Coexpression of gastrin and gastrin receptors (CCK-B and ΔCCK-B) in gastrointestinal tumour cell lines

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Abstract

***Background*—The peptide hormone gastrin is a recognised growth factor for gastrointestinal (GI) tumour cells. Carboxyamidated gastrins bind to the cell surface gastrin/cholecystokinin B (CCK-B) receptor which can be expressed as either a normal or a truncated isoform (ΔCCK-B).**

***Aims*—To compare gastrin gene expression with ΔCCK-B and total CCK-B (both isoforms) gene expression in both GI and non-GI tract derived human tumour cell lines.**

***Methods*—Total RNA was extracted and gene expression was assayed by the reverse transcription-polymerase chain reaction followed by Southern blotting and hybridisation with specific oligo probes.**

***Results*—Gastrin was expressed by 5/5 gastric and 7/8 colorectal cell lines. Coexpression of gastrin CCK-B isoform was found in 80% of gastric and 75% of colorectal cell lines. Non-GI cell lines, with the exception of a lymphoblastic leukaemia cell line, showed no coexpression. The truncated receptor, ΔCCK-B, was shown in 3/5 gastric and 5/8 colorectal cell lines and was always coexpressed with gastrin.**

***Conclusions*—The truncated gastrin receptor, ΔCCK-B, is coexpressed with gastrin in 8/13 GI tumour cell lines. Gastrin and CCK-B receptor isoforms may be involved in maintaining autocrine/paracrine growth pathways in GI cancer cells.**

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Keywords: gastrin; CCK-B; ΔCCK-B; RT-PCR; gastrointestinal cell lines

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The peptide hormone gastrin is secreted into the circulation by G cells in the gastric antrum and acts on gastric fundic parietal cells to stimulate acid secretion. It is also a well characterised growth factor for gastrointestinal (GI) tumours.[1] The mitogenic gastrin species were originally thought to be the carboxyamidated gastrins, of which G17 is the most plentiful in the antrum, but a post-translational processing intermediate, glycine extended gastrin (G-Gly) has also been found to be secreted into the circulation by the normal antrum and act as a growth factor.[2 3] Both precursor and carboxyamidated gastrin forms have been shown to be synthesised by a proportion of colorectal[4] and gastric tumours.[5] Tissue culture studies on GI tumour cell lines have shown that antigastrin antibodies can inhibit basal growth,[6] suggesting a gastrin autocrine loop.

The gastrin/cholecystokinin B (CCK-B) receptor is a member of the seven transmembrane domain superfamily. It binds cholecystokinin and G17 with high affinity and is expressed in the cortex and the gastric fundus.[7] In the latter tissue, CCK-B mediates acid secretion and the proliferative effects of G17 are exerted on gastric epithelium per se. It has been shown that CCK-B antagonists can inhibit growth of GI tumour lines and in vivo studies have shown an enhanced survival rate in xenograft models treated with these antagonists.[1] Expression of CCK-B mRNA has also been shown in some colorectal cell lines and tumour samples.[8] Recently, an alternative first exon (exon 1b) of the CCK-B gene was discovered.[9] Usage of this exon during transcription results in expression of a truncated isoform, termed ΔCCK-B, which shares an identical amino acid sequence with the normal receptor but lacks the N-terminal extracellular domain. The role of both CCK-B receptor isoforms in the malignant scenario is not known. In the present study, reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting have been used to assay various GI and non-GI cancer cell lines for expression of gastrin, total CCK-B isoform expression, and the ΔCCK-B isoform.

Materials and methods

CELL CULTURE

Cells were grown in RPMI media (Gibco BRL, Paisley, UK) containing 10% heat inactivated newborn calf serum (Sigma, Poole, Dorset, UK) at 37°C and in 5% CO_2.

RNA EXTRACTION

Total RNA was extracted from 10^6 cells using RNAzol-B (Biogenesis, Poole, Dorset, UK). Cells were lysed in RNAzol-B (1 ml) with chloroform (110 µl; Sigma). An aqueous phase was separated by a 13 000 *g* spin and eluted. RNA from the eluate was precipitated using isopropanol and washed twice in 75% ethanol. The RNA pellet was then resuspended in diethylpyrocarbonate (DEPC; Sigma) treated sterile water (50 µl).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Reverse transcription was performed as previously described.[10] Total RNA preparations were divided into two halves (25 µl) and

Table 1 Primer and probe sequences

Primer/probe	*Sequence (5'–3')*
Gastrin	
Probe	AGCACTGCGGCGGC
Upper primer	GCCCAGCCTCTCATCATC
Lower primer	GGGGACAGGGCTGAAGTG
CCK-B	
Probe	AGTTGCACGTAGCAGCCAT
Upper primer	TCTCGCGAGCTCTACTTAGGG
Lower primer	GAAGTTGCACGTAGCAGCCA
ΔCCK-B	
Probe	TGGGGTACATCCACAAGTGC
Upper primer	CAGGAGAAGAACTGAACTGTCC
Lower primer	TCGCTGACTGCCAGTGAGAG
GAPDH	
Upper primer	GGTGAAGGTCGGAGTCAACGGA
Lower primer	GAGGGATCTCGCTCCTGGAAGA

GAPDH primers are from MacLaughlin *et al.*[10] ΔCCK-B probe/primer sequences and code names are from Miyake.[9]

randomly primed. Both halves were incubated at 25°C for 10 minutes, 37°C for one hour, and 95°C for 10 minutes in reverse transcription reaction buffer (1× Dynazyme PCR buffer (Flowgen, Sittingbourne, Kent, UK), 30 µmol dNTPs (Pharmacia, St Albans, Herts, UK), 0.01 M dithiothreitol (Gibco BRL), 200 U Superscript reverse transciptase (Gibco BRL, UK)). One half was reverse transcribed into cDNA while the other half was used as a negative control and the reverse transcriptase was replaced with water. PCR was performed using a similar protocol to that described previously[10] except that different cDNA volumes were utilised for individual reactions. Reactions (50 µl) were prepared in 1.0 U Dynazyme (Flowgen) in 1× Dynazyme PCR buffer (Flowgen) with 40 µmol dNTPs (Pharmacia) and 10 µmol of upper and lower primers. The different volumes of cDNA preparations used for each PCR were as follows: GAPDH, 1 µl; gastrin, 5 µl; CCK-B and ΔCCK-B, 10 µl. One drop of mineral oil was layered on top of the reaction mixture which was incubated in a thermal cycler (Hybaid, Teddington, Middlesex, UK) at 95°C for five minutes before 40 cycles of 95°C for 45 seconds, 60°C for 90 seconds, and 72°C for 90 seconds. This was followed by a single stage of 60°C for 120 seconds and 72°C for 180 seconds.

SOUTHERN BLOTTING

Polymerase chain reaction products (10–20 µl) were electrophoresed on 2% (wt/vol) agarose gels and transferred on to Hybond-N nylon membrane (Amersham International, Aylesbury, Buckinghamshire, UK) by capillary blotting using an alkaline transfer buffer (0.5 M NaOH, 0.6 M NaCl). Blots were neutralised in 2× saline sodium citrate (2×SSC; Sigma) and ultraviolet fixed.

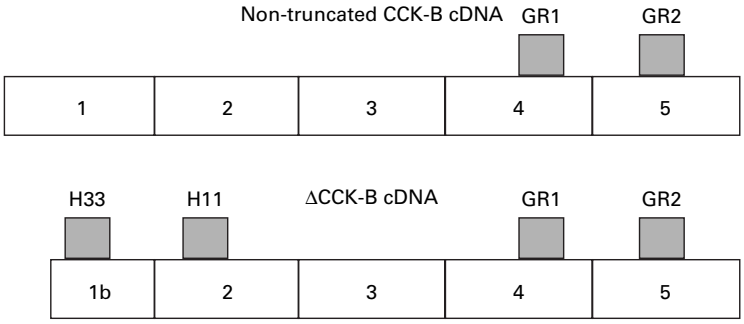

Figure 1 The "total CCK-B" RT-PCR assay using primers GR1 and GR2 detects both isoforms, while primers H33 and H11 specifically detect ΔCCK-B, the truncated CCK-B receptor.

PROBE LABELLING AND PRIMER SEQUENCES

Primers (H11 and H33) and the probe (H34) for ΔCCK-B are identical to the sequences published by Miyake.[9] All other primers and probes were designed on the OLIGO program (Medprobe a/s, Oslo, Norway) based on cDNA sequences taken from Seqnet (Daresbury Laboratory, UK). The gastrin probe was synthesised and 5'-digoxygenin (DIG) labelled by R&D Systems (Abingdon, Oxon, UK). The CCK-B and ΔCCK-B probes were synthesised as unlabelled oligos and 3' tailed with poly dATP and multiple DIG labelled dUTP using a DIG tailing kit (Boehringer Mannheim). All primers and the CCK-B and ΔCCK-B probes were synthesised by the Biopolymer Synthesis Unit (Department of Biochemistry, University of Nottingham). Table 1 and figure 1 present all sequences.

HYBRIDISATION

Blots were prehybridised in 5×SSC (Sigma), 1% (wt/vol) Blocking Reagent (Boehringer Mannheim, Lewes, East Sussex, UK), 0.1% (wt/vol) N-lauroyl sarcosine (Sigma), and 0.02% (wt/vol) sodium dodecyl sulphate (SDS; Sigma) for 30 minutes and then hybridised for 60 minutes with specific oligo probes. The hybridisation temperatures varied depending on the probes used. For gastrin, hybridisation proceeded at 34°C whereas the CCK-B and ΔCCK-B probes were used at 55°C with the inclusion of poly(A) (Boehringer Mannheim) to block non-specific binding. Post-hybridisation washes were performed at room temperature for gastrin and 50°C for CCK-B and ΔCCK-B. First washes were with 2×SSC + 1% (wt/vol) SDS for 10 minutes twice. Second washes were with 2×SSC for gastrin for 10 minutes twice or 0.5×SSC for CCK-B and ΔCCK-B for 10 minutes twice. Probe detection and chemiluminescence was carried out using anti-DIG-AP conjugate and CSPD (Boehringer Mannheim) following the manufacturer's instructions. After a 15 minute incubation at 37°C, the blots were exposed to a light sensitive *x* ray film (X-OMAT; Sigma) for between 45 and 90 minutes.

SEQUENCING

Polymerase chain reaction products were excised from agarose gels and purified using the Bandprep Kit (Pharmacia Biotech). Agarose gel slices were melted and DNA allowed to bind to Sephaglas (Pharmacia Biotech). Samples were washed and allowed to dry before resuspension in sterile water prior to sequencing. Sequencing was performed on an A373A sequencing machine (Advanced Biotechnologies, Warrington, UK) using the upper primer of each product, by the Biopolymer Synthesis Unit (Department of Biochemistry, University of Nottingham).

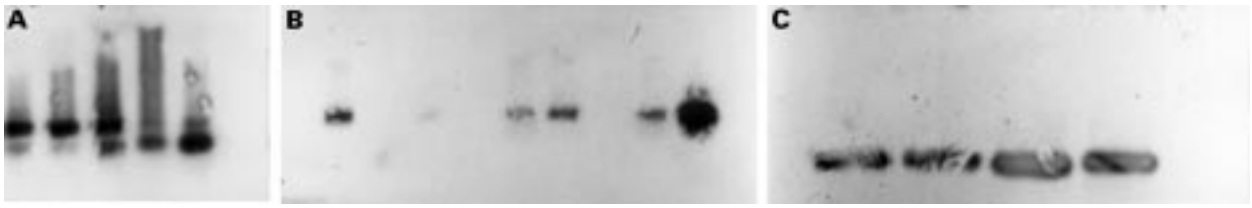

Figure 2 (A) Southern blot of gastrin RT-PCR products from cell lines showing 215 bp and 345 bp bands. From left to right: AGS, A431, HT29(L), Colo205, 791T. (B) Southern blot of ΔCCK-B RT-PCR products showing a 281 bp band in those cell lines where it was detected. From left to right: HCT116, LIM1215, C170HM2, MCF-7B, AGS, AGS, MKN45, C170HM2, LoVo. (C) Southern blot of CCK-B RT-PCR products. From left to right: AGS, MCF-7B, HCT116, ST16 ascites.

Results

GAPDH PCR PRODUCTS

All cDNA preparations gave an expected PCR product for GAPDH, verifiying successful RNA extraction and reverse transcription.

GASTRIN PCR PRODUCTS

Normal human gastric antrum cDNA samples gave an expected gastrin PCR product of 215 bp which was detected on Southern blots by a specific oligo probe. The PCR product was sequenced and was found to correspond to the published gastrin cDNA sequence.[11] Table 2 presents results of RT-PCR. Gastrin was expressed in 5/5 and 7/8 human gastric and colorectal cell lines respectively and was also detected in lymphoblastic leukaemia, fibrosarcoma, epidermoid, and osteosarcoma derived cell lines. Gastrin PCR products from tumour cell line samples showed a characteristic two band pattern (fig 2A) as reported previously in colon tumour cells.[12 13] The expected product, derived from a mature mRNA, of 215 bp was coexpressed with a higher molecular weight band of 345 bp. Negative control RT-PCR lanes showed no hybridisation on Southern blots, verifiying that the 345 bp band was derived from a gastrin RNA species. The gastrin PCR primers bind to regions on exons 2 and 3 of the cDNA and so the extra band is derived from retention of intron 2 as previously reported.[12 13]

EXPRESSION OF ΔCCK-B AND TOTAL CCK-B

The total CCK-B RT-PCR assay detected a region common to both ΔCCK-B and non-truncated CCK-B. Expected total CCK-B RT-PCR products of 169 bp to 184 bp were detected on agarose gels and comigrated and specifically hybridised with an oligo probe on Southern blots (fig 2C). LoVo cells were used as a positive control for a specific ΔCCK-B RT-PCR and a product of the expected molecular weight, 281 bp, was observed on agarose gels and specifically hybridised on Southern blots (fig 2B).

The assay for total CCK-B receptor detected expression in 4/5 and 6/8 human gastric and colorectal cell lines respectively (table 2), as well as the breast (MCF7) and lymphoblastic leukaemia (Molt 4) derived cell lines. The truncated isoform, ΔCCK-B, was expressed in 3/5 gastric and 5/8 colorectal cell lines (table 2) and the one lymphoblastic leukaemia derived cell line (Molt 4). This is the first study showing widespread expression of ΔCCK-B in GI tract tumour cell lines. The GI cell lines RD19 and LIM 1215 as well as the breast line MCF7 gave PCR products for total CCK-B but not ΔCCK-B. They therefore exclusively express non-truncated CCK-B receptor molecules.

Table 2 Results of RT-PCR

		Gastrin bands		*Total CCK-B*	*ΔCCK-B*
Origin	*Cell line*	*215 bp*	*345 bp*	*169–185 bp*	*281 bp*
Gastric	MGLVA1 ascites	Y	Y	Y	Y
	ST16	Y	Y	Y	Y
	MKN45	Y	N	N	N
	RD 19	Y	Y	Y	N
	AGS	Y	Y	Y	Y
Colorectal	HCT 116	Y	Y	Y	Y
	C170HM2	Y	Y	Y	Y
	AP5LV	N	N	N	N
	LoVo	Y	Y	Y	Y
	HT29 (L)	Y	Y	Y	Y
	LIM 1215	Y	Y	Y	N
	Colo 205	Y	Y	N	N
	KN12HM	Y	Y	Y	Y
Breast	MCF7	N	N	Y	N
	BR293	N	N	N	N
Fibrosarcoma	HT 1080	N	Y	N	N
Epidermoid	A431	Y	Y	N	N
Bladder	RT112/21	N	N	N	N
Osteosarcoma	791T	Y	Y	N	N
Leukaemia	Molt 4	Y	Y	Y	Y

Discussion

Gastrin is an important growth factor for GI cancer, being expressed almost ubiquitously in GI tract tumours.[1 4] In this study we report gastrin gene expression in 5/5 gastric and 7/8 colorectal tumour cell lines, coupled with an almost ubiquitous accumulation of gastrin pre-mRNA. Gene specific pre-mRNA accumulation, and also export to the cytosol, has been reported for the CD44 gene in GI tract tumours.[14] It is not known whether gastrin and CD44 pre-mRNAs are retained via the same mechanism, but if so then gastrin pre-mRNA might also be exported from the nucleus and then translated into a novel protein product where any potential biological activity may be assayed. Its high prevalence in GI tract tumour cells would be consistent with it imparting some advantage on tumour growth.

In the present study, it has been shown that expression of CCK-B receptor isoforms is always coupled with coexpression of the gastrin gene in GI tumour cell lines, implying that a gastrin/CCK-B autocrine loop may be maintained. Production of mature, carboxyamidated gastrin has been reported in the gastric cell line AGS,[5] and also in 69% of colorectal tumours,[4] and it is these species that stimulate the CCK-B receptors. This study is also the first to show expression of ΔCCK-B on several GI tract cell lines, with only LoVo and AGS having previously been characterised.[9] All of the tumour cell lines expressing ΔCCK-B

coexpressed gastrin. Any autocrine/paracrine gastrin growth pathways in these cell lines may be mediated by ΔCCK-B, and this possibility needs to be investigated. In non-GI tract tumour cell lines coexpression of gastrin and CCK-B was not found, except in the lymphoblastic leukaemia cell line Molt 4. Leukaemia cell lines have recently been reported to coexpress gastrin and CCK-B mRNA and maintain an autocrine growth pathway.[15]

The alternative exon, 1b, of the CCK-B gene may also be linked to alternative promotor sequences than exon 1. Both truncated and non-truncated receptor isoforms may therefore undergo a degree of tissue specific expression. Both isoforms are coexpressed in normal human brain and fundus, suggesting that they may be involved in the same normal physiological processes but exclusive expression of ΔCCK-B was found in the AGS gastric tumour cell line.[9] This study has found exclusive non-truncated CCK-B expression in three of the cell lines assayed (LIM 1215, RD 19, and MCF 7B), raising the possibility that tumour cells may uncouple the coexpression of receptor isoforms and that their malignant functions may differ. The ΔCCK-B receptor has a lower affinity for gastrin than its non-truncated counterpart[9] and would potentially only bind G17 at high concentrations. If human GI tumours are expressing ΔCCK-B and also producing and secreting carboxyamidated gastrins locally then they could function to maintain an autocrine/paracrine pathway.

The ΔCCK-B receptor has no unique amino acid sequences and assays designed to detect CCK-B expression must control for this, or else the interpretation of results may be confounded. Specific immunological detection of only ΔCCK-B will be impossible, unless differences in receptor configuration occur, and nucleic acid assays may codetect both isoforms unless designed to be specific for the alternative first exons. Ligand binding assays may not be able to distinguish between ΔCCK-B and other low affinity gastrin receptors such as CCK-C or even CCK-A (reviewed in Wank[16]). Our RT-PCR method specifically assayed ΔCCK-B but not non-truncated CCK-B and so identification of this molecule only occurred when ΔCCK-B was absent from the sample. Consequently, we did not investigate the possibility of exclusive ΔCCK-B expression.

The evidence from this study strongly implies the maintenance of a gastrin autocrine pathway in these cells acting via CCK-B isoforms. Patients with GI tract tumours may benefit from antigastrin directed therapies, such as Gastrimmune,[1 17] which potentially may eliminate both tumour and serum gastrin levels. Work is currently underway to quantify CCK-B isoform mRNA levels and also specifically identify the gastrin receptors expressed in tumours.

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