the mitogenic growth factor inhibits cell growth (22).Similarly antiCGRP antibodies inhibit F9 cell proliferation.The inhibition of cell proliferation by anti-CGRP antibodies is most probably due to specific binding to secreted CGRP,since the addition of exogenous CGRP to the culture medium abolished this effect, while the addition of CGRP antibodies to cells not producing CGRP did not affect their growth. Recently a proliferative action of CGRP has also been reported in human endothelial cells (23).

Human CGRP at 10^{-6} M increased F9 adenylate cyclase activity and non-mammalian cCGRP was more potent in stimulating cyclic AMP synthesis, at 10^{-9} M (19). We found that stimulation of the adenylate cyclase of F9 cells occurred at concentrations higher than the CGRP concentration accumulated in the culture medium. These results may be explained by the fact that we are not using the homologous mouse CGRP to stimulate the adenylate cyclase system. Another hypothesis is the possible involvement of a different signal transduction pathway, such as phospholipids, in mediating the mitogenic effect, of CGRP which could be stimulated at lower concentrations. However, in endothelial cells CGRP, which stimulates adenylate cyclase activity, has no effect on inositolphosphate formation (21).

In conclusion, CGRP, which has a chemiotactic effect (20), can regulate the growth of embryonal carcinoma cells by an autocrine-type process. Our results suggest that CGRP, previously considered as a neuropeptide acting principally on the nervous and cardiovascular system (22-23), may be involved in early embryonic development. Of particular interest in this context are recent reports involving CGRP in development as a regulator of acetylcholine receptors (24-25) and dopaminergic phenotypes in the mouse olfactory bulb (26).

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