Targeting of cholecystokinin B/gastrin receptor in colonic, pancreatic and hepatocellular carcinoma cell lines

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Abstract. Gastrin is a growth factor for both gastrointestinal and non-gastrointestinal tumours. Endocytosis of gastrin has been demonstrated in tumour cell lines expressing cholecystokinin-B/gastrin receptor (CCK-BR); this has raised the possibility of receptor targeted therapy. The aim of this study was to examine endocytosis of gastrin and CCK-BR in tumour cell lines. A small gastrin analogue, RG-G7, and the anti-CCK-BR antibody, anti-GRE1, were fluorescently labelled and uptake by cancer cell lines including AR42J, HepG2, and C170HM2 as well as transfected NIH3T3 fibroblast cells was assessed using standard and confocal fluorescence microscopy. CCK-BR expression of cell lines was assayed by reverse transcription-polymerase chain reaction and Western blotting. Apoptosis was detected using a fluorescent TUNEL method. RG-G7 and anti-GRE1 antibody were specifically taken up by all cell lines expressing CCK-BR. In addition to cytoplasmic uptake with RG-G7 and anti-GRE1 the latter also showed specific uptake into the nucleus. A coincidence of anti-GRE1 and apoptosis was seen. Targeting CCK-BR by peptide or antibody may offer therapeutic opportunities for some cancers.

Introduction

Gastrin is a peptide hormone which has long been known as a stimulant of gastric acid secretion (1). It also exerts a trophic effect on normal and malignant gastrointestinal cells both in vitro and in vivo (2-7), and can act as a growth factor for some non-GI tumours (8,9). In the post-translational processing of gastrin the precursor form preprogastrin is cleaved by enzymes to glycine-extended gastrin and finally the mature amidated gastrin (10). Amidated gastrin exerts its effect via the cholecystokinin (CCK)-B/gastrin receptor, a G-protein coupled receptor with seven hydrophobic transmembrane domains (11). There are two types of CCK receptors, type A and type B. CCK-A receptor was first characterised on pancreatic acinar cells (12), and CCK-B receptor was initially characterised in the brain (13), and has subsequently been shown to be identical to the gastrin receptor (11,14,15). The CCK-A and -B receptors show 48% amino acid homology (16), and can be differentiated by their agonist binding. CCK-A receptor binds sulphated CCK8 with high affinity and gastrin with low affinity, whereas CCK-B receptor (CCK-BR) recognises gastrin and both sulphated and non-sulphated CCK8 with approximately equal affinity (17). CCK-B/gastrin receptors are expressed in a number of human neoplastic cell lines and tissues of GI and non-GI origin (18-26).

Our group has been studying the expression of CCK-B/gastrin receptor in a number of tumours, using an anti-gastrin receptor antibody (anti-GRE1 antibody). We have previously demonstrated in addition to membrane and cytoplasmic expression of CCK-BR that there is nuclear expression of the receptor in some cancers including hepatocellular and pancreatic carcinoma (27-29). These studies were principally performed using immunocytochemistry and immuno-electron microscopy.

The aim of this study was to examine with confocal microscopy endocytosis of gastrin and CCK-B/gastrin receptor, using the anti-CCK-BR antibody, anti-GRE1. A fluorescently labelled anti-gastrin receptor antibody (anti-GRE1) and gastrin peptide derivative (RG-G7) (30) were used to observe co-localisation of gastrin and gastrin receptor and uptake of the anti-receptor antibody by tumour cells. The secondary aim was to determine the difference between uptake of antibody and of RG-G7 which may be of practical importance in the consideration of targeted therapy using antibody versus peptide. The studies were performed on cancer cell lines of colonic, pancreatic and hepatic origin (31-33), as well as on a mouse fibroblast cell line NIH3T3, NIH3T3 cells, transfected with either a classical or spliced variant of CCK-BR, were used as a positive control (34). Sub-cellular localisation was determined using confocal scanning laser microscopy (CSLM).
Materials and methods

Gastrin and anti-gastrin receptor antibody. Gastrin-7 (Sigma, Poole, Dorset, UK) was labelled with rhodol green (RG-G7) according to the method of Czerwinski et al (35). This short form of labelled gastrin has previously been shown to react specifically with the CCK-B/gastrin receptor whilst retaining the biological properties of gastrin (30).

Anti-GRE1 is an affinity purified rabbit polyclonal antibody targeting the amino terminal extra-cellular domain of human CCK-BR, raised using a peptide consisting of residues 5-21 of the CCK-B/gastrin receptor as previously described (29). The specificity of this antibody for the CCK-B/gastrin receptor has been confirmed by Western blotting, ELISA and radioligand inhibition binding studies (29). Anti-GRE1 antibody (Aphoton, CA, USA) was labelled with a red fluorescent marker (Alexa-546; Molecular Probes, OR, USA). This label was chosen because it is more fluorescent and more photostable than other commonly used markers (36), and is particularly suitable for confocal fluorescence microscopy. Briefly, the antibody was concentrated by centrifugation using centricron-SR3 vials (Amicon, MA, USA) to a final concentration of 2 mg/ml. Antibody (0.5 ml) was added to a vial of dye, mixed, and incubated overnight at 4°C, then labelled antibody was separated from unincorporated dye using a gel column. The staining characteristics of the fluorescently labelled antibody were compared to those of unlabelled antibody by staining of formalin fixed, paraffin embedded material from a gastrinoma. Binding of unlabelled antibody was detected using alkaline phosphatase conjugated sheep anti-rabbit antibody (Sigma). Specificity of binding of Alexa 546-labelled anti-GRE1 antibody was confirmed by preabsorption of antibody with antigenic peptide. F(ab) and F(ab)2 fragments of anti-GRE1 antibody were labelled with rhodamine, using standard techniques (37). The staining characteristics of these fragments of GRE1 antibody were confirmed in the same way.

Tumour cell lines. Tumour cell lines studied included AR42J, a rat pancreatic acinar carcinoma cell line (31); HepG2, a human hepatoma cell line (32); and C170HM2, a human colon carcinoma cell line (33). For a positive control, NIH3T3 cells stably transfected with expression vector containing either the classical or spliced variant form of the human CCK-B/gastrin receptor cDNA were used (34). Non-transfected NIH3T3 cells were used as a negative control. AR42J, HepG2 and NIH3T3 cell lines were obtained from the European collection of cell cultures (ECACC, Porton Down, UK); C170HM2, a recombinant colon cancer cell line, was from University of Nottingham, UK; transfected NIH3T3 cells were a gift from Professor T. Matsui (Kobe University, Kobe, Japan).

AR42J, C170HM2, HepG2 and non-transfected NIH3T3 cells were grown in RPMI-1640 medium containing 10% foetal calf serum and 2 mM glutamine (Sigma). Transfected NIH3T3 cells were grown in the same medium plus Geneticin (1 mg/ml; Sigma) to select for clones expressing the plasmid. RG-G7 was added at a concentration of 50 nM for 1 h at 37°C. Cells were then rinsed in 0.01 M phosphate buffer containing 0.0027 M KC1 and 0.137 M NaCl, pH 7.4 (PBS), fixed for 5 min in 10% neutral buffered formalin, rinsed again in PBS and then mounted in Vectashield (Vector, CA, USA).

To demonstrate any membrane binding of RG-G7, cells were incubated with Alexa-594 conjugated concanavalin A (Molecular Probes) for 3 min at 4°C before fixation. Results were viewed using both standard and confocal fluorescence microscopy. Fluorescent staining of cells was visualised on a Zeiss standard 14 epifluorescence microscope with an HBO 50 W high pressure mercury source and Zeiss filter set no. 9. Confocal laser scanning was performed using an Olympus BH2 microscope with a Bio-Rad (Hemel Hempsted, UK) MRC 600 confocal laser imaging system with an Argon-krypton laser, and COMOS 7.0A image acquisition software.

Co-localization of gastrin and gastrin receptor. After incubation with RG-G7 for 1 h, AR42J cells were fixed, then incubated with Alexa-546 conjugated anti-GRE1 antibody (20 μg/ml in PBS) for 1 h at room temperature. Slides were then rinsed in PBS for 5 min, mounted, and viewed by standard and confocal fluorescence microscopy.

Uptake of anti-CCK-B/gastrin receptor antibody (anti-GRE1 antibody). Cells were grown onto glass four-well slides as for RG-G7. Alexa-546 conjugated anti-GRE1 antibody was added to the cells for 1 h at 37°C at a concentration of 20 μg/ml. Cells were rinsed in PBS, then fixed in formalin and mounted in Vectashield (Vector). Anti-GRE1 antibody was added to tumour cell lines (described above) and to transfected and non-transfected NIH3T3 cells. Rhodamine-labelled F(ab) and F(ab)2 fragments of anti-GRE1 antibody were also added to all cell lines, under the same conditions. As a negative control, rhodamine-conjugated rabbit anti-mouse immunoglobulin (Dako), and rhodamine-conjugated rabbit IgG, F(ab), fragment (Jackson Immunoresearch Labs, PA, USA) were added to cell lines at the same concentration (20 μg/ml) as anti-GRE1 antibody, for 1 h at 37°C.

Detection of apoptosis. GRE1 antibody was added to all cell lines as described above. After incubating for 1 h at 37°C, cells were rinsed in PBS and fixed in 1% paraformaldehyde/PBS (Sigma). Apoptotic cells were detected using the TUNEL method (Apoptag fluorescein in situ detection kit, Intergen, USA), according to the manufacturer’s instructions.

Immunoblotting. Lysates of all cell lines were prepared in 80 mM Tris buffer pH 6.8 containing 5% sodium dodecyl sulphate and 1 mM phenylmethyl-sulphonyl fluoride (Sigma). Protein content in the lysates was measured using a modified Lowry assay (Bio-Rad).

A volume of lysate corresponding to 10 μg total protein from each sample was loaded onto a 10% acrylamide gel (Sigma). After separation, the proteins were electrotransferred to nitrocellulose membranes (Bio-Rad).

The membranes were incubated in PBS containing 5% bovine serum albumin/0.1% Tween-20 (Sigma) for 1 h at room temperature followed by 2-h incubation with the same
was obtained with both fluorescently labelled and unlabelled embedded gastrinoma. Nuclear staining of the gastrinoma confirmed by staining sections from a formalin-fixed paraffin

using anti-GRE1 antibody labelled with Alexa-546 was

The specificity and sensitivity of immunostaining obtained

The specificity and sensitivity of immunostaining obtained using anti-GRE1 antibody labelled with Alexa-546 was confirmed by staining sections from a formalin-fixed paraffin-embedded gastrinoma. Nuclear staining of the gastrinoma was obtained with both fluorescently labelled and unlabelled anti-GRE1 antibody; this staining could be abolished by pre-absorbance with epitope. This staining pattern has previously been described (27). F(ab) and F(ab)2 fragments of anti-GRE1 labelled with rhodamine gave the same staining pattern on this material; this staining could also be abolished by pre-absorbance of the antibody with the epitope.

buffer containing either anti-GRE1 antibody (1:200) or anti-GRE1 preabsorbed with the antigenic peptide. The membranes were washed with PBS/0.1% Tween-20 and incubated with biotinylated goat anti-rabbit/mouse antibody (Dako; 1:200) in 0.1% Tween-20/PBS for 1 h. After washing with PBS, the membranes were incubated with streptavidin-biotin complex (Dako) for 1 h. Labelling was revealed using diaminobenzidine (Vector).

Detection of gastrin receptor mRNA. To detect expression of CCK-B/gastrin receptor in these cell lines, reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect CCK-B/gastrin receptor mRNA. Total RNA was isolated from all cell lines. Cell suspensions were prepared using trypsin-EDTA, and total RNA isolated from 1-3x10⁶ cells using the SV total RNA isolation system (Promega) according to the manufacturer's directions. DNAase I digestion was performed to reduce the possibility of amplification of genomic sequences. Yields were estimated by UV spectrophotometry and RNA integrity was confirmed using denaturing gel electrophoresis. Reverse transcription and PCR were carried out using the one-step Access RT-PCR system (Promega). Specific primers for both gastrin receptor (19) and ß-actin (24) were used; these span introns enabling the distinction of genomic and mRNA amplifiers.

RT-PCR was performed on 1-2 μg of total RNA, using 50 pmol of each CCK-BR primer, or 25 pmol of each ß-actin primer. Reverse transcription was carried out at 48°C for 1 h, followed by 94°C for 2 min. On duplicate samples, reverse transcriptase was omitted as a negative control. PCR was 40 cycles of 94°C for 45 sec, 60°C for 90 sec, 68°C for 2 min, followed by a final cycle of 68°C for 7 min. A second round of PCR was performed using the Taqbead hot start polymerase system (Promega), on 2 μl of the primary reaction, under the same cycling conditions. Products were analysed by agarose gel electrophoresis.

Results

The specificity and sensitivity of immunostaining obtained using anti-GRE1 antibody labelled with Alexa-546 was confirmed by staining sections from a formalin-fixed paraffin-embedded gastrinoma. Nuclear staining of the gastrinoma was obtained with both fluorescently labelled and unlabelled anti-GRE1 antibody; this staining could be abolished by pre-absorbance with epitope. This staining pattern has previously been described (27). F(ab) and F(ab)2 fragments of anti-GRE1 labelled with rhodamine gave the same staining pattern on this material; this staining could also be abolished by pre-absorbance of the antibody with the epitope.

Uptake of RG-G7. Binding and internalisation of gastrin (RG-G7) was seen in AR42J, HepG2 and C170HM2 cells (Table I; Fig. 1a-c). Gastrin was taken up into the cytoplasm of these cancer cells. Binding was also seen in NIH3T3 cells stably transfected with gastrin receptor (Fig. 1d), but not in non-transfected NIH3T3. Fixed AR42J cells showing gastrin uptake were stained for gastrin receptor using Alexa-546 conjugated anti-GRE1 antibody, co-expression of gastrin and gastrin receptor was seen (Fig. 2). Gastrin receptor was detected on the membrane and within the cytoplasm of these cells.

Uptake of anti-GRE1 antibody. In the tumour cell lines AR42J, C170HM2 and HepG2, addition of anti-GRE1 antibody to live tumour cells resulted in binding and internalisation of the antibody into the cytoplasm and the nucleus of cells (Figs. 3 and 4). F(ab) and F(ab)2 fragments of anti-GRE1 antibody were also incubated with live cells from these tumour lines; uptake into the cytoplasm and the nucleus was seen. No uptake of rhodamine-conjugated irrelevant antibodies [rabbit anti-mouse antibody; rabbit IgG, F(ab)2 fragment] by these cell lines was seen. Uptake of anti-GRE1 antibody was not seen in non-transfected NIH3T3 cells. In NIH3T3 cells transfected with human gastrin/CCK-B receptor, addition of anti-GRE1 antibody to live cells resulted in binding to the membrane of cells (Fig. 4). This pattern of uptake differed from that seen in the tumour cell lines in that binding was confined to the membrane whereas tumour cells also demonstrated translocation to the nucleus.

Detection of apoptosis. Cells which showed anti-GRE1 antibody uptake were also found to be positive for apoptosis (Fig. 5). This coincidence of uptake and apoptosis was seen in tumour cell lines, but not in NIH3T3 cell lines.

Immunoblotting. The results of Western blotting of whole cell lysates is shown in Fig. 6. Specific immunoreactive bands of

<table>
<thead>
<tr>
<th>AR42J</th>
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<th>NIH3T3 (transfected)</th>
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<td>m</td>
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<td>GRE1</td>
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m, membrane; c, cytoplasmic; n, nuclear
Mr 241,000, 184,000 and 124,000 were seen in AR42J cells. Bands of Mr 184,000 and 124,000 were also seen in NIH3T3 cells transfected with the classical and spliced variant of CCK-B/gastrin receptor. In C170HM2 and HepG2 cell lines, specific bands were seen of Mr 151,000 and 112,000. No specific immuno-reactive bands were detected in non-transfected NIH3T3 cells.

SAVAGE et al: CHOLECYSTOKININ B/GASTRIN RECEPTOR

Figure 1. Fluorescently labelled gastrin (RG-G7) was added to (a) AR42J cells, (b) HepG2 cells, and (c) C170HM2 cells. Internalisation of gastrin was seen in all cell lines, as it was in NIH3T3 cells stably transfected with CCK-B/gastrin receptor (d). No binding or internalisation of RG-G7 was seen in non-transfected NIH3T3 cells.

Figure 2. Confocal photomicrograph of AR42J cells incubated with RG-G7 (green), then fixed and stained with anti-CCK-B/gastrin receptor antibody, anti-GRE1 (red). Gastrin was taken up into the cytoplasm of these cells; colocalisation of gastrin and gastrin receptor (yellow) can be seen; gastrin receptor alone can be seen on the surface of one cell. Optical sectioning of the cells using the confocal microscope confirms gastrin is taken up into the cytoplasm but not the nucleus of cells.

Figure 3. Live AR42J cells incubated with Alexa-546 labelled anti-CCK-BR antibody anti-GRE1 showed uptake of the antibody into the cell. Optical sectioning of the cell at 2-μm intervals using confocal microscopy shows that antibody is taken up into the nucleus as well as the cytoplasm of the cell (confocal photomicrograph).

Figure 4. Anti-GRE1 antibody added to (a) C170HM2 cells and (b) HepG2 cells can be seen within the cytoplasm and the nucleus of cells. In contrast, NIH3T3 cells transfected with classical (c) and spliced variant (d) of CCK-B/gastrin receptor show binding of anti-GRE1 antibody to the membrane of the cell, but no uptake into the nucleus (standard fluorescence photomicrograph; magnification, x660).

Figure 5. AR42J cell showing apoptosis, demonstrated by TUNEL assay (a) in a cell which has taken up anti-GRE1 antibody into the nucleus (b). Co-localisation can be seen (c) (fluorescence photomicrograph; x660).

Figure 6. Western blotting on whole cell lysates from all cell lines. Blotting was performed using anti-GRE1 antibody (a), and on duplicate blots with antibody preabsorbed with epitope (b). AR42J cells (1) showed specific bands of Mr 241,000, 184,000 and 124,000. NIH3T3 cells transfected with the spliced variant (2) and classical form (3) of the CCK-B/gastrin receptor showed specific bands of Mr 184,000 and 124,000. No specific bands were seen in non-transfected NIH3T3 cells (4). In C170HM2 (5) and HepG2 cells (6), specific bands of Mr 151,000 and 112,000 were seen.
in non-transfected NIH3T3 cells, i.e. not expressing CCK-BR. No uptake of irrelevant antibodies, added under identical conditions, was seen in any of the cell lines.

Confocal microscopy showed that anti-GRE1 antibody is internalised by tumour cells and can be detected within the cytoplasm and the nucleus of cells. Nuclear uptake of the antibody was seen in the pancreatic, colonic and hepatic cancer cell lines. This translocation of anti-GRE1 antibody to the nucleus of tumour cells is interesting in the light of previous observations using immunohistochemistry in which nuclear expression of the CCK-B receptor was seen in human resection specimens of hepatocellular carcinoma, pancreatic carcinoma, and gastric carcinoid (27,28,38). The internalisation of anti-GRE1 antibody demonstrated in this study is specific; it is not mediated via the Fc receptor as F(ab) fragments of anti-GRE1 antibody were internalised in a similar way, and no internalisation of irrelevant antibodies by these cell lines was detected under identical conditions. Most interestingly, a coincidence of nuclear uptake and apoptosis was seen in a high percentage of tumour cells; the relevance of this needs to be further investigated. In contrast to the nuclear uptake of anti-CCK-BR antibody, labelled peptide was taken up only into the cytoplasm of cells expressing CCK-BR.

A different pattern of uptake/binding of RG-G7 and GRE1 antibody was seen in the transfected NIH3T3 cells compared to the tumour cell lines. This binding was also specific as it was not seen in non-transfected NIH3T3 cells not expressing CCK-B/gastrin receptor mRNA (negative control). In cells transfected with the classical and spliced variant of CCK-BR (34), GRE1 antibody appeared to bind to the membrane, and was not translocated to the nucleus, as in the tumour cell lines. Similarly, the short gastrin RG-G7 bound only to the membrane of transfected NIH3T3 cells and was not taken up into the cytoplasm. This may represent some inherent difference between naturally expressed and cloned CCK-BR, or it may be a demonstration of differential isomer expression of the receptor in tumours. Watson et al (18) have reported that NIH3T3 cells transfected with classical CCK-BR only express a plasma membrane bound isomer of CCK-BR of Mr 70,000, whereas C170HM2 cells (which showed nuclear uptake of anti-GRE1 in these experiments) showed expression of this isomer of Mr 70,000 and an isofrom of Mr 40,000, which was only found in an intracellular location. This could also reflect a difference in autocrine production of gastrin and its precursors. NIH3T3 cells do not express progastrin, glycine extended gastrin or amidated gastrin (18), whereas AR42J cells (39), and C170HM2 cells express these precursor forms of gastrin (18). Whilst HepG2 cells have not been tested for expression of gastrin precursor forms, studies in hepatocellular carcinoma have shown that many of these tumours express progastrin and glycine extended gastrin (27). Progastrin and glycine extended gastrin are thought to have their own distinct receptor(s), but may have some effect through CCK-BR (isomers) (18).

Western blotting carried out on these cell lines using anti-GRE1 antibody gave specific bands in all cell lines positive for CCK-BR mRNA, but not in non-transfected NIH3T3 cells, negative for CCK-BR mRNA by RT-PCR. The bands were of Mr 241,000, 184,000 and 124,000 in AR42J cells; of Mr 184,000 and 124,000 in transfected NIH3T3 cells, and of
Mr 151,000 and 112,000 in C170HM2 and HepG2 cells. This variation in the weight of CCK-BR is most likely because CCK-BR is known to exist as several isoforms (18,40). Early studies on gastrin binding proteins in guinea pigs reported molecular weights varying from Mr 33,000 to 250,000 (41); the degree of glycosylation of the receptor can also affect molecular weight (42). Ito et al (34) have described a splice variant of CCK-BR which differs from classical CCK-BR by a 5 amino acid insertion; Miyake et al (43) and McWilliams et al (19) have detected a truncated isoform of the receptor in several tumour cell lines. These differences may reflect a variation in technique, as blotting was performed on whole cell lysates, whereas other studies analysed subcellular membrane fractions (18); the larger molecular weight proteins detected here may represent an undegraded complex of CCK-BR.

Whilst other studies have reported recycling of CCK-BR to the plasma membrane after release of gastrin into lysosomes (30), translocation of G protein coupled receptors to the nucleus is not unique. Insulin (44), IL-1 (45) and epidermal growth factor receptors (46) are all reported to translocate to the nucleus; indeed, translocation of anti-epidermal growth factor receptor antibody to the nucleus of cells expressing surface receptor has also been described (47). It has been suggested that chromatin binding of these and other growth factors, including basic fibroblast growth factor, nerve growth factor, and platelet derived growth factor, may be an important step in growth factor induced gene activation (46,48). We have previously described uptake of anti-GRE1 antibody and binding to chromatin in AR42J cells by immuno-electron microscopy (29); this may represent a mechanism by which gastrin receptor mediates the trophic effect of gastrin.

Nuclear translocation of the antibody/receptor complex may have important therapeutic implications. Animal studies (49) have shown that anti-GRE1 antibody has an inhibitory effect on growth of liver metastases of colon cancer in mice. A higher uptake of antibody was detected in tumour versus background liver, and a decreased tumour burden was seen in mice treated with this (unconjugated) antibody. Apoptosis was seen within the treated tumours, and a slight increase in necrosis over untreated animals was seen. Our observations of a coincidence of apoptosis in cells taking up anti-GRE1 antibody into the nucleus suggest that nuclear translocation of the antibody could be the mechanism of this observed effect.

The anti-CCK-BR antibody could be useful in the diagnosis and treatment of CCK-B expressing tumours. Likewise, the specific uptake of the RG-G7 gastrin peptide by cells expressing gastrin receptor suggests that this may also be useful for imaging and therapy of tumours overexpressing CCK-BR (50,51). Further study needs to be carried out to examine whether the observed nuclear uptake of anti-GRE1 antibody is a general function of tumour as opposed to non-tumour cells. Nuclear uptake has been reported with profile targeted therapy for example with the 111Indium-labelled tumour cells. Nuclear uptake has been reported with profile

References


