Antiserum Raised against an Epitope of the Cholecystokinin B/Gastrin Receptor Inhibits Hepatic Invasion of a Human Colon Tumor

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INTRODUCTION

The CCKB/gastrin receptor is a member of the G protein-coupled 7 transmembrane domain receptor superfamily (1, 2) and is expressed on parietal and enterochromaffin-like cells of the gastric mucosa (3, 4). CCKB/gastrin receptor expression has also been confirmed on gastrointestinal tract mucosa (5–8), as well as hepatomas and colorectal liver metastases (9).

Recent studies have shown that by using an antiserum directed against the NH2-terminal extracellular loop of the receptor, denoted GRP1, immunoreactive bands can be detected on both plasma membranes and at intracellular membrane locations of gastrointestinal tumor cell lines by Western blotting (10). The plasma membrane-associated bands were detected at Mr 74,000, the known molecular weight of the CCKB/gastrin receptor (11). The intracellular forms were of lower molecular weight and may have represented deglycosylated or degraded forms of the receptor (10).

A recent study has used the CCKB/gastrin receptor as a trafficking system for the delivery of cytotoxic drugs (12). By the use of heptagastatin linked to ellipticine via a succinoyl-substituted pentapeptide, a 1000-fold increase in cytotoxicity was found with the receptor-linked drug on CCKB/gastrin receptor-positive NIH 3T3 cells compared with receptor-negative cells. In addition, the drug receptor conjugate inhibited the growth of CCKB/gastrin receptor-positive cells in vivo with a delay in growth and a reduction in size (12).

Additional studies have followed the intracellular trafficking of the CCKB/gastrin receptor linked to the GRP1 antiserum described above (13). In these studies, using the rat pancreatic tumor AR42J, antibody-receptor complexes were localized to the nucleus, as confirmed by both light and electron microscopy, as well as Western blotting on isolated nuclear preparations (13).

The aim of the present study was to evaluate the therapeutic effect of passive immunization using the GRP1 antiserum in a human colorectal liver metastasis model. There is currently little to offer patients with advanced colorectal cancer. Blocking the CCKB/gastrin receptor would be an amenable therapeutic approach because serum gastrin levels have previously been found to be elevated in such patients, raising the possibility that up-regulation of CCKB/gastrin receptor expression may occur (14).

MATERIALS AND METHODS

Cell Line. C170HM2 is a human colorectal tumor cell line originally derived from a poorly differentiated tumor and was selected to invade the liver after peritoneal administration (15). C170HM2 has elevated levels of adhesion molecules and matrix metalloproteinase enzymes reflective of the levels seen in colorectal liver metastases (15, 16). It also expresses CCKB/gastrin receptors at the gene and protein level (10, 17).

The cell line was maintained at 37°C under humidified conditions in 5% CO2 and routinely passaged in RPMI 1640 tissue culture medium (Life Technologies, Inc., Irvine, United Kingdom) containing 10% heat-inactivated FCS (Sigma, Poole, United Kingdom).

Growth of C170HM2 Liver-invasive Tumors in Vivo. C170HM2 cells were harvested by using 0.025% EDTA and resuspended at 1 × 106 cells/ml in sterile saline (0.9%; pH 7.3). A 1-ml volume was then injected into the peritoneal cavity of male nude mice (6 weeks of age; bred within the Cancer Studies Unit (University of Nottingham, Nottingham, United Kingdom). This resulted in established liver tumors after approximately 10 days (15). Mice were killed at day 40–50, before the clinical condition of the mice was compromised. United Kingdom Coordinating Committee for Cancer Research guidelines were adhered to throughout all animal experimentation.

GRP1 Antiserum. GRP1 antiserum was directed against the NH2-terminal extracellular epitope of the human CCKB/gastrin receptor. The peptide was based on the known amino acid sequence of the human CCKB/gastrin receptor and comprised the epitope KLNRSVQGTGPGPGASL, which was linked to diphtheria toxoid (10). Rabbits were immunized with the peptide conjugated to diphtheria toxoid to derive a polyclonal antiserum (10).

Biodistribution of Radiolabeled GRP1 Antiserum. Polyclonal rabbit anti-GRP1 antiserum and normal rabbit serum were prepared at a protein concentration of 1 mg/ml. These sera were radiolabeled with Na125I (Sigma, Gillingham United Kingdom) by the Iodo-Gen method. Briefly, Iodo-Gen (Pierce, Chester, United Kingdom) was dissolved in methylene chloride at a concentration of 400 μg/ml and dispersed into tubes in 0.3-ml volumes. The contents of the tubes were then evaporated to dryness under a stream of nitrogen and stored desiccated at 4°C. A 0.5-ml volume of the antiserum preparation was placed in the tubes along with 15 MBq of Na125I (Amersham, Bucks, United Kingdom). The tube contents were mixed and incubated at room temperature for 10 min. A G25 Sephadex column (Pharmacia, Uppsala, Swe-

ABSTRACT

Serum gastrin is known to be elevated in patients with liver-metastasizing colon cancer; thus, cholecystokinin (CCK) B/gastrin receptors may also be up-regulated. A liver-invasive model of colon cancer was established with the human colonic cell line C170HM2, which expresses the CCKB/gastrin receptor at both the gene and protein level. An antiserum has been derived that is directed against the NH2-terminal 17 amino acids of the human CCKB/gastrin receptor coupled to diphtheria toxoid. The peptide was denoted gastrin receptor protein (GRP) 1. The therapeutic effect of GRP1 antiserum was evaluated on the liver invasion of C170HM2 tumors. Biodistribution studies revealed that GRP1 antiserum localized preferentially within the liver tumors when compared with normal liver tissue (1.5-fold increase after 24 h; P < 0.05). Antiserum against GRP1 inhibited both tumor take rate and final liver tumor weight when compared with treatment with control serum in mice with an increasing tumor burden. Liver tumor weights were reduced from 0.37 to 0.10 gram (P = 0.0155), 1.25 grams to 0.76 gram (P = 0.003) and 1.89 grams to 0.76 gram (P = 0.0068, all Mann-Whitney nonparametric U test). Necrosis and apoptosis were increased in the GRP1 antiserum-treated tumors when compared with control serum-treated tumors. As shown by Western blotting, CCKB/gastrin receptor expression of C170HM2 xenografts after treatment with GRP1 antiserum shifted to a predominantly lower molecular weight form (Mr 45,000) that is known to be an internalized form of the receptor. In conclusion, targeting of the CCKB/gastrin receptor may yield a valuable therapeutic modality for the treatment of advanced colon cancer.
den) was prepared by washing with 10 ml of 0.9% saline. The radioiodine-antibody mixtures were added and eluted by adding 0.5-ml fractions of 0.9% saline. The associated radioactivity of each fraction was measured, and peak fractions were pooled. The antibody presence was confirmed by measuring the absorbance at 280 nm.

$^{125}$I-labeled rabbit anti-GRP1 antiserum and normal rabbit serum were administered to nude mice bearing established C170HM2 liver tumors by tail vein injection of 200 $\mu$l of serum containing $2 \times 10^7$ cpm radioactivity. Mice were treated with a single injection, and the biodistribution of radiolabeled into the liver and liver tumors was compared.

Mice were killed at 6 h and at 1, 3, and 5 days, and the number of cpm was monitored in liver tumor, normal liver tissue, and blood. Results were calculated as cpm/gram $^{-1}$ tissue and expressed as the ratio of liver tumor:normal liver.

**Therapeutic Effect of GRP1 Antiserum.** Nude mice were injected with increasing concentrations of C170HM2 cells ($8 \times 10^7$ to $1.5 \times 10^6$ cells/mouse; initial i.p. injection) to establish different liver tumor loads. The number of mice initiated per group is shown in Table 1.

Antisera, either normal rabbit serum or GRP1 antiserum, were administered daily (0.5 ml volume) by tail vein injection from day 0 to day 40. The stable serum level achieved is known to be at a dilution capable of displacing GRP1 from the rat pancreatic tumor cell line AR42J.4

The clinical condition of the mice was carefully assessed during the study by measuring animal weights and by clinical observation. Mice were palpated toward the end of the study to monitor liver tumor bulk. At sacrifice, the liver tumors were dissected free of normal liver tissue and weighed. Half of each tumor was then frozen for Western blotting analysis, and the other half was fixed and paraffin embedded for histological analysis.

**Western Blotting.** Extracellular membrane extracts were prepared from C170HM2 xenograft tissue, and Western blotting was performed as described previously (10). Immunoreactive bands were localized using GRP1 antiserum (1:50 dilution of a 2 mg/ml $^{-1}$ protein stock). Specificity was confirmed by preabsorption (24 h, 4°C) of the GRP1 antiserum with 20 mg/ml $^{-1}$ GRP1 protein and by the use of normal rabbit serum as a negative control. Detection of immunoreactive bands was as described previously (10).

**Image Analysis of Necrosis in C170HM2 Liver Tumors.** Two experienced observers who were blind to the treatment groups counted the level of necrosis by using Leica Qwin image processing and an analysis system run on a Leica Q5001W personal computer. A program was developed enabling necrotic areas to be expressed as a percentage of total tumor area using H&E-stained sections.

The interobserver variation and intraobserver variation was <10.0%.

**Apoptosis.** The presence of apoptotic cells in the xenografts was detected by using the Apoptag Plus in situ apoptosis detection kit (Oncor, Gaithersburg, MD). This method detects apoptotic cells by direct immunoperoxidase detection of digoxigenin-labeled genomic DNA (DNA fragmentation results in free 3' OH groups at the end of DNA fragments that are tailed with digoxigenin-labeled nucleotides) in 4-µm sections prepared from paraffin-embedded tissue. Rat mammary gland epithelium was used as a positive control.

**Statistics.** All statistics were performed using the Mintab package for a personal computer.

**RESULTS**

**Biodistribution of Radiolabeled GRP1 Antiserum.** Nude mice bearing C170HM2 liver tumors were given $^{125}$I-radiolabeled GRP1 antiserum. The results at increasing time points after a single injection of the radiolabeled agent are shown in Fig. 1. The results are calculated as cpm/gram of tissue and expressed as a liver tumor:normal liver ratio for both rabbit GRP1 antiserum and normal rabbit serum. Each point is representative of two to six animals with the SDs shown.

At the earliest time points (6 h), there was lower radioactivity in the liver tumor compared with the normal liver after treatment with both GRP1 antiserum and normal rabbit serum. After 24 h, there was a 1.5-fold increase in the level of GRP1 antiserum localized within the liver tumor in comparison to the normal liver tissue. Localization of normal rabbit serum in the liver tumor remained less than that present in the normal liver (Fig. 1: significance values shown on figure legends). Enhanced liver tumor localization of GRP1 antiserum was maintained for 5 days.

**Therapeutic Effect of GRP1 Antiserum on Establishment and Growth of C170HM2 Liver Tumors.** Three separate therapy experiments were performed with GRP1 antiserum in which liver tumors were established with different initial cell inocula. The distribution of liver tumor numbers for each experiment is shown in Fig. 2, A (initial inoculum, $8.0 \times 10^5$ cells/ml $^{-1}$), B (initial inoculum, $1.0 \times 10^6$ cells/ml $^{-1}$), and C (initial inoculum, $1.5 \times 10^6$ cells/ml $^{-1}$). All lesions were macroscopically visible because the tumors invade through the external liver capsule; the smallest lesion measured was $4 \text{mm}^2$ (weight, 0.05 gram).

With an initial cell inoculum of $8.0 \times 10^5$ cells/mouse, there were mice with an absence of liver tumors in both the control and GRP1 antiserum-treated groups. However, there was a greater percentage of mice without liver tumors in the GRP1 antiserum-treated group (70% compared with 27% in the normal rabbit serum-treated group). No mice in the test group had >1 liver tumor, whereas 46% of mice in the control group had 2–4 liver tumors (Fig. 2A). The distribution was significantly different when comparing the control group with the test group ($P < 0.0055$, $\chi^2$ test).

With a cell inoculum of $1.0 \times 10^6$ cells/mouse, there were non-tumor-bearing animals in both groups, and the number of non-tumor-bearing animals was again in the GRP1 antiserum-treated group (54% in the GRP1 antiserum-treated group compared with 12.5% in the normal rabbit serum-treated group, Fig. 2B). In the control group, 75% of mice had >1.0 liver tumor, whereas in the test group, none of the tumors were 3.0 liver tumors (Fig. 2C).

**Table 1 Numbers of experimental mice randomized to each treatment group at each tumor cell inocula**

<table>
<thead>
<tr>
<th>Cell no./mouse</th>
<th>Control (n)</th>
<th>GRP1 antiserum (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$8.0 \times 10^3$</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>$1.0 \times 10^6$</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>$1.5 \times 10^6$</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>46</td>
</tr>
</tbody>
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4 D. McWilliams, personal communication.
the animals had >1.0 liver tumor, and the distribution was significantly different between the two groups as determined by a $\chi^2$ test ($P < 0.0001$). When the cell inoculum was increased to $1.5 \times 10^6$ cells/mouse, all of the control mice had liver tumors, resulting in a 100% take rate, as compared with a 72% take rate in the test group (Fig. 2C). In the control group, 70% of mice had >1.0 tumor/liver, and 9% of mice in the GRP1 antisem-treated group had >1.0 tumor/liver. This distribution was significantly different in the control versus test group ($P < 0.005$, $\chi^2$ test).

Tumor weight at therapy termination was measured in normal rabbit serum control- and GRP1 antisem-treated groups for all tumor cell inocula. The results are shown in Fig. 3. With increasing cell inocula, the final tumor weight within the liver rose accordingly in the control groups from a mean weight of 0.37 gram to 1.89 grams. In all experiments, treatment with GRP1 antisem resulted in a significant reduction in tumor burden: (a) a 73% reduction was seen with a cell inocula of $8.0 \times 10^5$ cells/mouse ($P = 0.0155$, Mann-Whitney nonparametric $U$ test); (b) a 64% reduction was seen with a cell inoculum of $1.0 \times 10^6$ cells/mouse ($P = 0.003$, Mann-Whitney nonparametric $U$ test); and (c) a 60% reduction was seen with a cell inoculum of $1.5 \times 10^6$ cells/mouse ($P = 0.0068$, Mann-Whitney nonparametric $U$ test).

The weight of the animals was measured throughout all three therapy experiments. Fig. 4 shows animal weights over time for mice treated with a cell inoculum of $1.5 \times 10^6$ cells/mouse. The treatment had no effect on the normal weight gain or clinical condition of the mice.

**Histological Analysis.** The levels of necrosis were assessed in H&E-stained sections from five to seven tumors from each treatment group from the first two therapy experiments. There was a small but significant ($P < 0.05$) increase in the level of necrosis in tumors from the GRP1 antisem-treated mice as compared with normal rabbit serum-treated control mice (necrosis cross-sectional area/tumor cross-sectional area), ranging from 23.5% (SD 8.5) to 32.0% (SD 12.5). Fig. 5, a and b, shows representative areas from the normal rabbit serum- and GRP1 antisem-treated liver tumors, respectively.

Apoptosis was also monitored using rat mammary gland epithelium (Fig. 6c) as a positive control. No apoptosis was evident in the tumors treated with normal rabbit serum (Fig. 6a), whereas apoptotic cells were evident in viable leading edge tumor tissue in the GRP1 antisem-treated tumors (Fig. 6b).

**Western Blotting Analysis to Determine GRP1 Immunoreactivity.** Western blotting was performed on xenograft tissue pooled from five mice from each treatment group from the first two therapy
experiments. An example of the immunoreactive bands generated by staining with GRP1 antiserum is shown in Fig. 7. Fig. 7a shows the immunoreactive bands of C170HM2 cells grown in vitro (10). Fig. 7b details the GRP1 immunoreactive bands from C170HM2 xenografts treated with either normal rabbit serum or GRP1 antiserum. In normal rabbit serum-treated xenografts, there is an immunoreactive band at $M_r$ 74,000 and a fainter band at around $M_r$ 45,000 that is known to be an intracellular form of a receptor previously exposed on the plasma membrane (10).

After treatment with GRP1 antiserum, there appear to be three immunoreactive bands, one at $M_r$ 74,000, a more intense band at $M_r$ 45,000, and an intermediate immunoreactive band that has not previously been observed in C170HM2 cells grown in vitro or in the control xenografts.

**DISCUSSION**

Colorectal tumor cells are known to express CCKB/gastrin receptors (5, 9, 18). Because serum gastrin levels have been shown to be elevated in patients with colorectal liver metastases (14), they may exist in an up-regulated state that has previously only been confirmed in gastric mucosa (19). Thus, they offer an attractive therapeutic
target. The C170HM2 liver-invasive xenograft model has previously been shown to closely parallel the liver invasion observed with a human colonic cancer (15, 16), and C170HM2 liver tumors have also been shown to be sensitive to liver-targeted cytotoxic therapy (20). Therefore, the C170HM2 liver-invasive xenograft model is a therapeutically amenable model to evaluate agents directed against liver metastases.

C170HM2 cells express the CCKB/gastrin receptor at both the mRNA level (17) and the protein level by Western blotting (10). They also express gastrin mRNA and secrete gastrin-like peptides (17, 10). Thus, C170HM2 is a potentially gastrin-sensitive colonic tumor. Antiserum directed against the CCKB/gastrin receptor had a strong therapeutic effect on liver-invasive growth at the level of tumor take and on tumor proliferation. Thus, such an antiserum may block the effect of serum and tumor-associated gastrin peptides.

GRP1 antibody-receptor complexes have previously been shown by electron microscopy to be internalized within the cell and to localize to the nucleus (13), which has been confirmed by confocal microscopy (21). In the present study, GRP1 antiserum has been shown to localize within C170HM2 tumors to a greater extent than in normal liver tissue. This would indicate tumor uptake in a specific manner and may represent a second mechanism by which GRP1 antiserum may be mediating a tumor-inhibitory effect. Thus, by internalizing within the cell and following transport to the nucleus, the antibody may have nonspecific inhibitory effects. Indeed, apoptosis was shown in the present study in the viable tissue within GRP1 antiserum-treated tumors. This indicates that in addition to the cytostatic effects mediated by the removal of a potent growth factor, the cellular machinery may have been induced to undergo programmed cell death in response to nuclear disturbance caused by the presence of an antibody. The level of cell death could potentially be further increased by linking the antibody to a cytotoxic agent, as has been described previously (12).

Fig. 6. Assessment of apoptosis within C170HM2 liver tumors from GRP1 antiserum-treated mice. Apoptosis of C170HM2 liver-invasive xenografts was determined by in situ hybridization using the terminal deoxynucleotidyl transferase-mediated nick end labeling method. A, normal rabbit serum-treated control tumors. B, GRP1 antiserum-treated test tumors. C, positive control (rat mammary gland epithelium). Magnification, ×125. The presence of apoptotic cells in the xenografts was confirmed by the use of direct immunoperoxidase detection of digoxigenin-labeled genomic DNA in 4-μm sections prepared from paraffin-embedded tissue.
Necrosis was modestly increased in the GRP1 antiserum-treated tumors compared with tumors from control serum-treated mice. This may reflect the fact that all tumors in the GRP1 antiserum-treated groups were smaller than the corresponding tumors in the normal rabbit serum-treated groups. The latter develop necrosis in response to poor vascularization over a certain size, whereas the small tumors in the test group potentially lack this nonspecific onset of necrosis.

Western blotting performed on xenograft tissue revealed that in the normal rabbit serum control-treated xenografts, the predominant receptor expressed was the $M_r$ 74,000 isoform, which is the reported molecular weight of the classical CCKB/gastrin receptor (11). The lower molecular weight receptor ($M_r$ 45,000) was also evident. This is an intracellular form of the CCKB/gastrin receptor expressed previously on the cell membrane (10). In xenografts treated with GRP1 antiserum, the $M_r$ 45,000 form of the receptor appeared to be expressed at a greater level. This would indicate that plasma membrane-associated receptors were internalizing to a greater extent, which may have been due to antibody binding and antibody-receptor complex internalization. Therefore, it would appear that in addition to direct competition for the binding of plasma gastrin, down-regulation of external receptors might also have occurred. The intermediate molecular weight isoform detected by Western blotting has not been observed previously. The significance of this observation is not known, but it may be artifactual because the receptor antibody complex may mask degradation sites responsible for complete cleavage to the $M_r$ 45,000 form.

In conclusion, antiserum directed against the NH$_2$-terminal portion of the human CCKB/gastrin receptor has localized within and caused growth inhibition and apoptosis of a human liver-invasive colorectal tumor in vivo. Because there is little to offer patients with advanced disease in terms of therapeutic agents, treatment with antibodies raised against the GRP1 epitope would be a potential therapeutic modality either alone or linked to cytotoxic/radiolabeled agents in a targeted approach.

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