

The cholecystokinin gene is abundantly co-expressed with gastrin-releasing peptide, enkephalin and neuropeptide Y genes in a clonal human neuroepithelioma cell line

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The cholinergic human neuroepithelioma cell line SK-N-MCIXC expressed mRNAs for the neuropeptides cholecystokinin (CCK), neuropeptide Y, gastrin-releasing peptide (GRP) and enkephalin. The CCK transcript of about 800 nt was present at very high levels and CCK-like peptides immunoreactive to a C-terminal CCK octapeptide antiserum were present in the cell line and its medium. This clonal neuronal cell line provides a unique model system to identify *cis*- and *trans*-acting factors responsible for neuron-specific expression and regulation of the CCK gene. Furthermore, the pluripotent properties of the undifferentiated cell line may open studies on neuronal differentiation at the level of co-expression of neuropeptides and transmitters.

Cholecystokinin; Neuropeptide co-expression; Neuronal cell line; Gene expression; Enkephalin; Gastrin-releasing peptide

1. INTRODUCTION

Cholecystokinin (CCK) is a biologically active peptide of brain and gut [1–3]. Characteristic is the strict tissue specificity of CCK gene expression and the differential processing of the CCK precursor. CCK is one of the most abundant neuropeptides. It occurs in nearly all brain regions, predominantly as the octapeptide CCK-8, and has a variety of central effects.

The upper intestine is the major site of CCK synthesis in the gastrointestinal tract, in which various forms of CCK exist, including CCK-8, -22, -33, -39 and -58 [3,4]. Furthermore, CCK-like peptides are sometimes produced by neoplasms. CCK peptides and mRNA have previously been detected in medullary thyroid carcinomas, pancreatic islet cell tumors and in rat cell lines derived from these [5–7]. Recently, CCK has also been found in two cell lines of neuronal origin one of which is the neuroepithelioma cell line SK-N-MC [8]. When expressed at high levels, neuronal cell lines are potentially useful models to study brain-specific expression and regulation of neuropeptide genes. Here we report that the clonal human neuroepithelioma cell line SK-N-MCIXC co-expresses at least four neuropeptide genes of which the CCK gene at very high levels.

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2. MATERIALS AND METHODS

2.1. Cell culture

The human neuroepithelioma cell line SK-N-MCIXC was cultured in Eagle's medium (minimal essential medium (MEM)) supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids (Eagle's formulation), penicillin (100 IU/ml), and streptomycin (100 µg/ml) as described [9]. Cells were harvested by trypsinization 12 h after replacement of fresh medium at a density of 10^6 cells/cm³. For determination of the peptide content in the medium, cells were kept in medium containing 0.5% fetal calf serum for 16 h. The human neuroblastoma cell lines LA-N-1, SMS-MSN, and SMS-KAN were grown as described [9].

2.2. Northern blot analysis

Total RNA was prepared from washed cell pellets by homogenization in the presence of guanidine isothiocyanate followed by phenol extraction and precipitation as described before [10]. Alternatively, DNA-free cellular RNA preparations were obtained by the method of Wilkinson [11]. Total RNA (20 µg) was glyoxylated, subjected to electrophoresis in a 1.4% agarose gel and transferred to a nylon filter (Hybond-N, Amersham International, Amersham UK). Filters were prehybridized in 50% formamide, 6 × SSC, 1% SDS, 1% defatted milk powder, 100 µg/ml denatured herring sperm DNA, at 50°C for 6 h and hybridized to ³²P-labeled DNA probes, synthesized using random hexamer primers [12], in the same solution at 50°C for 16 h. Filters were washed in 1 × SSC, 0.1% SDS at room temperature twice for 5 min, 0.1% SDS, 0.1 × SSC at 50°C twice for 15 min and in 0.1 × SSC, 0.1% SDS 2 mM EDTA at 65°C for 30 min and exposed to X-ray film. The CCK probe was a 550 bp *AluI*-*AluI* fragment of exon 2 of the human CCK gene [13]. The pro-enkephalin probe was the rat cDNA clone pYSEA [14], the neuropeptide Y probe was a 280 bp fragment of exon 2 of the rat gene [15] and the gastrin-releasing peptide (GRP) probe was a 850 bp human cDNA [16].

2.3. Radioimmunoassay

Washed cell pellets (5×10^7 cells) were extracted according to

Rehfeld and Dockray [17,18]. Briefly, cells were boiled in water for 10 min, acidified to 1 M acetic acid, sonicated and centrifuged. The supernatant was freeze-dried, dissolved in radioimmunoassay (RIA) buffer and centrifuged again. Media were collected, boiled for 10 min, acidified and prepared for RIA as described above. The RIA employed antiserum L48, which is directed against the C-terminus of CCK [18], ^{125}I -iodinated CCK-8 as tracer and CCK-8 as standard. Therefore, CCK-immunoreactivity is expressed in equivalents CCK-8.

2.4. Southern blot analysis

DNA was prepared from isolated nuclei of SK-N-MCIXC cells and human placenta as described [19]. Twenty μg was completely digested with the restriction endonucleases *Eco*RI and *Hind*III, subjected to electrophoresis in 0.7% agarose and transferred to nylon filters (Zeta probe, BioRad, Richman, CA) in 2 M sodium hydroxide for 5 h. Prehybridization was performed in 1.5 \times SSPE (SSPE is 0.18 M sodium chloride, 10 mM sodium phosphate, pH 7.0, and 1 mM EDTA), 1% SDS, 0.5% defatted milk powder and 0.5 mg/ml herring sperm DNA. Hybridization was done in the same solution containing 10% dextran sulfate with the ^{32}P -labeled human CCK probe (3×10^6 cpm/ml) (see above). Blots were finally washed in $0.1 \times$ SSC, 1% SDS at 50°C for 30 min and exposed to X-ray film overnight.

3. RESULTS AND DISCUSSION

The human neuronal cell lines LA-N-1, SMS-MSN, SMS-KAN and SK-N-MCIXC were analyzed for expression of the CCK gene by Northern blot analysis of total RNA using a probe containing exon 2 of the human CCK gene [13]. CCK expression was only seen in the neuroepithelioma cell line SK-N-MCIXC (Fig. 1). The content of the CCK transcript in the SK-N-MCIXC cell line was extremely high; it was easily detected after 30 min exposure of the filter containing 20 μg total cellular RNA (Fig. 1). The CCK transcript had a length of approximately 800 nt and comigrated with CCK mRNA of human brain cortex (not shown).

The selectivity of expression was further evaluated by hybridization of filters with probes for several other neuroendocrine peptides. No signals were obtained with human vasopressin, oxytocin and pro-opiomelanocortin probes. Using probes for pro-enkephalin, GRP and neuropeptide Y, pro-enkephalin mRNA (~ 1200 nt), GRP mRNA (~ 900 nt) and neuropeptide Y mRNA (~ 700 nt) were detected in cellular and total RNA of SK-N-MCIXC cells (Fig. 1). The signals of these mRNAs were weaker than those of CCK mRNA on the same filters (Fig. 1). Based on the signal intensities and exposure times the ratio of CCK mRNA:GRP mRNA:enkephalin mRNA:neuropeptide Y mRNA was roughly estimated to be 200:20:2:1.

The SK-N-MCIXC cell line contained CCK-immunoreactivity as determined by RIA. Serial dilutions of extracts of SK-N-MCIXC cell pellets showed displacement of the tracer parallel to the CCK-8 standard. The cellular content of CCK-immunoreactivity experiments was low (15 ± 8 pg CCK-8 equivalents per 10^7 cells), but the media contained 227 ± 18 pg CCK-8 equivalents per 10^7 cells, indicating that CCK-like material was released at high rate into the medium.

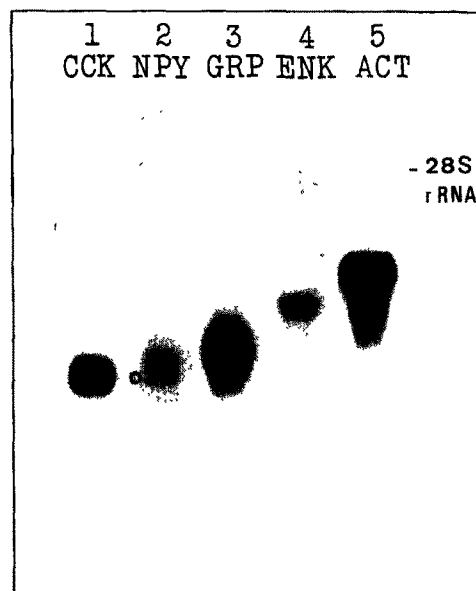


Fig. 1. Detection of neuropeptide mRNAs in the human neuroepithelioma cell line SK-N-MCIXC. Twenty μg of total RNA was analyzed on the Northern blot. The same blot hybridized to CCK, GRP, neuropeptide Y (NPY) and enkephalin (ENK) probes is shown. In lane 1 (CCK mRNA), the exposure time was 25 min, in lane 2 (GRP) 20 h, in lane 3 (ENK) 72 h and in lane 4 (NPY) 24 h. Lane 4 also shows the remaining signal of CCK appearing as the lower band (○).

Taken together, the findings of an abundant ~ 800 nt CCK transcript and CCK-immunoreactivity in the SK-N-MCIXC cell line indicates that this neuroepithelioma cell line contains an authentic human CCK mRNA and produces CCK-like peptides. The cellular content of CCK peptides may be lower than expected from the high level of CCK mRNA. It has been shown recently that the parent cell line SK-N-MC produces pro-CCK and pro-enkephalin in unprocessed form [8,20,21]. Similarly, most of the translation product of the CCK mRNA in SK-N-MCIXC may be partially unprocessed as well and was therefore undetectable to the antiserum used. The precise forms of CCK-immunoreactivity and the extent of processing of the CCK precursor awaits further immunological and biosynthetic characterization.

The human neuroblastoma cell line SK-N-MCIXC is a unique cell line of neuronal origin which expresses the human CCK gene at very high level. The CCK gene has been localized on chromosome 3 [22,23]. A normal, intact chromosome 3 appeared absent during cytological examination of the genome of SK-N-MCIXC, while minichromosomes occurred [9]. Therefore, DNA of this cell line was analyzed on Southern blots. Single bands of approximately 8 kb and 4 kb, were detected by the CCK exon 2 probe on Southern blots of DNA cut with *Eco*RI and *Hind*III, respectively. The size of fragments and the intensity of signals were the same as in normal human DNA (not shown). The results show that the human CCK gene in SK-N-MCIXC is present

as a single copy gene with a normal internal organization. Thus, no major amplification or rearrangement of the CCK gene has taken place. No instability of CCK expression with subsequent passage of cells was observed.

Neuroepithelioma cells are thought to be stem cells or immature intermediates to neuroblasts and epithelial cells [24,25]. The SK-N-MCIXC cell line was established after twice cloning the heterogeneous human neuroepithelioma cell line SK-N-MC [9]. Both cell lines display cholinergic properties, including the presence of acetylcholin and acetylcholinesterase [24,26]. The expression of the cholinergic phenotype and the co-expression of several neuropeptide genes (CCK, GRP, pro-enkephalin and neuropeptide Y) suggests that the undifferentiated SK-N-MCIXC cell line behaves like a pluripotent neuronal cell. Therefore, the SK-N-MCIXC cell line is not only useful in analyzing cell specificity and regulation of CCK gene expression and the transacting factors involved, but may also provide a valuable model for studies on peptide and transmitter co-expression during neuronal differentiation.

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